The Importance of Interleukin-2 and its Soluble Receptor in Patients with Multiple Sclerosis and Other Inflammatory Diseases of the Central Nervous System

by

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To my Parents

ABSTRACT

Interleukin-2 (IL-2) was originally identified in 1976 as a growth factor for T lymphocytes. Since that time it has become an important mediator of immune functions through its effects on the growth, development, and activity of T and B lymphocytes, natural killer cells, and lymphokine-activated killer cells. Although the role of IL-2 in the systemic immune response is well-established, the importance of IL-2 and soluble IL-2 receptor (sIL-2R) in the intrathecal immune response is still unclear, probably due to the lack of sensitive and standardised methods to detect these cytokines in body fluids. Another factor may be the shortage of longitudinal studies that correlate the local release of IL-2 and sIL-2R with other pathological and clinical features of inflammatory disorders of the central nervous system (CNS).

This study describes a sensitive method that detects IL-2 and sIL-2R in human cerebrospinal fluid (CSF) and serum samples, and is able to evaluate intrathecal synthesis of these cytokines *in vivo*. Using this method, intrathecal release of IL-2 and sIL-2R was evaluated in 148 patients with clinically definite multiple sclerosis (MS), 28 patients with other inflammatory neurologic diseases, 36 patients with post-polio syndrome, and 128 neurologic controls and normal subjects. Intrathecal synthesis of IL-2 and sIL-2R was predominantly found in patients with relapsing-remitting MS and other inflammatory CNS diseases. No evidence of *in vivo* antagonism was detected between IL-2 and sIL-2R. Detection of IL-2 and sIL-2R in CSF provided an objective indicator of poliovirus activation in post-polio syndrome.

Intrathecal levels of IL-2 and sIL-2R in MS correlated with local CNS synthesis of IgD and IgM, indicating that these cytokines are involved in the intrathecal humoral immune response in MS. Furthermore, IL-2 and sIL-2R demonstrated an outstanding predictive value for subsequent development of MS in 45 patients with acute isolated brainstem or spinal cord syndromes, who were prospectively followed-up for 30 months. High IL-2 and sIL-2R levels correlated with the degree of blood-brain barrier damage in patients with MS, primarily through the release of tumour necrosis factor-α. Moreover, the degree of disability and overall disease activity of MS correlated with high intrathecal levels of IL-2 and sIL-2R.

This study demonstrates that IL-2 and sIL-2R are valuable indicators of intrathecal immune activation, and that *in vivo* evaluation of these cytokines is imperative in understanding the pathogenesis of some CNS inflammatory diseases.

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ABBREVIATIONS

ACTH, adrenocorticotrophic hormone, **AIDS**, acquired immune deficiency syndrome; **ALS**, amyotrophic lateral sclerosis; **APC**, antigen-presenting cells; **bp**, base pair; **BSA**, bovine serum albumin; **cDNA**, complementary deoxyribo-nucleic acid; **CNS**, central nervous system; **Con A**, concanvalin A; **CSF**, cerebrospinal fluid; **CTLL**, cytolytic T lymphocyte lines; **CV**, coefficient of variation; **EDSS**, expanded disability status scale, **EEG**, electro-encephalography; **ELISA**, enzyme-linked immunosorbent assay; **FS**, functional scales, **GA**, glutaraldehyde; **G-CSF**, macrophage colony-stimulating factor; **Gd**, gadolinium; **Gly-PI**, glycosyl-phosphatidylinositol; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **HEEO**, high electroendosmosis; **HLA**, human leucocyte antigen; **Hr**, hours; **HRP**, horseradish peroxidase; **HSV-1**, herpes simplex type-1 virus; **HTLV-1**, human T lymphocyte virus type 1; **Ia**, *i*mmune response *a*ssociated antigen; **ICAM-1**, intercellular adhesion molecule 1; **IFN-γ**, interferon-gamma; **Ig**, immunoglobulin; **IL-2**, interleukin-2; **IL-2R**, interleukin-2 receptor; **kD**, kilo-dalton; **kd**, dissociation constant; **L**, litre; **LAK**, lymphokine-activated killer cells; **LGL**, large granular lymphocytes; **M**, molar; **M-CSF**, macrophage colony-stimulating factor; **ME**, medium electroendosmosis; **MHC**, major histocompatibility complex; **min**, minutes; **ml**, millilitre; **MND**, motor neuron disease; M_r, molecular weight; **MRI**, magnetic resonance imaging; **MS**, multiple sclerosis; **NK**, natural killer cells; **OD**, optical density; **OPD**, *o*-phenylenediamine hydrochloride; **p55**, low-affinity IL-2 surface receptor; **p75**, intermediate-affinity IL-2 surface receptor; **PAGE**, polyacrylamide gel electrophoresis; **PBS**, phosphate-buffered saline; **PHA**, phytohemagglutinin; **PVC**, polyvinyl chloride; **PVFM**, polyvinyl difluoride membrane; **RPM**, revolutions per minute; **SD**, standard deviation; **SEM**, standard error of the mean; **SDS**, sodium dodecyl phosphate; **sec**, seconds; **sIL-2R**, soluble interleukin-2 receptor; **SPSS**, statistical package for social sciences; **Tac**, T Cell Activation antigen; **TCGF**, T cell growth factor; **TGF-β**, transforming growth factor-β; T_H, helper T lymphocytes; **U**, unit; **vol**, volume; **w**, weight.

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Chapter 1. GENERAL INTRODUCTION

The vertebrate immune system has a virtually unlimited capacity to recognise and distinguish different molecular configurations and thus to bind to a myriad of potential antigens. Two broad lineage of cells that react specifically with antigens, B cells and T cells, are involved in this immune response. B cells are precursors of the antibody-secreting cells of the humoral immune system, while T cells consist of an array of different subtypes of cells, including those that serve critical regulatory and effector functions. The interaction of appropriately presented antigens with receptors of B or T lymphocytes initiates the immune response. However, this interaction is not sufficient to signal the initiation of effector functions, but provides the signal required for the induction of cytokines and their receptors. These cytokines play a major role in intercellular communication and are involved in both proliferative clonal expansion and in differentiation to specialised functions.

Interleukin-2 (IL-2), the first of a series of lymphocytotrophic hormones to be recognised and completely characterised, is pivotal for the generation and regulation of the immune response. The IL-2 and IL-2 receptor system is probably the best characterised of lymphocytotrophic hormones, both in terms of function and molecular biochemistry. This chapter is intended to: (*a*) present a resumé of the main events thought to be involved in the current knowledge of IL-2; (*b*) discuss, in reasonable detail, the structure, origin, biological functions, and significance of IL-2 and its receptors; and (*c*) provide an overview on the complex nature of the cytokine network and the immune functions of the brain. Particular emphasis will be directed towards the structure of IL-2 and its receptors, as this is important in understanding the biological functions related to this cytokine.

1.1 Historic background

Ever since Edward Jenner introduced vaccination in 1797 as a mean of preventing smallpox, biologists have been fascinated and mystified by the immune system. Throughout the past two centuries, investigators have slowly learned that the activity of the immune system depends on a symphony of highly specialised cells in the blood and tissues, each cell type performing a unique function. Yet until the late 1970s, it

was unclear how this unconnected multitude of cells was able to orchestrate its activities into a selective defence against diseases.

1.1.1 Historic shift

The discovery of IL-2 and the investigations that established it as the lymphocyto-trophic hormone responsible for signalling T cell proliferation have had an extraordinary impact on the discipline of immunology. The demonstration that the immune system is regulated by hormones (interleukins) in much the same way as most other organ systems has transformed the whole field of immunology. It reflects a historic shift away from the cellular immunology of the 1960s and 1970s which had focused on antigens as solely responsible for the intracellular biochemical reactions that promote cellular proliferation and differentiation. Indeed, physical contact among T cells, B cells, and macrophages had been considered an essential prerequisite for effecting intercellular communication. Consequently, antigen-receptor idiotypic net-works, cell-to-cell interaction, suppressor T cells, and antigen-specific helper and suppressor factors were believed to account for the comprehensive regulation of the immune response. Now, intercellular communication is firmly established as being driven by cytokine molecules, hence the name interleukin which means between leucocytes[1].

1.1.2 Between leucocytes

The beginning of the current understanding of the mechanisms responsible for stimulating lymphocyte growth can be traced back to 1965, when two independent teams of investigators simultaneously reported that culture media "conditioned" by proliferating lymphocytes contained an unidentified substance, which enhanced lymphocyte growth when antigen was present (Kasakura and Lowenstein 1965; Gordon and MacLean 1965). Many reports about these mysterious growth-enhancing substances appeared during the next decade, but most immunologists ignored them and continued to maintain that antigens were the sole agents responsible for

[1]An alternative explanation to the emergence of this term is that a soluble factor that promotes the communication between leucocytes was first discussed in a meeting held in *Interlaken*, Switzerland. Thus, this factor was given the name interleukin.

lymphocyte proliferation and that there should not be any mediator between the immune cells. The mysterious factors were thought simply to amplify proliferation that had already been triggered by an antigen. The term lymphokine was introduced in 1969 by Dumonde *et al* (1969) to describe non-antibody mediators of cellular immunity produced by activated lymphocytes.

In late 1975, Doris Morgan, a haematologist working in Robert Gallo's laboratory at the National Cancer Institute, Maryland, USA, reported that normal human T cells could be cultured without antigen for up to nine months, provided that a lymphocyte-conditioned medium was added to the culture at regular intervals (1976). Although Morgan's report appeared in *Science*, its significance was lost on most of the immunology community, probably because she and her co-workers were unfamiliar to most immunologists. The title of her report stressed that T cells had come from the bone marrow, which meant that they might be immature or unrepresentative of most T cells. In addition, the cultured cells were not shown to perform any antigen-specific functions, and immunologists were traditionally indifferent to phenomena that lacked antigen specificity.

Unencumbered by immunological dogmas, Kendall Smith (also a haematologist by training) reported in 1977 the long-term growth of tumour-antigen-specific cytotoxic T lymphocytes in conditioned medium (Gillis and Smith 1977). The immunologic community was very interested in his report, probably because it emphasised the culture of functional, antigen-specific T cell. Armed with a rapid and quantitative assay, Smith and co-workers published several reports between 1978 and 1981 (Gillis et al 1978 a & b; Baker et al 1978 & 1979; Smith et al 1980 a & b; Leonard et al 1982; Smith et al 1983) that collectively described for the first time the biological and biochemical characteristics of the *T Cell Growth Factor*, now known as interleukin-2. During this brief span, it became evident that the immune system uses hormonal mechanisms to direct virtually all aspects of the response to antigen. Accordingly, the vocabulary of immunologists and the kind of work they perform became familiar to those from other biologic disciplines. Lymphocytes and lymphokines became the cell and molecule of choice for an array of physiologic, biologic, biochemical, pathologic, and genetic studies directed toward comprehending how cells perform in response to separate and diverse external stimuli.

By 1982, it was evident that in order to understand the molecular mechanisms regulating T cell proliferation, the structure of IL-2 should be known. An important first step toward that goal was the isolation of the gene for IL-2 by Tanigushi and his colleagues in 1983 (Tanigushi et al 1983). Gene cloning technology allowed the production of large quantity of pure IL-2 which eventually permitted David McKay's group to deduce the three-dimensional structure of IL-2 molecule by X-ray crystallography in 1987 (Brandhuber et al 1987).

1.1.3 Characterisation of the IL-2 receptor

Although immunologists became aware that only antigen-activated T cells responded to IL-2, the mechanism of that response was not clearly understood. Experiments from Smith's group showed that activated T cells could absorb IL-2 activity, as would be expected if they bore IL-2 receptors. These findings encouraged them to produce radioactively labelled IL-2 which showed clear binding of IL-2 to cells through a high affinity receptor on the cell surface (Robb et al 1981). The application of this hormone-receptor concept to the immune system has had another extraordinary impact on models of immune regulation. Cellular immunology had previously maintained that macrophages, B cells, and T cells signalled one another exclusively through intimate contact. After the advent of the idea that the expression of IL-2 receptor determines which cells participate in the immune response, the interactions of the immune cells lost their mystical enigma. It was possible to understand them through principles borrowed from endocrinology; principles that describe the interactions of hormones and their receptors.

In 1984 Warren Leonard and others in Maryland, USA, and Toshio Nikaido and colleagues in Kyoto, Japan, simultaneously reported the isolation of a gene for a putative IL-2-receptor protein (Leonard et al 1984; Nikaido et al 1984). This protein chain reacted with a monoclonal antibody against the IL-2 receptor developed by Uchiyama and co-workers (1981). However, the chain's small size (55 kD) and low affinity for IL-2 meant that it could not be the complete IL-2 receptor. Then, in 1986, Keisuke Teshigawara of Kyoto University working in Kendall Smith's laboratory, and Mitsuru Tsudo, also of Kyoto University but working in Thomas Waldmann's laboratory, independently made the startling discovery of a second, larger (75 kD) IL-2-receptor chain (Tsudo et al 1986; Teshigawara et al 1987). By the end of 1986, most

workers in the field began to race toward the characterisation of the new 75-kD chain. Tsudo eventually won this race after taking a new position in Tokyo. By mid-1988 he had derived monoclonal antibodies that reacted with the 75-kD chain (Tsudo et al 1989). A young haematologist, collaborating with Tsudo, was able to isolate the gene encoding the 75-kD chain (Hatakeyama et al 1989).

1.1.4 The IL-2 system in neurology

Clinical neurologists and neuroimmunologists were slow to appreciate the impact of the IL-2 system on the field of clinical medicine. The first study on the role of cytokine in neurologic diseases was conducted in 1982, about five years after the discovery of IL-2, when two independent groups reported a role of IL-2 in the transfer of experimental allergic encephalomyelitis in laboratory animals (Holda et al 1982; Ortiz and Weigle 1982). A year later, a group of neuroimmunologists led by Dale McFarlin, further extended these results by reporting a potential relevance of IL-2 to the pathogenesis of multiple sclerosis (MS) (Richert et al 1983). The first evidence of IL-2 release by glial and astrocytic cells was provided at the same time by Fontana and colleagues (1983).

By 1984, there was little doubt that extensive communications between the immune system and the central nervous system (CNS) do exist, partly through the cytokine network (Fontana and Grob 1984). At the same time, it was evident that cerebrospinal fluid (CSF) lymphocytes from MS patients were able to produce elevated amounts of IL-2 (Burns et al 1984; Merrill et al 1984). In 1985, research work led by Alan Davison at the Department of Neurochemistry, Institute of Neurology, London, provided conclusive evidence that lymphocytes containing IL-2 as well as lymphocytes expressing IL-2 receptors were present in MS brain lesions (Bellamy et al 1985). It soon became clear that intrathecal release of IL-2 or expression of IL-2 receptors were not restricted to patients with MS since these findings were detected in various infectious and inflammatory neurologic disorders (Sobel et al 1986; Schluesener and Lassmann 1986; Giulian et al 1987, Boutin et al 1987; Gurney 1987). However, there is still considerable uncertainty regarding the clinical significance of IL-2 or its receptors in patients with neurologic diseases (see section 1.7).

1.2 The human Interleukin-2

Interleukin-2, originally called T-cell growth factor (TCGF), is both sufficient and necessary for the expansion of most T-cells, natural killer (NK) cells, macrophages, and B cells during certain phases of their response. An indicator to the importance of IL-2 in immunology is the rapidity of progress in research work related to this cytokine. In only five years from the first experiments describing the biologic characteristics of TCGF, its molecular properties had been defined, monoclonal antibodies reactive with it were developed, it had been purified to homogeneity, and cDNA as well as genomic DNA clones had been isolated and sequenced. IL-2 finally made the front cover of *Newsweek* in 1986, and it is now widely acknowledged as a central mediator of many aspects of the immune response.

Although IL-2 is a protein of only 133 amino acid residues for which a low resolution X-ray structure does exist, the complexity of its receptor system has provided an additional challenge to structure-function studies. As a result, the next two sections will discuss in reasonable detail the structure and biologic functions of both IL-2 and its membrane-bound receptors.

1.2.1 Structure of IL-2

Early experiments suggested that the structure of IL-2 could show molecular heterogeneity (Watson et al 1979). However, further biochemical analyses revealed that IL-2 is a glycoprotein with a molecular size of 15.5 kD and a single isoelectric point (*p*I 8.2) after removal of charged sialic acid residues (Robb and Smith 1981). Thus, the apparent molecular heterogeneity could be explained by variable glycosylation and sialylation of a single protein. Further evidence that IL-2 activity is attributable to a single molecule came after the development of IL-2-reactive monoclonal antibodies which provided a reliable immunoaffinity purification of IL-2 to enable NH_2-terminal sequence determination (Smith et al 1983). IL-2 has a carbohydrate-binding (lectin) domain with specificity for high-mannose glycopeptides, which may play a critical role in the clearance and intracellular routing of this molecule (Sherblom et al 1989).

Human interleukin-2 is a polypeptide chain of 133 amino acid residues (Fig. 1.1) plus a signal sequence of 20 residues. It has been crystallised and analysed by X-ray diffraction to 3.0 angstroms resolution (Brandhuber et al 1987). It revealed that IL-2 is a novel alpha-helical protein with a core structure of six anti-parallel short

helical segments (Fig. 1.2). These segments have amino acid residues 11-19, 33-56, 66-78, 83-101, 107-113, and 117-133 respectively. A disulfide bridge is formed by cysteine residues at positions 58 and 105 (Fig. 1.2). The overall helical content is about 65% of the whole structure of IL-2, and there are no segments of β secondary structure in the molecule. Helices B, C, D, and F shown in Fig. 1.2 form an apparent antiparallel alpha helical bundle which differs significantly from the classical four-helix bundle represented by cytochrome c, cytochrome b, and myohaemerythrin (Brandhuber et al 1987).

As could be seen form the three-dimentional structure of IL-2 depicted in Fig. 1.2, the alpha helical bundle differs significantly from the classical four-helix bundle. The packing regions of the helices are shorter, involving only three to four turns of helix, while classical four-helix bundles usually have at least five turns in each helix. Furthermore, the packing angles all fall in the range of 25° to 30°, and hence are somewhat larger than the average of 18° found in classical four-helix bundles.

Following extensive data on site-specific amino acid substitution and antibodies to peptides that cross-react with IL-2 (Ju et al 1987; Collins et al 1988), it has been suggested that helices B, C, D, and F form a structural scaffold while helices A, B′, and E contain the residues that interact with the IL-2 receptor (Fig. 1.3). Helix A is required for biological activity, and its normal conformation is required for the binding to the β subunit of IL-2 receptor. A neighbouring amino acid (Asp^{20}) is required for effective binding to the β IL-2-binding subunit. Specific mutations at this site exhibit a very low level of bioactivity yet retain normal overall protein conformation and are still bound by the α (Tac) subunit of IL-2 receptor (Collins et al 1988). Conversely, helix B′ and helix E are required for binding to the Tac subunit.

Finally, the carboxy terminal residues 131-133 and two of the three cysteine residues (Cys^{58} and Cys^{105}) are required for full biologic activity and binding (Ju et al 1987). Reduction of the disulfide bond destroys biologic activity (Yamada et al 1987), thereby indicating that the tertiary, folded structure of IL-2 is essential for stimulating growth. Indeed, truncated segments of the IL-2 sequence cannot promote T cell growth (Ju et al 1987).

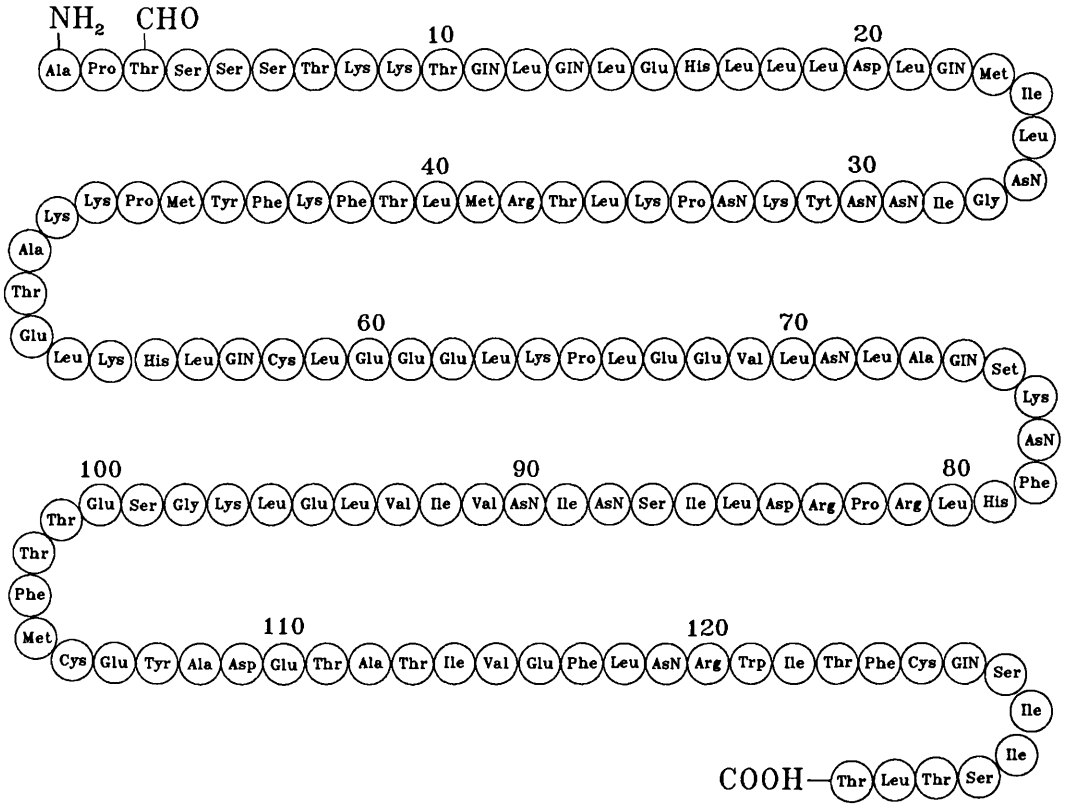

Fig. 1.1. The primary amino acid sequence of human interleukin-2. Sites of the two cystiene residues (58 and 105) that mark the position of a disulphide bond can be seen. As will be explained in the text, this disulphide bond seems to be essential for full biological activity and binding of IL-2. The signal sequence of the IL-2 molecule is not depicted in this diagram. Residues for the signal sequence are as follows:
IVEFLNR WITFCQSIIS TLT.

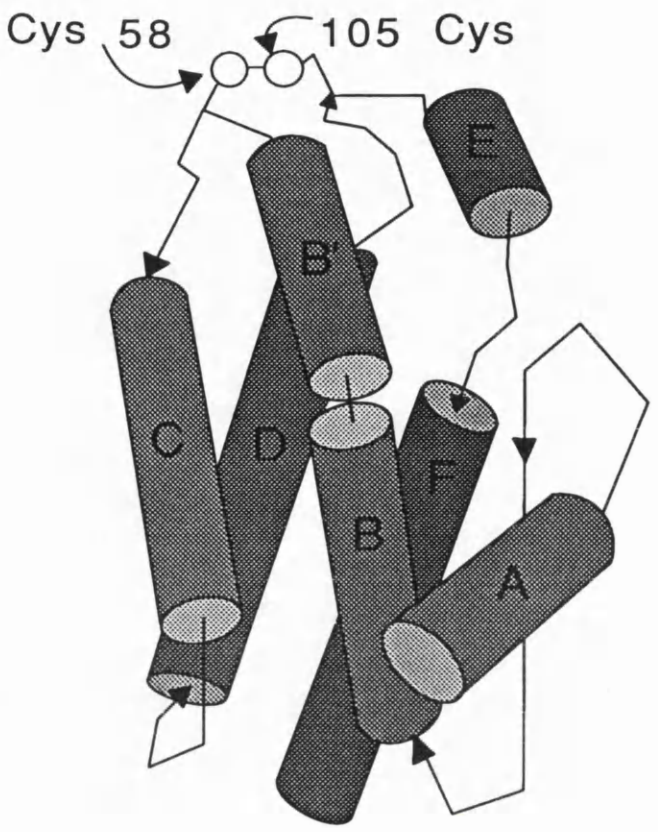

Fig. 1.2. Schematic stereo drawing of IL-2 based on data obtained by x-ray crystallography. The alpha helices are represented as cylinders and are lettered sequentially from the amino terminus. A short helical segment near the amino terminus (residues 11 to 19; helix A) is followed by an extended loop (residues 33 to 56) which forms a helix interrupted near the middle by Pro^{47} (hence the two segments are referenced as B and B′). Following Cys^{58} of the disulfide bond are helix C (residues 66 to 78) and helix D (residues 83 to 101). Following Cys^{105} is a short E helix (residue 107 to 113) which leads into the carboxyl-terminal helix F (residues 117 to 133). [modified from Brandhuber et al 1987].

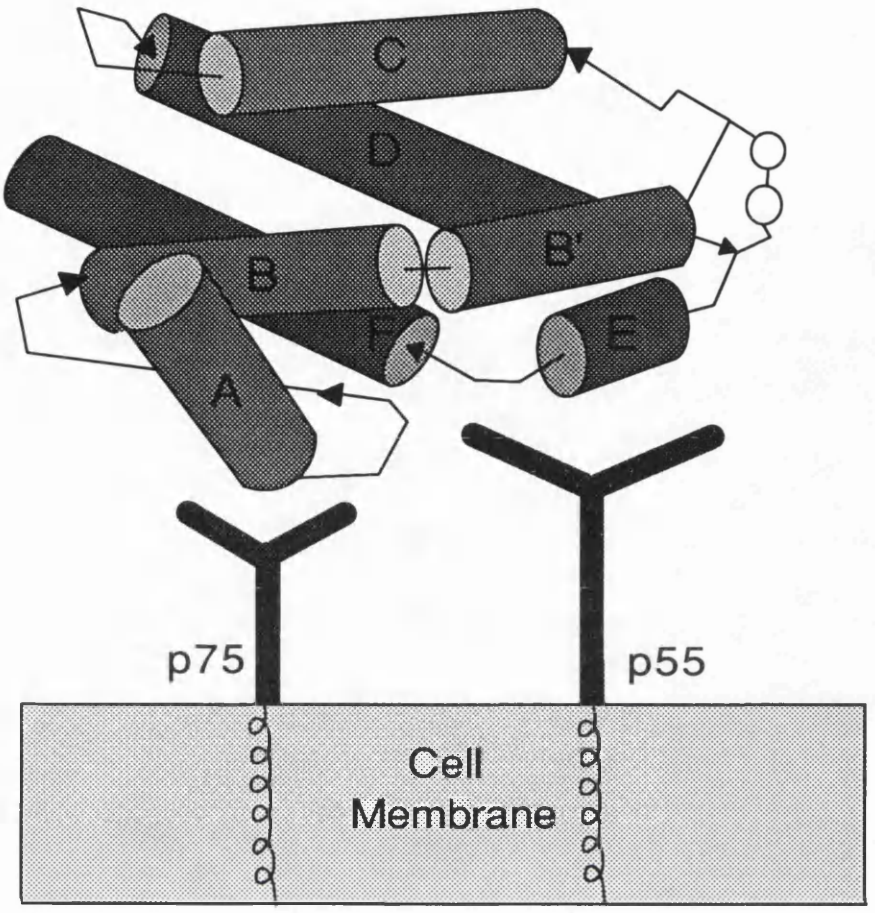

Fig. 1.3. Schematic drawing showing a possible mode of interaction of IL-2 with the two distinct subunits of the IL-2 receptor.

1.2.2 Physio-chemical properties of IL-2

The molecular weight, amino acid length, and isoelectric point of IL-2 have been described above. Similar to hormones of the endocrine system, IL-2 is produced in trace quantities. Similar to most cytokines, human and mouse IL-2 are equally active in the human system *in vivo*, but human IL-2 is several-fold less active in the mouse system. Probably there is an evolutionary basis for this kind of behaviour. However, this behaviour differs from that of interferons, where human interferons are active in the human system only and not on cells from other species, and vice versa. Even though a significant structural homology has been observed between human and murine IL-2, they are antigenically distinct and do not crossreact immunologically.

In general, the biological activity of IL-2 is rather stable under highly diverse conditions such as pH and temperature extremes. IL-2 can be stored at 4°C for several months or exposed to 56°C for 10-60 min. It is also stable at pH 2. However, IL-2 activity is quite labile to various proteolytic enzymes (Aggarwal et al 1984). Unlike interferons, IL-2 has been shown to exist in monomeric form.

1.2.3 Genetic regulation of IL-2 production[1]

Adequate understanding of the genetic regulation of IL-2 production is imperative for planning effective immunosuppressive therapy (see Chapter 6). The complementary DNA for human IL-2 has been cloned from a number of human cell sources (Taniguchi et al 1983; Clark et al 1984; Rosenberg et al 1984) and the gene for IL-2 has been mapped to chromosome 4 (Seigel et al 1984). The overall genomic organisation of IL-2 is encoded by four exons separated by two long introns (Fujita et al 1983). This structure is remarkably similar to the genomic organisation of other cytokines, including IL-4 (Arai et al 1989); IL-5 (Campbell et al 1987); interferon (IFN)-γ (Gray and Goeddel 1983); and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Stanley et al 1985). Consequently, it appears that a family of similar molecules could have evolved from a set of common ancestral genes. In addition, such a genomic structural homology suggests that each exon may well encode distinct elements that are common to a single family of cytokines. As a result, structural similarities and functional cross-reactivity among individual cytokines could occur even without obvious primary amino acid sequence homology (Smith 1988 a&b).

The synthesis of IL-2 is regulated at least in part at the level of DNA transcription (Efeat and Kames 1986). Both an increase of cytoplasmic free calcium $[Ca^{2+}]_i$ and activation of protein kinase C are required to activate transcription of the IL-2 gene in resting T cells. Detailed analysis of the IL-2 gene has demonstrated that a 276 base pair (bp) segment upstream from the transcription initiation site contains the sequences that regulate its transcriptional activation (Durand et al 1987). This

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[1]**Glossary:**

Exon	Any segment of an interrupted gene that is represented in the mature RNA product
Transcription	Synthesis of RNA on a DNA template
Upstream	Identifies sequences proceeding in the opposite direction from expression: e.g., the promoter is upstream from the transcription unit

region is responsive to the synergistic actions of stimuli which increase $[Ca^{2+}]_i$ and induce the activation of protein kinase C (Kern et al 1988). These two events will in turn stimulate the production of as yet unidentified gene products which may bind to the IL-2 regulatory region or influence the binding of other regulatory proteins to this sequence (Shaw et al 1988). The latter stage, the downstream event of protein kinase C activation, remains elusive, yet the signal must be transmitted to the nucleus to activate transcription of IL-2 gene (Arai et al 1990).

The minimal IL-2 enhancer contains recognition sites for several DNA binding proteins, including two octamer motifs (Kamps et al 1990). Evidence has been presented recently that the homeodomain-containing protein Oct-1 and an inducible auxiliary protein participate in activating transcription of the IL-2 gene (Ullman et al 1991). Oct-1, unlike other homeodomain-containing proteins, also regulates commonly expressed genes such as those encoding H2b and small nuclear RNAs. However, more work is needed to define the complete mechanism of activation of IL-2 gene transcription.

1.2.4 IL-2 release

Interleukin-2 is produced by T cell precursors at a circumscript developmental stage (after intrathymic immigration and before acquisition of cell surface expression of CD4 and/or CD8). Among peripheral T cells, significant IL-2 secretion appears to be confined to activated T helper cells (T_H: the human $CD4^+$). Freshly isolated resting T cells do not usually express IL-2 mRNA, nor do they contain IL-2 protein, although this has been disputed recently (see below). T cells activation by antigens or mutagens, and ligand binding to CD2 surface receptors usually stimulate IL-2. Antigens also induce IL-2 release by T_H cells. Similarly, potent polyclonal mitogens stimulate CD8 T lymphocytes and medullary thymocytes to secrete moderate amounts of IL-2. In addition, a subset of large granular lymphocytes (LGL) that bear the CD2 and CD6 markers can be induced by lectin or by IFN-γ to produce IL-2.

Recent advances of T cell cloning have allowed the classification of T_H cells in terms of the cytokines they secrete. In 1986, Mosmann and co-workers proposed a major subdivision of murine CD4+ T_H cells based on differences in their pattern of cytokine production. T cell clones of the T_H1 subset synthesise and secrete IL-2 and IFN-γ, whereas T cell clones of the T_H2 subset produce IL-4, IL-5, IL-6, and IL-10 but

not IL-2. Such segregation undoubtedly plays a key role in the regulatory mechanisms of the immune response (Mosmann and Coffman 1989 a&b), although several researchers did not find a clear-cut patterns among existing panels of human T cell clones. However, recent reports have provided some evidence that T cells with stable T_H1 or T_H2 patterns could exist in humans (Wierenga et al 1990; Del Prete et al 1991; Romagnani 1991).

It has been reported that the two types T_H cells have different functions: T_H2 cells are much more effective B cell helpers, especially in the production of IgE (Coffman et al 1988), whereas T_H1 cells mediate a delayed-type hypersensitivity reaction (Cher and Mosmann 1987). Responses involving these two types of T cell were said to be mutually inhibitory (Mosmann and Koffman 1989), especially when strong immune responses are considered. Although T_H1 and T_H2 cells are the best characterised subsets, additional cytokine-secreting phenotypes do exist (reviewed by Mossman and Moore 1991). In particular, most resting T cells probably only secrete IL-2 when first stimulated, and then rapidly differentiate into cells secreting multiple cytokines.

1.2.5 The central role of IL-2 in the immune response

Interleukin-2 is a key immunoregulatory cytokine that controls the expansion of lymphocytes selected by antigen, and regulates the induction of their effector functions. Expansion of lymphocytes is necessary for effective immune response, because T or B cells with specificity for a given antigen are very infrequent. The responding precursors are not fully differentiated initially, and in general do not mediate appropriate function until they become effector cells. It is now well established that IL-2 plays central role in this process of differentiation (Swain 1991). In contrast, the effects of other cytokines, such as IL-4, IFN-γ, and TGF-β, tend to be directive in that each promotes the development of particular type of immune response and suppresses other pathways (Swain 1991).

The induction of lymphocyte proliferation involves two main steps. The first step, called competence, is initiated by exogenous signals delivered to the quiescent T cell by mitogens or antigens. This leads to partial activation of T cells, resulting in an increase in size, induction of transcription of certain genes, and passage from the G_0 phase to the early G_1 phase in cell cycle. However, the original signal by itself is

not sufficient to stimulate growth. A second endogenous signal is needed to induce cell cycle progression through an entire series of events recognised as G_1 progression, which ultimately leads to cell division. It is now well-established that IL-2 is required for the progression of the T cell through G_1 progression (Cantrell and Smith 1984; Stern and Smith 1986).

IL-2 is secreted mainly by T_H 4 to 12 h following the binding of antigen (or mitogen) to the T cell receptor. The release of IL-2, and its subsequent binding with IL-2 receptors, results in proliferation of T cells, enhanced secretion of other cytokines, heightened expression of membrane receptors for other growth factors (e.g. transferrin receptor, insulin receptor), and the expression of induced class II MHC. IL-2 receptors are distributed over a wide spectrum of immunologically relevant cells (see section 1.3.6). Thus, the biological effects of IL-2, although originally defined on the basis of the growth of T cells in culture, have expanded to include interactions with macrophages, activated B cells (Kishi et al 1985), LGL (Henny et al 1981), NK (Ortaldo et al 1984), and other cytotoxic cells (Gillis et al 1979; Zarling and Bach 1979) (Fig. 1.4). In activated B cells, IL-2 directly favours switching the μ heavy-chain mRNA transcription from the membranous to the secretory form, which may eventually lead to the secretion of pentameric IgM (see Chapter 3).

Monocytes are stimulated by IL-2 in their cytotoxic function, and NK cells, both in their growth and in their effector function. Virtually all T cell subsets that display the high affinity IL-2 receptor respond to IL-2 by undergoing cell division (Smith 1988b). As an exception to this rule, some HTLV-1-transformed T cell lines respond to IL-2 with a negative signal, i.e. inhibition of DNA synthesis. Although it was widely believed that IL-2 does not induce proliferation of resting T cells, a number of investigators have recently demonstrated that highly purified preparations of both natural and recombinant IL-2 induced high levels of T cell proliferation in the absence of any known mitogens or antigens (reviewed by Mookerjee and Pauly 1990). The specificity and duration of the immune response is usually regulated by IL-2 receptors (see section 1.3). A summary of the biological properties of IL-2 is presented in Table 1.1.

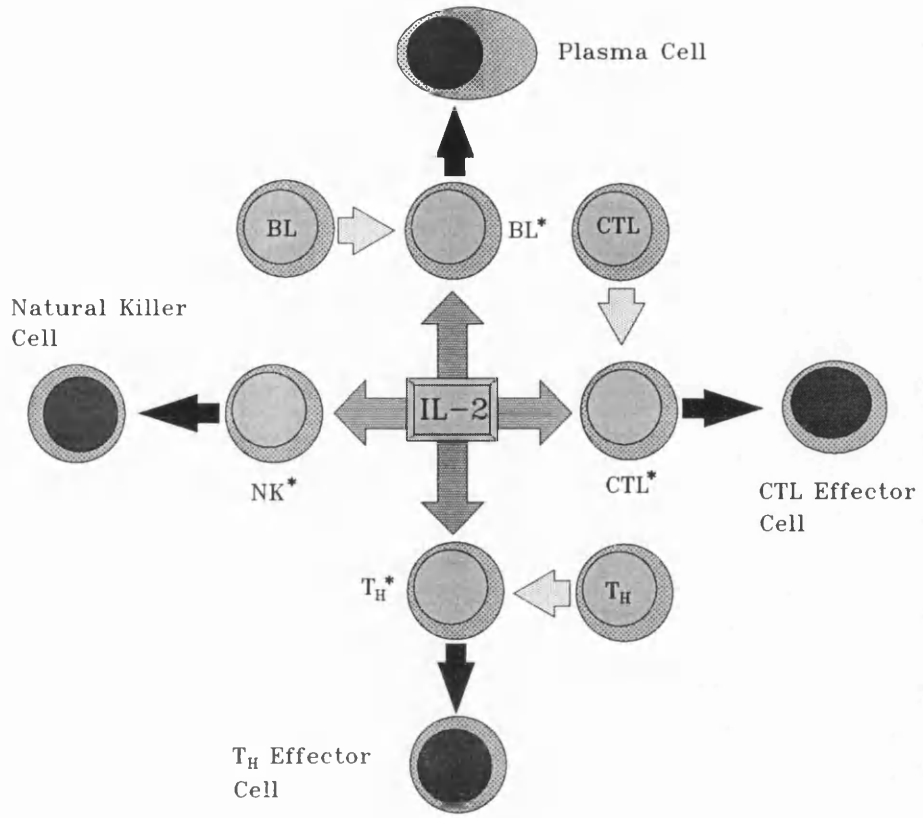

Fig. 1.4. The central role of IL-2 in the immune response. IL-2 acts on lymphocytes that have already been activated (*) by antigen or mitogen. It could also induce proliferation of resting T cells in the absence of antigens or mitogens (see text). IL-2 causes expansion and participates in the differentiation into effector cells. BL, B lymphocytes; CTL, cytotoxic T lymphocytes; NK, natural killer cells; T_H, helper T cells.

Because of the heterogeneity of T lymphocytes, high doses of exogenous IL-2 may stimulate a panel of distinct, in part even mutually antagonistic, immune functions. Therefore, IL-2 potentiates the function of cytotoxic T effector cells, stimulates or down-regulates T cell help provided to humoral effector cells, enhances suppressor cell function, and elicits the production of other cytokines (see below). Accordingly, depending on the experimental model, IL-2 may exert paradoxical, stimulatory as well as inhibitory effects on *in vivo* immune responses. It will be discussed later that this complexity is crucial to the immune mechanism against pathogens.

Table 1.1. Biologic activities of IL-2.

Target Cell Specificity for Proliferative Stimulus

1. Activated mature T cell subsets
2. Resting T cells [see Mookerjee and Pauly (1990) for full references]
3. Activated large granular lymphocytes
4. Activated B cells

Biologic Effects

1. Binds to specific receptors on T cells, B cells, and monocytes
2. Promotes entry into the *S* phase of the cell cycle (except in monocytes)
3. Enhances macrophage cytocidal state
4. Activates natural killer activity of large granular lymphocytes
5. Stimulates lymphokines secretion (see Table 1.2)
6. Augments immunoglobulin production

Two lines of evidence support the notion that IL-2 has predominantly immunostimulatory properties *in vivo*. (*i*) Antibodies directed against the inducible p55 Tac subunit of the IL-2 receptor have immunosuppressive, but never, stimulatory effects (Diamantstein and Osawa 1986; Söulillöu et al 1987). (*ii*) A variety of different states of immunodepression are associated with a deficient IL-2 production. Inherited states of immunodeficiency such as severe combined immunodeficiency, Nezelof syndrome, agammaglobulinaemia, and Wiskott-Aldrich syndrome are characterised by a defective IL-2 secretion from peripheral blood lymphocytes. Similarly, acquired immunodeficiencies provoked by total lymphoid irradiation, thermal injury (Teodorezyk-I et al 1987), graft-versus-host reactions (Welte et al 1984), immunosuppressive therapy (Merluzzi et al 1983), advanced tumours (Kay and Kaplan 1986), monoclonal gammopathy (Massaia et al 1990), or HIV infection (McElrath et al 1990) are accompanied by defects in IL-2 secretion. In many of these states of immunodeficiency, IL-2 ameliorates *in vitro* parameters of cellular immune functions (e.g., mitogen responsiveness and cytotoxic function). In humans, IL-2 has thus been proposed for the therapy of severe combined immunodeficiency and AIDS (McElrath et al 1990).

The ability of IL-2 to evoke the cytolytic functions of lymphoid cells and to induce growth and activation of antigen-independent NK and LAK cells has led to the successful use of recombinant IL-2 (rIL-2) in immunotherapy of cancer and infectious diseases (Fahey et al 1987; Balmer 1991; Kintzel and Calis 1991). In addition, IL-2 is likely to be of therapeutic value in a wide range of pathological conditions including autoimmune disorders, chronic infections, and organ transplant rejection (reviewed by Smith 1988).

In addition to its biological effects on various cell subtypes, IL-2 induces production of various cytokines. Indeed, the IL-2/IL-2 receptor system is embedded into an interactive network in which multiple factors that regulate IL-2 and IL-2 receptor expression are themselves influenced by IL-2, or they may exert postreceptive antagonistic and agonistic effects on IL-2 (Table 1.2). T lymphocytes activated by IL-2 produce other cytokines including IFN-γ; lymphotoxin; B cell growth and differentiation factors such as IL-4 and IL-6; haematopoietic growth factors such as IL-3, IL-5, and GM-CSF; and TGF-β. IL-2-activated T cells can also exhibit enhanced cytotoxicity. These various functions are amplified even further by the capacity of IL-2 to clonally expand the pool of antigen-reactive cells.

Table 1.2. The central position of IL-2 in the cytokine network.[a]

Property	Cytokine
Induced by IL-2 *in vivo*	IL-6, IFN-γ, TNF-α
Induced by IL-2 *in vitro*	IL-1α, IL-1β, IL-5, IL-6, IFN-γ, TNF-α, TNF-β, GM-CSF
Synergises with IL-2	IL-6, IL-7, TNF-α (thymocyte proliferation)
Induces IL-2 production	IL-1β, IL-6, IL-7
Inhibits IL-2 production	IL-10
Inhibits IL-2 action	IL-4, TGF-β
Induces IL-2 receptor	IL-1β, IL-5, IL-7, IFN-γ, TNF-α
Down-regulates IL-2 receptor	IL-4

[a]The table summarises interaction discussed throughout the text and is not intended to encompass all interactions between the IL-2/IL-2 receptor system in the cytokine network. Moreover, interactions between this system with peptide mediators classified as hormones (e.g., endorphins, ACTH, etc) are not reported. Only one predominant effect per cytokine is listed.

1.2.6 Known effects of IL-2 in the CNS

Several lines of evidence have confirmed the existence of IL-2 and IL-2-binding sites in multiple areas of the brain in experimental animals and humans (Bellamy et al 1985; Boutin et al 1987; Nieto-Sampedro and Chandy 1987; Araujo et al 1989). In addition, by means of *in situ* hybridisation, IL-2 mRNA was localised in discrete areas of the brain where it was found both in neuronal cell bodies and astrocytes (Farrar 1988; Farrar et al 1989).

There is considerable evidence that the growth of glial cells can be influenced by IL-2 and other cytokines (Benveniste and Merrill 1986). Human IL-2 was shown to influence the growth, proliferation and differentiation of oligodendrocytes (Benveniste and Merrill 1986) and other CNS cells (Okamoto et al 1990). In addition, IL-2 can activate T cells which specifically trigger proliferation of oligodendrocytes, and affects differentiation of brain cells through enhanced gene expression (Merrill 1987 and 1990). IL-2 also regulates the expression of myelin basic protein by enhancing transcription of mRNA and accelerating differentiation of primary oligodendrocytes (Benveniste Merrill 1987). Recent studies from several laboratories have demonstrated that brain cells such as astrocytes; oligodendrocytes; microglia; motor, sensory and sympathetic neurones; and pituitary cells respond to IL-2 (Haugen and Letourneau 1990; Merrill 1990).

In a recent series of experiments, evidence has been provided that IL-2 has profound effects on signal transmission and neuronal performance within the CNS. When applied through cannulae that have been chronically implanted in several areas of rat brain by a stereotactic technique, IL-2 was able to affect gross behaviour, producing dose-dependent soporific effects, and an increase in EEG spectrum power (De Sarro et al 1990; Nisticò and De Sarro 1991). Different behavioral effects, such as asymmetric body posture with ipsilateral turning behaviour and periodic ipsilateral circling, were observed when IL-2 was injected into the caudate nucleus or substantia nigra (Nisticò and De Sarro 1991). This suggests that IL-2 unilaterally inhibits the nigrostriatal dopaminergic pathway, thus allowing the predominance of the contralateral dopaminergic mechanisms. High doses of IL-2 introduced into the third cerebral ventricle produced epileptiform activity consisting of discharges of high amplitude (300-400 μV) spikes, sharp waves or spike wave complexes.

The electrophysiological and behavioural effects of IL-2 seem to be due to activation of specific receptors (De Sarro et al 1989 and 1990). When monoclonal antibodies against IL-2 receptors were injected into the brain 15 minutes before administration of IL-2, they antagonised or reduced the behavioural and EEG power spectrum effects of IL-2 (Merrill 1990). Similarly, naloxone (an antagonist at opioid receptors) was able to block the behavioural and EEG effects of IL-2, suggesting that opiate and IL-2 receptors might be functionally coupled. In contrast, when yohimbine (a specific α_2 adrenergic antagonist) was injected into the brain, it could not prevent the behavioural and spectrum power changes that follow IL-2 injection at the same site, thereby confirming that such effects are mediated by specific receptors (Nisticò and De Sarro 1991).

Therapy with IL-2 has provided an opportunity to study some of effects of this cytokine on human central nervous system. IL-2 produces some troublesome but reversible CNS side effects, including somnolence, headaches, convulsions, stupor, and coma (Fent and Zbinden 1987). Dose related neuropsychiatric changes, including disorientation, behavioural changes, delusions, and hallucinations occur in about one-third of patients receiving IL-2 (Balmer 1991). IL-2 could also cause several haemodynamic changes which include peripheral vasodilatation, increased vascular permeability, and vascular leak syndrome. Pathological effects of IL-2 on blood-brain barriers are discussed in details in Chapter 5.

1.3 The multichain IL-2 receptor

It is now well established that IL-2 transmits its biological signals to specific cells within haematopoietic, lymphoid or other organ systems via interaction with specific high-affinity membrane receptors (Waldmann 1989). Resting cells do not express high affinity IL-2 receptors, but receptors are rapidly expressed on T cells after interaction with antigen or mitogen. Thus, the interaction of an antigen with its receptor on resting T cells induces the expression of both IL-2 and its ligand-binding receptor. IL-2 binding to its high-affinity receptor provides the signals that lead to T cell proliferation and ultimately to the generation of specific regulatory and effector cells. Both IL-2 synthesis and IL-2 receptor expression are transient events, and the decline in expression of these proteins plays an important role in the normal termination of

the immune response. This section describes the structure of IL-2 receptor and discusses the mechanisms involved in initiation, and subsequent termination, of the immune response.

1.3.1 Structure of the high-affinity IL-2 receptor

In contrast to the growth factor receptors, the IL-2 receptor is unique. Human high-affinity functional IL-2 receptor (~ 10 pM) is composed of at least two distinct polypeptide subunits (Fig. 1.5), the extensively characterised α subunit of M_r 55 kD (p55 or Tac antigen) and a β subunit of M_r 75 kD (p75), initially identified in ^{125}I-labelled IL-2 cross-linking studies (Sharon et al 1986; Robb et al 1987; Saragovi and Malek 1987; Teshigawara et al 1987).

There is recent evidence for a more complex subunit structure of the high affinity receptor that involves other peptides in addition to p55 and p75 binding proteins. With the use of coprecipitation analysis, radiolabelled IL-2 cross-linking procedures, and flow cytometric resonance transfer measurements, a series of additional proteins of 22, 35, 40, 95, and 105 kD have been associated with the two IL-2 binding subunits (Szollosi et al 1987; Edidin et al 1988; Waldmann 1989). Furthermore, at least some mouse p75 was reported to exist as a disulfide-linked heterodimer with a subunit of M_r 22 kD (p22) (Saragovi and Malek 1987).

In humans, a putative p64 component of IL-2 receptor has been tentatively named IL-2Rγ chain (Takeshita et al 1990). A p70 molecule was also described which could be a precursor of p75 that lacks post-translational processing such as N-linked glycosylation and sialyation (Asao et al 1990). In addition, an association between IL-2 receptor and class I HLA as well as intercellular adhesion molecule-1 (ICAM-1) has been demonstrated (Sharon et al 1988; Burton et al 1990). Moreover, ICAM-1 has been shown to interact physically with p55 Tac subunit within the cell membrane (Burton et al 1990) and may speculatively focus IL-2 receptors to the site of monotopic T-T cell interactions mediated by ICAM-1 and its ligand.

Despite the above evidence of a more complex IL-2 receptor structure, the biological effects of the additional proteins are not well-established at present. Therefore, this section will only consider the extensively characterised p55 and p75 subunits of the IL-2 receptor.

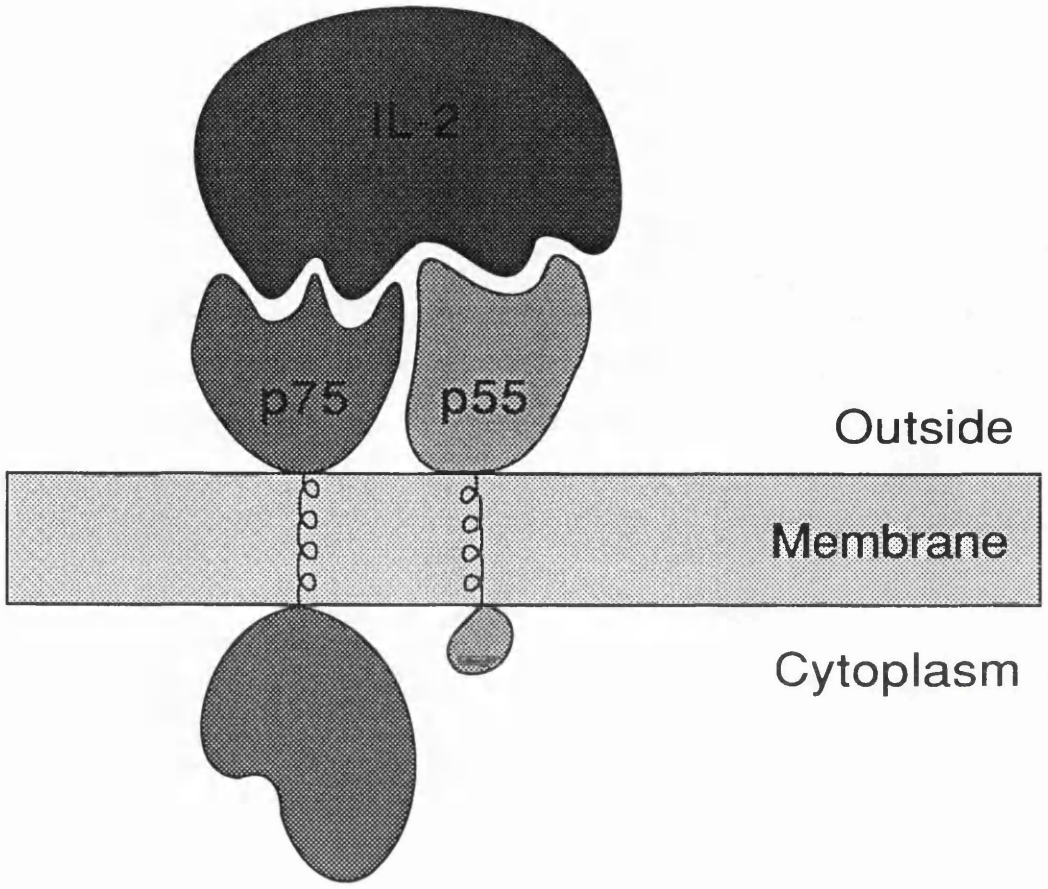

Fig. 1.5. The bimolecular structure of high-affinity IL-2 receptor. The two subunits of the receptor are not connected by covalent disulfide bonds and their cooperative binding of IL-2 is easily disrupted by monoclonal antibodies reactive with the p55 Tac subunit (Wang and Smith 1988). The cytoplasmic domain of p75 subunit (286 amino acids) is depicted as being larger than the small cytoplasmic domain of p55 Tac subunit (13 amino acids) to emphasise the fact that the p75 subunit contains the structures responsible for signalling internalisation and cell growth. The area of contact between IL-2 and the two IL-2-binding proteins is only diagrammatic and does not depict actual distance.

1.3.2 Biochemical properties of the p55 Tac subunit

The p55 Tac subunit, which is capable of binding IL-2 with low affinity (~ 10 nM), was characterised as a 55 kD glycoprotein (Leonard et al 1982 and 1983). Using a combination of pulse-chase and tunicamycin experiments, the p55 was shown to be composed of a 33 kD peptide precursor after cleavage of the hydrophobic leader sequence. This precursor was cotranslationally N-glycosylated to 35 kD and 37 kD forms. One hour after the addition of unlabelled amino acids, the 55 kD mature form of the receptor was observed, suggesting that O-linked carbohydrate was added to the IL-2 receptor subunit. Furthermore, the p55 subunit was shown to be sulfated and phosphorylated on a serine residue (Shackelford et al 1984; Gaulton et al 1986).

The p55 Tac subunit is composed of 251 amino acids plus a 21-amino acid signal peptide (Leonard et al 1984) (see Table 1.3 below). The 219 NH_2-terminal amino acids make up an extracellular domain. This domain contains two potential N-linked glycosylation sites (Asn-X-Ser/Thr; where X is any amino acid) and multiple possible O-linked carbohydrate sites. Multiple cysteine residues have been identified that participate in the formation of intramolecular disulfide bonds required for the IL-2 binding site. A second domain contains a single hydrophobic region near the COOH-terminal of the protein of 19 amino acids, which is presumably a membrane spanning region. The third and final domain is a very short (13 amino acids) cytoplasmic domain that contains several positively charged amino acids, presumably involved in the anchoring of the protein. Potential phosphate acceptor sites (serine and threonine, but not tyrosine) are present within this domain. However, this cytoplasmic domain appears to be too small for enzymatic function and signal transduction (Greene et al 1985; Kondo et al 1988). Thus, the p55 subunit differs from many other growth factor receptors that have large intra-cytoplasmic domains with tyrosine kinase activity. This finding raised the suggestion that the p55 Tac subunit serves only to bind to the p75 subunit to form the high affinity heterodimer thereby increasing the sensitivity and rapidity of the lymphocytic response (Kuziel and Greene 1990). Nonetheless, the presence of p55 Tac subunit on the cell surface could augment IL-2 sensitivity, i.e. it shifts IL-2 dose-response curve to lower concentration by virtue of its ability to augment the receptor affinity.

1.3.3 Biochemical properties of the p75 subunit

Several observations challenged the previously held view that the p55 Tac subunit was the only molecule involved in IL-2 binding and triggering of immunological response. These observations included; *(a)* the great difference in affinity (> 10^3 in Kd) between high- and low-affinity receptors; *(b)* the inability of the short cytoplasmic domain of the p55 Tac subunit to transduce receptor signals to the nucleus; *(c)* the ability of certain Tac-negative cells (such as LGL that are precursors of the NK and LAK cells) to produce non-proliferative responses to IL-2 (Ortaldo et al 1984); *(d)* the finding that MLA-144 cell line, which was Tac-negative, could manifest 4,000 IL-2-binding sites with intermediate affinity (Waldmann 1986); and *(e)* the generation of only low-affinity receptors when mouse non-lymphoid cells were transfected with cDNAs encoding human p55, whereas similar transfectants of mouse T-cell lines display both high and low-affinity receptors (Sabe et al 1984; Hatakeyama et al 1985). These observations led to the supposition, and eventually the characterisation of the p75 subunit of the IL-2 receptor.

Cloning of human p75 has demonstrated (Hatakeyama et al 1989) that this protein consists of 541 amino acids with an extracelluar region of 214 amino acids, a single hydrophobic transmembrane region, and a relatively large cytoplasmic domain of 286 amino acids (Fig. 1.6) that mediates IL-2 signal transduction even in the absence of p55 (Robb et al 1987), although the exact mechanism of signal transduction remains incompletely defined. The consensus sequence for tyrosine kinase is absent in the p75 subunit, but a triplet, Ala-Pro-Glu, that is believed to be the consensus motif for a catalytic domain of some kinases is present. In fact, several lines of evidence indicate that tyrosine phosphorylation is involved in the IL-2 signal transduction pathway (Morla et al 1988; Saltzman et al 1988) (Fig. 1.7).

There is a significant homology among the IL-2 receptor p75 subunit and IL-6 receptor, the IL-3 receptor, the IL-4 receptor, and erythropoietin receptor (Arai et al 1990). The same consensus sequence was also found in the prolactin receptor and the growth hormone receptor (Bazan 1989). In fact, all the above receptors were thought to be members of the haematopoietin receptor-gene family (Gillis 1991). Receptor structure of members of this gene family is characterised by specifically conserved cysteine residues that may be found in either single or multiple copies in the extracelluar domains of the receptor. These cysteines may contribute to the tertiary

structure by forming a disulfide bridge. Another characteristic feature, particularly in the region close to the transmembrane domain, is the presence of serine-tryptophan-X-serein-tryptophan (WSXWS, where X is any amino acid) motif that is often present as a single copy just proximal to the membrane spanning region of the receptor. In addition, p75 (like many other cytokine receptors of the haematopoietin receptor-gene family) contains multiple copies of fibronectin type 3 binding motifs throughout the extracelluar domain.

1.3.4 Affinity characteristics of IL-2 receptors

Kinetic binding studies with IL-2 have provided an interesting perspective on the formation of the high affinity IL-2 receptor. Details of binding characteristics of the two separate subunits are shown in Table 1.3. These details became possible only after the identification and isolation of leukaemic cell clones that express solely p55 or p75 subunits (Tsudo et al 1986; Teshigawara et al 1987). The p75 subunit binds IL-2 with an intermediate affinity and forms a high affinity receptor complex when the p55 subunit is coexpressed. It is especially noteworthy that the p75 and p55 subunits are not connected via a covalent disulfide bond. Instead, these two subunits apparently interact via non-covalent forces to form a heterodimeric high affinity IL-2 receptor. Also, the p75 subunit IL-2-binding site is separate and distinct from the IL-2-binding site on the p55 subunit.

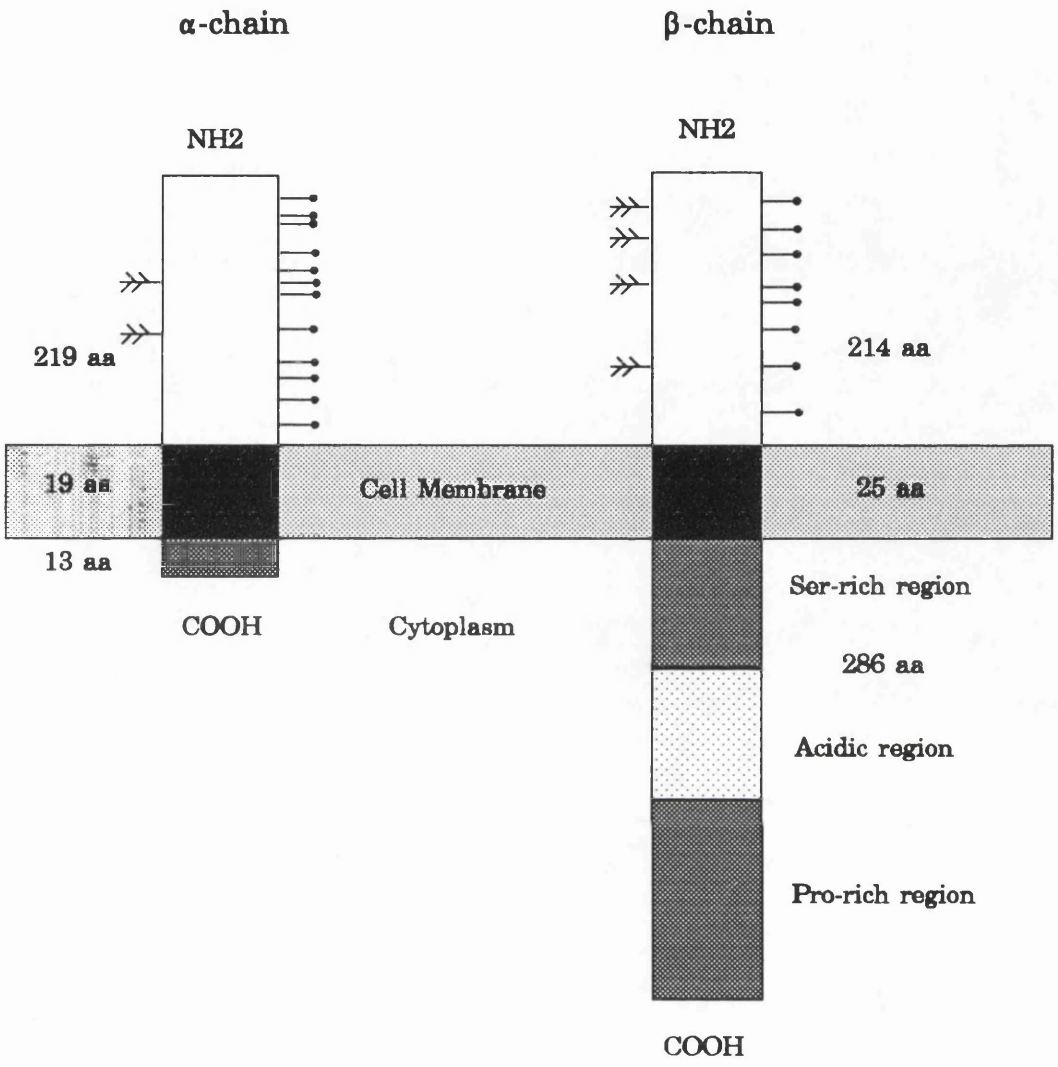

Fig. 1.6. Structure of the human IL-2R p55 and p75 chains. Symbols on the right and left sides of each column represent the positions of cysteine residues and N-glycosylation sites, respectively. See text for details of p75 cytoplasmic region.

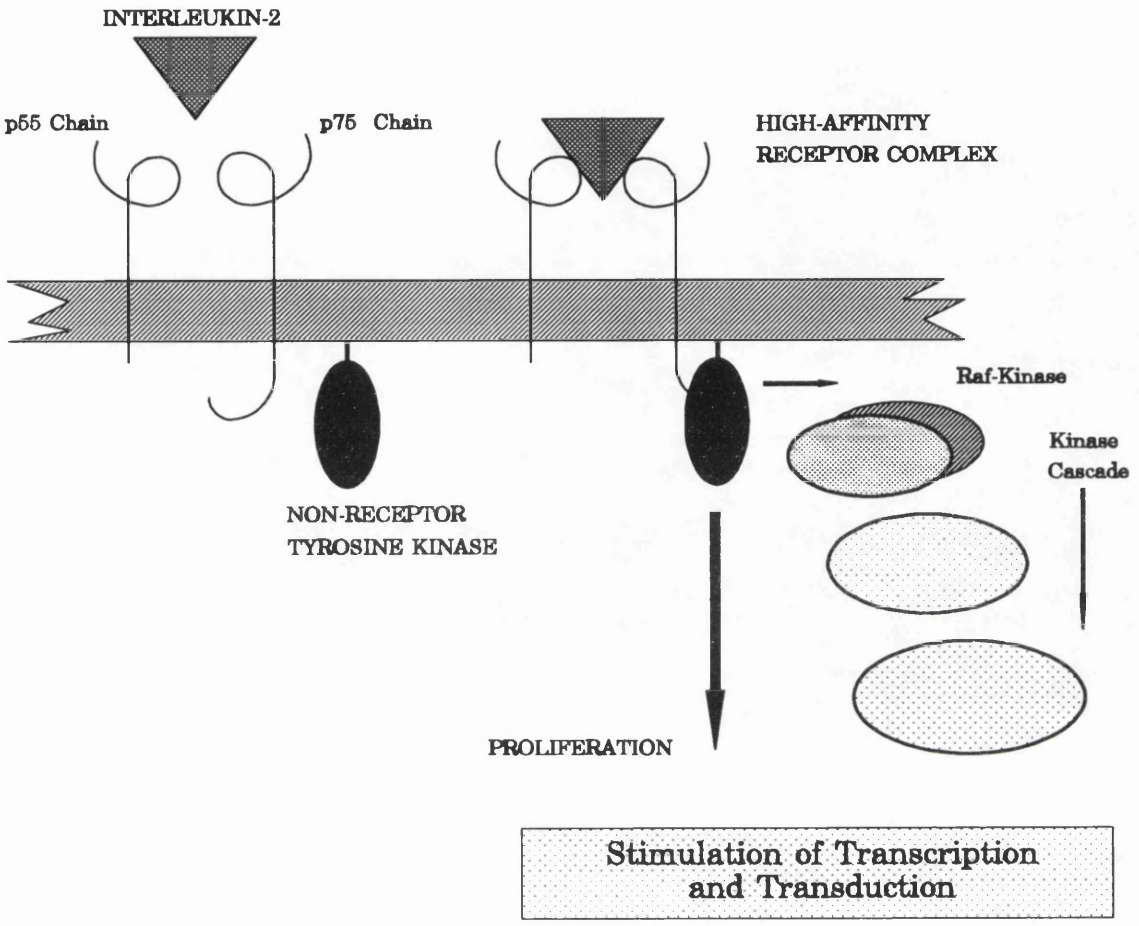

Fig. 1.7. The proposed mechanism of signal transduction in IL-2-stimulated cells. Components of IL-2-receptor complex are also shown. The signal transducing, high-affinity, IL-2 receptor consists of p55 α-chain and a p75 β-chain, both of which bind IL-2. A non-receptor tyrosine kinase is thought to associate with the p75 chain to instigate the tyrosine phosphorylation of a set of cytoplasmic protein substrates, which compose the transduction pathway for IL-2. (see Guy *et al* 1990 for details).

A dynamic view of the structure-function relations of the two separate IL-2-binding proteins and how they cooperate to form high affinity receptors derives from kinetic binding studies (Lowenthal and Greene 1987; Wang and Smith 1988). When expressed individually, each chain reacts with IL-2 very differently, as displayed by the kinetic and equilibrium binding constants shown in Table 1.3. The on and off for IL-2 binding to the p55 Tac subunit is very rapid (less than 5 seconds) while the on and off rate for IL-2 binding to the p75 subunit is markedly slower (20-50 minutes) (Lowenthal and Greene 1987; Smith 1988a; Wang and Smith 1988). As a result, IL-2 binds to and dissociates from the p75 subunit relatively slowly, whereas it reacts very rapidly with the p55 subunit. Even more interesting are the kinetic binding data obtained when the high affinity receptor is analysed. The association rate of the heterodimer is contributed by the fast-reacting p55 subunit, whereas the dissociation rate is derived from the slow-reacting p75 subunit. Since the affinity of binding at equilibrium is determined by the ratio of the dissociation rate constant ($k' \sim 10^{-4}\ s^{-1}$) and the association rate constant ($k \sim 10^{7} M^{-1}\ s^{-1}$), this unique kinetic cooperation between the low- and intermediate-affinity ligand binding sites results in a receptor with very high affinity ($K_d = k'/k \approx 10^{-11}\ M$). Thus far, the high affinity IL-2 receptor is the only example of this kind of receptor formed by making use of distinct binding sites on two separate subunits (Smith 1988b).

1.3.5 Genetic regulation of IL-2 receptor

Synthesis of IL-2 receptor is regulated at least in part at the level of DNA transcription (Leonard et al 1985; Efeat and Kames 1986; Weiss et al 1986). Reagents that activate protein kinase C are sufficient and more potent than those that only increase $[Ca^{+2}]_i$ in the induction of IL-2 receptor gene expression (Waldmann 1986; Cross et al 1987). IL-2 itself up-regulates the expression of IL-2 receptor via a transcriptional mechanism (Depper et al 1985; Smith and Cantrell 1985). The induction of IL-2 receptor by phrobol esters can not be inhibited by cyclosporin A, a potent inhibitor of IL-2 transcription (Kronke et al 1984). Compared to the regulation of IL-2, it seems that the regulation of IL-2 receptor is less stringent than the regulation of its ligand.

Table 1.3. Features and kinetics constants of IL-2 receptor subunits.

Chain	Affinity	Number of Amino Acids			Kinetic Binding Constants *(Mean Values)*			Equilibrium Dissociation Constants	
		Extra-cellular	Cyto-plasmic	Total	Dissociation (k')		Association (k)	Kinetic (k'/k)	Equilibrium
					s^{-1}	$t_{½}$	$M^{-1}\ s^{-1}$		
p55 α	Low	219	13	272	4.0×10^{-1}	1.7 sec.	1.4×10^{7}	2.9×10^{-8}	1.4×10^{-8}
p75 β	Intermediate	214	286	541	2.5×10^{-4}	46 min.	3.8×10^{5}	0.7×10^{-9}	1.2×10^{-9}
α+β	High	—[a]	299	—[a]	2.3×10^{-4}	50 min.	3.1×10^{7}	0.7×10^{-11}	1.3×10^{-11}

[a]Additional peptides may participate in the high affinity form of IL-2 receptor (see section 1.3.1).

The gene that encodes IL-2 receptor subunits consists of eight exons and seven introns spanning a minimum distance of 25 kb. *In situ* hybridisation studies have localised the receptor gene to chromosome 10p band 14→15 (Waldmann 1989). Transcriptional induction of the genes encoding IL-2 receptor subunits has been shown to be mediated by a sequence element that is homologous to the nuclear factor-κB binding site of the Ig kappa gene enhancer (Pomerantz et al 1989). More recently, an additional sequence motif located ~ 10 bp downstream of the previously identified site has been described (Boumpas et al 1991).

1.3.6 Distribution of IL-2 receptors

The IL-2 receptors are found on a wide array of immunologically relevant cells. Most resting T cells, B cells, and monocytes in the circulation do not display IL-2 receptors. However, the majority of T cells can be induced to express IL-2 receptors by antigen or alloantigen stimulation, by interaction with lectins, or by the addition of monoclonal antibodies to the T-cell antigen receptor complex (Waldmann 1986; Baroja 1988; Waldmann 1989). The p55 Tac subunit has also been demonstrated on activated B cells. Normal peripheral blood B cells activated by *Staphylococcus aureus*, pokeweed mitogen, phrobol myristate acetate, or anti-μ immunoglobulins can be induced to express IL-2 receptors (Tsudo et al 1984; Maraguchi 1985).

IL-2 receptors have been detected on activated cells of the monocyte/ macrophage series, including the Reed-Sternberg cells in Hodgkin's disease, Kupffer's cells of the liver, cultured lung macrophages, Langerhans' cells of the skin, and normal human peripheral blood monocytes stimulated with lipopolysaccharide or IFN-γ (Herrmann et al 1985; Holter et al 1987). IL-2 receptors have also been identified on cells other than those of the lymphocyte/monocyte series. A proportion of blast cells from almost half of the cases with acute myelogenous leukaemia expressed IL-2 receptors. In addition, IL-2 receptors could be induced on granulocytic cells with various agents, including IFN-γ and GM-CSF. IL-2 receptor expression was also reported in cells infiltrating acute brain lesions in patients with MS (Bellamy et al 1985; Hofman et al 1986). Stimulation via a variety of different mechanisms induces functional IL-2 receptors in oligodendrocytes, lymph-borne dendritic cells, and murine pituitary cells.

1.3.7 IL-2 receptors and the specificity of the immune response

As the function of T cells and B cells appear to be controlled by multiple cytokines operating through a network, the bimolecular structure of IL-2 receptor provides an interesting insight into how the immune system maintains specificity for the original antigen. Antigen-specific receptors on T and B cells may play a key role in this process. T cells activated by a specific antigen express high levels of p55 Tac subunit, which can form the high affinity IL-2 receptors. These T cells may be selectively amplified because antigen-activated T cell clones carrying the high affinity IL-2 receptor proliferate in response to low levels of IL-2, whereas bystander T cells, which bear only p75, require higher levels of IL-2 for growth. Likewise, clonal amplification and maturation of B cells may be achieved if resting B cells, which bear antigen-specific Ig on their cell surface, become selectively activated to enter the cell cycle. These antigen-primed B cells may proliferate and differentiate into plasma cells that produce specific antibody in response to B cell-trophic cytokines. Cytokines that have the ability to induce expression of the p55 subunit on resting bystander T cells may help polyclonal amplification of T cells. Similarly, cytokines that have the ability to activate resting bystander B cells may augment polyclonal B cell responses.

Lymphocytes express maximal levels of high affinity IL-2 receptors for only a brief period following exposure to specific antigen. A subsequent decline in receptor expression ensues which is independent of the presence of IL-2, indicating that there are other mechanism(s) of receptor regulation beside IL-2-mediated receptor downregulation. It also means that there is only a finite period for immune T cell clones to expand. Simultaneously, another mechanism facilitates the disappearance of IL-2 receptors, one that operates via an effect of IL-2 itself. By binding to high affinity receptors, IL-2 evokes a reciprocal change in the type and character of the binding sites expressed (Smith and Cantrell 1985). Within 1 to 2 hours of IL-2 receptor occupancy, the density of high affinity receptors decreases by as much as 50%. Consequently, T lymphocyte proliferation ceases and the cells arrest in the resting phase of the cell cycle (G_0 or early G_1), even in the presence of saturating levels of IL-2. Re-addition of antigen causes re-appearance of optimal numbers of high affinity receptors on the lymphocytes, enabling cells to grow again in response to IL-2. These events can be repeated, thereby emphasising the constant need of stimuli to induce IL-2 receptors necessary for continuous proliferation of T cell clones.

The above mechanisms lead to the conclusion that the intracellular signals generated by the T cell antigen-receptor complex and by IL-2 receptors function to switch membrane IL-2 receptor expression in a reciprocal fashion. Thus, a critical interplay operates between the positive effect of antigen receptor activation and the negative influence of IL-2 receptor triggering, both harmonising to regulate precisely the density of high affinity IL-2 receptors and the final proliferative potentials of activated T cells (Smith and Cantrell 1985). Such a reciprocal receptor regulatory system provides a secure form of intrinsic control over antigen-initiated but IL-2-dependent T cell clonal expansion. Since high affinity IL-2 receptors are expressed only upon antigen receptor activation, as antigen is cleared *in vivo*, the IL-2-directed accelerated internalisation of p75 subunit favours the disappearance of functional high affinity receptors and return to the state of resting unresponsive cells.

Evidence has been recently presented of a second mechanism for the regulation of the immune response through differential modification of the expression of the two IL-2 receptor subunits (Loughnan and Nossal 1989). IL-4 and IL-5 were reported to be able to independently induce the expression of the p75 and p55 Tac subunits respectively on murine B cells. Such distinct regulation of two subunits of a single receptor by two different cytokines permits a coordinated appearance of functional high-affinity receptors. This subtlety of regulation permits a novel and sophisticated level of control. IL-2 usually exerts a powerful influence early in B cell activation. If it alone induced the full IL-2 receptor, a B cell might be driven down an IL-4+ IL-2 differentiation pathway prematurely or inappropriately (Loughnan and Nossal 1989). On the other hand, if IL-5, with its own capacity to induce growth and differentiation, is also required, the effects of a later-acting IL-2 would thereby be constrained. A tight and complex regulation of receptor expression might also limit unwanted "bystander" effects in antibody responses, thus helping to maintain the specificity of the immune response (Loughnan and Nossal 1989 and 1990).

In addition to the above mechanisms, the bioavailability of IL-2 may contribute to the specificity of the immune response by restricting IL-2 production. The signals that induce IL-2 gene transcription and its mRNA are short-lived. Similarly, the half-life of IL-2 is short (Mier and Gallo 1982), and it is rapidly cleared by the kidney. Finally, IL-2 is subjected to proteolytic processes, and is also "consumed" by IL-2 receptor-bearing cells, which may serve as a "cytokine sink" (Lo et al 1989).

1.4 The soluble IL-2 receptor

One of the most interesting observations regarding cytokine receptors, particularly IL-2 receptor, has been the discovery that they can exist either in membrane bound or soluble forms. The soluble IL-2 receptor (sIL-2R) is released by activated normal peripheral blood mononuclear cells and by HTLV-1-infected leukaemic cell lines. Studies using ELISA have detected sIL-2R in normal human and murine sera (Osawa et al 1986 a&b). Increased levels of sIL-2R have been identified in biological fluids (such as plasma, urine, synovial fluid, and CSF) in a variety of disease states. This section discusses recent knowledge of the structure and biological function of the soluble IL-2 receptor.

1.4.1 Structure and origin of sIL-2R

The sIL-2R is a truncated form of the p55 Tac chain of the cell membrane-associated receptor (Osawa et al 1986a; Loughnan and Nossal 1990). Soluble IL-2 receptor has been shown by SDS-PAGE molecular mass determination to be ≈10 kD lighter than p55 Tac subunit while retaining all tested epitopes (Rubin et al 1985). Like its cellular counterpart, sIL-2R is capable of binding IL-2 with a low affinity of 10^{-8} M.

Two mechanisms have been proposed for the release of sIL-2R. A probable mechanism is that cell surface-associated IL-2 receptors are cleaved from the cell surface. An alternative mechanism is that sIL-2R could be translated as a transmembrane-domain-deficient protein and released from the cell. The latter mechanism could arise from transcription from a separate gene lacking the transmembrane coding region or from alternate splicing of normal full-length mRNA producing a truncated mRNA.

Both putative mechanisms of generating a soluble IL-2R have been detected before, for example, with secretory immunoglobulin (Early et al 1980), the insulin receptor (Berhanu and Olefsky 1982), and class I MHC antigens (Robinson 1987). However, there is mounting evidence that cleavage of the soluble IL-2R from the cell surface is the most likely mechanism of sIL-2R release. An increase in the serum level of sIL-2R has been demonstrated in patients within 15 min of intravenous injection of IL-2 (Lotze et al 1987). It seems that cleavage of the sIL-2R from the cell surface would be more likely to occur in a 15-minute period than transcription, translation, and release of a transmembrane-domain-deficient soluble IL-2 receptor. In addition,

no cDNA clone corresponding to mRNA encoding a transmembrane-domain-deficient receptor has yet been isolated. Moreover, sIL-2R released from cell-surface radioiodinated-IL-2 receptor cells was found to be radiolabelled to a specific radioactivity similar to cell-surface IL-2R (Loughnan et al 1988), indicating that cleavage of IL-2R from the cell surface is the usual mechanism of sIL-2R release.

Given that sIL-2R is cleaved from the cell surface, two mechanism of cleavage appear possible. The cell surface IL-2 receptor could be cleaved from the cell close to the junction of the extracelluar and transmembrane domain by a site-specific protease. The observation that the low-affinity, but not high affinity, IL-2 receptors are pronase-sensitive (Robb and Rusk 1986) would appear to support this hypothesis. A second possible mechanism of cleavage would be for post-translational linkage of IL-2 receptor through glycosyl-phosphatidylinositol (Gly-PI) to the cell membrane with subsequent cleavage for the receptor from the cell surface by phospholipase C (Loughnan et al 1988). Such mechanism of anchorage and release has been demonstrated with Thy-1 and several other cell-surface proteins (Low 1987).

1.4.2 Regulation of sIL-2R production

The release of sIL-2R is under the control of IL-2 (Loughnan et al 1988), probably through the binding of IL-2 to p55 Tac subunit (Burkhardt et al 1989). Alternatively, binding of IL-2 only to the high-affinity receptors or to small numbers of low-affinity receptors may be sufficient to induce accelerated shedding of other unbound low-affinity receptors (Burkhardt et al 1989; Loughnan and Nossal 199). Either of these mechanisms may also involve the *de novo* synthesis of cell-surface low-affinity receptors as Smith and Cantrell (Smith and Cantrell 1985) have previously demonstrated that high-affinity receptor binding leads to increased expression of low-affinity receptors. Both hypotheses would require the prior expression of cell-surface receptors. Thus, it seems plausible that the accelerated release of sIL-2R by interleukin-2 that was reported earlier (Burkhardt et al 1989) may be due to the ability of IL-2 to up-regulate the expression of its receptors on preactivated T cells.

1.4.3 Biologic functions of sIL-2R

It was demonstrated that sIL-2R can bind to IL-2 with low affinity similar to that of its cellular counterpart (K_d of 5-20 x 10^{-8} M) (Jacques et al 1987). This observation

raised the possibility that the sIL-2R may play a role in immune regulation by competing with membrane-associated high affinity receptors for available IL-2 (Rubin et al 1985; Jacques et al 1987; Burkhardt et al 1989). This hypothesis holds that by such an action, sIL-2R could be immunosuppressive by removing biologically active IL-2 from the microenvironment of responsive cells, thereby restricting unwanted, non-specific (bystander) activation of cells by IL-2.

Another possible mechanism for a putative immunoregulatory effect of sIL-2R could be a competition for IL-2 with the cell surface-associated p75 (medium-affinity) receptor (Loughnan and Nossal 1990) although no data supporting this theory has been presented to date. However, it is almost universally agreed that the release of sIL-2R appears to be a consequence of activation of various cell types, particularly T cells, that may play a role in the regulation of the immune response (Waldmann 1989). The analysis of levels of sIL-2R in body fluids appears to provide a very valuable non-invasive method for the analysis of both normal and disease-associated lymphocyte activation *in vivo*.

1.5 An overview on the complexity of the cytokine network

The field of cytokines grew out of the recognition of interleukins as mere mediators between leucocytes. By the end of the 1970's more than 100 biological activities of cytokines had been identified; and by the end of the 1980's the application of monoclonal antibody technology and recombinant DNA methodology has now led to the gene cloning of at least 9 cytokines (IL-1 to IL-6; G-CSF; GM-CSF and M-CSF).

To the immunologist, cytokines are viewed as effecting both intrinsic and extrinsic pathways of immunregulation. Accordingly, as *cell cooperators, traffic regulators*, and *messengers of defence*, cytokines have assumed major importance in clinical immunology as well as various disciplines of clinical medicine. In effecting immune responses, their multiple activities underlie local and systemic reactions of delayed hypersensitivity, rejection of allografts and parasitised cells, tissue damage of autoimmune diseases, and restriction of tumour growth. In immune induction, cytokines act as mediators or even "adjuvants" of T cell and B cell sensitisation; they contribute to feedback interactions between subpopulations of lymphocytes and other mononuclear leucocytes; and their controlled release may well balance the need for immunological diversity against the risks of phenotypic restriction.

Although certain cytokines such as IL-5 have limited functions, acting on only one or two cell types, the majority of cytokines have a variety of effects on many different cells. For example, IL-2 is active on T and B cells, NK, haematopoietic stem cells, macrophages and other cells. Because they are macromolecules, it is possible that certain cytokines may be polyfunctional, depending upon the microenvironment in which they are generated. It has been reasoned that even though a given class of cytokine can be generated by specific cell type, this is immaterial to the central concept that antigen-specific or alternative pathways of lymphocyte activation provide the physiological stimuli which lead to cytokine production (Arai et al 1990). Therefore, there is considerable overlap of function between cytokines, e.g. proliferation of T cells can be enhanced by IL-2, IL-4, IL-6, IL-7 and IL-9. In spite of this overlap, the cytokine functions are not identical and each of these cytokines induces proliferation of distinct subsets of T cells or has different effects on proliferation within a particular subset. There are also several synergistic, antagonistic and cascade interactions between different cytokines.

Current understanding of cytokine function indicates that there is a very complex network of interactions within the immune system. This complexity has raised doubts among some immunologists as to the significance of the multitude of activities that have been described. However, a more constructive way to view this complexity is to consider that it represents a very sophisticated and versatile regulatory system for overcoming various defence strategies of the invading pathogens. If intercellular communications within the immune system were relatively simple, it might be possible for an infectious agent to synthesise a product that interfered with an essential link between cells. Because pathogens can evolve more rapidly than their mammalian hosts, it would be very difficult for any single immune system mechanism to perform a given function without possibility of interference. The strategy of multiple interlocking regulatory mechanisms offers extra insurance against this interference as it is difficult for a pathogen simultaneously to evolve several different processes that are able to interfere with all of the necessary components of a complicated interlocked system. As a result, the very complexity of the cytokine system may be a defence mechanism against pathogen interference.

1.6 The Immune logical brain

The concept that the CNS represents an immunologically privileged site has evolved over the past two decades. Although the fact that this privilege is not absolute has been pointed out in several earlier reports (Barker and Billingham 1977; Geyer and Gill 1979; Freed 1983), it is now possible to examine the immune functions of CNS in the light of our recent understanding of the immune system.

There are several differences between the CNS and the majority of peripheral sites. First, lymph drainage from the brain to the peripheral lymph nodes is not well defined; although experiments have shown that macromolecules deposited within the brain can reach deep cervical lymph nodes. Second, with regard to classical antigen-presenting cells (APCs), the dendritic leucocytes that reside in almost all tissues of the body are absent from the brain. However, cells with many APC surface markers were detected within the choroid plexus. Third, the blood-brain barrier, when intact, might prevent surveillance of the CNS by immune cells, although several reports have demonstrated that it does not represent a barrier to activated lymphocytes. Finally, MHC antigens, which play a key role in the generation and propagation of the immune response, are not expressed at detectable levels in healthy unactivated CNS tissue, apart from endothelial cells. Nonetheless, most CNS cells do have the capacity to express certain of these antigens under pathological conditions as explained below.

Despite the above differences, surprising molecular parallels between the brain and the immune system have emerged in recent years. Most surface antigens on T cells or B cells that are important for the recognition of antigen or for cell interaction (e.g. the T and B cell antigen receptors, the products of the MHC loci, and the CD4 and CD8 markers of T cell subtypes) are members of the immunoglobulin supergene family (Hunkapiller and Hood 1986). Some members of the family, notably Thy-1 and the MRC OX2 antigen, are expressed on both lymphocytes and neurones (Williams and Gagnon 1982). The Ig gene motif is also present in two proteins important for cell adhesion within the nervous system, the neural cell adhesion molecule and myelin associated glycoprotein (Arquint et al 1987). The utilisation of the Ig gene motif by both the brain and the immune system possibly indicates a common evolutionary origin for the two systems. Perhaps primitive sensory cells were the precursors of both the nervous system and the immune system, and indeed one could view the immune system as an internal "sensory" system for antigens.

Maybe it is not so surprising that the logic of their construction and the recognition molecules these systems use have many common features.

In addition to molecular similarities between neuronal and immune cells, soluble mediators of the immune response have a well recognised activity on neuronal cells (Fontana and Fierz 1985). IL-2 for example, stimulates the growth and differentiation of various glial cell types including astrocytes and oligodendrocytes (see section 1.2.6). Similarly, IL-1 is a mitogen for astrocytes (Giulian and Baker 1985), and IFN-γ induces Ia expression on astrocytes, which then enables them to function as antigen-presenting cells (Fontana and Grob 1984). Antigen presentation by IFN-γ-stimulated astrocytes is both MHC-restricted and antigen-specific. Immunologic functions of astrocytes may be important for the clearance of viruses from the brain, and are significant for inflammatory diseases of the CNS, such as MS (Waage et al 1989). Astrocytes, in fact, are capable of generating class II MHC molecules and this capacity could be related to the pathogenesis of MS (Fontana and Fierz 1985).

One of the more recently ascribed functions of the astrocytes relevant to this chapter has been that of a resident immunocompetent cells of the CNS. Astrocytes present antigens in an MHC-restricted fashion to antigen-specific T cells, resulting in T cell activation. Another manner in which astrocytes and other glial cells can function as an immunocompetent cells is through the ability to synthesise various immunoregulatory cytokines. As discussed above, glial cells can secrete various cytokines including IL-1, IL-2, IL-6, colony stimulating factors, and TNF-α.

Recent evidence has suggested that some neurokines, such as ciliary neurotrophic factor and cholinergic differentiating factor, and cytokines are topologically similar, and comprise a newly recognised family of ligands (Hall and Rao 1992). Likewise, receptors of neurokines and cytokines reflect significant structural homology. These characteristics may identify the basis for the similarities of action between the ligands, and a potential capacity to affect the differentiation of precursors in both neuropoiesis and haemopoiesis (see the recent review by Hall and Rao 1992). Because of the structural similarities between cytokines and neurokines, and between their receptors, the potential for functional crossover between these molecules is high. Thus, it is likely that, during development, a single ligand may have effects on the differentiation of both maturing neurones and blood cells.

The above data provide convincing evidence that cytokines play a crucial role in immune regulation within the CNS. The reciprocity of the production of neuroendocrine hormones and cytokines and their receptors in brain and immune cells was clearly demonstrated by Johnson and Torres (1985). These authors reported that the neurohypophyseal hormones vasopressin and oxytocin were capable of replacing IL-2 requirement for T cell growth and mitogen induction. In fact, IL-2 and its receptor are thought to represent a model of hormone-receptor system utilised by both the nervous and immune systems for intersystem communication. However, although IL-2 is a recognised growth factor for neuronal cells (see section 1.2.6), there is still much to be done in order to attribute a definite physiological or pathological role of IL-2 or other cytokines in the brain. Indeed, one aim of the current study is to detect possible correlations between IL-2 and pathologic changes within the CNS.

1.7 Aims of the study

In inflammatory and infectious neurologic disorders the question naturally arises of whether IL-2 and its receptor are involved in the pathogenesis of CNS lesions or the development of characteristic clinical and laboratory features. Although IL-2 and sIL-2R have been detected in the CSF and serum from patients with various inflammatory neurologic diseases, considerable confusion still remains as to the clinical significance of their presence. An example of this confusion is the work conducted by Wallace Tourtellotte and his group on the clinical relevance of IL-2 and sIL-2R in patients with multiple sclerosis. In a study of 32 MS patients and 77 patients with other diseases published in August 1990 (Kittur et al 1990), they reported that:

> *"The quantitation of soluble IL-2R in CSF may be useful as an indication of T-cell activation events in an inflammatory CNS process"*. They then concluded that *"the data suggest the presence of a T-cell activation-inflammatory process in the CNS of individuals with MS and aseptic meningitis. The assay of IL-2R may provide a marker for the presence of activated T cells in the CNS."*

However, in January 1991, Tourtellotte's group published another major study (Peter et al 1991) in which they used a rather unorthodox title which read: *Serum and CSF levels of IL-2, sIL-2R, TNF-α, and IL-1β in chronic progressive MS: Expected lack of clinical utility.* In this latter study, they commented that the lack of significance of IL-2 and sIL-2R in patients with MS is *"not surprising"*, and they totally avoided any mention

of their study of August 1990. Such discrepancy is most probably due to lack of standardisation of the methodology for detecting IL-2 and sIL-2R, as well as differences in patients selections or other related factors.

As a result, the first purpose of this study was to develop a sensitive and reproducible method for the detection of intrathecal synthesis of IL-2, which would be suitable for routine diagnostic purposes. At the time this work was started, bioassay systems and commercial enzyme-linked immunosorbent assays (ELISAs), all of which had average sensitivity and limited reproducibility were the procedures available for the detection of IL-2 in biological fluids. The sensitivity of these assays was usually inadequate to detect borderline elevation of IL-2, particularly in the CSF.

Another purpose of this study was to examine the distribution of IL-2 and its soluble receptor in CSF and serum samples from a large heterogenous neurologic population. The purpose was to determine whether high levels of IL-2 and sIL-2R could discriminate between patients who suffer from putative inflammatory disorders and those who do not. A laboratory test that can evaluate the state of the immune system *in vivo* would be invaluable in routine clinical practice.

The study then closely analysed the dynamics of IL-2 and sIL-2R in patients with multiple sclerosis, which is a major cause of neurologic disability among young adults. This disease is associated with well-established immunologic abberations that involve a variety of immune cells. The study of IL-2 system in acute MS had several objectives, such as (*a*) to examine the relation between the IL-2 system and intrathecal synthesis of immunoglobulins, (*b*) to evaluate the predictive value of high levels of IL-2 or sIL-2R in early clinical presentation for subsequent development of MS, (*c*) to correlate levels of IL-2 and sIL-2R with certain pathophysiologic events in MS, such as damage to the blood-brain barrier, and (*d*) to determine the relationship between high levels of IL-2 or sIL-2R and clinical features of disease activity and progression. The dynamics of IL-2 and sIL-2R in MS patients were correlated with that of TNF-α, which has been implicated in certain pathological and clinical changes observed in multiple sclerosis.

Finally, the study examined the importance of IL-2 and sIL-2R in poorly characterised neurologic disorders, such as neuro-muscular changes in patients with old poliomyelitis. The value of IL-2 and sIL-2R in the diagnosis of this condition and in understanding pathogenetic factors was closely evaluated.

CHAPTER 2. DETERMINATION OF IL-2 IN THE CSF

2.1 Introduction

Recent advances in the understanding of cytokine structure and function have indicated the need for more stringent cytokine assays. All known cytokines affect more than one cell type and often have diverse effects even on cells of the same linage (Mosmann and Coffman 1987; O'Garra et al 1988; Mosmann 1988). Further complexity is added by the fact that each type of cell in the immune system responds to more than one cytokine. These complexities extend to the tissue culture-adapted cell lines that are used to assay the biological activities of the cytokines, and it is doubtful if any cell line exists that is reliably specific for any individual cytokine.

Because of this multiplicity of cytokine action, monospecific bioassays have been difficult to establish (Mosmann and Fong 1989). The multifunctional nature of cytokine responses requires that the basic bioassay must be modified to obtain satisfactory specificity. Ideally, the bioassay for one cytokine could respond only to that cytokine, and no other cytokine should either give a signal in the assay or inhibit the effect of the cytokine being measured. In other words, the assay should be monospecific and dominant. Monoclonal antibodies are invaluable to improve the specificity of bioassays by blocking unwanted activities in the assay. In addition, a monoclonal antibody specific for the cytokine of interest can be used to confirm that the signal in the assay is due to the correct cytokine.

The development of a solid-phase ELISA for the detection of cytokines was a significant advance in the rapid and reliable quantification of cytokine levels in biological samples. The availability of monoclonal antibodies specific for individual cytokines has ensured the monospecificity and dominance described above. The two-antibody sandwich-ELISA is often as sensitive as the corresponding bioassay, has very high specificity that is not interfered with by other cytokines, and results should be easily standardised between laboratories. Another advantage of ELISA over bioassays is the ability to detect soluble IL-2 receptor in biological fluids. As a result, different commercial ELISA systems have been developed in the past few years to provide simple and reliable detection of cytokines in routine laboratories.

Although commercial ELISA systems for the detection of IL-2 are now widely used in research laboratories, they lack the sensitivity needed to detect IL-2 in the

CSF, and usually have poor reproducibility (personal observations). Therefore, a more sensitive and reproducible ELISA is needed to detect IL-2 in CSF samples from patients with neurologic diseases. In addition, standardisation of IL-2 and sIL-2R levels in CSF and corresponding serum samples is of paramount importance in order to differentiate between passive leakage of these cytokines from serum to CSF and *de novo* intrathecal synthesis within the intrathecal compartment. This chapter describes the development of a sensitive, specific, and reproducible double-antibody sandwich ELISA for the detection of IL-2 in human CSF and serum samples. The chapter also describes a standardisation method for the detection of *de novo* synthesis of IL-2 within the intrathecal compartment.

2.2 Patients

Paired CSF and serum samples from 25 patients (age range 4 to 53 years; 11 females) with acute untreated meningitis were utilised for the detection of IL-2. Samples were obtained within 4 hours of hospital admission and were prepared as detailed below. Clinical diagnosis of meningitis was confirmed bacteriologically where CSF isolates included 10 *N. meningitides*, 6 *S. pneumoniae*, 5 *H. influenzae*, and 4 *E. coli*.

Control CSF and serum samples were obtained from two groups. The disease control group included 15 patients with active rheumatoid arthritis who had no neurologic involvement but had a lumbar puncture to investigate vertebral complaints. The second group is the normal controls which comprises 8 subjects who presented with non-specific neurotic symptoms or tension headache. Extensive laboratory investigations in this group failed to detect any neurologic cause for their symptoms.

2.3 Methods

2.3.1 Sample preparation

Five millilitres (ml) of blood were collected in a sterile glass tube containing no anti-coagulant and were allowed to clot spontaneously over a period of 10-15 minutes. Serum was then collected by centrifugation at 8,500 RPM at room temperature. Cells in CSF samples were separated by cyto-centrifugation and all CSF and serum samples were filtered through a 0.22 μm disposable sterile filter (Millipore) to remove contaminating particulate materials. One thousand kallikrein inhibitory units of

protease inhibitor (Aprotinin, A4529, Sigma Chemical Company, UK) were added to each ml of CSF and serum samples immediately after collection to prevent protein degradation. Fifty µl of sodium azide were also added to test samples to prevent bacterial growth. Samples were then frozen in aliquots at -70°C shortly after collection and thawed just before use. Repeated thawing was avoided.

2.3.2 Preparation of horseradish peroxidase (HRP) anti-IL-2 conjugate

Affinity purified polyclonal rabbit anti-human IL-2 antibody (EP-100, Genzyme, Suffolk) was used to prepare the enzyme conjugate. Glutaraldehyde (GA) was utilised as a cross-linking agent in the HRP labelling of the anti-IL-2 antibody. Preliminary experiments using one- and two-step GA as well as periodate conjugation procedures showed that the two-step GA method is the most efficient technique for the coupling of HRP to anti-IL-2 antibody (data not shown).

The coupling process was performed as described by Avrameas and Ternynk (1971). In brief, 10 mg peroxidase (type VI, Sigma, UK) in 0.2 ml 100 mM phosphate buffered saline (PBS), pH 6.8, was activated by excess GA for 24 h at 24°C then GA was removed by passage through a Sephadex G-25 column (Pharmacia, UK) equilibrated with 0.9% NaCl. The polyclonal anti-human IL-2 antibody equilibrated with 0.9% NaCl in 0.2 ml of 0.5 M sodium carbonate buffer, pH 9.5, was incubated with the activated peroxidase for 24 h at 4°C then the remaining activated groups were blocked with 0.1 ml 1 M lysine, pH 7.0, for 4 h. The conjugate was subsequently dialysed overnight against PBS, filtered through 0.22 µm Millipore membrane, then separated from free peroxidase by passage on protein-A (Pharmacia UK) Sepharose column. Separation of free antibody from the HRP-conjugate was achieved by affinity chromatography on Con A-Sepharose (Pharmacia, UK). The conjugate was finally desorbed with α-methyl-D-manno-pyranoside at 100 mM in PBS, pH 7.2, and 100 mM NaCl and stored in dark containers below 8°C.

2.3.3 Immunoassay for IL-2

All assays were performed blind to clinical data. Wells of polyvinylchloride microplates (Falcon MicroTest III) were coated with 100 µl of 1% gelatin in PBS containing 20 units/well monoclonal murine anti-human IL-2 antibody (DMS-1, Genzyme, Suffolk), incubated for 1 h at 37°C and left overnight at 4°C. Plates were

washed 4 times with 200 µl of wash buffer (0.05% Tween 20 in PBS, pH 7.4) and incubated with 150 µl of 2% gelatin in PBS for 1 h at room temperature to block non-specific binding. The wells were emptied, filled with 150 µl of wash buffer and the plates were stored at 4°C until use.

A standard calibration curve was generated on each assay by freshly prepared serial dilutions of the international standard for IL-2 (preparation code 86/504, NIBSC, UK) in PBS containing 2% gelatin. Results were expressed in international units (IU)/ml as recommended by the Expert Committee on Biological Standards of the World Health Organization (Gearing and Thorpe 1988). Another standard calibration curve was simultaneously generated on each plate using freshly prepared serial dilutions of lyophilised human rIL-2 (Lymphocult, Biotest, Germany) in PBS containing 2% gelatin, and results were expressed in U/ml. This step was included in the assay to permit comparison of results from this study with results already reported by other research groups. Although the use of international unitage in research work ensures standardisation between laboratories, most research groups still use the old U/ml system. One unit of IL-2 is defined in this study as the amount of IL-2 which is required to support half-maximal incorporation of tritiated thymidine [^{3}H]-TdR by murine CTLL cells. Aggregation of the rIL-2 standard, which could interfere with immunologic activity, was avoided by gel filtration prior to use.

One hundred µl of diluted standards, CSF and 1:100 diluted serum samples were added to the wells in duplicates. Control negative and control positive samples were added to each assay. Wells of the first row of each plate served as blank where 100 µl of 2% gelatin in wash buffer were added. Optical density values (OD) for the blanks were subtracted from all test values. Plates were incubated for 4 h at 37°C and washed 4 times. Then 80 µl of HRP-antibody conjugate (diluted 1:1000) were added to the wells. The plates were incubated for 1 h at 37°C and washed 4 times. 100 µl of chromogen solution[1] was added to the wells and the reaction was allowed to proceed for 20 min in the dark at room temperature. The reaction was stopped by 40 µl of 2 mol/L hydrochloric acid. Plates were read at 420 nm in an ELISA plate reader (Dynateck Microplate Reader MR 700, UK).

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[1]The chromogen solution contains *o*-phenylenediamine dihydrochloride (Sigma, P-1526) and $5x10^{-3}$% H_2O_2 in 100 ml of 0.02 M acetate buffer.

2.3.4 IL-2 bioassay

Results of IL-2 assay obtained by ELISA were compared with levels detected by IL-2 bioassay. Bioassay of IL-2 was performed on all test samples as previously described (Gillis and Smith 1977). In brief, IL-2 dependent mouse CTLL cells were used as an indicator system for determination of IL-2. Indicator cells (100 µl; 1 x 10^4) were placed in 96-well, round-bottomed microtitre plates (Microtest III) with serial dilutions of either the test samples, IL-2 international standard, or human rIL-2. Fifty µl/well of a monoclonal antibody to IL-4 (11B11; 10 µg/ml) were added to inhibit the activity of IL-4 on the detector cells. IL-2 activity was assessed by [^{3}H]thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) uptake of CTLL cells after a 6-hour pulse at the end of a 24-hour culture period.

2.3.5 Evaluation of intrathecal IL-2 synthesis

Levels of IL-2 in CSF samples are usually low and may be difficult to detect when relatively insensitive methods are used. Moreover, detectable CSF levels of IL-2 may represent passive transudation from the systemic circulation across blood-CSF barriers (Saris et al 1988). Thus, levels of IL-2 in CSF and corresponding serum were standardised by calculating their ratios to albumin concentration (Adachi et al 1989 and 1990) to correct for passive IL-2 transudation. As a result, standardised CSF IL-2 levels which are higher than concomitant serum levels are taken as indicative of intrathecal synthesis of this cytokine. Albumin concentration in CSF and serum specimens were assayed by electroimmunoassay (Ganrot and Laurell 1974).

2.4 Results

2.4.1 Antibodies to IL-2

The DMS-1 monoclonal and EP-100 polyclonal antibodies used in this assay were shown to be neutralising to IL-2 bioactivity (Smith et al 1983). Thus, the risk of detecting degradation products of IL-2 which are not biologically active was greatly minimised. These antibodies were also found to neutralise the biological effects of both recombinant IL-2 and natural IL-2 of various origins (Dr. R. Hentges, personal communication).

2.4.2 Precision and specificity of the assay

Duplicate determination of 20 positive samples were run in 25 different microtitre plates to calculate the within-run variance. The coefficient of variation (CV) was less than 7.5% in 22 plates and less than 11% in the remaining 3 plates. The between-run variance was estimated by repeated measurement of five positive dilutions of the positive samples, each assayed 8 times in total. The CV was less than 8.5%.

Standard calibration curve using serial dilutions of the international standard for IL-2 resulted in an excellent linear correlation (Fig. 2.1). A dose-response test was performed in serial dilutions on 10 positive CSF and serum samples (Fig. 2.2) to establish the minimal detectable concentration of IL-2. The assay was highly sensitive, detecting IL-2 amounts as low as 1.5 IU/ml (equivalent of $85x10^{-3}$ U/ml). As also shown in Fig. 2.2, negative samples yielded a constant OD less than 0.2 regardless of the dilution. The addition of a protease inhibitor, to prevent protein degradation, improved the stability of IL-2 and consequently enhanced the detection limit of the assay (Fig. 2.3).

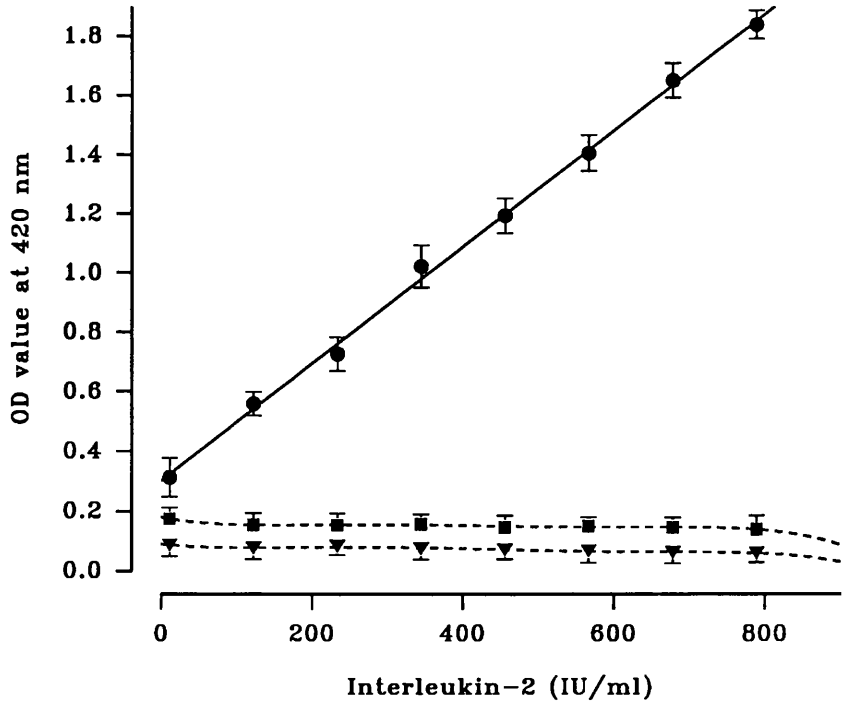


Fig. 2.1. Standard curve for human IL-2 (●—●) compared to standard curves of human IL-6 (■– –■) and tumour necrosis factor-α (▼– –▼) applied at the same concentration. Values represent mean±3 SEM.

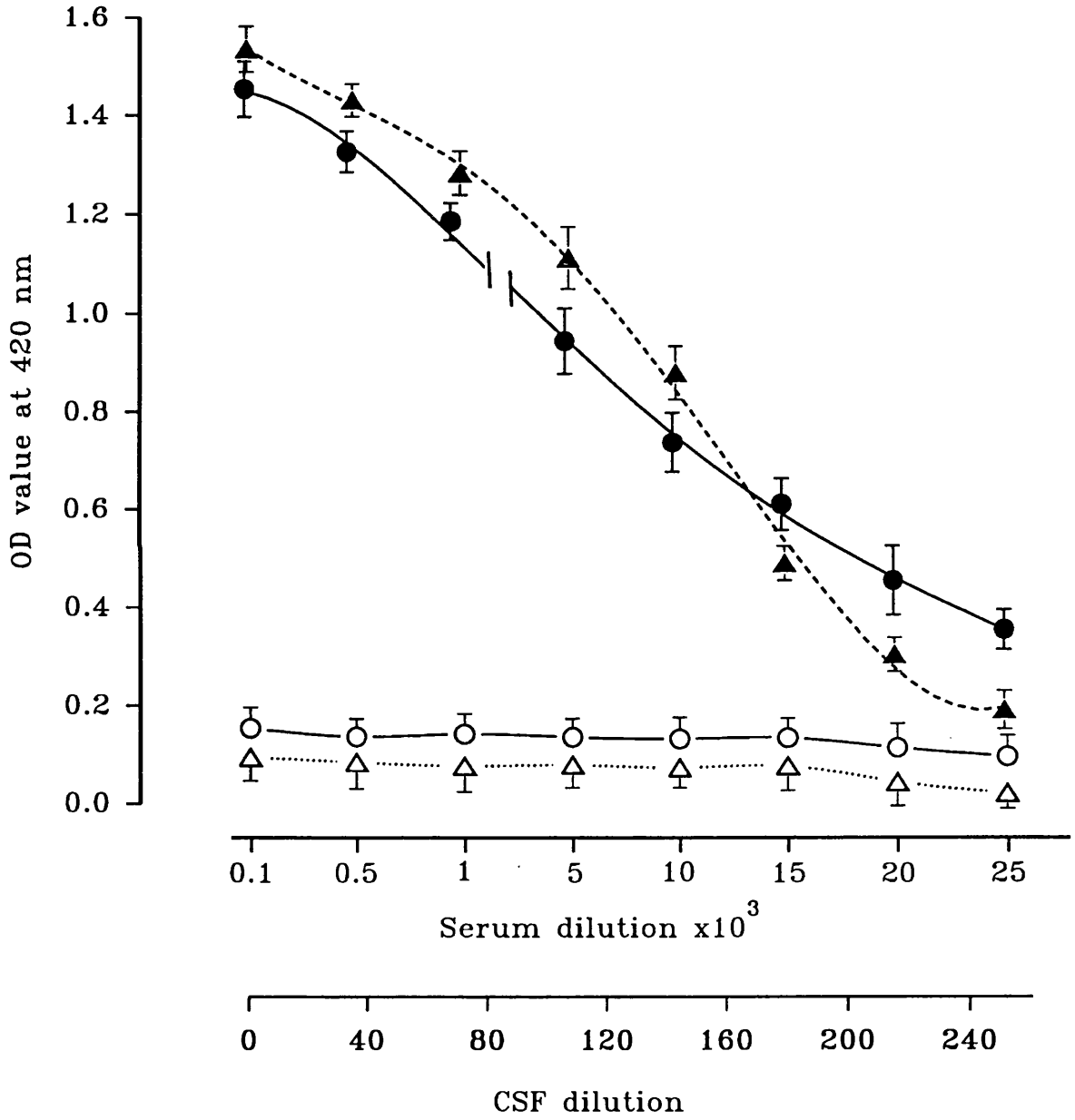


Fig. 2.2. Effect of serum (●—●) and CSF (▲– –▲) dilution on absorbance values in 10 positive samples compared to values form 10 negative serum (o—o) and CSF (▵– –▵) controls.

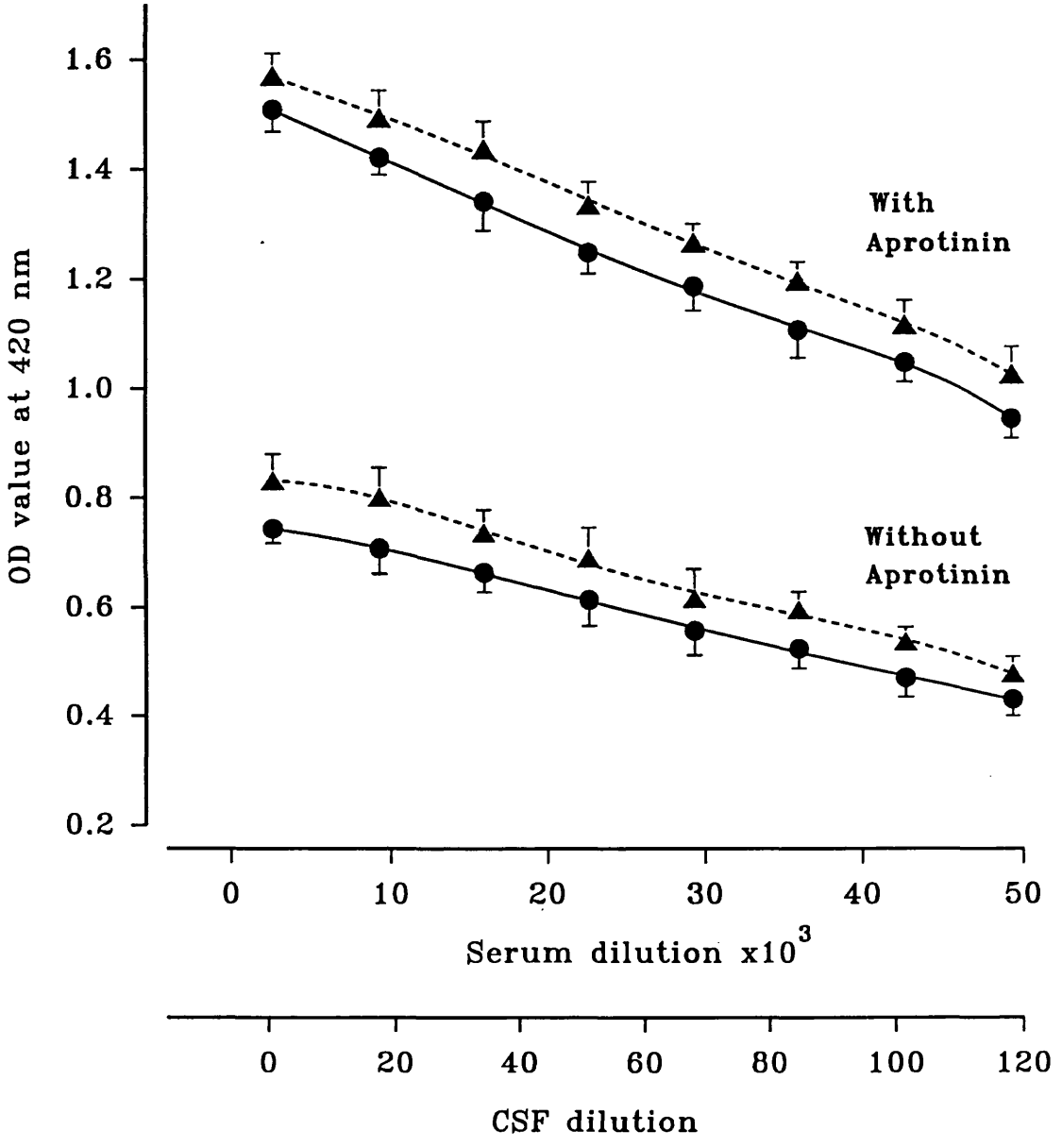


Fig. 2.3. Influence of protease inhibitor (Aprotinin) on absorbance values in 10 positive serum (●—●) and CSF (▲– –▲) controls. Values represent mean±3 SEM.

2.4.3 Correlation of ELISA with bioassay

Results obtained by the immunoassay were correlated with IL-2 concentrations detected by the bioassay to determine whether these levels reflect biologically active IL-2 in body fluids. The concentrations of IL-2 in serum samples as determined by ELISA correlated remarkably well with biologically active IL-2 which was detected by bioassay (Fig. 2.4). As also shown in the figure, there was a good correlation between the ELISA and bioassay in detecting levels of IL-2 in the CSF.

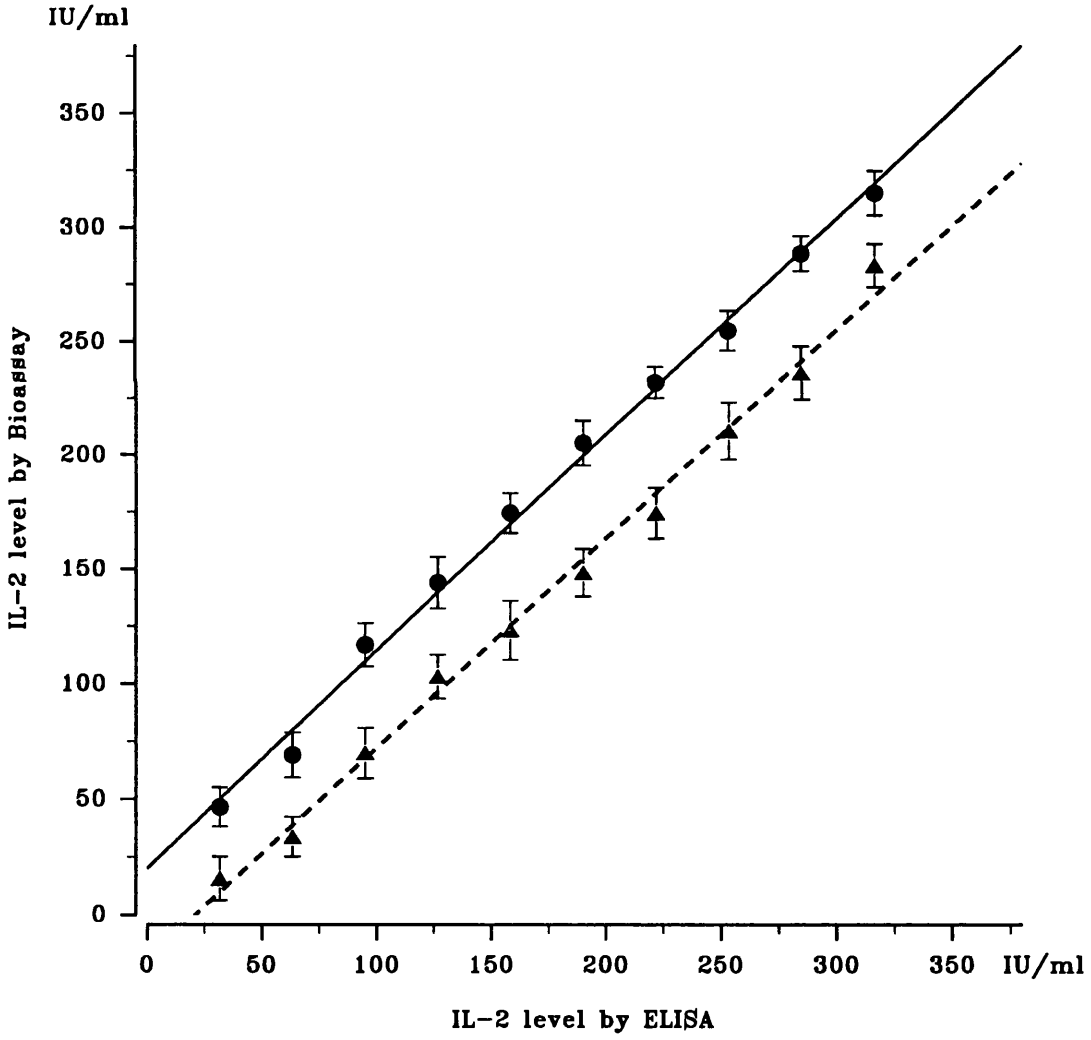


Fig. 2.4. Comparison of ELISA and bioassay in detecting IL-2 in 20 serum (●—●) and CSF (▲– –▲) samples.

2.4.4 Optimal conditions for the assay

The optimal dilution of the HRP-conjugated IL-2 antibody was found to be 1:1000 (Fig. 2.5). Lower dilutions increased the absorbance values of both the test and background. Satisfactory results were obtained when the HRP-conjugate was incubated for one hour (Fig. 2.5). Longer incubation time (up to 3 h) did not result in any significant increase in background activity. Best results were produced when HRP-conjugate was incubated at 37°C. Loss of activity was shown at 24°C, but slightly less activity was lost after incubation at 30°C.

2.4.5 Intrathecal IL-2 production

In patients with acute bacterial meningitis, the absolute concentration of IL-2 in CSF (mean±SD= 258.2±93.7 IU/ml) was significantly higher than concomitant serum concentration (mean±SD= 62.3±44.1 IU/ml; $p< 0.01$). However, IL-2 concentration in the CSF and serum samples was related to albumin concentration as blood-CSF barrier dysfunction could occur in patients with CNS infections (Quagliarello et al 1986; Tunkle and Scheld 1989). Figure 2.6 shows standardised values of IL-2 obtained in the study population. Twenty two (88%) of the 25 patients with CNS infections had significantly higher IL-2 levels in CSF when compared to corresponding serum levels ($p< 0.001$) indicating intrathecal IL-2 production. In contrast, no patient from the disease control group showed evidence of intrathecal IL-2 synthesis (Fig. 2.6). No detectable IL-2 reactivity was found in CSF from the normal control subjects. CSF levels of IL-2 in CNS infections were higher than those detected in the controls ($p< 0.0001$). A good correlation was detected between CSF to serum ratios of IL-2 and albumin (Fig. 2.7).

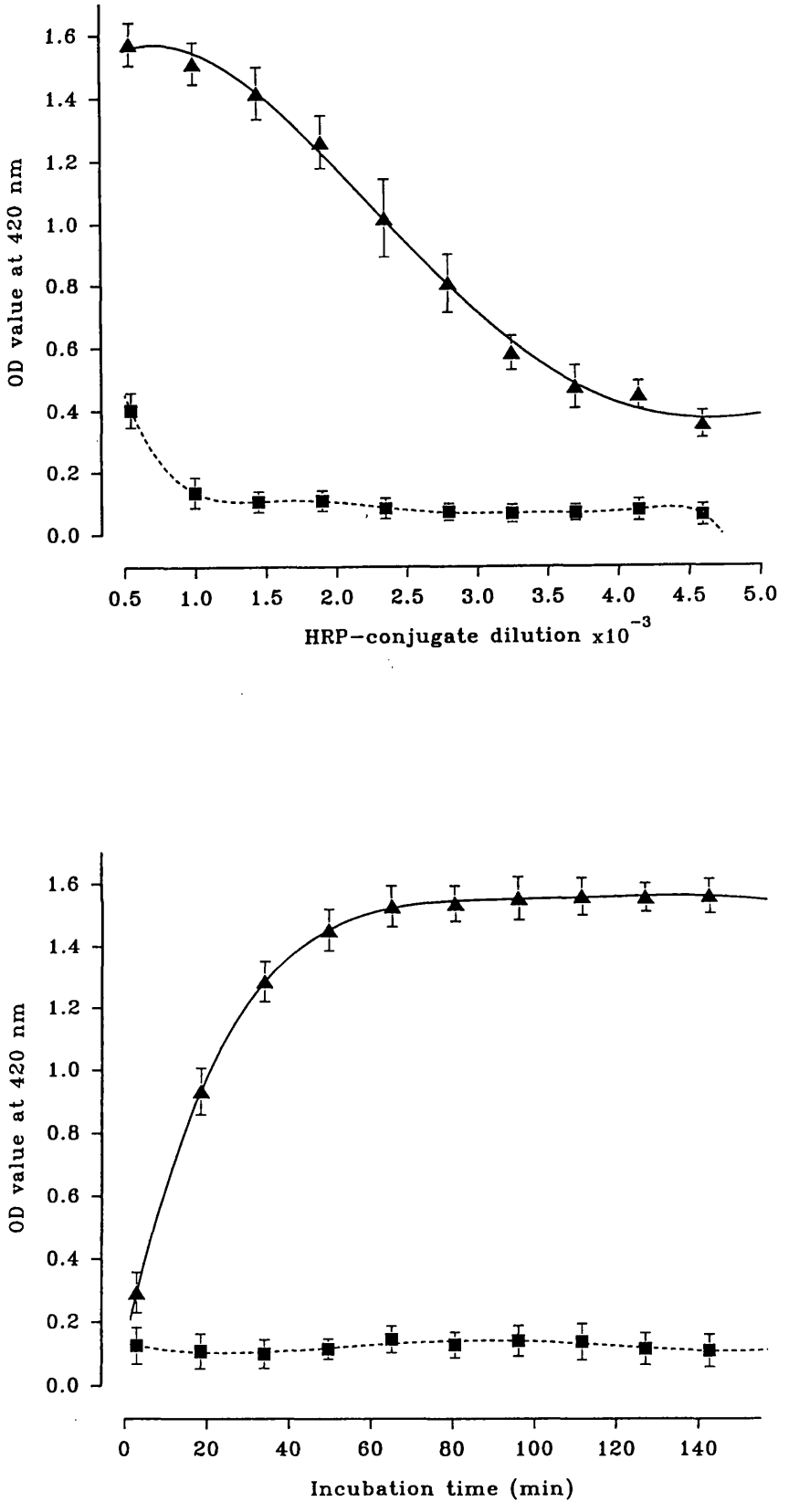


Fig. 2.5. Effect of dilution (**top**) and incubation time (**bottom**) of HRP-conjugate on absorbance values in 10 positive (●—●) and 10 control negative (■– –■) CSF samples. Values represent mean±3 SEM.

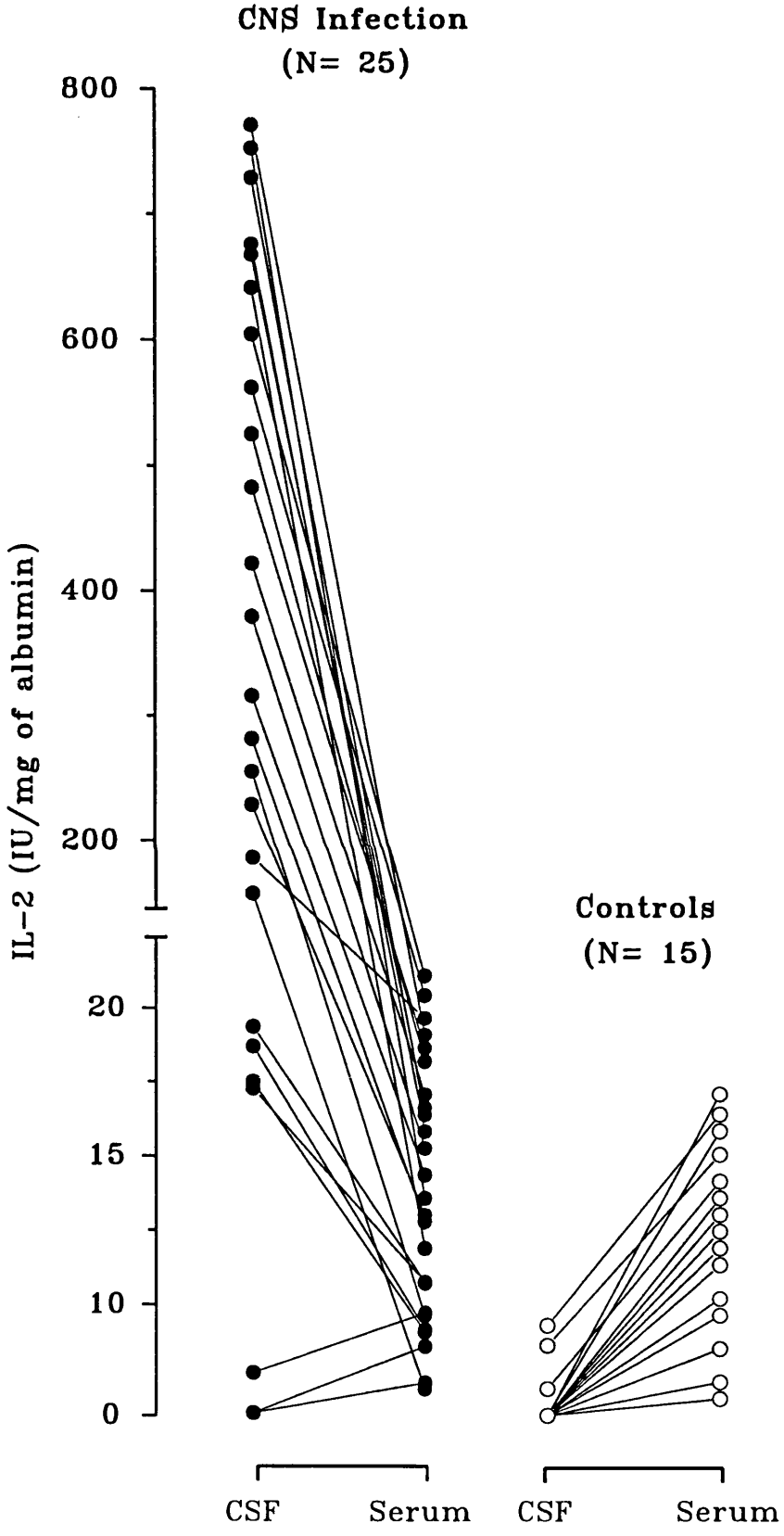


Fig. 2.6. Standardised CSF and serum levels of IL-2 in patients with acute CNS infections compared to those obtained from controls. Statistical analysis is presented in the text.

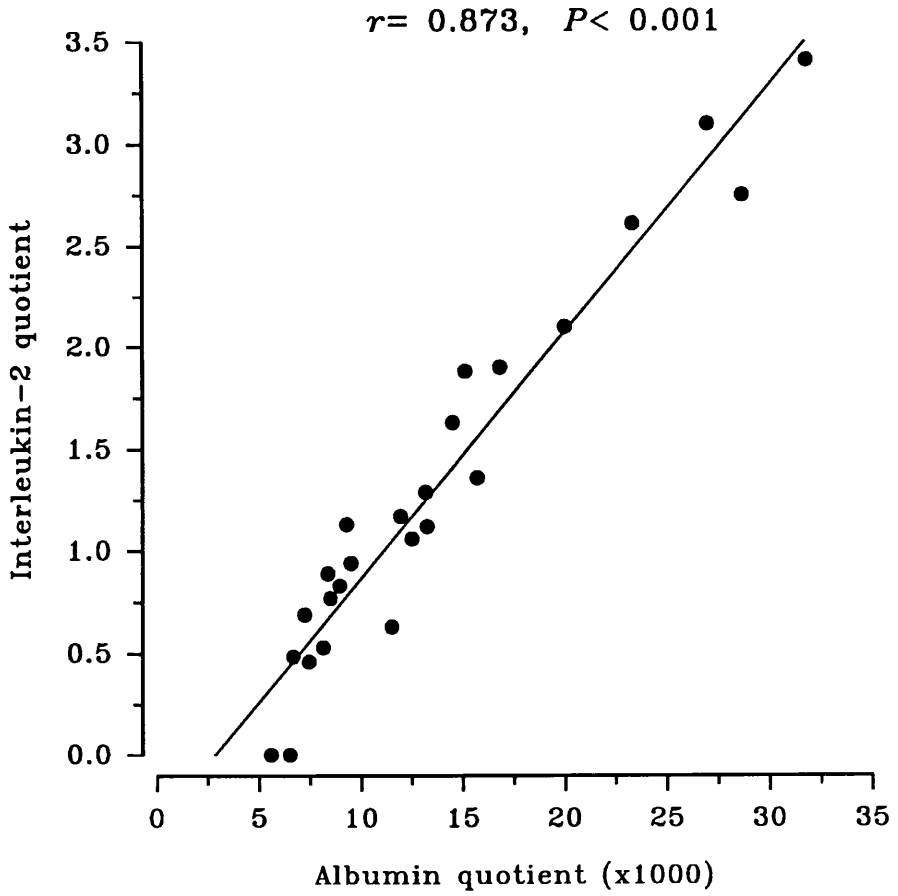


Fig. 2.7. Relationship between CSF to serum quotient of interleukin-2 and CSF to serum quotient of albumin in 25 patients with CNS infections.

2.5 Discussion

The ELISA technique described here was developed for the quantitative determination of IL-2 synthesis within the intrathecal compartment. The technique proved to be specific for IL-2 (Fig. 2.1) and reproducibility was found to be satisfactory. Moreover, the technique was found to be more sensitive than most commercially available ELISA kits, which usually detect IL-2 levels higher than 10 IU/ml.

Traditionally, IL-2 concentrations have been determined by bioassay using IL-2 dependent indicator cells which proliferate in the presence of IL-2 and become quiescent in its absence (Gillis et al 1978). However, bioassay procedures may have some disadvantages when compared with immunoassays. The bioassay is both labour and material intensive and is critically dependent upon maintaining pathogen-free, IL-2 sensitive indicator cells. It is also possible that non-IL-2 components present

in test supernatants, such as IL-4 and residual lectin or phorbol ester, may influence proliferation of the indicator cells (Grabstein et al 1986, Hu Li et al 1987, Gearing and Thorpe 1988). The rapidity of immunoassay procedure is another advantage since biological assays usually take 24-30 hours. The ELISA has an additional advantage over bioassays in that it is much less sensitive to inhibitory or interfering effects from column buffers or complex biological samples such as serum (Mossman and Fong 1989). It should also be remembered that sIL-2R can not be detected by bioassays.

One of the drawback of ELISA is the fact that they measure antigenetically active protein, not necessarily biologically active IL-2. Although a satisfactory correlation between ELISA and bioassay was detected in this study, occasional bioassay might be useful to confirm that the IL-2 detected by ELISA is biologically active. The requirement of coupling anti-IL-2 antibody to peroxidase may be considered another disadvantage of the ELISA. However, no major problems were encountered in the preparation of HRP-labelled antibody. Furthermore, a large quantity of labelled antibody may be prepared at one time since the HRP-conjugate can be stored for at least 6 months without loss of avidity.

The addition of a protease inhibitor immediately after sample collection is an important step in the detection of IL-2 in body fluids. Secreted IL-2 is susceptible to rapid proteolysis, both *in vitro* and *in vivo*, by various proteolytic enzymes, which include trypsin; chymotrypsin; and *Staph. aureus* V8 protease (Aggarwal et al 1984). Therefore, the addition of an enzyme inhibitor of a wide spectrum, such as aprotinin, prevents spontaneous protein fragmentation. This fact may explain the successful detection of IL-2 in the CSF from a significant number of patients included in the study. As will be discussed in the next chapter, studies that failed to implement special precautions to avoid spontaneous cytokine degradation had unacceptably high interassay variability.

Standardisation of IL-2 levels in CSF and serum by relating them to albumin concentration is a crucial step in the objective determination of intrathecal IL-2 production, particularly in conditions associated with blood-CSF barrier impairment. Albumin is synthesised only in the liver and its presence in CSF is dependent on the function of the blood-CSF barriers (Link and Tibbling 1977; Tourtellotte and Ma 1978). Fig. 10 suggested that blood and CSF levels of IL-2 are regulated in a manner relatively similar to that of albumin. Therefore, taking into account the albumin and

IL-2 concentrations in CSF and serum should detect significant elevation of CSF IL-2 levels in patients who produce this cytokine in the intrathecal compartment. In clinical conditions not associated with severe barrier damage, calculations of CSF to serum ratio of IL-2 (Sharief et al 1991a) may be sufficient to detect intrathecal release of this cytokine.

Any discussion on the methodology of cytokine detection should refer to assays other than ELISA or bioassay that could be utilised in some research laboratories to determine IL-2 release. It has to be mentioned, however, that the assays which follow have not been standardised for routine laboratory use. IL-2 gene transcription may be measured by Northern blots. More sensitive assays that allow for the quantitation of IL-2 mRNA based on the polymerase chain reaction have been described (Murray et al 1990). This latter method is suitable for determining IL-2 production by *ex vivo* explanted cells that have not been stimulated by *in vitro* culture. For the estimation of frequencies of IL-2-producing cells, either limited dilution assays or *in situ* hybridisation techniques are available (McGuire and Rothenberg 1987). Furthermore, reverse haemolytic assays (Lewis et al 1990) and spot ELISAs (Skidmore et al 1989) have been developed to determine production of cytokines by individual cells.

In conclusion, the ELISA method presented here is a sensitive and specific procedure that allows routine detection of intrathecal synthesis of IL-2 in neurologic patients. Although anti-IL-2 antibodies were used to develop this method, antibodies against other cytokines, e.g. IL-1 and tumour necrosis factor, could be easily employed to detect their synthesis within the central nervous system.

CHAPTER 3. INTERLEUKIN-2 AND sIL-2R IN MS

3.1 Introduction

Multiple sclerosis, an inflammatory and demyelinating disease of the central nervous system, is a major cause of neurologic disability among young adults (Eber 1986). The disease follows a chronic, recurrent course and is associated with well-established immunologic aberrations that involve B lymphocytes (Tourtellotte et al 1988) and various subsets of T lymphocytes (Waksman 1984). The aetiology and pathogenesis of MS are still enigmatic. Investigations on peripheral blood and CSF as well as the target organ, the CNS, give strong support for immunological mechanisms involving B and T cell activities (reviewed by Calder et al 1989; Hafler and Weiner 1989). There is increasing evidence that MS may be an autoimmune disease, possibly initiated by a viral infection of the CNS (Weiner and Hafler 1988; Rudge 1991), with a T cell response associated with abnormalities in immunoregulation. A gene within the T cell receptor (TcR) β chain complex or a closely linked locus seems to influence the susceptibility for MS (Seboun et al 1989). Certain TcR α chain polymorphisms are also associated with MS (Oksenberg et al 1989). A selective loss of the suppressor-inducer T cell subset, and an increased expression of the T cell activation antigen Ta_1 have been demonstrated (Morimoto et al 1987).

Despite the presence of a lymphocytic infiltration in CNS tissue in MS, CSF lymphocyte levels are usually not elevated. Consequently, the appearance of easily measured soluble proteins in the CSF that would indicate the presence of activated T cells in the CNS would be of benefit, particularly in some *in vivo* studies of MS patients. As discussed in Chapter 1, the elevation of IL-2 or sIL-2R in biological fluids may indicate T cell activation. Therefore, the determination of IL-2 and sIL-2R levels in serum and CSF could be useful for the detection of CNS infectious or inflammatory processes that involve activation of T cell-mediated component. Similarly, the relationship of IL-2 to B cells is well established. IL-2 has been shown to induce both proliferation (Tsudo et al 1984) and differentiation of B-cells, and high affinity IL-2 receptor sites were demonstrated on cloned, activated B cell lines (Waldmann et al 1984). Moreover, high affinity IL-2 receptors were upregulated on activated B cell line in response to exogenous IL-2. SAC, which is a potent cross-linker of surface Ig, activates tonsillar B cells both to express IL-2 receptors and to proliferate in response

to IL-2 (Splawsky 1987). However, the *in vivo* relation of IL-2 and sIL-2R to B-cells, especially in CNS diseases, was not clearly defined before this study was conducted. Similarly, the relationship between these cytokines[1] and intrathecal immunoglobulin production in inflammatory conditions of the CNS, such as MS, was largely unknown.

Increased levels of IL-2 and sIL-2R have been reported in the serum and CSF of patients with CNS infections, such as subacute sclerosing panencephalitis (Fischer et al 1989), acute and subacute encephalitis (Boutin et al 1987), and HIV infections (Gallo et al 1989; Griffin et al 1990). Elevated levels were also detected in the serum and occasionally in the CSF of MS patients (Adachi et al; Gallo et al; Greenberg et al 1988; Trotter et al). However, the notion that high concentration of IL-2 could be detected in MS is not universally accepted as some researcher groups were unable to detect meaningful levels of IL-2 or sIL-2R in patients with active MS (Table 3.1).

Table 3.1. Reports of elevated IL-2 and sIL-2R in serum and CSF of patients with active MS. The table does not list studies that examined IL-2 or sIL-2R levels in serum samples alone.

Research Group	Interleukin-2		sIL-2R	
	Serum	CSF	Serum	CSF
Gallo *et al* 1988	6/21	9/21	—	—
Gallo *et al* 1989	20/36	11/36	15/36	0/36
Gallo *et al* 1991	15/20	10/20	20/20	7/20
Adachi *et al* 1989	6/7	2/2	8/10	8/8
Adachi *et al* 1990	6/10	4/6	11/13	11/11
Peter *et al* 1991[a]	14/50	1/50	5/50	0/50

[a]*This study included patients with chronic progressive MS.*

[1]The term *cytokines* in the text generally refers to both IL-2 and sIL-2R although the latter is a shed receptor and not a genuine cytokine.

It is obvious from the above table that the frequency of detectable IL-2 and sIL-2R in MS patients is variable even within the same group of researchers. Moreover, concentrations of IL-2 in MS patients varied extensively among different research groups (Table 3.2). For instance, Trotter and colleagues have published 4 different sets of values on what appears to be the same group of patients. Also, Gallo and others have published totally unrelated values on presumably the same group of MS patients. Interestingly, almost all workers listed in tables 3.2 have utilised commercial kits from the same sources, namely *Genzyme Corporation* for the detection of IL-2. Serum concentrations of sIL-2R in MS patients described in the literature have also varied widely among investigators (Table 3.3), despite the fact that all of them have used the same commercial ELISA kit supplied by *T Cell Science Inc.*

Table 3.2. Serum concentrations of IL-2 in patients with active MS reported by different research groups using commercial ELISA plates obtained from the same manufacturer (see text). Corresponding CSF levels are not available in most of these studies.

Mean ± SD level (Unit/ml)	Research Group	Year of Study
< 3.0[a]	Weller and others	1991
< 6.25[a]	Rudick and Barna	1990
42.3 ± 97.30	Trotter and others	1988
65.1 ± 115.8	Trotter and others	1989
84.7 ± 144.2	Trotter and others	1990
92.1 ± 134.9	Trotter and others	1991
37.6 ± 23.80	Gallo and others	1989
79.8 ± 156.4	Gallo and others	1991
27.1 ± 16.50	Adachi and others	1989
90[a]	Peter and others	1991

[a]*No details on mean ± SD levels were available in this report.*

Even more confusing than the discrepancies listed in Table 3.2 are results of *in vitro* synthesis of IL-2 by lymphocytes from MS patients. In these studies, the *in vitro* IL-2 production was measured upon activation of peripheral lymphocytes with non-

specific polyclonal stimuli (Con A, PHA, and anti-CD3 monoclonal antibodies), class II-expressing autologous cells, or alloantigens. Results from such studies showed that IL-2 activity in the conditioned media was significantly decreased (Merrill et al 1984; Fischer et al 1989). Another intriguing *in vitro* result was the fact that IL-2 production was low at the beginning of clinical exacerbation but slowly increased as the clinical state improved (Fischer et al 1988). A similar state of decreased *in vitro* production was reported in patients with other autoimmune diseases such as SLE (Alcocer-Varela and Alarcon-Segovia 1982), insulin-dependent diabetes mellitus (Zier et al 1983), chronic active hepatitis (Saxena et al 1986), Sjögren's syndrome (Miyasaka et al 1984), and rheumatoid arthritis (Kitas et al 1988). Further, a puzzling negative correlation was reported between serum IL-2 activities and *in vitro* production in lupus patients (Huang et al 1988).

The low *in vitro* production of IL-2 in MS and other inflammatory diseases was some what paradoxical in relation to the expected effects of IL-2 in these diseases. Several mechanisms were originally proposed to explain an alleged IL-2 'defect' (reviewed by Kroemer et al 1991). The IL-2 'defect' was considered to be caused by (*i*) an inability of macrophages to produce IL-1, (*ii*) an inability of T cells to respond to mitogens or IL-1, (*iii*) a primary inability of T cells to synthesise or secrete IL-2, (*iv*) an increase in the number of activated T cells dedicated to secreted cytokines other than IL-2, and (*v*) inhibitory influence of macrophages or suppressor T cells on IL-2 producing cells. Obviously, detailed *in vivo* studies of patients with inflammatory diseases were required to explain the discrepancy between the *in vitro* IL-2 'defect' and the apparent *in vivo* IL-2 hyperproduction.

It has already been demonstrated in chapter 2 that intrathecal levels of IL-2 and sIL-2R are significantly elevated in patients with acute bacterial meningitis. The current objectives of the chapter are to: (1) study the distribution of IL-2 and sIL-2R in a heterogenous neurologic population, (2) determine intrathecal levels of IL-2 and sIL-2R in patients with clinically definite MS who have different clinical presentations, (3) verify whether increased levels of IL-2 and sIL-2R may be considered a useful marker of inflammatory CNS diseases, (4) analyse whether *in vivo* values of IL-2 support the concept of an IL-2 'defect' in MS, (5) correlate these levels with other CSF features in MS including the local synthesis of immunoglobulin G, A, D and M isotypes. The relationship between CSF concentrations of IL-2 and sIL-2R and CSF

leucocyte count were also closely examined to explore the possibility that these cytokines may be released by CSF cells.

Table 3.3. Serum concentrations of sIL-2R in patients with active MS reported by different research groups using similar commercial ELISA plates. Corresponding CSF levels are not available in some of these studies.

Mean ± SD level (Unit/ml)	Research Group	Year of Study
190 ± 12	Weller and others	1991
278 ± 135	Rudick and Barna	1990
330 ± 100	Adachi and others	1989
361 ± 126	Adachi and others	1990
465 ± 122	Bansil and others	1991
477 ± 359	Peter and others	1991
550 ± 240	Capra and others	1990
1,104 ± 323.9	Trotter and others	1991

3.2 Patients and controls

Paired CSF and serum samples were obtained from different clinical groups and were prepared as described in Chapter 2. Samples were obtained from 148 patients with clinically definite MS (Poser et al 1983) and were utilised for the study. One hundred and ten patients had relapsing-remitting disease while the remaining 38 patients had chronic primary progressive MS in whom the disease process progressed steadily since onset (Fog and Linnemann 1970). Samples from patients with relapsing-remitting disease were obtained within 96 hours of an acute relapse. A relapse phase was defined as the interval within 2 weeks of worsening of existing symptom(s) provided the course has been stationary or has improved during the previous month (Poser et al 1983). No patient involved in the study had received immunosuppressive treatment for at least 8 months before the lumbar puncture.

Paired CSF and serum samples were obtained form 137 patients with various inflammatory and non-inflammatory neurologic disorders (Table 3.4) to serve as a neurologic control group. The group of inflammatory diseases of the CNS comprised

various non-MS inflammatory conditions, such as cerebral lupus (4 patients), neuro-sarcoid (11 patients), neuro-Behçet's (5 patients), post-infectious myelitis (5), and necrotising leukomyelitis of unknown cause (3 patients). Samples from the inflammatory neurologic group were obtained within one week of admission to hospital.

Degenerative CNS conditions (Table 3.4) comprised 14 patients with Alzheimer's disease and other dementias, 6 patients with motor neurone disease, 3 patients with Parkinson's disease, and 3 patients with multiple system atrophy. Cerebrovascular diseases included 7 patients with stroke, 4 patients with transient ischaemic attacks, and a patient with moyamoya disease (extensive basal cerebral *rete mirabile* with microaneurysm formation). Patients with CNS tumours consisted of 6 patients with meningioma, 4 with gliomas, and 2 patients with craniopharyngioma.

Paired CSF and serum samples from 21 normal subjects were also analysed to establish normal reference levels of CSF immunoglobulin levels. Those normal subjects presented with mild non-specific tension headache or neurotic syndromes, and neurological examination as well as detailed clinical investigations (magnetic resonance imaging, computerised tomography, evoked potentials, and CSF analysis) had excluded a known organic origin for their symptoms.

3.3 Methods

3.3.1 Assays of IL-2 and sIL-2R

All CSF and serum samples contained a protease inhibitor (Aprotinin, Sigma Chemical Company, UK) and were processed as described in section 2.3.1. CSF samples were selected to exclude those containing more that 1 erythrocyte per μL at the time of collection. Assays were performed in a blinded fashion on coded samples where the investigator was unaware of the clinical diagnosis. Results of all CSF assays were entered into a computer database file, and sample codes were broken after the completion of the study, to perform statistical analyses. Levels of IL-2 in unconcentrated CSF and diluted serum were detected by the capture ELISA described in Chapter 2.

Serial dilutions of two standard curves were utilised in each ELISA plate to maintain adequate standardisation. The first standard curve used recombinant human IL-2 (Lymphocult, Biotest, Germany) while the second curve employed the

International Standard for IL-2 (86/504, NIBSC, England). However, results of IL-2 in this chapter will be presented only in U/ml to permit comparison with results from other research groups since no investigator, to date, has used the International Unitage in reporting the concentrations of IL-2 in neurologic diseases. The reason for using the International IL-2 Standard in this study was mainly to monitor inter-assay variability.

Table 3.4. Clinical features of the study population.

Clinical group	Total No. (Females)	Mean Age ± SD[a]	Mean Disease Duration ± SD[a]
Clinically Definite MS:			
Relapsing-remitting	110 (71)	33.2 ± 5.7	6.9 ± 4.2
Chronic progressive	38 (22)	37.1 ± 7.6	9.4 ± 5.1
Neurologic controls:			
Inflammatory CNS Diseases	28 (11)	37.2 ± 11.3	6.2 ± 5.1
Cerebrovascular Diseases	12 (5)	31.5 ± 8.2	0.3 ± 0.1
Degenerative CNS Diseases	26 (13)	53.7 ± 9.1	14.6 ± 8.4
CNS Tumours	12 (7)	44.6 ± 5.7	4.2 ± 3.2
Vertebral Lesions	36 (17)	35.2 ± 10.1	10.5 ± 8.6
Chronic Neuropathies	23 (12)	46.8 ± 6.4	12.1 ± 7.3
Normal Subjects	21 (10)	25.1 ± 9.6	—
Total	306 (161)	38.2 ± 8.2	—

[a] *in years*

Levels of sIL-2R were also measured by a commercial sandwich enzyme-immunoassay (Cell Free, T cell Sciences, Cambridge, MA) according to the manufacturer's instructions. The procedure uses two non-competing murine monoclonal antibodies to the α subunit of the IL-2 receptor, and has been thoroughly

described by Zucchelli *et al* (1989). Briefly, microtitre plates with 96 wells were coated with a monoclonal antibody to the IL-2R. Non-specific binding was blocked with 150 µl of 2% gelatin in PBS for 1 h. After the wells had been washed 4 times with wash buffer, unconcentrated CSF and 1:100 diluted serum samples were added in duplicate and incubated for 2 h at 37°C. Samples were then discarded, the plate washed and an antibody to IL-2R conjugated with HRP was added to each well. After another 2 h at 37°C the plate was washed and incubated with *o*-phenylenediamine HCl (OPD; P-1526, Sigma, UK) in acetate buffer containing $5x10^{-3}$ hydrogen peroxide. After 20 minutes at room temperature in the dark, the reaction was stopped by the addition of HCl and the plates were read at 420 nm with a microplate reader (Dynateck Microplate Reader MR 700, UK). Units of sIL-2R were calculated from a standard calibration curve generated on each assay by freshly prepared serial dilutions of recombinant human IL-2R (Genzyme, England) in PBS containing 2% gelatin.

3.3.2 Immunoglobulin assays

Immunoglobulin G concentration in the test samples was analysed by electroimmuno-assay (Tourtellotte et al 1971) with minor modifications. Test samples, serial dilutions of IgG standard, and quality control samples were electrophoresed overnight in agarose gels made from 1% HEEO agarose (SeaKem-50013, FMC, USA) and 0.5% ME agarose (SeaKem-61823, FMC, USA) containing $5x10^{-4}$ goat anti-human IgG antibody (ATAB-012-11, Atlantic Antibodies, England). At the end of electrophoresis, the gels were pressed, washed in PBS for 30 min, dried under a stream of warm air, then stained for 15 min in Coomassie Brilliant Blue. Gels were subsequently destained, washed, and dried under a stream of hot air. Peak heights were measured then the IgG concentration in the test samples was calculated by using a standard curve.

Levels of IgM in CSF and serum samples were measured by a modified capture enzyme-linked immunosorbent assay (Sharief et al 1990). Briefly, affinity purified goat anti-human IgM Fc (ATAB-013-11, Atlantic Antibodies, England) was adsorbed to PVC microtitre plate with 96 wells (Falcon Micro-Test III), then any unoccupied binding sites were blocked by 1% (w/vol) gelatin in PBS. Unconcentrated CSF and $1:10^4$ diluted serum were incubated for 2 h at room temperature. After washing, 1:1000 dilution of HRP-conjugated $F(ab')_2$ fragment of rabbit anti-human IgM antibody (Sigma-A4290, UK) was added. After incubation and washing, bound

enzyme activity was measured using OPD and hydrogen peroxide, and read in a dual wavelength ELISA plate reader. The use of $F(ab')_2$ fragment as detector markedly reduced non-specific background (Sharief et al 1989). Reproducibility, particularly of the serum assay, which uses a high dilution of sample, was aided by a liquid handling station (Probus Quatro, England).

Concentration of IgA in test samples was determined by a capture ELISA system described earlier (Lolli et al 1990). Briefly, 96-well PVC microtitre plates were coated with 100 µl/well of a dilution of 1:4000 of anti-human α chain antibody (ATAB-80260, Atlantic Antibodies, England). After washing, unconcentrated CSF and $1:10^4$ diluted serum samples were added in duplicates and incubated for 2 h followed by the addition of HRP-conjugated $F(ab')_2$ fragment of goat anti-human IgA antibody (Sigma-A4165, UK). Serial dilutions of IgA standard (ATAB-061-01, Atlantic Antibodies, England) and control CSF and serum samples were included in each plate. Optical densities of test samples were averaged, read from the standard curve, and corrected by the original dilution factor.

CSF and serum concentration of IgD were also detected by a similar ELISA (Sharief and Hentges 1991a) where PVC microtitre plates were coated with 100 µl/well of 1:4000 dilution of affinity purified antihuman δ-chain antiserum (Dakopatts A-093, Dako, Denmark), blocked by 2% BSA and 0.1% Tween (BDH Ltd, England) in PBS, then incubated with the test samples. After overnight incubation, IgD reactivity in the test samples was detected by rabbit HRP-conjugated anti-human δ-chain antibody (A-6281, Sigma, UK). Known amounts of human IgD standard (OTRD 03, Behring, Germany) and control CSF and serum and background control wells were included in each plate. Optical densities of test samples were dealt with as for IgA.

Intrathecal synthesis of immunoglobulins was measured through the calculation of the index values (Tibbling et al 1977) according to the following formula: *Ig Index= (CSF Ig x serum albumin) ÷ (serum Ig x CSF albumin).*

3.3.3 Detection of oligoclonal bands

Intrathecal synthesis of immunoglobulins could also be determined through the detection of oligoclonal bands. Electrophoretically restricted immunoglobulins commonly occur in CSF of patients with MS or other inflammatory diseases of the CNS in the form of discrete bands. Each band normally represents the product of a

single B lymphocyte clone. Intrathecal synthesis of an Ig is said to be present when respective oligoclonal bands are present in the CSF but are either absent from or substantially more in number than corresponding serum. In general, CSF oligoclonal bands are more reliable in detecting intrathecal synthesis of immunoglobulins than the Ig index (Sharief et al 1990). However, oligoclonal bands do not provide quantitative data on the extent of locally produced immunoglobulins. Thus details about the magnitude of intrathecal synthesis of immunoglobulins in the study population were obtained by calculating the Ig index, as explained above.

Oligoclonal IgG bands were detected by isoelectric focusing on agarose gel (Walker et al 1983). In brief, CSF and diluted serum containing approximately 100 ng of IgG were electrofocused in agarose gels containing ampholytes (Pharmacia) with a pH range of 3.0 to 10.5. Oligoclonal IgG bands were then detected using a double-antibody amplification immunostaining. Control positive and negative samples were included in each batch as quality control. Oligoclonal IgM reactivity was detected by the method of glutaraldehyde (GA)-enhanced immunofixation (Sharief et al 1989), which utilizes agarose gel electrophoresis, GA cross-linkage, and subsequent immunostaining by HRP-conjugated $F(ab')_2$ fragment of rabbit anti-human IgM antibody (Sigma-A4290, UK). CSF and diluted serum samples containing 10 ng total IgM were electrophoresed in agarose gel of high electroendosmosis (Miles Laboratories, London). Control positive and control negative samples were included in each gel to serve as quality control.

Oligoclonal IgA bands were also detected essentially by the method of GA-enhanced immunofixation (Sharief et al 1991). In brief, native CSF and diluted serum samples containing 15 ng of IgA were electrophoresed on agarose gel then the separated proteins were covalently linked to nitrocellulose membrane using GA. IgA bands were immunostained by a HRP-conjugated $F(ab)_2$ fragment of anti-human IgA antibody (Sigma-A4165, UK). No reliable method was available for the detection of CSF oligoclonal IgD bands at the time this study was conducted. Thus, details about intrathecal synthesis of IgD were obtained through the calculation of IgD index alone.

3.3.4 Other CSF assays

Concentration of albumin in CSF and serum samples was determined by electro-immunoassay (Ganrot and Laurell 1974). CSF white cell count was performed using

Fuchs-Rosenthal chambers (Hemocytometer, Aimer Products, London), and cytological examination was performed with a Giemsa stain from a cytocentrifuge (Cytospin 2, Shandon Products Ltd, England) preparation. The CSF leucocyte count corrected, according to the peripheral count, if red blood cells were also shown to be present. The presence of reactive lymphocytes (i.e., cells that are different in size and staining characteristics due to high cytoplasmic Ig content) was particularly noted. In view of their activated state, reactive lymphocytes could be a potential source of IL-2 release.

3.3.5 Statistical analyses

Nonparametric Wilcoxon sum rank, Mann-Whitney, and Pearson's correlation matrix tests were used, as appropriately, for statistical analysis. The distribution of IL-2 and sIL-2R in the study population was evaluated using confidence intervals for non-parametric data (Campbell and Gardner 1988). Statistical analyses were performed using SPSS/PC+ software program (SPSS Inc, Chicago, IL). The observed significance level (probability "*P*") will be rounded to decimal figures throughout the text. If *P* was small enough (≤ 0.05), the hypothesis that the two variables compared are independent was rejected. All *P* values are two-tailed.

3.4 Results

3.4.1 Distribution of IL-2 and sIL-2R

The distribution of IL-2 and its soluble receptor in CSF and serum samples of the study population is presented in Table 3.5. Interleukin-2 was not detected in the CSF of normal control subjects, whereas the mean±SD CSF level of sIL-2R in these subjects was 42.7±20.6 U/ml. Mean serum level of IL-2 in normal subjects was 2.07±0.6 U/ml, and mean serum level of sIL-2R was 58.2±31.4 U/ml. Detectable concentrations of IL-2 and sIL-2R in normal subjects probably represent continuous low level of cell proliferation induced by IL-2 (Greene and Leonard 1986).

In patients with MS, IL-2 levels were considerably elevated in the CSF of 80 patients (73%) with relapsing-remitting MS and in only 7 patients (18%) with chronic progressive MS ($P< 0.01$). Similarly, CSF levels of IL-2 in patients with relapsing-remitting MS were significantly higher than CSF levels of IL-2 in neurologic controls ($P< 0.001$). High CSF levels of IL-2 were detected in 15 patients (54%) with non-MS

inflammatory neurologic diseases (7 with neuro-sarcoidosis; 4 with neuro-Behçet's; 2 with cerebral lupus; and 2 with post-infectious myelitis). High CSF levels of IL-2 were detected in 3 (14%) non-inflammatory controls: 2 patients with gliomas and a patient with a stroke.

Interleukin-2 was not detected in CSF from patients with degenerative CNS diseases, vertebral lesions or chronic neuropathies (Table 3.5). As also shown in Table 3.5, levels of CSF IL-2 in relapsing-remitting MS were higher than corresponding serum levels. In contrast, CSF IL-2 levels in patients with chronic progressive MS were relatively similar to corresponding serum levels.

Levels of sIL-2R in CSF and serum of patients with MS showed similar relationships to those seen in IL-2 levels (Table 3.5). Detectable levels of sIL-2R were found in CSF of 89 patients (81%) with relapsing-remitting MS and 23 patients (61%) with chronic progressive MS. In patients with relapsing-remitting MS, CSF levels of sIL-2R were higher than serum levels. Similarly, CSF levels of sIL-2R in patients with relapsing-remitting MS were higher than matching levels in patients with chronic progressive MS. The relationship between CSF levels of IL-2 and sIL-2R is presented below (section 3.4.3). Levels of sIL-2R in CSF of relapsing-remitting MS were higher than CSF levels in the neurologic controls ($P< 0.0001$).

Although CSF levels of IL-2 and sIL-2R were clearly higher than corresponding serum levels in patients with relapsing-remitting MS, standardised levels were calculated to correct for passive transudation of these cytokines from blood to CSF. The standardised levels were determined by calculating ratios of IL-2 and sIL-2R to albumin as described in Chapter 2. This standardisation confirmed that levels of IL-2 and sIL-2R in patients with MS are significantly elevated in the intrathecal compartment when compared with peripheral blood (Figs. 3.1 and 3.2).

It is obvious from Table 3.5 that high intrathecal levels of IL-2 and sIL-2R could be seen in inflammatory CNS diseases other than MS. In this regard, it could be argued that although high cytokine levels within the intrathecal compartment are more or less specific for inflammatory neurologic disease, such levels do not distinguish between multiple sclerosis and other inflammatory conditions. In view of the fact that multiple sclerosis is the commonest inflammatory condition of the CNS, the rest of this chapter will focus on the dynamics of IL-2 and sIL-2R in this disease.

Table 3.5. Levels of IL-2 and sIL-2R in the study population. Numbers in brackets represent the total numbar of patients who had abnormally elevated levels.

Clinical Group	95% Confidence Intervals for IL-2 (U/ml)		95% Confidence Intervals for sIL-2R (U/ml)	
	Cerebrospinal Fluid	Serum	Cerebrospinal Fluid	Serum
Clinically Definite MS:				
Relapsing-Remitting	9.7 to 14.4 (80)	5.85 to 10.3 (88)	118.6 to 161.9 (89)	96.1 to 125.2 (96)
Chronic Progressive	-0.45 to 4.5 (7)	1.24 to 3.6 (14)	24.3 to 56.4 (23)	37.6 to 60.6 (27)
Neurologic controls:				
Inflammatory CNS Diseases	2.5 to 11.2 (15)	2.3 to 13.6 (18)	85.4 to 132.2 (19)	54.2 to 104.3 (20)
Cerebrovascular Diseases	-0.68 to 2.1 (1)	-1.3 to 5.3 (3)	34.5 to 71.2 (2)	44.3 to 62.1 (2)
Degenerative CNS Diseases	0[a]	-0.23 to 3.2 (2)	12.7 to 32.1 (0)	32.4 to 53.1 (0)
CNS Tumours	-0.84 to 4.5 (2)	0.32 to 4.2 (3)	44.6 to 78.7 (3)	41.4 to 67.3 (4)
Vertebral Lesions	0[a]	-0.24 to 3.2 (1)	10.7 to 32.1 (0)	35.2 to 51.2 (0)
Chronic Neuropathies	0[a]	-0.54 to 2.5 (2)	12.4 to 37.3 (0)	37.2 to 61.3 (0)
Normal Controls	0[a]	-0.65 to 1.7	16.2 to 26.8	29.8 to 56.2

[a]*Below the detection limit of the assay.*

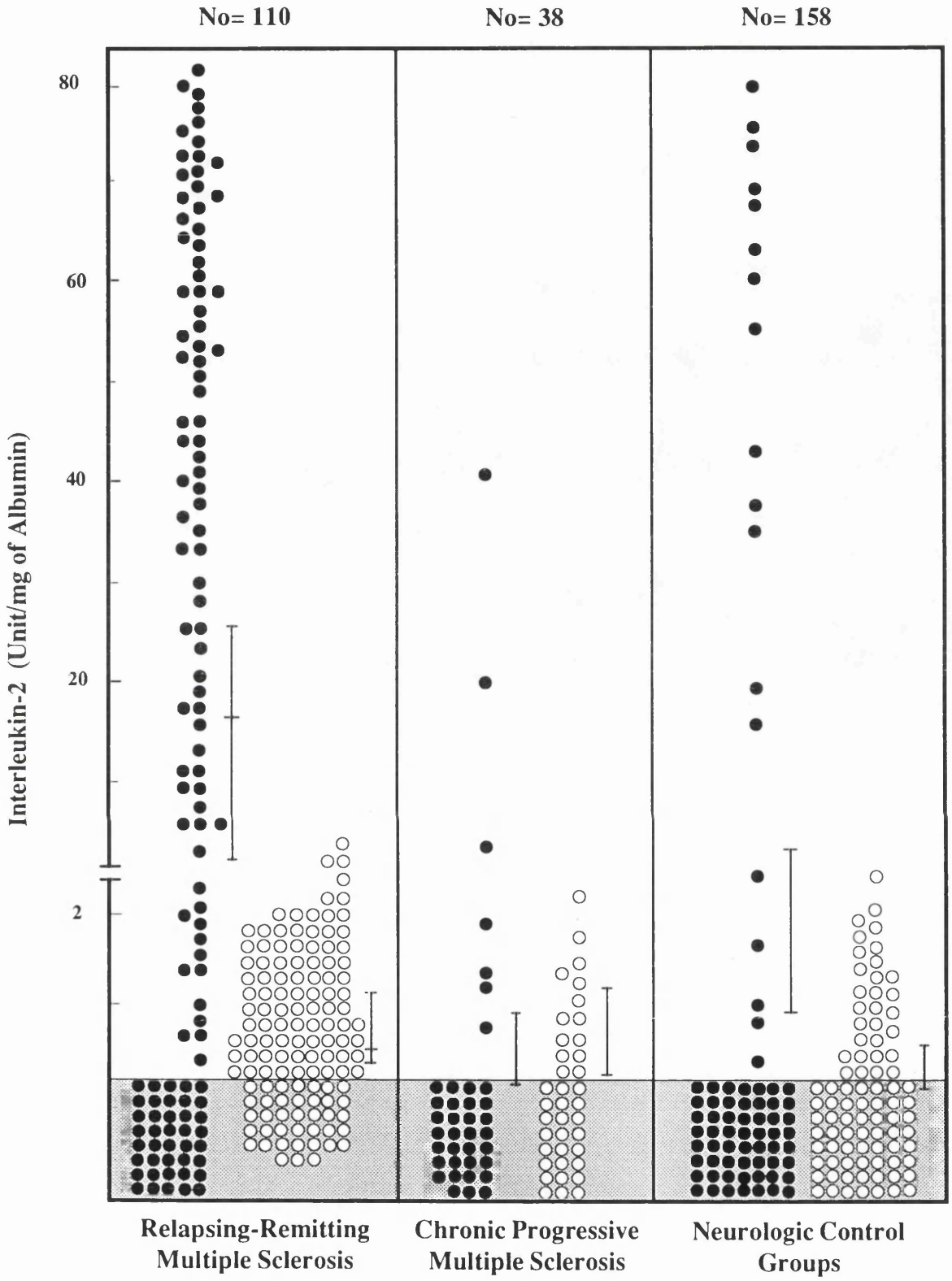


Fig. 3.1. Standardised levels of IL-2 in cerebrospinal fluid (●) and serum (○) in the study population. Shaded area represents values below the detection limit of the IL-2 assay. Bars represent medians and 95% confidence intervals. Absolute values of IL-2 in CSF and serum samples will be presented later (see Fig. 6.1).

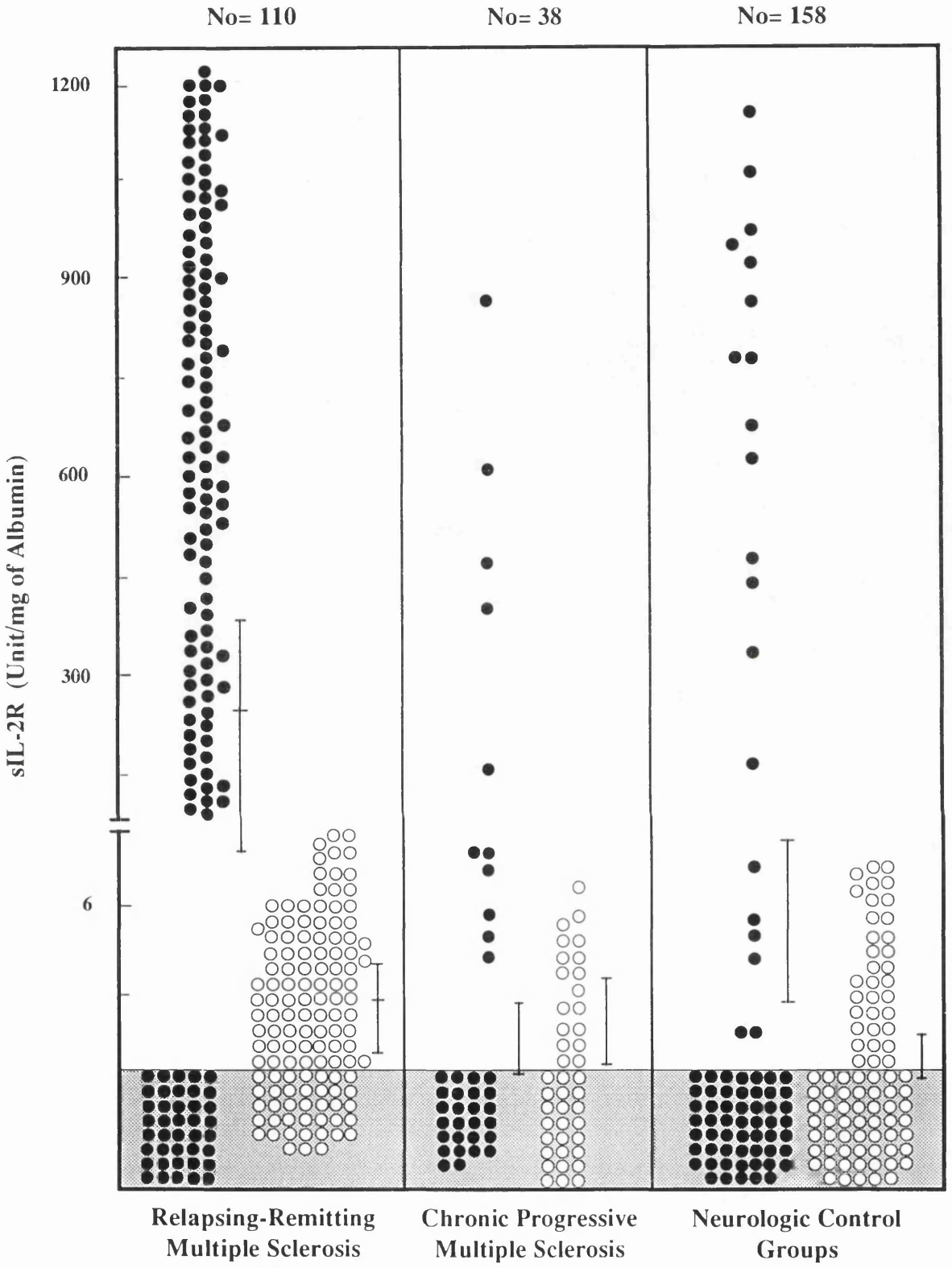


Fig. 3.2. Standardised levels of sIL-2R in cerebrospinal fluid (●) and serum (○) in the study population. Shaded area represents values below the detection limit of the sIL-2R assay. Bars represent medians and 95% confidence intervals.

3.4.2 Kinetics of serum and CSF levels

Individual serum and CSF levels of IL-2 and sIL-2R in MS patients were compared to determine if there was a correlation between cytokines levels in the intrathecal compartment and systemic circulation. No significant correlation between serum and CSF IL-2 levels was found (r= 0.34, P= 0.081). Similarly, there was no correlation between serum and CSF sIL-2R levels (r= 0.33, P= 0.094). Indeed, no correlation between serum and CSF levels was detected when standardised levels of IL-2 and sIL-2R were compared.

3.4.3 Comparison between IL-2 and sIL-2R

Table 3.6 depicts the relationship between the presence of IL-2 and sIL-2R in CSF of patients with MS. As shown in the table, the presence of IL-2 in CSF significantly correlated with the presence of sIL-2R in patients with relapsing-remitting MS. In contrast, no significant correlation was found between the presence of IL-2 and sIL-2R in patients with chronic progressive MS, probably because of the low number of patients who had detectable levels of cytokines in CSF. In patients with relapsing-remitting MS who had detectable levels of IL-2 in CSF, significant correlation was found between CSF levels of IL-2 and sIL-2R (Fig. 3.3). No correlation was detected between serum levels of IL-2 and sIL-2R in MS patients.

Table 3.6. Number of MS patients who have detectable IL-2 and/or sIL-2R in CSF.

IL-2 in CSF	sIL-2R in CSF	
	Absent	Present
Relapsing-Remitting MS		
Absent	13	17
Present	8	72[a]
Chronic Progressive MS		
Absent	12	19
Present	3	4

[a]*Corrected* χ^2= *15.6, P< 0.0001*

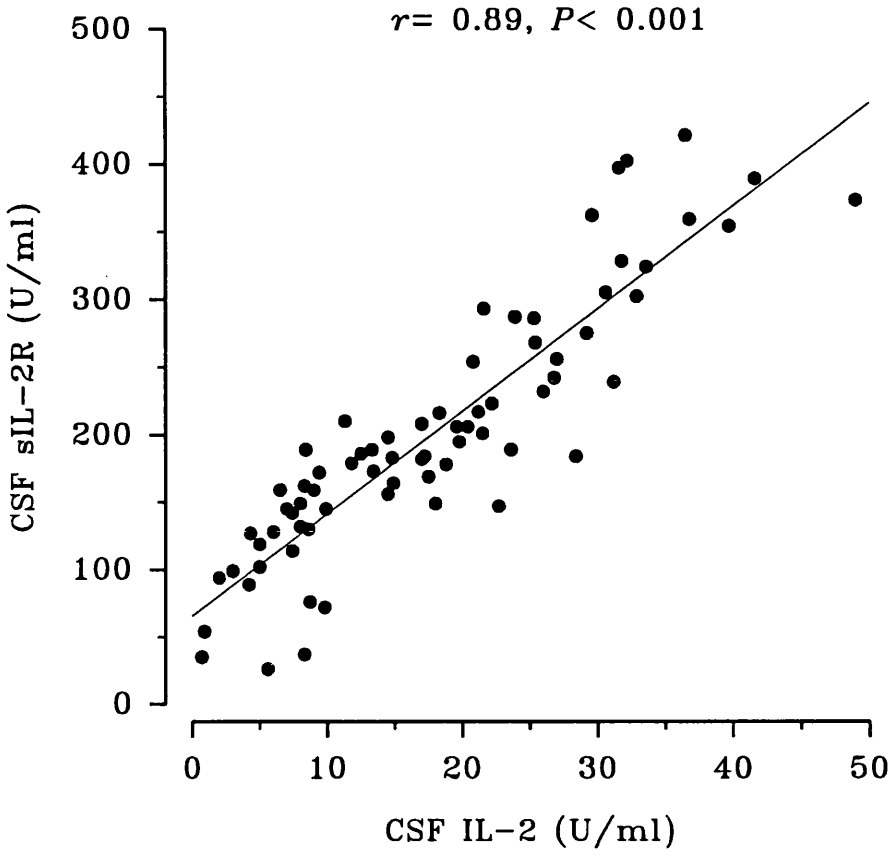


Fig. 3.3. Relation between CSF levels of IL-2 and sIL-2R in patients with relapsing-remitting MS. Only patients with detectable IL-2 in CSF were included in this analysis.

3.4.4 Relationship of IL-2 and sIL-2R to intrathecal Ig synthesis

Results of intrathecal synthesis of immunoglobulins in patients with MS are presented in Table 3.7. Abnormally high levels of IgG, IgA, and IgM indices were detected in similar frequencies in patients with relapsing-remitting and chronic progressive MS. However, IgD index values in patients with relapsing-remitting MS were significantly higher than values in chronic progressive MS (Table 3.7). Values of immunoglobulin indices in MS patients were significantly higher than neurologic controls ($P< 0.001$), including patients with other inflammatory CNS diseases ($P< 0.01$).

In view of the significantly high prevalence of IL-2 and sIL-2R in CSF of patients with relapsing remitting MS, levels of these cytokines in CSF and serum samples were correlated with values of IgG, IgA, IgD, and IgM indices. Two subgroups were analyzed for each immunoglobulin index: first group, those with abnormally high index value, i.e. more than the cut-off level (mean+2SD) of the group of 21 normal control subjects; and the second group, those with normal index value.

Levels of IL-2 in CSF were found to be significantly higher in patients with abnormally high IgD and IgM index values compared to levels seen in patients with normal index values, while no such relationship was observed for either IgG or IgA index (Fig. 3.4A). Similarly, levels of sIL-2R in the CSF were significantly higher in patients who had abnormally high IgD and IgM index values (Fig. 3.4B), whereas patients with high IgG and IgA index values did not demonstrate significant elevation of sIL-2R in the CSF. The serum levels of IL-2 and sIL-2R were not observed to be significantly related to any immunoglobulin index values (Fig. 3.4).

Table 3.7. Mean±SD of immunoglobulin indices in 21 normal control subjects and 148 patients with clinically definite MS.

Variable	Normal Controls	Multiple Sclerosis	
		Relapsing Remitting	Chronic Progressive
IgG Index	0.53 ± 0.14	1.13 ± 0.53 84 (76%)	0.86 ± 0.62 29 (76%)
IgA Index	0.19 ± 0.07	0.46 ± 0.28 67 (61%)	0.41 ± 0.29 21 (55%)
IgD Index	0.13 ± 0.10	0.50 ± 0.28[a] 59 (54%)	0.18 ± 0.16 3 (8%)
IgM Index	0.031 ± 0.022	0.11 ± 0.08 62 (56%)	0.09 ± 0.05 20 (53%)

Note. Data in the MS group include number (and percentage) of patients who show values above the cut-off levels (means+2SD) of the normal controls.

[a]*P< 0.001 compared with values of IgD index in patients with chronic progressive MS.*

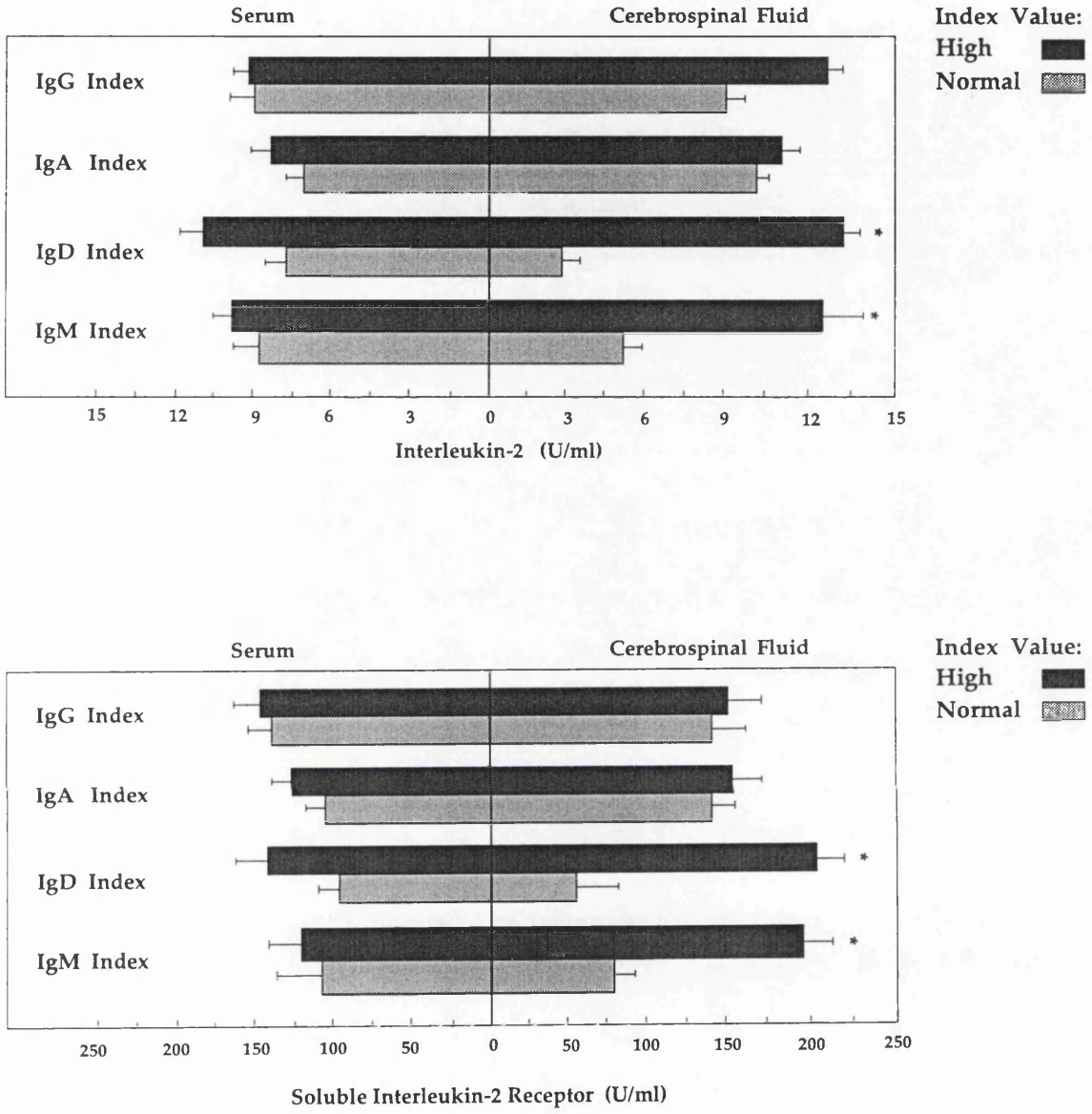


Fig. 3.4. Mean (±SEM) levels of serum and CSF interleukin-2 and soluble IL-2 receptor in relation to values of immunoglobulin indices in patients with relapsing-remitting MS. Normal index values are defined in the text.

*$P < 0.01$ relative to cytokine levels in patients with normal index values.

The correlation between index values of individual immunoglobulins and the intrathecal amounts of IL-2 and sIL-2R in MS patients who had detectable CSF levels of IL-2 or sIL-2R was then analysed. Such analysis was important to discern the relationship between CSF cytokine levels and intrathecal synthesis of immunoglobulins. Intrathecal cytokine concentrations were determined by calculating the CSF to serum ratios of IL-2 and sIL-2R. These ratios usually correct for passive transudation of the cytokines through the blood-CSF barriers (Keir and Thompson 1986; Sharief et al 1991a).

There was a significant correlation between intrathecal levels of IL-2 and both IgD and IgM index values, whereas no such correlation existed between intrathecal IL-2 and either IgG or IgA (Fig. 3.5). Similarly, intrathecal sIL-2R levels correlated with IgD and IgM index values (Fig. 3.6). Intrathecal levels of sIL-2R did not correlate with values of IgG or IgA indices (Fig. 3.6).

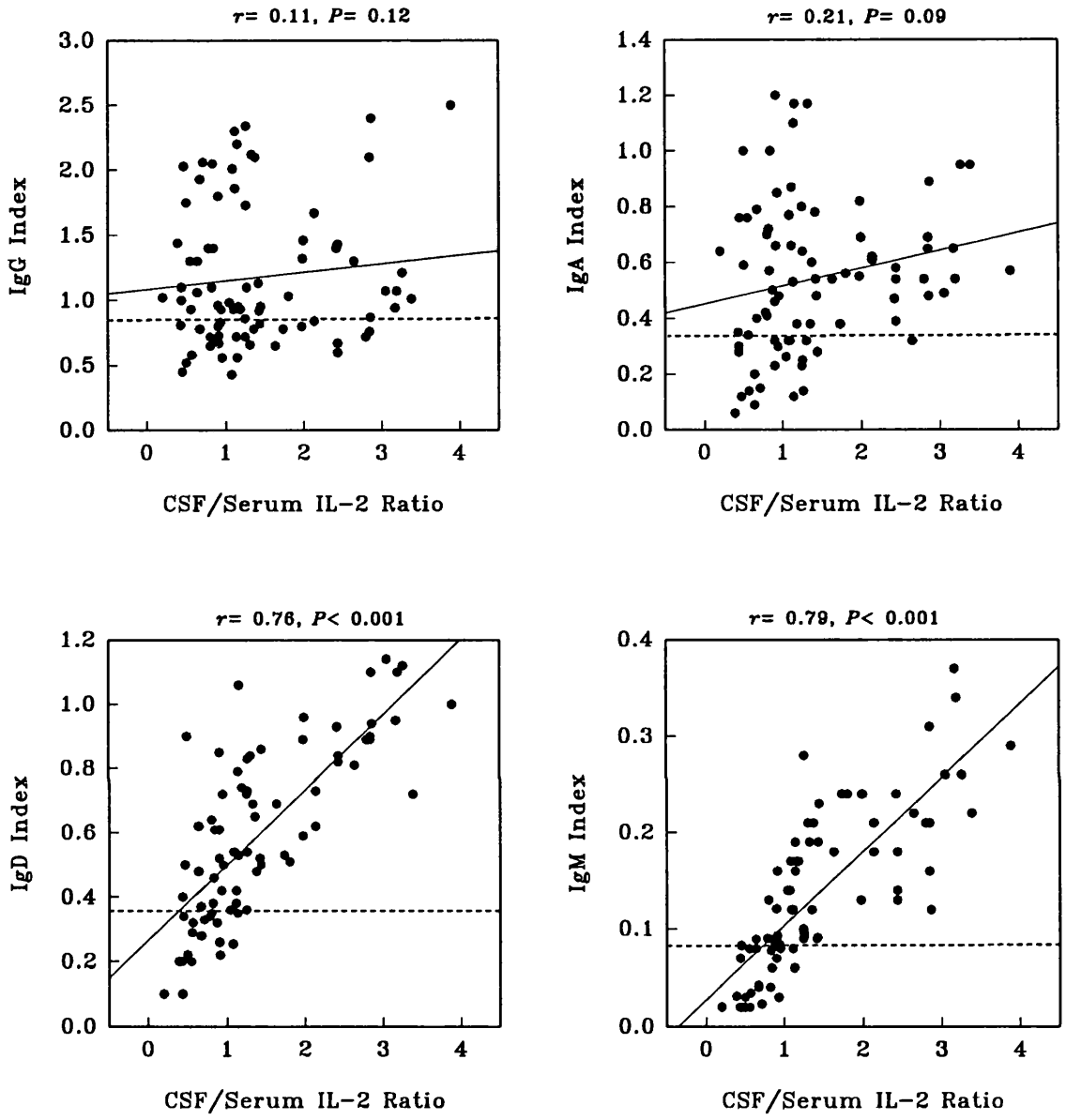


Fig. 3.5. Correlation between CSF to serum ratio of IL-2 and individual values of IgG, IgA, IgD, and IgM indices in patients with relapsing-remitting MS. Patients with undetectable IL-2 levels were excluded from this correlation. Interrupted horizontal lines represent upper range of index values in normal control subjects.

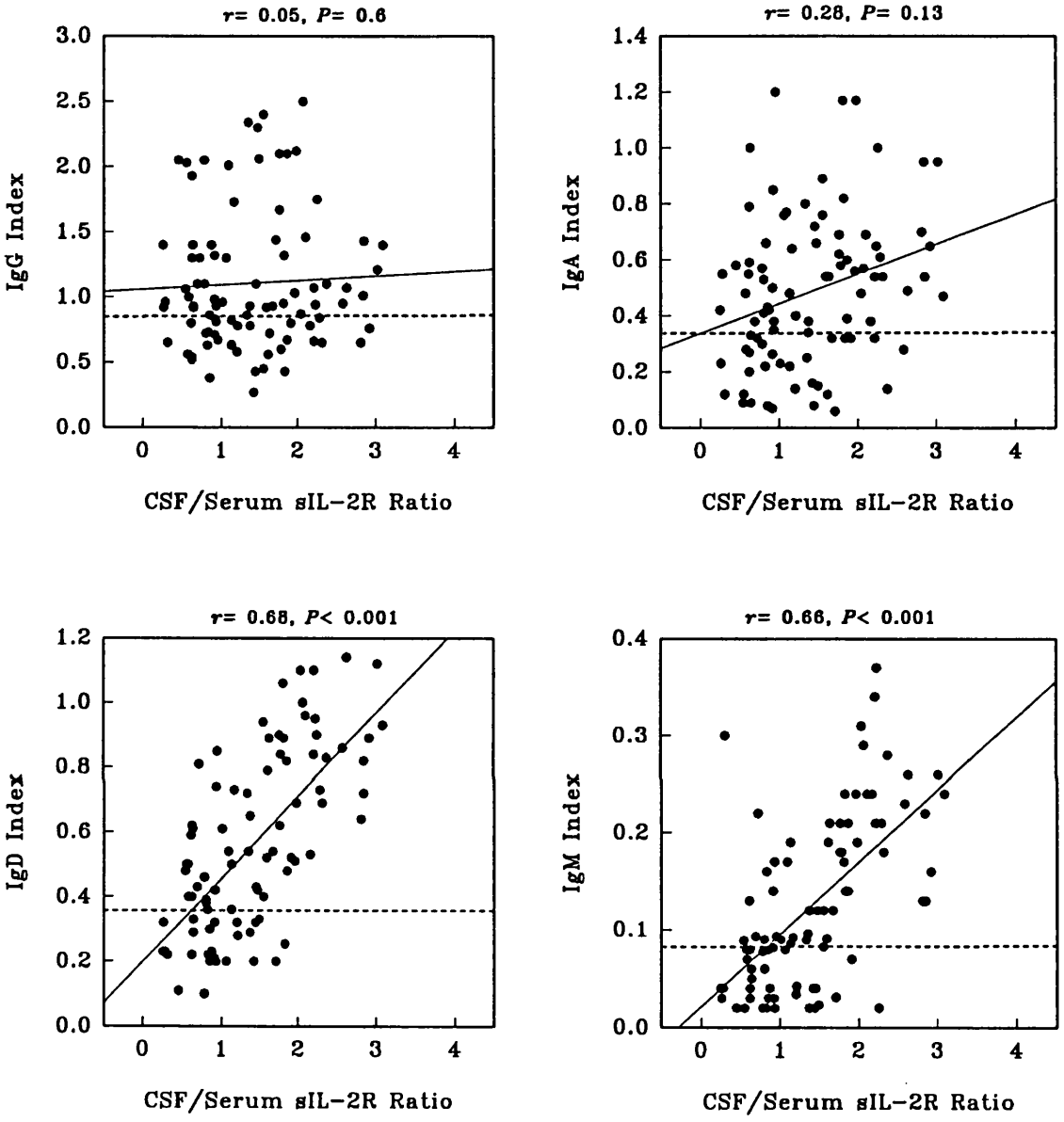


Fig. 3.6. Correlation between CSF to serum ratio of sIL-2R and individual values of IgG, IgA, IgD, and IgM indices in patients with relapsing-remitting MS. Patients with undetectable sIL-2R levels were excluded from this correlation. Interrupted horizontal lines represent upper range of index values in normal control subjects.

3.4.5 Relationship of IL-2 and sIL-2R to CSF oligoclonal bands

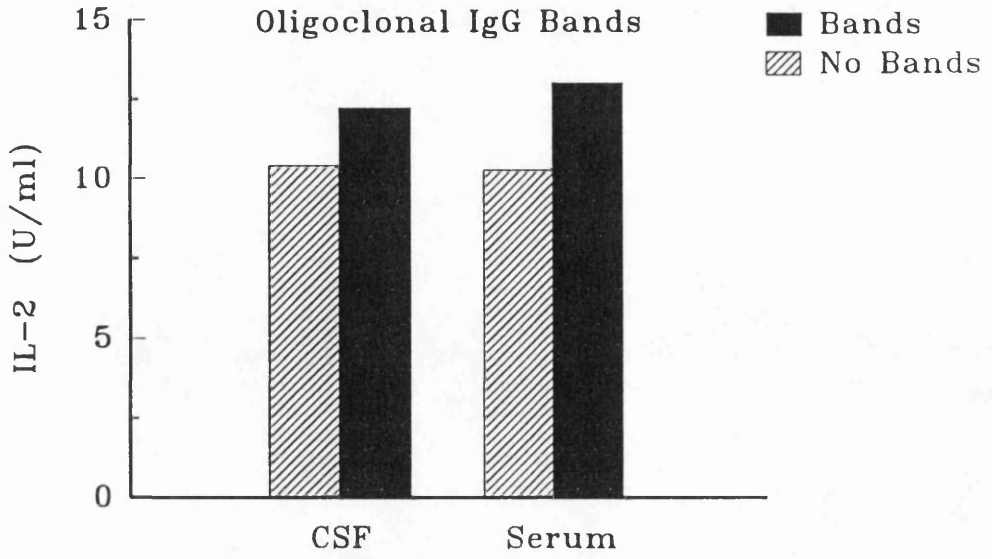
It was clear from results presented in section 3.4.4 that intrathecal levels of IL-2 and sIL-2R correlated with the quantity of intrathecally released IgD and IgM. However, previous reports have suggested that the presence of oligoclonal bands in CSF would be more reliable than Ig index in detecting intrathecal synthesis of immunoglobulins (Souverijn et al 1989; Sharief et al 1990; Souverijn et al 1991). Therefore, CSF levels of IL-2 and sIL-2R in patients with relapsing-remitting MS were related to the presence of oligoclonal bands in the CSF of these patients to corroborate the results presented in section 3.4.4. Table 3.8 illustrates the distribution of oligoclonal IgG, IgA, and IgM in patients with MS included in the study. The presence of oligoclonal IgD bands was not examined because of methodological difficulties in detecting oligoclonal IgD in unconcentrated CSF samples.

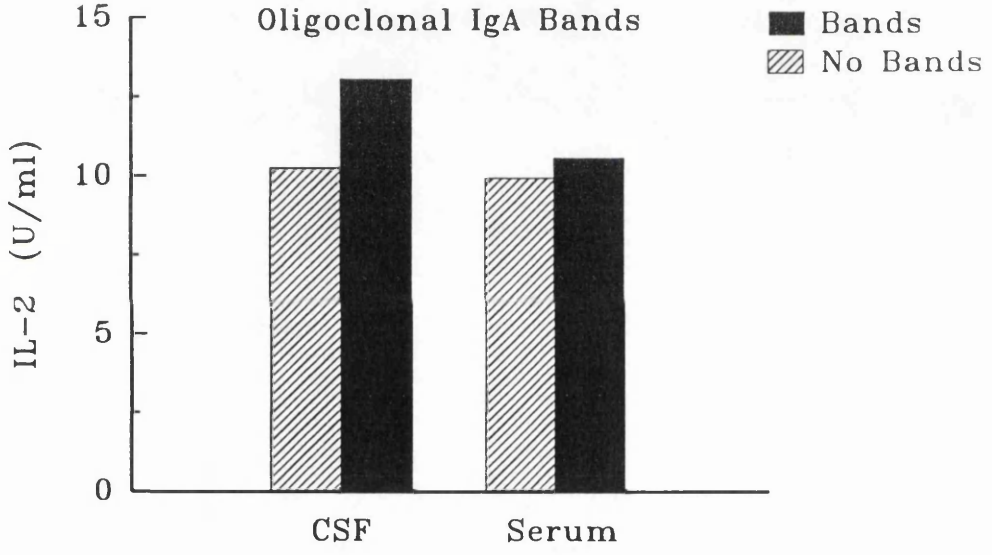
Table 3.8. Number (and %) of MS patients who had CSF oligoclonal Ig bands.

CSF Oligoclonal Bands	Multiple Sclerosis	
	Relapsing-Remitting	Chronic Progressive
IgG	107 (97%)	36 (95%)
IgA	71 (65%)	20 (53%)
IgM	69 (63%)	17 (45%)

No significant association was detected between CSF IL-2 levels in relapsing-remitting patients and the presence of oligoclonal IgG or IgA bands. However, significantly higher levels of IL-2 (mean= 14.6 U/ml) were detected in CSF of patients with oligoclonal IgM bands than in those without oligoclonal IgM bands (mean= 5.06, $P<$ 0.01). This significant relationship is depicted in Fig. 3.7. Similarly, CSF levels of sIL-2R were significantly higher in patients with oligoclonal IgM bands than in those without IgM bands (means= 183.2 U/ml vs. 68.3 U/ml, $P<$ 0.01; Fig. 3.8). Serum levels of IL-2 and sIL-2R did not correlate with the presence of oligoclonal bands in CSF of patients with relapsing-remitting MS.

Fig. 3.7. Mean levels of IL-2 in CSF and serum from patients with relapsing-remitting MS, according to the presence of oligoclonal bands in CSF. Statistical relationships are presented in the text.


Oligoclonal IgG Bands
Bands
No Bands
IL-2 (U/ml)
15
10
5
0
CSF
Serum


Oligoclonal IgA Bands
Bands
No Bands
IL-2 (U/ml)
15
10
5
0
CSF
Serum

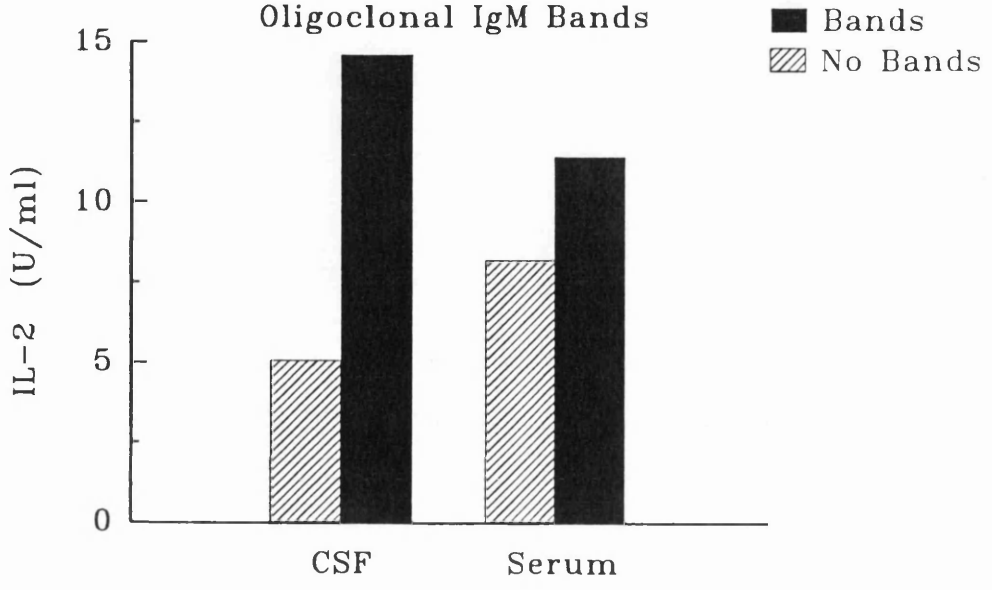
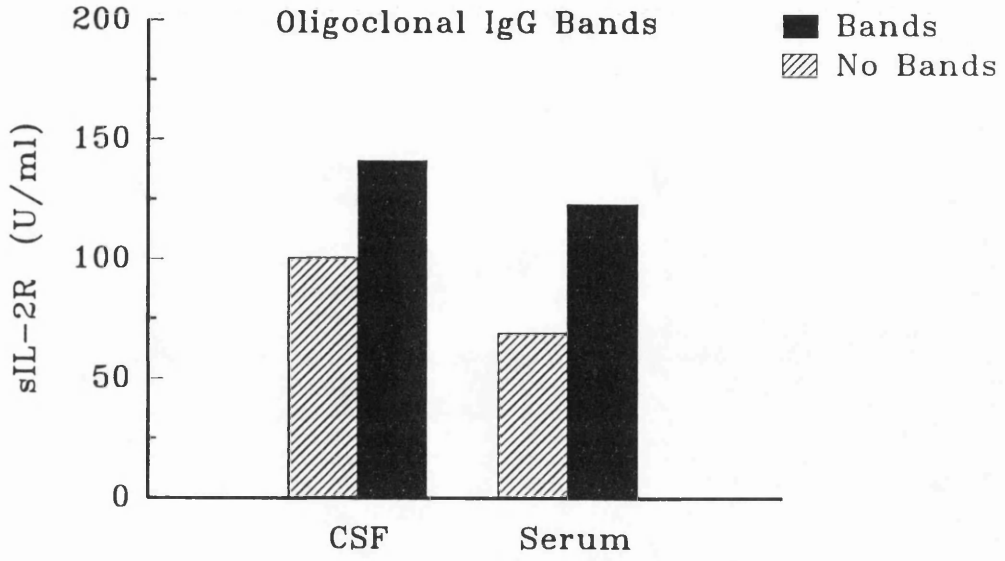
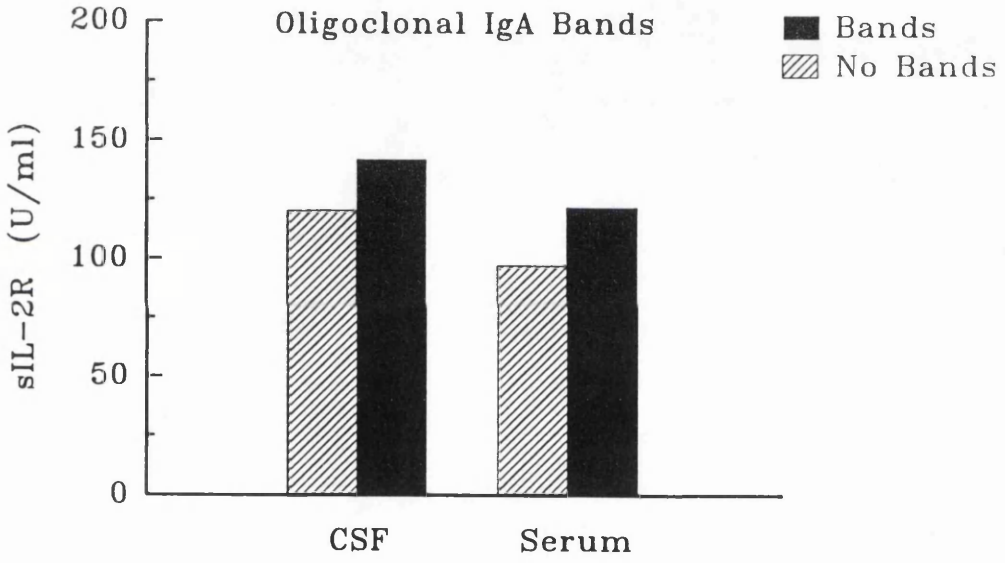
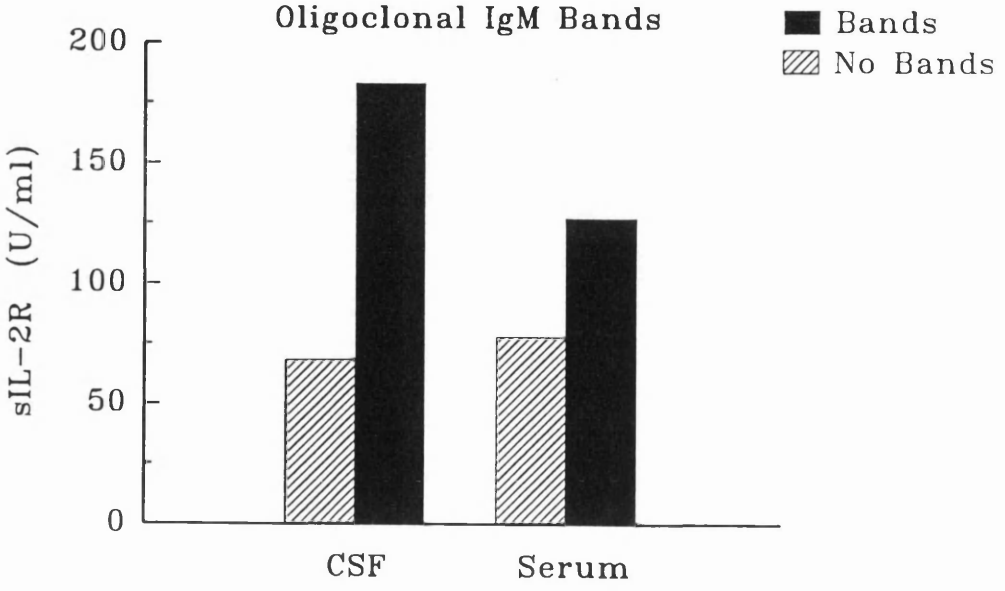

Oligoclonal IgM Bands
Bands
No Bands
IL-2 (U/ml)
15
10
5
0
CSF
Serum

Fig. 3.8. Mean levels of sIL-2R in CSF and serum from patients with relapsing-remitting MS, according to the presence of oligoclonal bands in CSF. Statistical relationships are presented in the text.


Oligoclonal IgG Bands
Bands
No Bands
sIL-2R (U/ml)
200
150
100
50
0
CSF
Serum


Oligoclonal IgA Bands
Bands
No Bands
sIL-2R (U/ml)
200
150
100
50
0
CSF
Serum


Oligoclonal IgM Bands
Bands
No Bands
sIL-2R (U/ml)
200
150
100
50
0
CSF
Serum

3.4.6 Relationship of IL-2 and sIL-2R to CSF leucocytes

The CSF leucocyte count was 0-4 cells/mm^3 in 57 patients (52%) with relapsing-remitting MS and in 20 patients (53%) with chronic progressive MS. CSF leucocytes were 5/mm^3 or more (up to 51 cells per mm^3) in 53 patients (48%) with relapsing-remitting MS and in 18 patients (47%) with chronic progressive disease. All cells were shown to be mononuclear using Rosenthal chambers. Reactive lymphocytes (up to 43/mm^3) were detected in CSF of 40 patients (36%) with relapsing-remitting MS and in CSF of 11 patients (29%) with chronic progressive disease. Such cells were present mainly in CSF samples with a pleocytosis (see Table 3.9).

The relationship of CSF leucocytes to the presence of IL-2 in patients with MS is depicted in Table 3.9. As shown in the table, no significant relationship exists between the presence of IL-2 and CSF pleocytosis or CSF reactive lymphocyte count in patients with MS. Indeed, the correlation between CSF IL-2 concentration and CSF cell count or the presence of reactive lymphocytes in relapsing-remitting MS was not statistically significant (Fig. 3.9). Similarly, no correlation was detected between CSF IL-2 concentration and CSF pleocytosis or reactive lymphocyte count in patients with chronic progressive MS.

Table 3.9. CSF leucocyte count related to the detection of IL-2 in CSF from patients with multiple sclerosis. Numbers in brackets represent patients with reactive lymphocytes.

CSF Leucocytes	IL-2 in CSF	
	Absent	Present
Relapsing-Remitting Patients[a]		
0-4 Cells/mm^3	19	38 (1)
5 or More Cells/mm^3	11 (9)	42 (30)
Chronic Progressive Patients[b]		
0-4 Cells/mm^3	17	3 (1)
5 or More Cells/mm^3	4 (8)	4 (2)

[a]*Corrected* $\chi^2 = 2.21$, $P = 0.14$
[b]*Corrected* $\chi^2 = 0.38$, $P = 0.53$

Table 3.10 illustrates the relationship between CSF cell count and the presence of sIL-2R in CSF of patients with MS. Like IL-2, the presence of sIL-2R in CSF was not related to CSF pleocytosis or the presence of reactive lymphocytes in CSF. Moreover, sIL-2R concentration in the CSF failed to correlate with CSF cells or reactive lymphocytes both in relapsing-remitting (Fig. 3.9) and chronic progressive MS.

Table 3.10. CSF leucocyte count related to the detection of sIL-2R in CSF from patients with multiple sclerosis. Numbers in brackets represent patients with reactive lymphocytes.

CSF Leucocytes	sIL-2R in CSF	
	Absent	Present
Relapsing-Remitting Patients[a]		
0-4 Cells/mm^3	16	38 (1)
5 or More Cells/mm^3	7 (4)	46 (35)
Chronic Progressive Patients[b]		
0-4 Cells/mm^3	11	9 (1)
5 or More Cells/mm^3	4 (3)	14 (7)

[a]*Corrected* $\chi^2 = 4.14$, $P = 0.07$
[b]*Corrected* $\chi^2 = 2.97$, $P = 0.09$

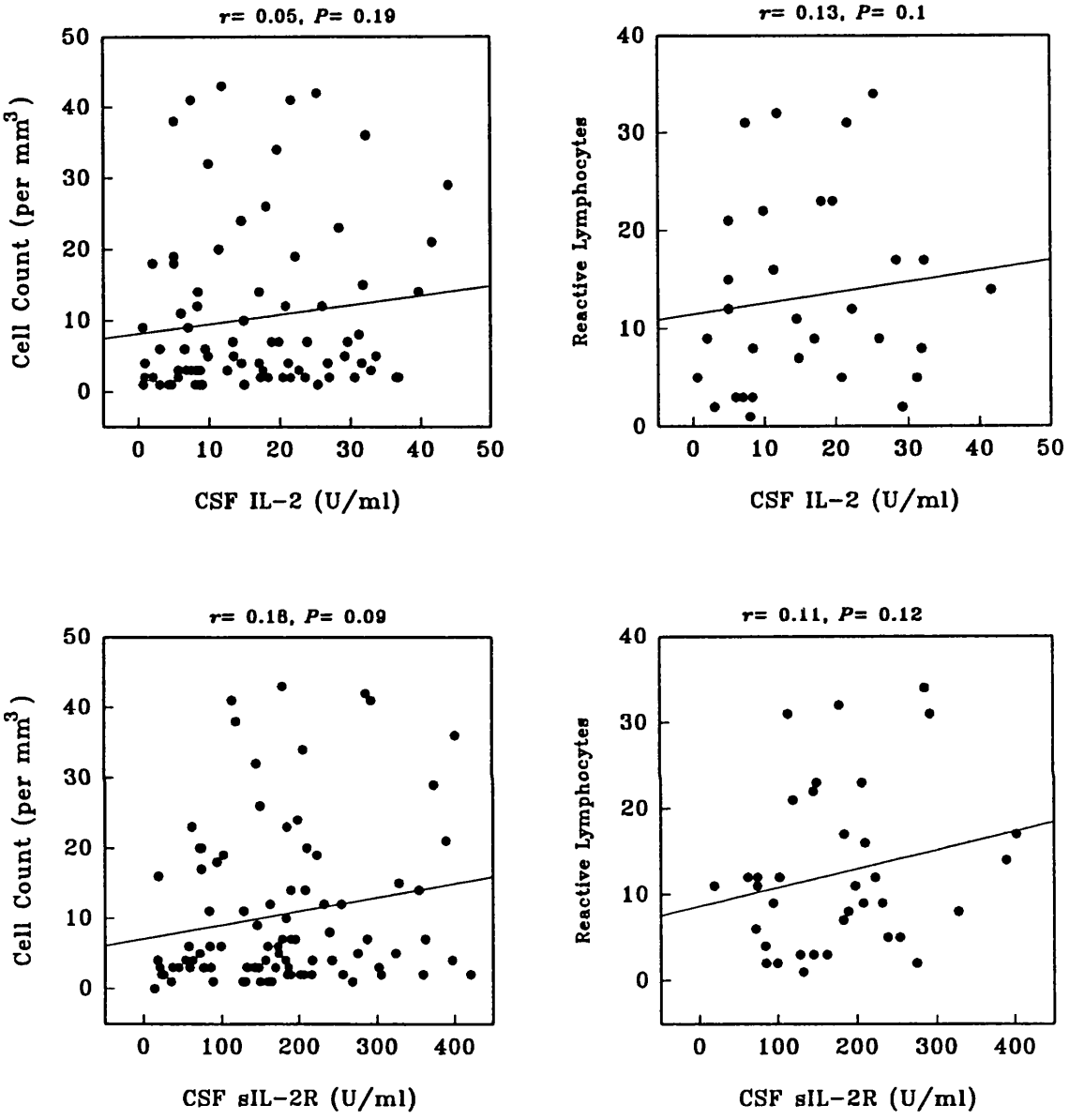


Fig. 3.9. Top. Correlation of CSF concentration of IL-2 with CSF leucocyte and CSF reactive lymphocytes in patients with relapsing-remitting MS. Patients with undetectable IL-2 in CSF were excluded from this correlation.

Bottom. Correlation of CSF concentration of sIL-2R with CSF leucocyte and CSF reactive lymphocytes in patients with relapsing-remitting MS. Patients with undetectable sIL-2R in CSF were excluded from this correlation.

3.5 Discussion

3.5.1 Levels of IL-2 and sIL-2R in MS

Increased levels of IL-2 or sIL-2R or both have been demonstrated in the serum (Selmaj et al 1986; Trotter et al 1988) and CSF (Gallo et al 1988 and 1989; Adachi et al 1989) samples of MS patients. However, wide discrepancies still exist amongst different research groups regarding the range of IL-2 and sIL-2R concentration in serum samples of MS patients (see Tables 3.2 and 3.3). It is noteworthy that all groups listed in Tables 3.2 and 3.3 have used the same commercial ELISA with remarkably differing results. Moreover, some research groups have reported totally different results in apparently the same group of patients without providing proper explanation for the discrepancy. For instance, Trotter *et al* (1988) in a preliminary report described a mean±SD serum IL-2 concentration of 42.3±97.3 U/ml in patients with MS. However, when they published the whole study (Trotter et al 1990), they reported a mean±SD serum IL-2 concentration of 84.7±144.2 without explaining the reason for this discrepancy. Therefore, it is quite difficult to compare results reported in this study with those listed in Table 3.2. It is plausible that such variation may be due to differences in patients selection.

Another explanation for the very high serum levels of IL-2 reported by some groups (e.g. Trotter *et al* 1990) could be a putative *in vitro* production of IL-2, particularly in the presence of contaminating antigens in the blood collecting equipments or storage tubes. It has recently been reported that various cytokines could be produced by blood cells within 24-36 h of storage even if kept at 4°C (Leroux-Roels et al 1990; Murch and McDonald 1990). Thus, stringent precautions were taken in this work to avoid *in vitro* production of IL-2 by (*a*) centrifugation of CSF and blood samples immediately after collection to eliminate cellular elements that could produce IL-2, (*b*) filtration of the test samples through 0.22 μm sterile filter to remove contaminating particulate materials, (*c*) addition of sodium azide to kill any remaining cells that could release IL-2, and (*d*) storage of the test samples below -70°C if immediate assay of IL-2 was not feasible. These steps may explain the relatively large difference between serum concentration of IL-2 reported here and concentrations reported by previous groups.

Some research groups have failed to detect IL-2 or sIL-2R in CSF from patients with active MS. For example, Gallo *et al* (1989) failed to detect sIL-2R activity in MS CSF, probably because of differences in patients selection or the use of less sensitive methods. The detection of high levels of sIL-2R in the CSF of MS patients reported here, however, is in full agreement with the findings of Adachi et al (1989) and Kittur et al (1990). Similarly, higher CSF levels of IL-2 compared to serum levels corroborate an earlier study by Gallo *et al* (1988) who detected IL-2 in the CSF of 43% of patients with active MS while serum IL-2 was detected in only 29%.

Serum and CSF concentrations of IL-2 and sIL-2R reported here are relatively higher than results previously published by this author (Sharief et al 1991). This difference could be due to several factors. First, samples utilised in the previous study did not contain a protease inhibitor and may have endured spontaneous IL-2 degradation. Second, commercial ELISA kits were used to detect IL-2 in the previous study whereas a more sensitive immunoassay was employed in this study. Third, differences in patients selection may have contributed to the variation in IL-2 levels as this study included 148 patients with MS while the previous study utilised 70 patients.

3.5.2 Differences between *in vivo* and *in vitro* IL-2 results

It is obvious from results of this study that MS patients demonstrate abnormally high CSF and serum IL-2 levels. Such results are in sharp contrast to the defective IL-2 production which has been reported in previous *in vitro* studies (Selmaj et al 1988, Fischer et al 1989). One hypothesis to explain this discrepancy is the "exhaustion theory" described by Huang *et al* in 1988. These authors suggested that the precommitment and preactivation of peripheral T cells *in vivo* could result in a transient exhaustion of IL-2 secretion. In support of this hypothesis, PHT responses of T cells from patients with active SLE were restored if cells were allowed to "rest" *in vitro*, a process that usually take 24-36 h.

As an alternative to the exhaustion theory, it is conceivable that production of IL-2 by a subpopulation of *in vivo*-activated lymphocytes elicits a feedback regulation pathways that attempt to restore the homeostasis of the IL-2/IL-2R system by nonspecifically suppressing IL-2 production. In support of this concept, patients

treated with high doses of IL-2 have been reported to exhibit depressed mitogen responses *in vitro*.

Regardless of why IL-2 production is reduced *in vitro*, the fact that CSF and serum IL-2 levels are elevated in MS patients suggests that extreme caution should be exercised if *in vitro* functional data are applied to any *in vivo* situation. Moreover, it has to be noted that reduced *in vitro* production of IL-2 discussed here should not be confused with the increased production of this cytokine in samples stored below 4°C mentioned above. Increased *in vitro* production is usually detected after 24-36 h of storage while the low IL-2 production observable in inflammatory diseases is noticed much earlier.

3.5.3 Intrathecal release of IL-2 and sIL-2R in MS

The findings reported here of significantly higher levels of IL-2 and sIL-2R in the CSF of MS patients relative to corresponding serum levels suggest that IL-2 and sIL-2R are locally produced within the intrathecal compartment (Boutin et al 1987; Kittur et al 1990). Some previous reports (Gallo et al 1989; Trotter et al 1990) suggested that the increased synthesis of IL-2 and sIL-2R in MS is a systemic, rather than local CNS immune response. The difference between these reports and findings reported in this study may be due to the degree of disease activity, total disease duration, degree of disability, or a combination of these or other factors in different MS groups. There are definite aberrations of intrathecal immune response in MS patients that involve both T lymphocytes (Waksman and Reynolds 1984) as well as B lymphocytes (Link et al 1987). However, the disease process is a dynamic one and MS patients are therefore, heterogeneous regarding intrathecal synthesis of immunoglobulins or other soluble products of the immune cells.

The increased local CNS synthesis of IL-2 in MS patients reported here suggests an intense activation of intrathecal immune cells. This increased local synthesis of IL-2 further extends previous reports which detected IL-2 in active plaques and white matter in MS brain (Hofman et al 1986; Cuzner et al 1988). The finding of increased sIL-2R levels in CSF of MS patients is also noteworthy because it further stresses an intrathecal immune activation in MS. High levels of sIL-2R are usually detected in conditions associated with lymphocyte activation, such as

infectious (Boutin et al 1987) or autoimmune states (Lawrence et al 1988). In fact, increased levels of sIL-2R was suggested to parallel the evolution of the demyelinating pathologic process in MS (Greenberg et al 1988).

The absence of a strong correlation between CSF lymphocytes and CSF levels of IL-2 or sIL-2R suggests that these cytokines may be derived from neural and not CSF cells. This fact is further confirmed by the lack of correlation between IL-2 or sIL-2R and the presence of reactive lymphocytes in the CSF. In the CNS, IL-2 and sIL-2R could be formed at multiple sites. IL-2 was detected in both active plaques and normal appearing white matter in MS brains (Hoffman et al 1986; Cuzner et al 1988). Similarly, IL-2R-bearing lymphocytes were found in the perivascular infiltrate in MS brain lesions (Bellamy et al 1985). Indeed, similar pattern of IL-2 production and the presence of IL-2R-expressing cells at the site of the immune response has been reported in non-neurological diseases. For example, IL-2 is produced in the salivary glands in patients with Sjögren's syndrome (Fox et al 1985), in the thyroid gland in autoimmune thyroiditis (Kroemer et al 1985), in ocular tissues in autoimmune diseases of the eye (Hook et al 1988), and in the synovial fluid of patients with rheumatoid arthritis (Lemm and warnatz 1986).

Elevated sIL-2R levels in CSF of MS patients reported here may reflect a state of ongoing lymphocyte activation, leading to commensurate induction and expression of IL-2R on inflammatory cells locally implicated in the disease. The pathogenic role of activated immune cells is substantiated by a more marked elevation of sIL-2R in CSF relative to levels in corresponding serum. As discussed in chapter 1, the physiological significance of sIL-2R is a matter of debate. sIL-2R has been speculated to function as an IL-2 antagonist by possible binding of sIL-2R to its ligands. However, the occupancy theory of receptor binding in a steady state system (Clark 1933) states that K_d= [ligand][receptor]÷[ligand bound to receptor]. Therefore, for binding of equal amounts of ligand (i.e., IL-2) to both the high- and low-affinity receptors, a ratio of 1,000 low- to high-affinity receptors would be needed. It follows that for the sIL-2R to bind sufficient IL-2 to affect appreciably the amount of IL-2 available for the binding to the high-affinity receptors, a concentration in the order of 10^4 to 10^5 fold higher than that of the high-affinity receptor would be needed. Given the observed release rates for both T and B cells (Loughnan et al 1987) and the

average serum levels of sIL-2R detected by various research groups (see Chapter 3), such a concentration within the cellular micro-environment would seem very unlikely. Moreover, as IL-2 has a very fast rate of dissociation from the low-affinity receptor, in the order of seconds (Wang and Smith 1988), it is unlikely that sequestration of IL-2 by sIL-2R could occur. Thus, it seems most unlikely that sIL-2R has an immuno-regulatory role through competition for IL-2 with the high-affinity receptor. Results from this study corroborate the hypothetical argument presented above. The elevated levels of both IL-2 and sIL-2R in patients with acute relapsing-remitting MS argue against an inhibitory role of sIL-2R. In fact, elevated sIL-2R may be secondary to an overproduction of IL-2, since this cytokine stimulates expression and secretion of its own receptor. Alternatively, high CSF levels of IL-2 and sIL-2R may reflect abnormal antigenic and/or polyclonal stimulation of CNS immune cells expressing both IL-2 and IL-2R. It has to be noted that no inhibitory role of sIL-2R was detected in this study. Irrespective of the inhibitory theory, the measurement of sIL-2R could be considered a valuable noninvasive approach in the analysis of disease-associated immune activation *in vivo*. Detection of high sIL-2R concentrations in the CSF may, therefore, provide an objective indication of ongoing immune activity within the central nervous system.

3.5.4 Relationship of IL-2 and sIL-2R to the clinical types of MS

The elevated IL-2 levels in CSF from patients with relapsing-remitting MS compared to chronic progressive disease corroborate similar results reported by Gallo et al (1989). Such finding suggests basic immunological differences between relapsing-remitting and chronic progressive MS. In fact, the two disease processes have already been reported to have differences in genetic (Olerup et al 1987), epidemiological (Larsen et al 1985), clinical (Confavreux et al 1980; Larsen et al 1985), and radiological (Hawkins et al 1991) features. Moreover, another immunological difference between the two clinical types of MS has been described by this author concerning intrathecal IgD synthesis (Sharief and Hentges 1991a). The relationship between the intrathecal levels of IL-2 and sIL-2R and that of IgD and IgM is discussed below.

The quantitative variation in the concentrations of IL-2 and sIL-2R in the two clinical groups of MS described above may have important implications in

understanding the immunopathology of MS. It has long been known that HLA histocompatibility factors play a major part in the predisposition to certain neurological diseases. It is also well-established that a clear association exists between MS and certain HLA antigens particularly MHC class II molecules (Govaerts 1985). Moreover, certain MHC molecules, such as HLA-DR2 DQw6 haplotype, are associated with susceptibility to both chronic progressive and relapsing-remitting MS, whereas the HLA-DR4 DQw8 haplotype has been associated with chronic progressive MS. Interestingly, increased IL-2 receptor affinity has been recently reported in individuals who were positive for certain MHC class II molecules (Pomier et al 1990). This finding suggests that IL-2 receptor synthesis could be modulated by MHC class II molecules and that their affinity as well as their ability to initiate proliferation driven by IL-2 is dependent on the MHC class II molecules. The difference in levels of IL-2 and sIL-2R in relapsing-remitting when compared to chronic progressive MS patients may be due to the MHC class II molecules themselves, determining the expression of IL-2, or may be due to linkage disequilibrium between HLA genes and closely located genes such as those for IL-2R.

Alternatively, IL-2 could be one of the factors that mediate the genetic predisposition of patients positive for particular MHC class II molecules to develop a relapsing-remitting disease. This author tends to prefer this theory since IL-2 was found to positively correlate with MS disease severity and relapse activity (see Chapter 6). Obviously, further studies are needed to elucidate the precise relationship between IL-2 the and genetic predisposition to MS. It will be discussed later that subsequent studies have provided crucial information on the relation of IL-2 release with the clinical type of MS (section 3.5.6).

3.5.5 Relationship of IL-2 and sIL-2R to intrathecal immunoglobulin release

Results of this study show that intrathecal IL-2 and sIL-2R are significantly related to the local CNS production of IgM and IgD. Although the *in vivo* role of IL-2 in B-cell differentiation remains unknown, these results suggest that IL-2 or sIL-2R or both may play an important role in intrathecal immunoglobulin production in MS. *In vitro*, IL-2 has been demonstrated to cause an 8-16 fold increase in the levels of mRNA for the secretory form of μ heavy chains when used on B-cells co-cultured with anti-IgM,

although high concentrations (100 U/ml) of this cytokine were required for the induction of IgM synthesis. Furthermore, IL-2 induced proliferation of anti-μ-stimulated B-cell populations (Nakagawa et al 1985). These responses were abolished when anti-Tac, which recognizes IL-2 receptor was added to the cultures.

The importance of the IL-2/IL-2 receptor system in the humoral response is further demonstrated in other reports (Depper et al 1985; Waldmann 1989) when anti-Tac antibody inhibited immunoglobulin production by B-cell activated by polyclonal activators. Because IL-2 stimulates T-cells as well as B-cells, findings reported in this study could theoretically reflect either a synthesis of T-cell derived cytokines that act on B-cells, or a direct IL-2 action on B-cells themselves, or both. Data presented in this study do not allow a clear distinction between these two possibilities, but suggest that the relationship of intrathecal synthesis of IgM to IL-2 is similar to that of IgD.

No significant correlation was detected between IL-2 or sIL-2R and the intrathecal synthesis of IgG or IgA. In B-cell ontogeny, the process of Ig class switching is important for the success of the humoral immune response. IgM is the first isotype expressed on the surface of B-cells followed by the subsequent coexpression of IgD. Immunoglobulin class expression switches during activation of B-cells from IgM to IgG, IgA, or IgE. It has been suggested that modulation of isotype pattern involves a particular subset of T helper lymphocytes (Esser and Radbruch 1990). The lack of correlation between IL-2 or sIL-2R and intrathecal synthesis of either IgG or IgA may be due to the concomitant release of other cytokines. A candidate for regulating IgG, particularly IgG2a isotype is IL-4. Interleukin-4 as well as IFN-γ have been shown to affect isotype selection in both T cell-dependent and T cell-independent systems, enhancing IgG2a production (Stevens et al 1988). Similarly, three cytokines: IL-4, IL-5 and transforming growth factor-β have been reported to enhance production of IgA isotype (Coffman et al 1988). Conversely, the role of these cytokines in enhancing secretion of IgM or IgD is still controversial (reviewed by Finkelman et al 1990). It is noteworthy that IL-2 is usually released by T_{H1} subset of T helper lymphocytes while both IL-4 and IL-5 are predominantly secreted by T_{H2} subpopulation (Mosmann and Coffman 1989).

The significant correlation between IL-2 and intrathecal release of IgM and IgD provides more insight into the ontogeny of the immune response in patients with MS. Intrathecal synthesis of IgM in MS has been associated with relatively early and active disease processes (Sharief and Thompson 1990). Similarly, recent studies have shown that intrathecal production of IgD is a putatively important component of the immune response in clinically active MS (Sharief and Hentges 1991a). Local production of immunoglobulins D and M seems to be a principal event in intrathecal humoral immune activation in active MS. Thus, IL-2 and its soluble receptor could play an active role in the regulation of intrathecal immune response in this disease and, thereby, may determine the severity as well as the outcome of the disease process.

Although the role of IL-2 in immunoglobulin production is still largely unknown, data reported here support the view that IL-2 may play a role in the differentiation of activated B-cells into immunoglobulin-secreting cells in patients with MS. Longitudinal in vivo studies of intrathecal immune response in MS are required to further elucidate the mechanisms by which IL-2 and its receptor contribute to the generation of local CNS immune response and to the control of immunoglobulin isotype selection.

3.5.6 Further studies on IL-2 and IgM or IgD synthesis

Findings reported here were submitted for publication in September 1990 (Sharief et al 1991a), when the role of IL-2 in IgM and IgD production was largely unknown. Data on the relationship between IL-2 and IgM synthesis were corroborated by a report that appeared in January 1991 (Eardley and Koshland 1991), which indicated that IL-2 is primarily involved in the synthesis of IgM. This report demonstrated that IL-2 drives antigen-activated B cells to synthesise IgM through a unique signal transduction mechanism, which involves the hydrolysis of Gly-PI molecules. B cells synthesise a Gly-PI that is sensitive to hydrolysis by PI-phospholipase C and is rapidly degraded ($\leq$ 1 min) in response to IL-2. The products of hydrolysis, myristylated diacylglycerol and PI-glycan, function as second messengers for the intracellular relay of the IL-2 signal. Finally, McFadden and Koshland reported in December 1991 that IL-2 delivers a signal that induces the cells to transcribe the gene for the pentamer IgM joining component, the J chain, which is required for the

assembly and secretion of IgM. They identified two lymphokine-responsive elements in J-chain gene promoter, which provide the final protein component necessary for the assembly and secretion of pentameric IgM antibody.

Data reported here were also corroborated by a further study that appeared in September 1991 (Splawski and Lipsky 1991), which demonstrated that IL-2 appears to be essential in promoting immunoglobulins, particularly IgM, production. The suggestion that IL-2 may produce some of its effects on B cells through the release of other cytokines such as IL-4 (see section 3.5.5) has also been confirmed by this report (Splawski and Lipsky 1991), which indicated that the IL-4-induced secretion of immunoglobulins, notably IgG and IgA, was dependent on the presence of IL-2. Finally, data on IL-2 role in IgD secretion were corroborated by a novel study, which was published in October 1991 (Svetić et al 1991).

The dichotomy of IL-2 profile between relapsing-remitting and chronic progressive MS may have important implications in understanding the pathogenesis of the disease process. When this dichotomy was first published in February 1991 (Sharief et al 1991a), its clinical significance was not entirely clear. However, a subsequent report that appeared in *Science* in October 1991 provided a better understanding of the relation between IL-2 synthesis and separate clinical presentations of a given disease process. Yamamura and others (1991) have reported that lesions of tuberculoid leprosy, which is usually a self-limiting form of leprosy, have high percentage of IL-2 and IFN-γ. In contrast, the chronic progressive type of leprosy (i.e. lepromatous form) had abundant mRNAs coding for IL-4, IL-5, and IL-10, while mRNA coding for IL-2 was conspicuously absent. Although it is difficult to differentiate whether this cytokine pattern is the cause or result of leprosy, such pattern is likely to contribute to the pathogenesis of other chronic diseases of man. Further longitudinal studies should define the dynamics of IL-2 patterns in the pathogenesis of some chronic human diseases.

CHAPTER 4. THE PREDICTIVE VALUE OF IL-2 AND sIL-2R FOR THE DEVELOPMENT OF MS

4.1 Introduction

Having established that IL-2 and sIL-2R are involved in the early intrathecal immune response in MS (see Chapter 3), the next objective of this study was to determine whether these cytokines could predict eventual progression to MS in patients presenting with acute monosymptomatic syndromes. Isolated lesions of the brainstem or spinal cord are frequently seen in patients with MS, and may be the presenting feature in about one third of them (Mathews 1985). From a clinical point of view, particularly for management purposes, it would be helpful if the future development of MS could be predicted during the early stages of the disease process.

As stated in the previous chapter, MS has definite aberrations of the immune system, which include an exaggerated intrathecal synthesis of IL-2 and sIL-2R as well as increased intrathecal production of various Ig isotypes (Thompson 1977; Thompson 1988). Therefore, the determination of CSF immunoglobulin changes should be included in any study that analyses the early risk of MS. The evaluation of intrathecal IgM synthesis, in particular, has a considerable clinical relevance in monitoring recent immunological stimulation in MS (Sharief and Thompson 1989).

A recent combined clinical, CSF oligoclonal IgG bands and magnetic resonance imaging (MRI) study (Miller et al 1989) reported that progression to MS occurs in about half of patients who presented with isolated brainstem or spinal cord lesion. However, the predictive value of intrathecal synthesis of IL-2, sIL-2R, and IgM for the subsequent development of MS was unknown when this study was conducted.

This chapter presents data obtained from a 30-month prospective study to determine whether the presence of IL-2 and sIL-2R in CSF of patients presenting with acute monosymptomatic lesions of the brainstem or the spinal cord is a prognostic indicator for the development of MS. Data concerning IL-2 and sIL-2R are compared with the predictive value of other CSF features and magnetic resonance imaging at presentation for subsequent development of MS.

4.2 Patients

Paired CSF and serum samples were obtained from 60 patients who presented with clinically monofocal acute lesion of either the brainstem or the spinal cord. The study was restricted to patients below the age of 50 years who exhibited unequivocal objective evidence of unifocal white matter disease and who had adequate follow-up for at least 30 months. Patients with evidence of vascular diseases were excluded. Nine patients did not fulfil such criteria and were excluded from the study. Four patients were dropped from the study because a non-MS cause of their presenting symptoms was determined within 6 months of follow-up. Another 2 patients became claustrophobic and were unable to continue the MRI follow-up. No patients had received immunosuppressive or glucocorticoid treatment within the study period.

The clinical features of the study population are shown in Table 4.1. Twenty patients had isolated brainstem lesions and complained of diplopia, ataxia, or vertigo. Clinical signs included nystagmus, various gaze palsies and trigeminal and facial nerve involvement with concomitant long tract signs. The 25 patients who presented with clinically isolated acute spinal cord lesions developed maximal neurologic deficit within 2 weeks of the onset of symptoms. All underwent myelography to exclude compressive lesion and no patient was suffering from transverse myelitis.

Table 4.1. Clinical features of the study population.

Characteristics	
Total Number (females)	45 (28) Patients
Mean age ± SD	37.3 ± 8.2 years
Age range	19 to 47 years
Isolated Brainstem Lesions	20 Patients
Isolated Spinal Cord Lesions	25 Patients

On initial presentation, full history was documented and findings of neurological examination were recorded in the patient's clinical notes. Clinical and MRI follow-up data were obtained during subsequent assessments at regular intervals. Multiple sclerosis was diagnosed at follow-up according to the criteria of Poser *et al* (1983).

Clinically definite MS requires evidence of further lesions involving different parts of the CNS after an interval of at least 1 month. Paraclinical evidence of new CNS lesions allows the diagnosis of probable MS. Cerebrospinal fluid data were evaluated blind and patients were divided into two main groups according to the presence of IL-2 in the CSF. Clinical, pathological and imaging features in patients with detectable IL-2 in the CSF and in those who had no detectable IL-2 concentrations in CSF were then compared to test the hypothesis that intrathecal IL-2 synthesis predicts progression to MS. Similar comparisons were performed in analysing the predictive values of sIL-2R, oligoclonal IgG and IgM, and MRI of the brain.

4.3 Methods

4.3.1 Cerebrospinal fluid studies

Cerebrospinal fluid was obtained during the acute episode within 7 days of the MRI study. Matching serum samples were obtained at the same time of CSF collection. Samples form 42 patients were treated with the protease inhibitor (as described in section 2.3.1), while samples from the remaining 3 patients did not contain a protease inhibitor. All samples were stored in -70°C and thawed just before analysis.

Assays of immunoglobulins G, A, D, and M in the CSF and serum samples were carried out as described in section 3.3.2. The extent of intrathecal synthesis of these immunoglobulins was evaluated through the calculation of Ig index as explained above. The detection of oligoclonal IgG, IgA, and IgM bands in test samples was carried out as described in section 3.3.3. IgG bands were detected by agarose isoelectric focusing, while IgA and IgM bands were detected by agarose gel electrophoresis. Control negative and positive samples were included in each batch of electrophoretic or isoelectric focusing procedure to achieve quality control. In the present study, the presence of two or more separate IgA and IgM bands in the CSF that have no counterpart in homologous serum was regarded as evidence of intrathecal immunoglobulin production (Sharief et al 1989 and 1990). Due to the higher resolution power of isoelectric focusing method, intrathecal IgG synthesis required the presence of four or more bands in the CSF that had no counterpart in corresponding serum.

4.3.2 Neuroimaging

All patients underwent MRI studies of the brain and spinal cord at presentation as part of the routine diagnostic investigations, and they also had a follow-up scans 4-19 months after the initial presentation. MRI was performed on a Picker 0.27 T superconducting imager. A spin-echo sequence ($SE_{2000/60}$) with 5-10 mm thick contiguous slices was obtained in all cases. A pulse repetition rate (TR) of 1.5 sec. was used with an echo delay time of 80 ms, producing a relatively T_2-weighted scan to detect any pathology. A second sequence was performed with a TR of 0.5 sec. and an echo delay time of 40 ms, producing a T_1-weighted scan to depict the anatomy.

The scans were reviewed by neuro-radiologists who reported the number, anatomical location, and extent of MRI lesions blind to the CSF data. The presence of three or more asymmetrical cerebral white matter (i.e. multifocal) lesions, at least one of which is periventricular in location, was considered strongly suggestive of MS. Lesions accounting for the presenting syndrome, if detected by MRI, were excluded from the analysis of MRI findings.

4.3.3 Statistical analyses

The significance of differences between patients who had detectable IL-2 in CSF and those who did not was compared by χ^2 analysis combined with Yates' continuity correction. The predictive values of IL-2, sIL-2R, oligoclonal bands and MRI for progression to MS were calculated by the multivariate technique of discriminant analysis (Goldstein and Dillion 1978), using SPSS/PC$^+$ software. The probability that a patient belongs to the MS group at follow-up (Predictive Value) was estimated according to Bayes' theorem (Hische et al 1982) using the formula:

$$\text{Relative Predictive Value} = \frac{p}{p + (1 - p)/C} \times 100$$

where p, "prior probability", is estimated from observed proportion of patients who subsequently develop MS, and C, "conditional probability", is calculated from the assumption that a patient belongs to MS group and the probability of the observed score given membership in the group is estimated. The obsrved significance level (i.e., P) will be rounded to decimal figures throughout the text.

4.4 Results

4.4.1 Distribution of IL-2 and sIL-2R at presentation

High IL-2 levels were detected in the CSF of 24 patients (53%) and in the serum of 32 patients (71%) (Fig. 4.1). No IL-2 concentration was detected in the paired samples that did not contain the protease inhibitor. Abnormally high sIL-2R concentrations were detected in the CSF of 31 patients (69%) and in the serum of 36 patients (80%) (Fig. 4.2). No significant relationship was detected between patients age or sex and the presence of IL-2 or sIL-2R in CSF (Table 4.2). Similarly, CSF distribution of IL-2 and sIL-2R was relatively similar in patients who presented with acute brainstem lesions and those who presented with acute spinal cord lesions (Table 4.2).

Table 4.2. Clinical features in patients with isolated syndromes related to the presence of IL-2 and sIL-2R in the CSF at presentation.

Feature at Presentation	CSF IL-2		CSF sIL-2R	
	Absent	Present	Normal	High
Total Number (females)	21 (14)	24 (14)	14 (10)	31 (18)
Mean age ± SD (years)	33.9±7.5	33.6±6.4	36.4±6.9	32.6±2.1
Isolated Brainstem Lesions	10	10	6	14
Isolated Spinal Cord Lesions	11	14	8	17

The relationship between IL-2 or sIL-2R and other CSF features at presentation is shown in Table 4.3. CSF concentrations of IL-2 and sIL-2R significantly correlated with IgM index and with the presence of oligoclonal IgM in CSF at presentation. Similarly, significant correlation existed between CSF IL-2 and sIL-2R at presentation (r= 0.86, P< 0.001). However, no correlation was detected between CSF concentrations of IL-2 or sIL-2R at presentation and corresponding serum levels (r= 0.38, P= 0.08 and r= 0.45, P= 0.06 respectively).

Fig. 4.1. Distribution of IL-2 in CSF and serum samples from patients with acute isolated syndromes of brainstem (**A**) and spinal cord (**B**) at presentation. The figure does not include 4 patients with acute syndromes of brainstem and 5 with acute syndrome of spinal cord who had no detectable IL-2 in either CSF or serum samples. A patient who had a serum IL-2 concentration of 41 U/ml is indicated. Upper range of serum IL-2 in normal controls subjects is 3.1 U/ml.

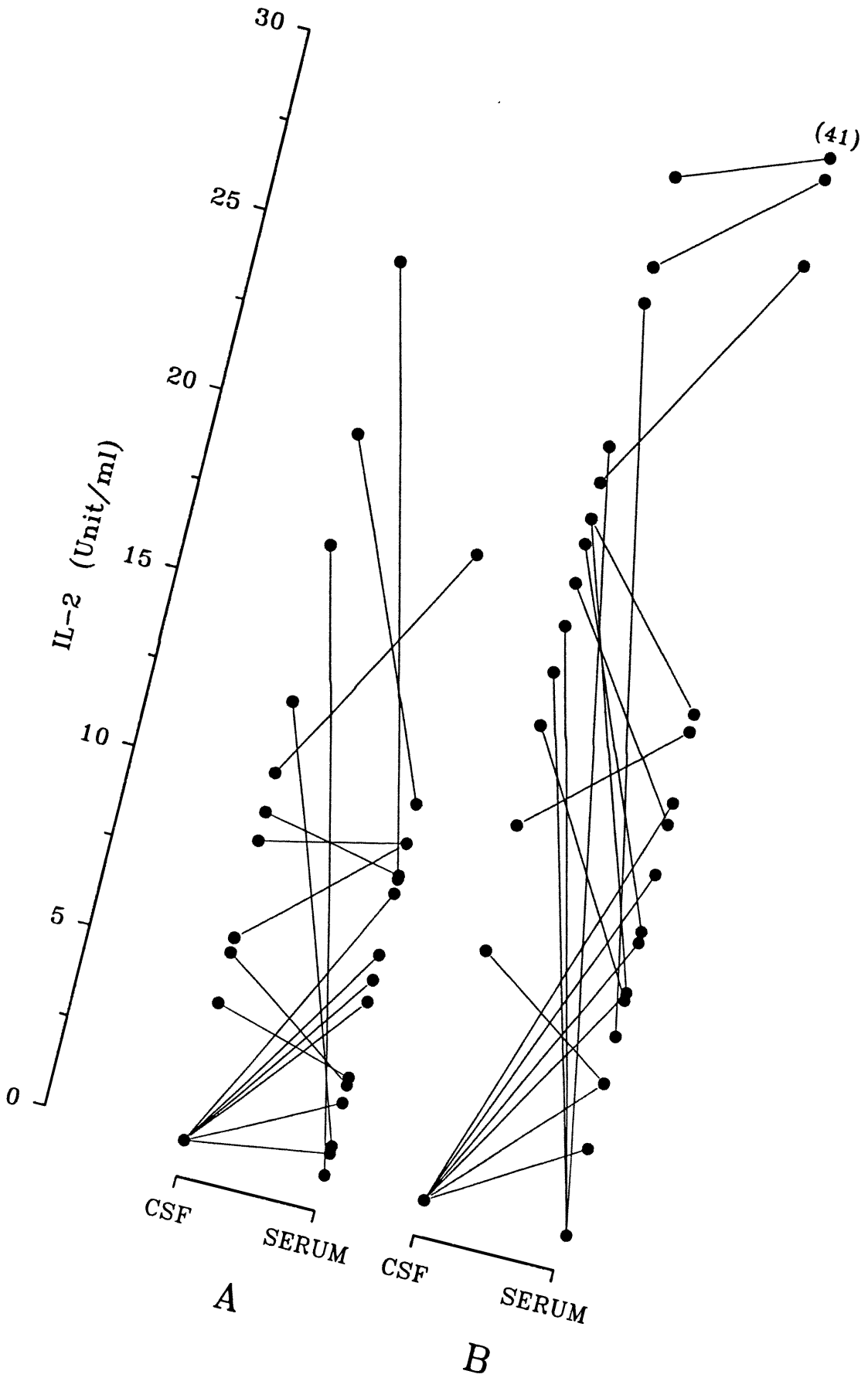

30
25
20
15
10
5
0
IL-2 (Unit/ml)
(41)
CSF
SERUM
A
CSF
SERUM
B

Fig. 4.2. Distribution of soluble IL-2 receptor (sIL-2R) in CSF and serum samples from patients with acute isolated syndromes of brainstem (**A**) and spinal cord (**B**) at presentation. Two patients (one with acute syndrome of brainstem and one with acute syndrome of spinal cord) who had no detectable IL-2 in either CSF or serum samples are not included. Interrupted line represents upper range of CSF sIL-2R in normal control subjects. The upper normal range of serum sIL-2R is 130 U/ml.

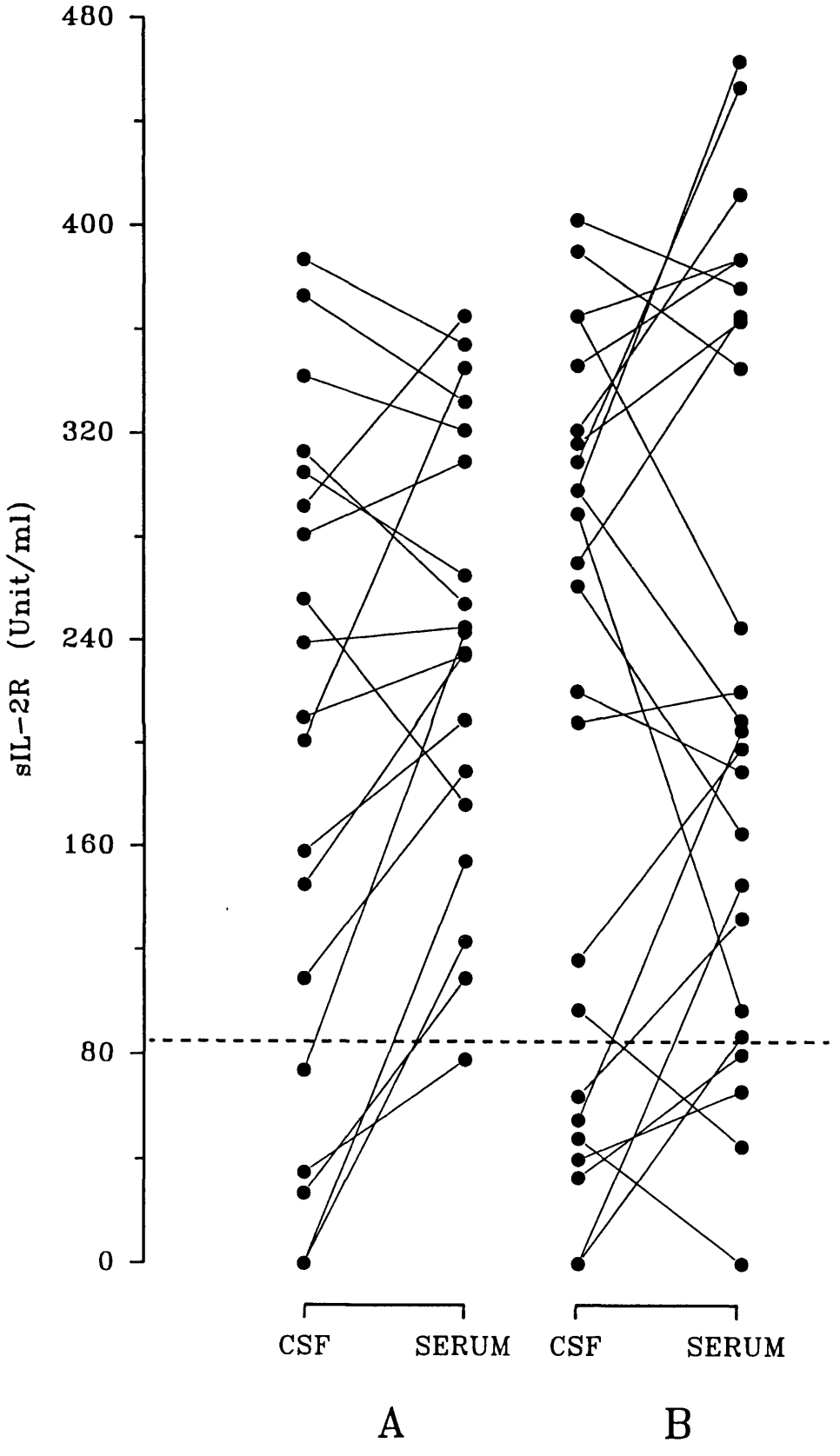

480
400
320
240
160
80
0
sIL-2R (Unit/ml)
CSF
SERUM
CSF
SERUM
A
B

Table 4.3. Cerebrospinal fluid features in patients with isolated syndromes related to the presence of IL-2 and sIL-2R in CSF at presentation.

Feature at Presentation	CSF IL-2		CSF sIL-2R	
	Absent	Present	Normal	High
mean±SD IgG Index (No. with High value)	0.85±0.4 (9)	0.88±0.4 (12)	0.84±0.4 (6)	0.88±0.4 (15)
mean±SD IgA Index (No. with High value)	0.42±0.2 (8)	0.37± 0.3 (8)	0.46±0.3 (7)	0.36±0.3 (9)
mean±SD IgD Index (No. with high value)	0.31±0.2 (3)	0.36±0.3 (7)	0.35±0.2 (3)	0.34±0.3 (7)
mean±SD IgM Index (No. with high value)	0.06±0.03 (8)	0.09±0.02 (17)	0.06±0.03 (6)	0.09±0.03 (19)
No. of positive CSF IgG OCB	4	18	3	19
No. of positive CSF IgA OCB	7	7	6	8
No. of positive CSF IgM OCB	3	23	1	25
mean±SD CSF Leucocyte Count (cells/mm^3)	3.1±1.6	2.9±1.2	3.0±1.3	3.0±1.5

The patients included in this study were followed-up for 30 months to determine the outcome of their initial illness. According to the diagnostic criteria of MS (poser et al 1983), 22 patients (49%) developed clinically definite MS or laboratory-supported definite MS. No significant relationship existed between the development of MS and Ig indices, CSF pleocytosis, or the presence of oligoclonal IgA bands at presentation. Therefore, these parameters will not be further analysed in this chapter. Data analysis in this chapter will concentrate on the predictive values of IL-2, sIL-2R, MRI, and oligoclonal IgM and IgG bands for subsequent development of MS. Results of the 30-months follow-up study are reported herein.

4.4.2 Interleukin-2 and progression to MS

As judged from both clinical and MRI criteria, 22 (92%) of the 24 patients who had detectable IL-2 in CSF at the original episode progressed to MS after a mean interval of 12.8 months (Table 4.4). It is noteworthy that no patient negative for CSF IL-2 at presentation progressed to MS within a follow-up period of 30 months. Ten patients who progressed to MS presented initially with an isolated brainstem syndrome, whereas the remaining patients had a spinal cord syndrome on presentation.

Two patients with acute isolated spinal cord lesions who did not have detectable IL-2 in CSF at presentation developed intrinsic spinal cord tumours after a mean follow-up interval of 25 months. CSF levels of sIL-2R were within normal limits in these 2 patients. Another patient who had no detectable IL-2 or sIL-2R in CSF at presentation developed a pontine lymphoma after 28 months of follow-up.

Table 4.4. Number of patients who progressed to MS at follow-up related to the presence of IL-2 in CSF at presentation.

Outcome at Follow-up	CSF IL-2 at Presentation	
	Absent	Present
Multiple Sclerosis	0	22
No Progression to Multiple Sclerosis	21	2

Corrected $\chi^2 = 34.1$, $P < 0.0001$

As already demonstrated in Fig 4.1, detectable concentrations of IL-2 were present in the serum of 32 patients at presentation. Of those 32 patients, 5 had a serum concentration of IL-2 within the normal range (i.e., below 3.1 U/ml). The presence of abnormally high IL-2 concentration in serum was not significantly related to the outcome after 30 months of follow-up (Table 4.5). As shown in the table, of the 19 patients who did not show abnormally high serum levels of IL-2 at presentation, 6 (32%) developed MS at follow-up.

Table 4.5. Number of patients who progressed to MS at follow-up related to the presence of IL-2 in serum at presentation.

Outcome at Follow-up	Serum IL-2 at Presentation	
	Normal	Abnormal
Multiple Sclerosis	6	16
No Progression to Multiple Sclerosis	13	10

Corrected $\chi^2 = 2.8$, $P = 0.09$

After a mean period of 13.6 months, 6 of the 10 patients who presented with an isolated brainstem syndrome developed clinical relapses outside the brainstem, and were therefore classified as clinically definite MS; another 2 had a brainstem relapse; and MRI follow-up revealed new cerebral white matter lesions in the remaining 2 patients. Similarly, 7 patients who had presented with an isolated spinal cord syndrome had clinical relapses outside the spinal cord within a mean interval of 15.2 months (i.e. clinically definite MS). Three other patients developed new relapses in the spinal cord at a level different from the presenting lesions and 2 patients had new cerebral white matter lesions after a mean MRI follow-up period of 14.5 months.

4.4.3 Correlation between IL-2 and MRI findings

The relationship between IL-2 and MRI findings at presentation is depicted in Table 4.6. A significant relationship was detected between CSF IL-2 and the presence of MRI-detected cerebral white matter lesions at presentation. In contrast, serum IL-2 levels at presentation were not significantly related to the MRI findings (Table 4.6). The significant relationship between CSF IL-2 and MRI findings was analysed further. In patients with detectable IL-2 in CSF, individual IL-2 levels positively correlated with the number of MRI-detected cerebral white matter lesions at presentation (Fig. 4.3), whereas the number of cerebral lesions did not correlate with serum IL-2 levels. In patients who progressed to develop MS at follow-up, a significant correlation existed between CSF IL-2 levels at presentation and the time interval required for the development of MS (Fig. 4.3). As depicted in the figure, patients who had higher concentrations of IL-2 in CSF at presentation developed MS earlier than patients who had relatively lower CSF concentrations at presentation.

Table 4.6. Relationship between IL-2 levels and MRI findings at presentation in patients with acute isolated syndromes of the brainstem and spinal cord.

IL-2 Levels at Presentation	MRI findings at Presentation	
	Negative	Positive
In CSF[a]		
Absent	14	7
Present	7	17
In serum[b]		
Normal	10	9
High	11	15

Note. Normal IL-2 concentration in serum is < 3.1 U/ml.

[a]*Corrected* $\chi^2 = 4.9$, $P < 0.02$
[b]*Corrected* $\chi^2 = 0.2$, $P = 0.71$

4.4.4 Soluble IL-2 receptor and the risk of MS

As judged from both clinical and MRI criteria, 22 (71%) of the 31 patients with abnormally high sIL-2R in CSF at the original episode progressed to MS after a mean interval of 12.8 months (Table 4.7). Similar to the findings with CSF IL-2, no patient who had a CSF concentration of sIL-2R within the normal range at presentation progressed to MS within a follow-up period of 30 months. Interestingly, high sIL-2R levels in the serum at presentation carried a significant, albeit small, risk of developing MS during the follow-up period (Table 4.7). The mean±SD CSF sIL-2R at presentation in patients who progressed to develop MS was 318.2±48.5 U/ml, while mean±SD CSF sIL-2R in patients who did not develop MS was 80.5±77.9 U/ml. The mean±SD initial serum concentration of sIL-2R in patients who developed MS at follow-up was 318±68 U/ml, whereas the mean±SD serum sIL-2R concentration in those who did not progress to MS was 142±81 U/ml.

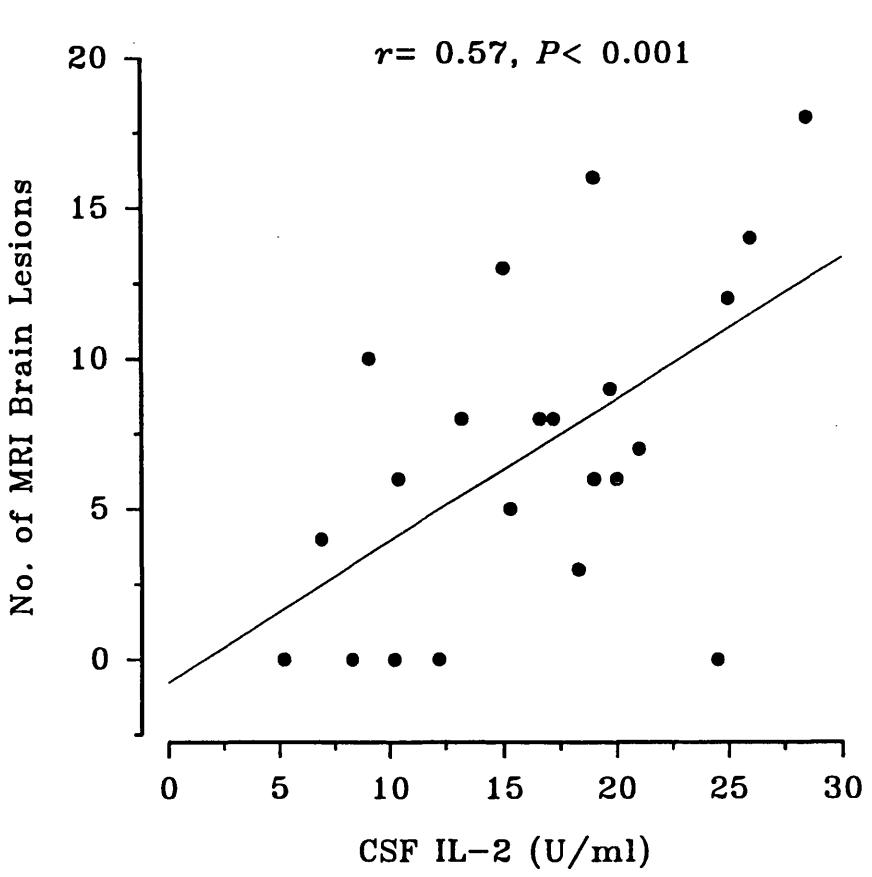
Table 4.7. Number of patients who progressed to MS at follow-up related to the presence of sIL-2R at presentation. Normal CSF level of sIL-2R is usually below 82 U/ml, and normal serum level of IL-2 is below 130 U/ml.

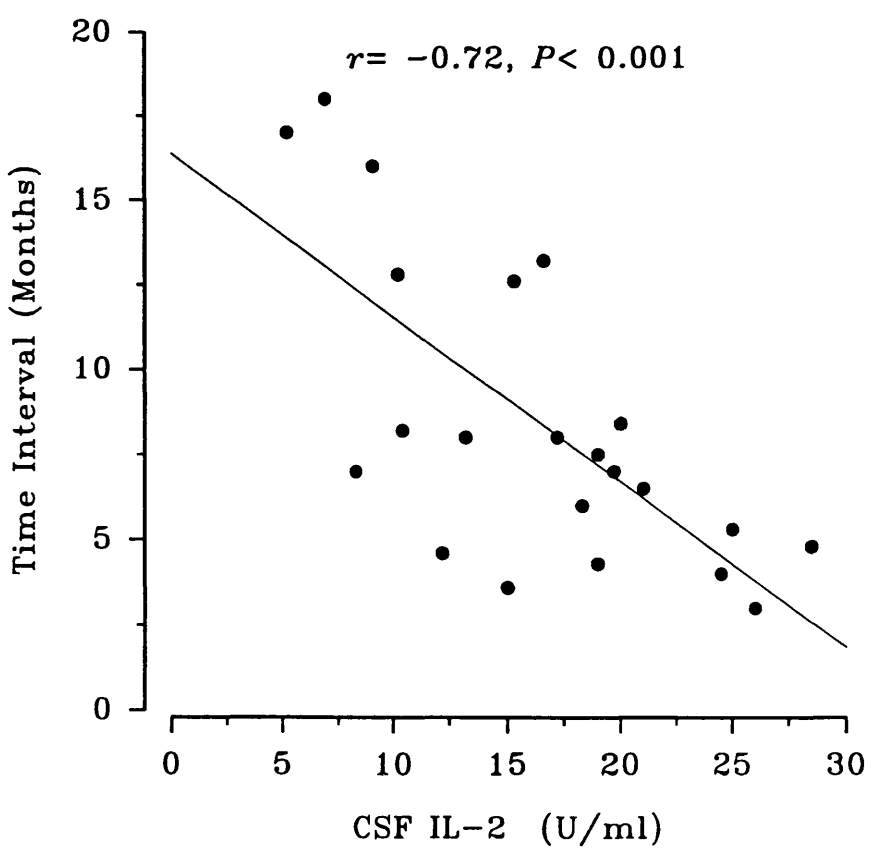
Soluble IL-2 Receptor at Presentation	Outcome after 30 months follow-up	
	Multiple Sclerosis	No Progression to Multiple Sclerosis
In CSF[a]		
Normal	0	14
High	22	9
In serum[b]		
Normal	1	8
High	21	15

[a]*Corrected* χ^2= *16.7, P< 0.0001*
[b]*Corrected* χ^2= *4.1, P= 0.041*

Fig. 4.3. **A.** Correlation between CSF IL-2 and MRI-detected cerebral lesions at presentation in patients with acute isolated brainstem or spinal cord syndromes. Patients who had no detectable CSF IL-2 at presentation are not included.

B. Correlation of CSF IL-2 at presentation with the time interval between presentation and development of multiple sclerosis. Patients who had no detectable CSF IL-2 at presentation are not included.


A
r= 0.57, P< 0.001
No. of MRI Brain Lesions
20
15
10
5
0
0
5
10
15
20
25
30
CSF IL-2 (U/ml)


B
r= -0.72, P< 0.001
Time Interval (Months)
20
15
10
5
0
0
5
10
15
20
25
30
CSF IL-2 (U/ml)

Ten patients who progressed to MS presented initially with an isolated brainstem syndrome, whereas the remaining patients had an isolated spinal cord syndrome on presentation. High levels of sIL-2R in CSF were not significantly related to MRI-detected cerebral white matter lesions at presentation (Table 4.8). Similarly, high serum levels of sIL-2R were not related to MRI findings at presentation (χ^2= 1.61, *P*= 0.21). The relationship between sIL-2R levels and the presence of MRI lesion at presentation was further analysed by *Student's t-test*. The mean±SD concentration of CSF sIL-2R at presentation in patients who were MRI-positive was 241.6±139.1 U/ml, while mean±SD of CSF sIL-2R in MRI-negative patients was 145.5±116.1 U/ml, *P*< 0.02. In patients who progressed to MS at follow-up, high levels of sIL-2R in CSF or in serum were not significantly related to the time interval between presentation and development of MS.

Table 4.8. Relationship between CSF levels of sIL-2R and MRI findings at presentation in patients with acute isolated syndromes of the brainstem and spinal cord.

CSF sIL-2R Levels	MRI findings at Presentation	
	Negative	Positive
Normal	8	6
High	13	18

NOTE. Normal sIL-2R concentration in CSF is 82 U/ml.
[a]*Corrected* χ^2= *4.9, P< 0.02*

4.4.5 Oligoclonal IgM and progression to MS

Using both clinical and MRI criteria, 22 (85%) of the 26 patients who had oligoclonal IgM bands at the original episode progressed to MS after a mean interval of 12.8 months (Table 4.9). No patient negative for oligoclonal IgM at presentation progressed to MS within a mean follow-up period of 18.3 months. A significant relationship existed between oligoclonal IgM bands and CSF IL-2 similar to that detected in patients with clinically definite MS (reported in Chapter 3). The mean±SD

concentration of IL-2 in CSF of oligoclonal IgM-positive patients was 14.1±8.2 U/ml compared to a mean±SD CSF IL-2 concentration of 1.2±0.9 U/ml in oligoclonal IgM-negative patients ($P< 0.001$).

A significant correlation was also detected between oligoclonal IgM bands and CSF concentrations of sIL-2R at presentation in patients with acute isolated syndromes of brainstem and spinal cord. The mean±SD concentration of sIL-2R in CSF of oligoclonal IgM-positive patients was 287.3±91.4 U/ml compared to 73.8±76.1 U/ml in patients who were negative for oligoclonal IgM bands ($P< 0.0001$).

Table 4.9. Number of patients who progressed to MS at follow-up related to the presence of oligoclonal IgM bands in CSF at presentation.

CSF Oligoclonal IgM Bands	Outcome after 30 months follow-up	
	Multiple Sclerosis	No Progression to Multiple Sclerosis
Negative	0	19
Positive	22	4

Corrected $\chi^2= 28.1$, $P< 0.0001$

4.4.6 Oligoclonal IgG bands and the risk of MS

Eleven (50%) of the 22 patients who demonstrated CSF oligoclonal IgG bands presented with an isolated brainstem syndrome and the other 11 had isolated spinal cord syndrome. Table 4.10 shows the relationship between the presence of oligoclonal IgG bands in CSF at presentation and progression to MS. Eighteen (82%) of the 22 progressed to multiple sclerosis within a mean interval of 15.1 months. Four patients (2 with isolated spinal cord syndrome) who had no detectable oligoclonal IgG bands in the original episode progressed to MS within a follow-up period of 13.7 months. Two of the 4 patients progressed to clinically definite MS within a mean of 16.2 months; one patient progressed to develop new multifocal MRI brain lesions; and another patient had new relapse at a different spinal cord level.

4.4.7 MRI and the risk of MS

Magnetic resonance imaging demonstrated disseminated cerebral white matter lesions in 24 patients at initial presentation; 7 of these patients had no detectable IL-2 in CSF, and 6 did not have high sIL-2R concentration in CSF. The 7 patients who had no detectable IL-2 in CSF were without oligoclonal IgG bands, and 6 did not demonstrate oligoclonal IgM bands. As judged from clinical and paraclinical criteria, 17 (71%) of the 24 patients progressed to MS within a mean interval of 14.3 months (Table 4.11).

Table 4.10. Number of patients who progressed to MS at follow-up related to the presence of oligoclonal IgG bands in CSF at presentation.

CSF Oligoclonal IgG Bands	Outcome after 30 months follow-up	
	Multiple Sclerosis	No Progression to Multiple Sclerosis
Negative	4	19
Positive	18	4

Corrected $\chi^2 = 16.1$, $P < 0.0001$

Table 4.11. Number of patients who progressed to MS at follow-up related to the findings of brain MRI at presentation.

MRI of Brain	Outcome after 30 months follow-up	
	Multiple Sclerosis	No Progression to Multiple Sclerosis
Multifocal Lesions	17	7
No Lesions	5	16

Corrected $\chi^2 = 8.1$, $P < 0.005$

Progression to MS occurred in the 17 patients (7 with isolated brainstem syndrome) who demonstrated multifocal MRI brain lesions and who had detectable IL-2 in CSF at presentation. None of the 7 patients who had undetectable IL-2 in CSF progressed to MS during the follow-up period, although MRI was positive at presentation. This fact demonstrates the higher specificity of CSF IL-2 in predicting the risk of progression to MS (see below).

Eleven patients who initially had disseminated brain lesions progressed to clinically definite MS; 4 patients subsequently developed clinical relapses at a level different from that of the original lesion; and 2 patient demonstrated new periventricular MRI lesions at follow-up.

Clinical multiphasic disease developed in 2 patients who had normal brain MRI scan, but had high IL-2 and sIL-2R concentrations in CSF at presentation after a follow-up intervals of 15 and 16.5 months respectively. Another 3 patients who did not have multifocal MRI cerebral lesions at presentation developed new brain white matter lesions within a mean MRI follow-up period of 15.4 months. All 5 patients who progressed to MS, despite normal MRI brain scans at presentation, had high concentrations of IL-2 and sIL-2R in CSF during the original episode. This fact further illustrates the high specificity of CSF IL-2 and sIL-2R in predicting the risk of developing MS, compared to the predictive ability of MRI. No evidence of MS was detected in 7 patients who had MRI brain lesions at presentation (Table 4.11) after a mean follow up interval of 27.1 months.

4.4.8 Correlation of oligoclonal Ig bands with MRI findings

The correlation of MRI findings with oligoclonal Ig bands in the CSF at presentation is shown in Table 4.12. It is interesting to note that patients with isolated brainstem or spinal cord syndromes who did not demonstrate oligoclonal IgG bands at presentation were more likely to have a normal MRI brain scan than patients who did not demonstrate oligoclonal IgM bands.

Table 4.12. The relation between MRI brain scan and the presence of oligoclonal IgG and IgM bands in the CSF at presentation in patients with isolated brainstem or spinal cord syndromes. Data represent number of patients in each group.

MRI Findings	Oligoclonal IgG Bands[a]		Oligoclonal IgM Bands[b]	
	Absent	Present	Absent	Present
Negative	16	5	13	8
Positive	7	17	6	18

[a]*Corrected* χ^2= *8.1, P< 0.005*
[b]*Corrected* χ^2= *4.8, P< 0.03*

4.4.9 The predictive value of CSF and MRI studies

The highest rate of progression to MS was detected in patients who had detectable IL-2 and sIL-2R in CSF at presentation. The detection of multiple cerebral lesion by MRI or the demonstration of oligoclonal Ig bands in CSF did not improve the predictive capacity for subsequent development of MS. The predictive values of cerebrospinal features and MRI at presentation are shown in Table 4.13.

Findings depicted in Table 4.13 suggest that the predictive value of CSF IL-2 at the initial presentation is higher than that of CSF sIL-2R, oligoclonal Ig bands, or MRI brain lesions. Two of the 5 patients in whom oligoclonal IgG bands and MRI were negative, but who had high CSF concentrations of IL-2 at presentation, subsequently developed several periventricular MRI lesions after a mean interval of 16.5 months. Another one of those 5 patients developed a single new cerebral white matter lesion after 16 months, but the diagnosis of MS was not made because of the strict criteria adopted for MRI interpretation (see discussion).

Table 4.13. Statistical significance and predictive value of CSF parameters and MRI brain lesions at presentation for subsequent development of multiple sclerosis.

Parameter	*F* Value[a]	*P* Value	Relative Predictive Value
CSF Interleukin-2	123.3	< 0.0001	93%
CSF Oligoclonal IgM Bands	109.9	< 0.0001	89%
CSF Soluble IL-2 Receptor	87.4	< 0.0001	83%
CSF Oligoclonal IgG Bands	24.3	< 0.001	78%
MRI of the Brain	11.3	< 0.01	70%
All the above	134.2	< 0.0001	96%

[a]Calculated from one-way analysis of the variance with MS as the grouping variable.

4.5 Discussion

4.5.1 The predictive value of IL-2 and sIL-2R

An important prognostic issue in patients with acute monofocal syndromes concerns progression to MS. As more therapeutic procedures are advocated for the treatment of MS, the chances for an effective therapy would be enhanced if the putative treatment is administered at an early stage of the disease process. The ability to predict subsequent development of MS would also help in planning a proper support scheme. Results presented in this chapter clearly demonstrate that the detection of IL-2 and sIL-2R in CSF at the initial presentation is an important prognostic factor in determining the subsequent development of MS. The predictive power of IL-2, in particular, was superior to that of intrathecal IgM synthesis, which is commonly regarded as a reliable indicator of recent immunologic stimulation (Sharief 1991; Lolli et al 1991).

In the patient population studied here, CSF oligoclonal IgG and MRI findings at presentation had far less predictive value than either IL-2 or sIL-2R . This is somewhat expected in view of the findings presented in Chapter 3, which clearly demonstrated that IL-2 and sIL-2R are involved in the early intrathecal immune

response. Furthermore, immunological abnormalities involving various subsets of lymphocytes similar to those found in clinically definite MS are common in patients with isolated CNS lesions (Miller et al 1989; Koopmans et al 1989). While MRI is undoubtedly the best test for lesion dissemination in space, the study of immunological alteration in early acute episodes may be more appropriate when evaluating recent relapse or disease activity. Early MS lesions could be below the detection threshold of the technique or the particular MRI machine used while still manifesting intense immunologic activity.

Recent criteria (Poser et al 1983) allow the use of paraclinical evidence to demonstrate lesion dissemination in space for research protocols. Caution should be exercised when evaluating multifocal cerebral white matter MRI lesions for the diagnosis of MS as MRI techniques at their present status lack specificity, and lesions similar to those found in MS are seen in many disorders such as vasculitis; lacunar infarcts; Bingswanger's, Alexander's, and Fabry's diseases; systemic lupus erythematosus; leukodystrophies; post-infection syndromes; and radiation and chemotherapy reactions (reviewed by reference Paty 1988) as well as in healthy individuals (Hawkins et al 1991). As a result, the detection of a single MRI lesion could not be regarded as indicative of an inflammatory or demyelinating process. In this study, the interpretation of MRI results was very conservative in that only lesions strongly suggestive of MS (i.e. three or more lesions, at least one periventricular in location Paty 1988; Koopmans et al 1989) were considered as evidence of dissemination in space.

The findings of higher risk of progression to MS in patients with intrathecal IgG synthesis at presentation are in full agreement with previous longitudinal studies of monosymptomatic presentation (Paty 1988; Miller et al 1989). Further, Miller et al (1989), using one new MRI lesion as evidence of dissemination in space, failed to detect significant risk of MRI brain lesions at presentation on the progression to MS in patients with acute isolated brainstem syndromes. However, they reported that CSF oligoclonal bands significantly predicted subsequent development of MS in these patients. Cerebrospinal fluid oligoclonal bands are not only of better prognostic value than MRI, but their diagnostic importance in MS is well established. Paty (1988), in his review of the literature found that MRI was

positive in 86% of clinically definite MS and in 59% in suspected MS (73% overall). Oligoclonal CSF bands are usually positive in 95% of clinically definite MS and in 86% of probable MS, with 90% positive overall.

There has not been a previous study on the predictive value of IL-2 and sIL-2R or that of oligoclonal IgM bands in MS. Thus, findings reported in this study could not be compared with those of other research groups. Clearly, further prospective studies are needed to determine the significance of IL-2 and sIL-2R in other presenting syndromes of MS, e.g. optic neuritis and chronic progressive myelopathy. Miller and collaborators (1989) reported that none of 13 patients presenting with acute isolated syndromes who were negative for oligoclonal IgG and MRI brain scan had progressed to MS within 16 months. Their findings are further extended by the current study which demonstrates that IL-2 and sIL-2R are valuable predictive indicators of progression to MS in acute presenting syndromes even when oligoclonal IgG bands in CSF or MRI findings are negative.

Although results presented here clearly demonstrate the advantage of CSF IL-2 and its soluble receptor in predicting subsequent progression to MS, the follow-up period is relatively short, and MS is likely to develop in more patients as time progresses. Whether patients negative for CSF IL-2 or sIL-2R will ultimately develop MS is undetermined at present. Moreover, it is noteworthy that optic neuritis is generally more common than isolated syndromes of the brainstem or spinal cord as the first presenting lesion of MS. However, suitable samples from patients with optic neuritis were not available during the conduct period of this study, but prospective studies are currently being planned.

Hartard and colleagues (1988) failed to detect significant correlation between CSF oligoclonal bands and progression of MS. Their findings are difficult to explain as the method of detection of oligoclonal bands has not been fully reported. Cerebrospinal fluid methodology is of particular importance especially when prospective studies are undertaken. The notion that intrathecal production of immunoglobulins does not fluctuate in any meaningful way with disease activity has recently been disputed (Oger 1989). Multiple sclerosis is a dynamic process and intrathecally synthesised immunoglobulins are affected by varying metabolic activities in different stages of the disease. Binding of CSF immunoglobulins to

target structures can also lead to varying CSF levels in different clinical stages of the disease process. The electrophoretic or focusing process for the detection of oligoclonal bands should therefore be standardised according to the amount of immunoglobulin present in the test sample. Similarly, adequately standardised methods for the detection of IL-2 and sIL-2R are imperative in any prospective evaluation of the predictive role of these mediators.

4.4.2 Relationship between IL-2 and MRI lesions

In addition to establishing a strong predictive value of IL-2, this study provides some interesting information on the relationship between IL-2 and MRI lesion burden on presentation in patients who eventually developed MS. It seems that CSF IL-2 parallels the degree of cerebral lesion burden in potential MS patients, at least as it is manifested by MRI high signals. This is the first time a significant correlation has been described between intrathecal IL-2 synthesis and MRI-detected brain lesions. Such finding further extends pathological studies that detected IL-2R-bearing cells in active lesions of MS brains (Bellamy et al 1985). Since recent MRI lesions correspond very well to the extent of demyelination seen on pathological brain section (Paty 1988), the significant correlation between IL-2 and MRI lesions provides additional evidence for the usefulness of IL-2 detection in the *in vivo* monitoring of immune processes in active demyelinating conditions.

A plausible explanation for the correlation between IL-2 and MRI lesions is that the new MRI lesions, which normally represent acute plaques, are the sites of most inflammatory reactions that include IL-2 releasing cells. Since IL-2 release has been detected in acute demyelination plaques (Bellamy et al 1985, Selmaj et 1988), it is in accord that it would correlate with total cerebral plaque burden, represented by new MRI signals. Levels of sIL-2R also correlated with MRI lesion burden — a fact which suggests that the IL-2 release is due to a direct immune response. As the plaque edge is the site of most of the inflammatory reaction, one might anticipate a higher correlation if MRI lesion circumferences were measured instead of lesion numbers. However, such procedure could not be performed by the MRI machine used in this study.

This author could not detect a significant correlation between MRI lesions and intrathecal synthesis of IgD in long-standing MS (Sharief and Hentges 1991a), a finding that may seem inconsistent with the above discussion. However, there are basic differences between chronic MRI lesions and acute, relatively recent, lesions. Whereas the latter commonly results from acute demyelinating plaques and surrounding inflammatory response, chronic MRI lesions could be the result of gliosis or foci of remyelination (Sarpel et al 1987; Hunt et al 1989; Koopsmans et al 1989). Indeed, it is quite difficult to test for the relative number of new plaques in routine MRI (Koopsmans et al 1989). Therefore, until the correlation between the measured MRI high signals in chronic MS is standardised, one should not draw conclusions as to the precise relationship between intrathecal release of IL-2 and the number of MRI-detected lesions in well-established MS. It is tempting to argue that patients reported here, who presented with acute monofocal syndromes, have provided a unique opportunity to study the correlation between IL-2 and "acute" cerebral white matter lesions of MS. The *in vivo* correlation reported here between CSF IL-2 and cerebral plaque burden (as detected by MRI) corroborates and extends *in vitro* models that demonstrated persistent and widespread demyelination following recombinant IL-2 infusion (Ellison et al 1990).

In conclusion, intrathecal production of IL-2 is not only a parameter of disease activity in MS, but may also permit a prospective assessment of the subsequent evolution of the disease process. Further advantage of the predictive value of intrathecal IL-2 synthesis is best illustrated in patients who have negative CSF oligoclonal IgG bands or normal MRI findings at presentation.

CHAPTER 5. RELATIONSHIP OF IL-2 and sIL-2R TO BLOOD-BRAIN BARRIER DAMAGE IN MS

5.1 Introduction

The significant relationship of IL-2 and sIL-2R to CSF and MRI features of MS described in the previous chapters suggests that these cytokines may play an important role in the pathogenesis of MS. This chapter, therefore, analyses the relationship of intrathecal cytokine levels and blood-brain barrier dysfunction in MS.

There is considerable evidence that alterations of the blood-brain barrier are important in the pathogenesis of MS (James 1989; Koopmans et al 1989). Initial observations that early plaques of MS usually develop around small cerebral blood vessels have long been considered to be an important clue to the pathogenesis of that disease (Dawson 1916; Lumsden 1970). This notion has recently received new impetus from Gd-enhanced MRI which shows foci of blood-brain barrier leakage as an early detectable change in the CNS of patients with MS (Hawkins et al 1991). Furthermore, there is now direct evidence that these foci are inflammatory in origin (Gay and Esiri 1991), raising the important suggestion that they might represent the primary lesion in MS.

Immunocytological studies in MS brains have demonstrated that damage of blood vessels of acute plaques was closely associated with the presence of activated macrophages and other inflammatory cells (Gay and Esiri 1991). In addition, deposition of complement as well as IgG and IgM, but not IgA, was detected in all acute plaques examined (Gay and Esiri 1991). Such finding suggests that damage to the blood-brain barrier in early MS plaques is due to inflammatory changes, which are probably mediated by activated immune cells. The presence of IgM in these plaques further emphasises the acute association.

In view of the significant correlation between intrathecal IL-2 and early MS plaques (see Chapter 4), reports presented above suggest that IL-2 may be involved in endothelial damage in acute MS plaques. Indeed, IL-2R-bearing lymphocytes have already been detected in the perivascular infiltrate in MS brain lesions (see above). Further, pathologic studies have consistently shown that administration of recombinant IL-2 (rIL-2) leads to disruption of the blood-brain barrier (Ellison et al 1987; Watts et al 1989), a finding that could be relevant in the context of

pathophysiological changes seen in patients with MS. Clinical studies have also shown that administration of rIL-2 to patients with cancer who had no evidence of CNS involvement may result in disruption of the blood-brain barrier (Saris et al 1988). Recombinant IL-2 preparations are not glycosylated and differ very slightly in amino acid sequence from native IL-2, but have identical biologic activities (Roche 1989).

It could be argued that pathological and clinical adverse reactions of rIL-2 may merely represent some coincidental findings that do not necessarily implicate IL-2 in the damage observed in endothelial cells. However, firm evidence that human endothelial cells are directly affected by IL-2 has recently been provided (Hicks et al 1991). Such evidence is mainly based on the fact that human vascular endothelial cells express significant levels of functional IL-2 receptors, both of low and high affinity. Therefore, IL-2 may directly affect human endothelial cells both *in vitro* and *in vivo* (Hicks et al 1991). However, the relationship between IL-2 concentrations and damage to blood-brain barrier in patients with MS is currently unknown.

Interleukin-2 is not the only cytokine that could induce cerebral endothelial damage. It has been reported earlier that newly formed MS plaques contain T lymphocytes and macrophages at their active edge (Hauser et al 1986). Both cell types secrete TNF-α, a pleiotropic cytokine with a wide variety of biological functions on a broad range of cells (reviewed by Beutler and Cerami 1990). TNF-α is recognised to be an important mediator of several inflammatory and immunological responses in a number of tissues, including the CNS (Mustafa et al 1989; Mintz et al 1989). There is increasing evidence that TNF-α is capable of causing vascular endothelial damage both in experimental animals and humans. Effects of TNF-α on endothelial cells that are relevant to MS include modulation of endothelial cell functions, resulting in vascular endothelial damage, and an increase in vascular endothelial permeability leading to vascular leak syndrome (Tracey et al 1987).

TNF-α has already been identified in MS brain lesions (Hofman et al 1989; Selmaj et al 1991a). Moreover, independent research groups have provided evidence that TNF-α levels in CSF reflect disease activity in MS (Sharief and Hentges 1991b; Tsukada et al 1991). However, the pathogenic mechanisms of central nervous system damage caused by TNF-α remain incompletely defined. In addition, some research groups (Gallo et al 1989; Franciotta et al 1989; Peter et al 1991) have failed to detect TNF-α in the CSF of MS patients.

The aim of this chapter is to evaluate the *in vivo* relationship of the IL-2 system and TNF-α to alterations of blood-brain barrier functions in patients with active MS. Concentrations of IL-2, sIL-2R, and TNF-α were determined in paired CSF and serum samples from 110 patients with active relapsing-remitting MS and 54 control patients with non-inflammatory damage to blood-brain barrier. Values were then correlated with CSF to serum ratio of albumin which is a reliable indicator of barrier damage (see below).

5.2. Patients and controls

Paired CSF and serum samples from the 110 patients (71 females) with clinically definite MS described in Table 3.4 were utilised in this study. As stated in section 3.2, these patients were in clinical relapse during CSF collection, and no patient had received immuno-suppressive treatment for at least 8 months prior to CSF collection. Lumbar CSF was obtained from all patients and none had a reduced CSF flux rate, e.g. due to immobilization or vertebral disc protrusion.

CSF samples were selected to exclude those containing more than 1 erythrocyte per μL at the time of collection. All serum and CSF samples contained a protease inhibitor (Aprotinin, A4529, Sigma Chemical Company, UK) and were processed as described in section 2.3.1.

Paired samples were obtained from 54 patients with various non-inflammatory neurologic diseases in whom blood-brain barrier damage was detected, to serve as neurologic control group. Their diagnoses included meningioma (10 patients), craniopharyngioma (5 patients), intracranial arteriovenous malformation (9 patients), cerebrovascular diseases (16 patients), obstructive hydrocephalus (8 patients), and benign intracranial hypertension (6 patients). Protease inhibitor was also added to the control samples to prevent protein degradation. The neurologic control patients were selected to participate in the study on the basis of high CSF albumin concentration without prior knowledge of other CSF features. Paired samples from the 21 normal subjects who presented with non-specific headache or neurotic syndromes (see section 3.2) were also included to serve as normal reference controls.

5.3 Methods

5.3.1 CSF Assays

All assays were performed in a blinded fashion on coded sterile samples. Levels of IL-2 in unconcentrated CSF and 1:100 diluted serum samples were determined by the ELISA system described in Chapter 2. A commercially available ELISA kit (Cellfree, T cell Sciences, USA) was used for the determination of sIL-2R levels in unconcentrated CSF and diluted serum specimens as described in section 3.3.1. Albumin concentrations in CSF and serum specimens were determined by electroimmunoassay. In this study, the method had a coefficient of variation of 2.7%.

Levels of TNF-α in native CSF and 1:100 diluted homologous serum samples were determined by a sandwich-type ELISA described previously (Mitchie et al 1988). In brief, specific mouse anti-human TNF-α (121-00, Genzyme Diagnostics, Kent, England) was bound to a microtitre plate to create the solid-phase, then the unoccupied binding sites were blocked by 2% gelatin in PBS. Samples, standards, and controls were then incubated with the solid-phase antibody. Following several washing steps, the bound human TNF-α was incubated with polyclonal rabbit anti-human TNF-α (EP-600, Genzyme Diagnostics, Kent). The resulting immune complexes were then incubated with the amplification reagent; an HRP-conjugated goat anti-rabbit IgG (A-6667, Sigma Chemical Company, UK). Finally, the substrate peroxidase and the chromogen OPD were added.

The TNF-α ELISA had a coefficient of variation of 4.8% and a lower limit of detection of 0.01 Units/ml. A standard curve was run on each ELISA plate using recombinant human TNF-α (1293-00, Genzyme Diagnostics, Kent, England) in serial dilutions. A bioassay utilizing highly sensitive WEHI cells (Espevik and Nissen-Meyer 1986) was employed to verify results obtained by the ELISA.

5.3.2 Evaluation of the blood-brain barrier

The term blood-brain barrier in this study was generally applied to describe the overall exclusionary interfaces between circulating blood at one side and the extracellular as well as the CSF space at the other side (Felgenhauer 1986). These interfaces include the epithelium of the choroid plexus, the endothelial cells of cerebral capillaries, and the layer of cells lining the arachnoid membrane (Felgenhauer 1986) (Fig. 5.1).

The integrity of the blood-brain barrier was evaluated by calculating the CSF to serum albumin quotient (Q_{alb}) (Tibbling et al 1977), which is one of the best chemical indicators of barrier damage (Tibbling et al 1977; Schliep and Felgenhauer 1978). It is noteworthy, however, that measurement of Q_{alb} represents an approximation to blood-brain barrier breakdown as it commonly measures breakdown of blood-CSF barrier. The choroid plexus, in particular, has no significant blood-tissue barrier function.

5.3.2 Statistics

The distribution of cytokines in the study population was evaluated by confidence intervals for non-parametric data (Campbell and Gardner 1988). Relation of IL-2, sIL-2R, and TNF-α values to Q_{alb} was studied by Pearson's correlation matrix test. The relationship between cytokine levels and the degree of blood-brain barrier damage was analysed by Kruskal-Wallis one-way analysis of variance.

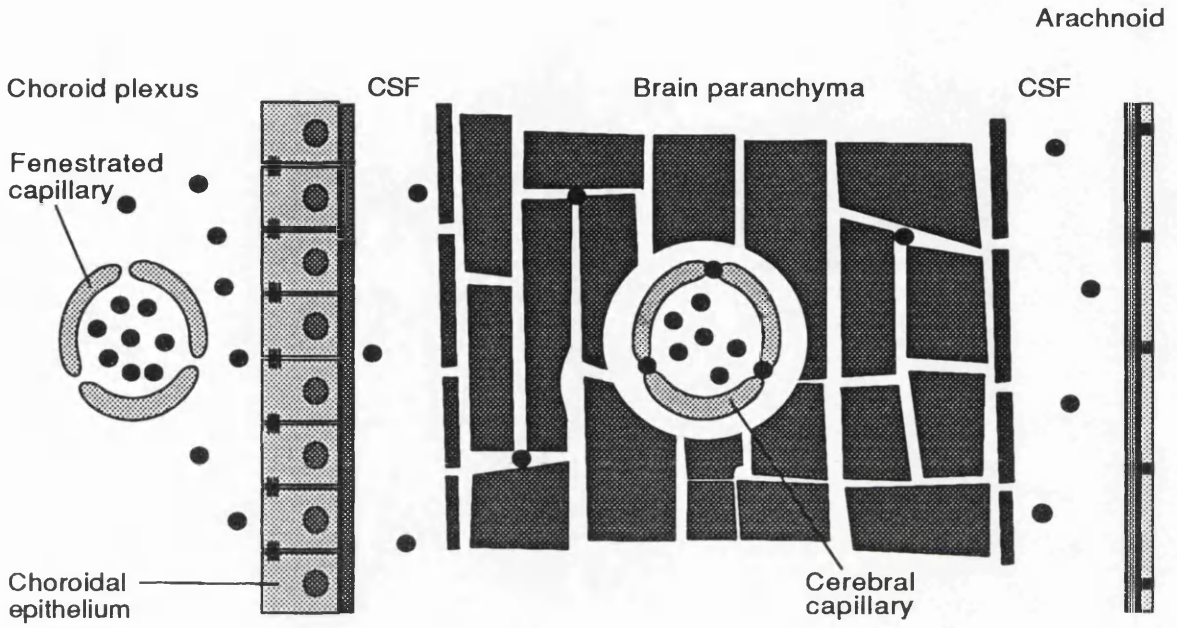


Fig. 5.1. Schematic representation of the blood-brain barrier. Protein particles are represented by ●. As demonstrated in the diagram, there are three barriers, each characterised by different permeability and flux conditions:

1. The blood-brain barrier (circulating blood/extracellular space)
2. The blood-CSF barrier (circulating blood/CSF space)
3. The paranchymal cell membrane barrier (extracellular space/intracellular space)

The essential anatomical structures of the blood-brain barrier are (*a*) the endothelia of arterioles, capillaries and venules; (*b*) the epithelial cells and the basement membrane of the choroid plexus; and (*c*) the layer of cells lining the arachnoid membrane.

5.4 Results

5.4.1 CSF to serum albumin quotient

The normal controls had a mean CSF albumin of 198 mg/L (range=132 to 295) and a mean Q_{alb} of $1.6x10^{-3} \pm 2.1x10^{-3}$. In the MS group, 69 patients (63%) had abnormally high Q_{alb} suggestive of barrier impairment, 61 of whom had elevated levels of CSF IL-2 (Fig. 5.2). No MS patient had a value of Q_{alb} higher than $13.4x10^{-3}$, while the neurologic control group had a median Q_{alb} of $11.64x10^{-3}$ (range= 6.9 to $51.95x10^{-3}$).

Barrier disruption in the MS and neurologic control groups was graduated according to values of Q_{alb} as previously described (Schliep and Felgenhauer 1978), to provide better information on the degree of barrier impairment. This rating scheme produced three groups of barrier damage (Table 5.1).

Table 5.1. The degree of blood-brain barrier damage in MS patients and neurologic controls. Values include number (and %) of patients in each group.

Clinical Group	Degree of Barrier Impairment[a]			
	No Damage	Mild	Moderate	Severe
Multiple Sclerosis *(Total No= 110)*	41 (37%)	36 (33%)	22 (20%)	11 (10%)
Neurologic Controls *(Total No= 54)*	0	12 (22%)	24 (44%)	18 (33%)

[a]*According to CSF/serum albumin quotient where values below $5.8x10^{-3}$ indicate no barrier damage, 5.8 to 7.4= mild; 7.5 to 10= moderate; and above $10x10^{-3}$= severe barrier damage.*

5.4.2 Distribution of IL-2 and sIL-2R

It has been described in Chapter 3 that high IL-2 levels were detected in CSF of 80 patients (73%) with MS, and high CSF sIL-2R levels were seen in 89 (81%) MS patients. In contrast, high CSF and serum levels of IL-2 were detected in 14 patients (26%) from the neurologic control group. Another 5 control patients (10%) had elevated serum IL-2 concentration (mean±SD= 5.8±4.2 U/ml), while no IL-2 was detected in homologous CSF. High CSF and serum levels of sIL-2R was detected in 17 neurologic control patients (32%).

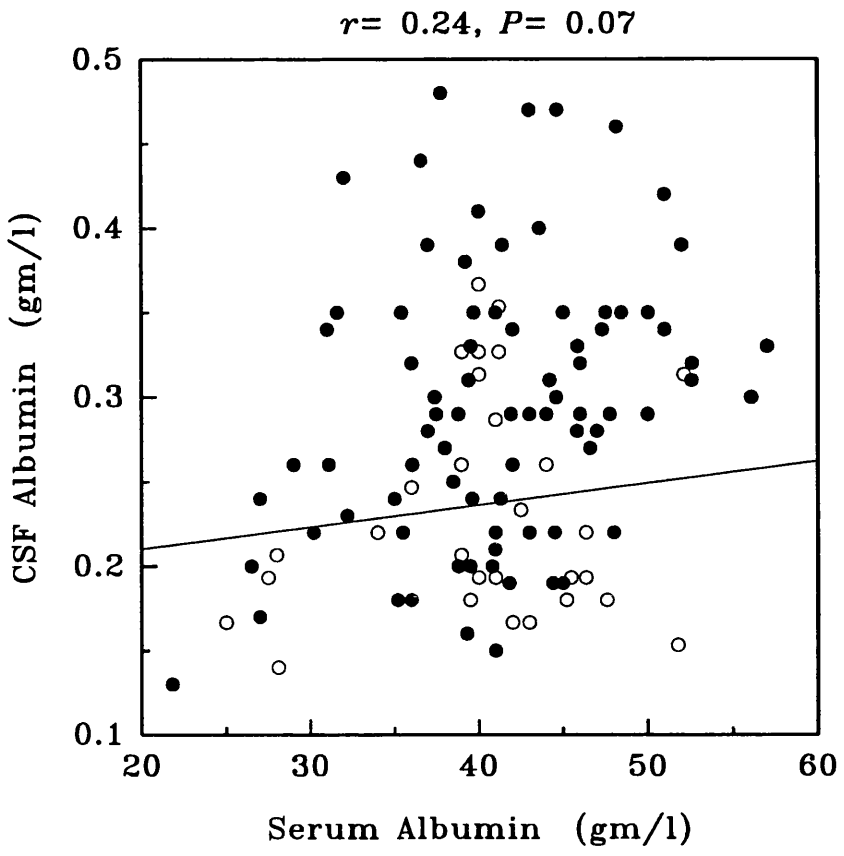


Fig. 5.2. Relationship of CSF to serum albumin concentration in 110 patients with active relapsing-remitting MS. Closed circles denote patients who have high CSF levels of IL-2 while open circles indicate those who have no detectable IL-2 in CSF. Values on the top of the figure are for the whole group of MS patients. There was no significant difference in the relationship of serum and CSF albumin between patients who were positive for CSF IL-2 and those who were not.

5.4.3 Correlation of IL-2 and sIL-2R with barrier impairment

In patients with MS, significantly higher incidence of blood-brain barrier damage was detected in patients who had high CSF levels of IL-2 or sIL-2R (Table 5.2). MS patients who did not show high IL-2 levels in CSF (no.= 8) had only minor barrier damage (mean±SD Q_{alb} value= 6.5±0.34 $x10^{-3}$). Similarly, patients who did not demonstrate abnormally high sIL-2R in CSF (no.= 9) had minor barrier damage (mean±SD Q_{alb} value= 6.4±0.42 $x10^{-3}$).

Table 5.2. Number (and %) of MS patients who showed signs of blood-brain barrier damage related to the presence of IL-2 and sIL-2R in the CSF.

Condition of Blood-Brain Barrier[a]	CSF IL-2[b]		CSF sIL-2R[c]	
	Absent	Present	Normal	High
Intact (no.= 41)	22 (54%)	19 (46%)	12 (29%)	29 (71%)
Damaged (no.= 69)	8 (12%)	61 (88%)	9 (13%)	60 (87%)

[a] *As evaluated by CSF to serum albumin quotient where values > $5.8x10^{-3}$ indicate barrier damage.*
[b] *corrected* χ^2= *27.2, P< 0.0001.*
[c] *corrected* χ^2= *18.1, P< 0.0005.*

Individual levels of IL-2 and sIL-2R were then correlated with Q_{alb} values to determine the precise relationship between these cytokines and features of barrier impairment. In MS patients who had high intrathecal IL-2 levels, CSF IL-2 concentrations significantly correlated with values of Q_{alb} (Fig. 5.3). Similarly, CSF concentrations of sIL-2R in these patients significantly correlated with Q_{alb} values (Fig. 5.3). In contrast, serum concentrations of either IL-2 or sIL-2R failed to correlate with Q_{alb} values in MS patients (Fig. 5.3), suggesting that intrathecal concentrations of IL-2 and sIL-2R are probably more important than systemic concentrations in investigating the relationship between IL-2 and blood-brain barrier impairment.

In the neurologic controls who had abnormally high IL-2 level, individual IL-2 levels in CSF or serum failed to correlate with Q_{alb} values (r= 0.11, P= 0.13; r= 0.15, P= 0.11 respectively). Similarly, sIL-2R levels in CSF or serum in neurologic controls did not correlate with Q_{alb} values (r= 0.21, P= 0.09; r= 0.27, P= 0.07 respectively).

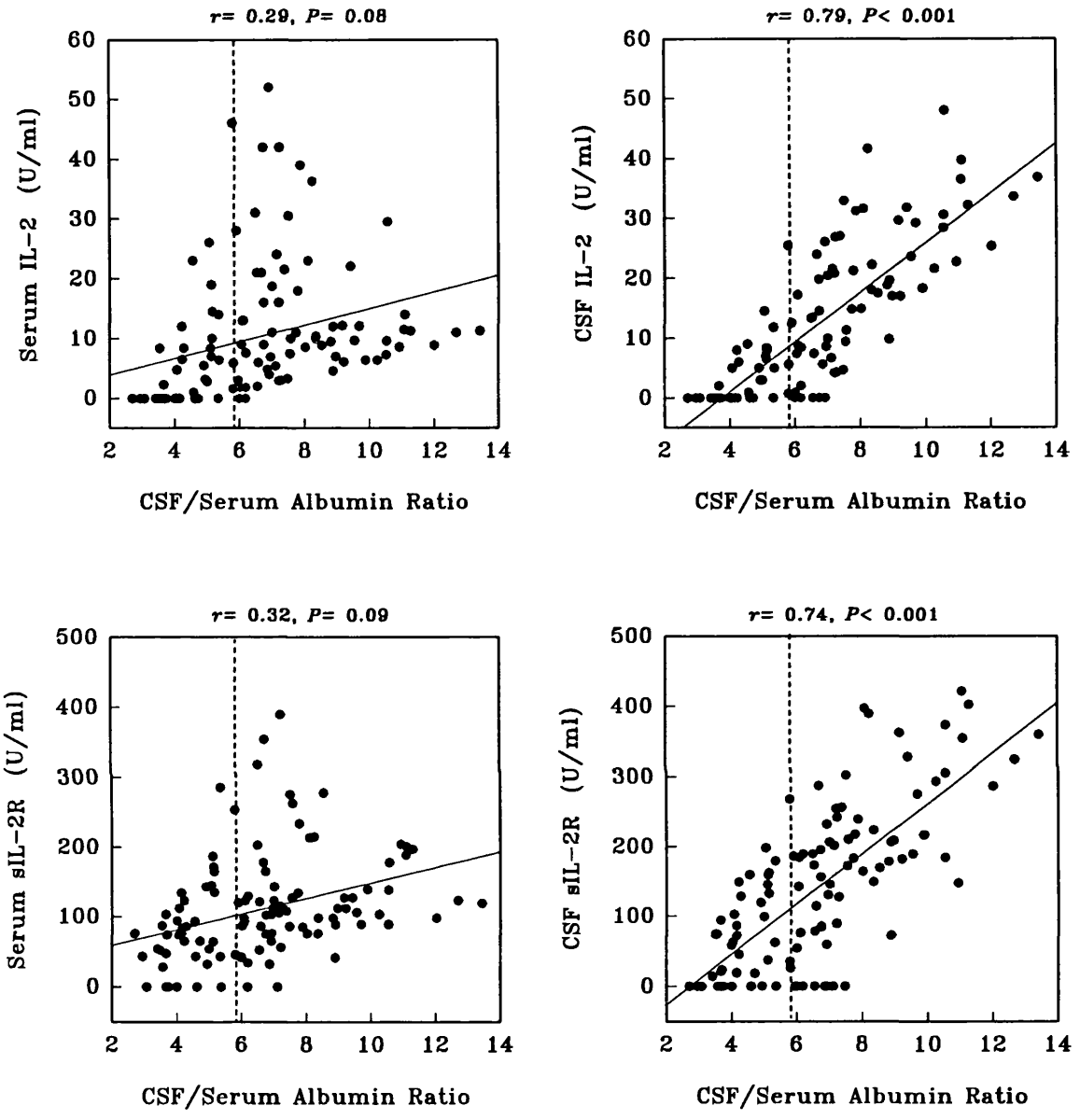


Fig. 5.3. The correlation between CSF to serum albumin ratio and the concentrations of IL-2 and sIL-2R in serum and CSF samples from patients with multiple sclerosis. Patients who have no abnormally elevated cytokine levels are not included. Values of CSF to serum albumin ratio in patients with no detectable cytokines are listed in the text. Vertical interrupted lines indicate cut-off value of the albumin ratio in normal control subjects.

Although the above results clearly demonstrated that Q_{alb} values in MS patients correlated with CSF but not serum levels of IL-2 and sIL-2R, the study sought to exclude passive transudation of these cytokines from the systemic circulation through damaged barriers. Therefore, CSF to serum ratios of IL-2 and sIL-2R were calculated, as described earlier (Sharief 1991), to correct for any passive leakage from the systemic to the intrathecal compartment. Such correction confirmed that intrathecal levels of IL-2 and sIL-2R significantly correlated with barrier impairment (Fig. 5.4).

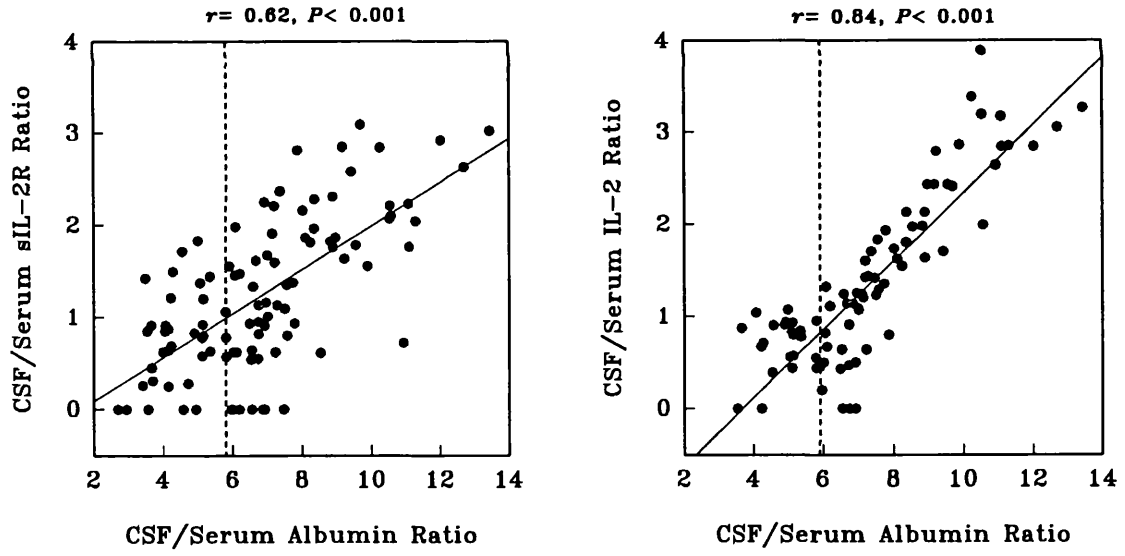


Fig. 5.4. Correlation of CSF to serum ratio of albumin with ratios of IL-2 and sIL-2R in patients with MS. Patients with no detectable cytokine levels in CSF and serum are not included. Vertical interrupted lines indicate the cut-off value of the albumin ratio in normal control subjects. The calculation of CSF to serum cytokines ratios is a reliable method for the determination of intrathecal contents of these cytokines (see text).

5.4.4 Intrathecal cytokines and the degree of barrier damage

Having established that intrathecal levels of IL-2 and sIL-2R correlated with signs of barrier impairment, as detected by high Q_{alb} values, the next purpose of this chapter was to determine the relationship between these cytokines and the extent of barrier impairment. The degree of blood-brain barrier damage was evaluated by the method described by Schliep and Felgenhauer (1978) as explained above.

Concentrations of IL-2 in CSF of patients with MS significantly correlated with the degree of barrier impairment (Fig. 5.5; $P< 0.001$ using Kruskal-Wallis one way analysis of variance). As also demonstrated in the figure, serum concentrations of IL-2 in MS patients failed to correlate with the extent of barrier impairment. This fact confirms the suggestion made in section 5.4.3 that CSF IL-2 content is more important than systemic levels in evaluating the impairment of blood-brain barrier in MS patients. The significance of intrathecal IL-2 content to the degree of barrier damage was further confirmed when CSF to serum ratios of IL-2 were calculated (see Fig. 5.10 below). In contrast to patients with MS, elevated levels of IL-2 in CSF and serum samples from the neurologic controls did not significantly correlate with the degree of blood-brain barrier damage (Table 5.3).

CSF levels of sIL-2R in MS patients also correlated with the degree of barrier damage, while serum sIL-2R levels did not correlate with the degree of barrier damage (Fig. 5.5). This fact provides further evidence that impairment of the blood-brain barrier is associated with a direct release of IL-2 in the intrathecal compartment since the presence of high sIL-2R levels generally implies activation of the IL-2 system. Therefore, it was not surprising to find that intrathecal sIL-2R content in MS patients (as determined by CSF to serum sIL-2R ratio) significantly correlated with the degree of barrier impairment (see Fig. 5.10 below). In contrast, no correlation was detected between CSF or serum sIL-2R levels and the degree of barrier impairment in the neurologic control group (Table 5.3).

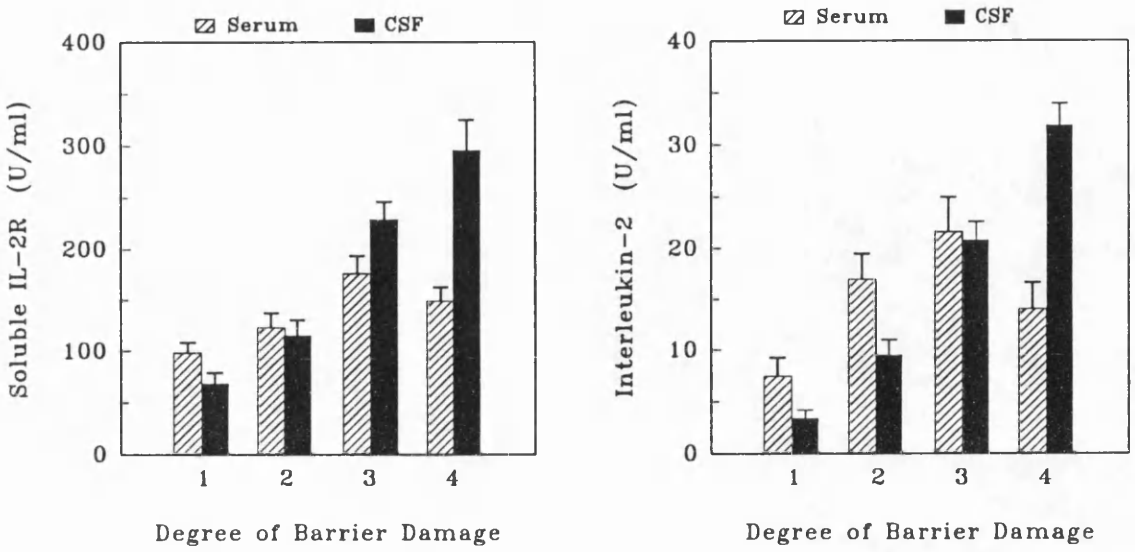


Fig. 5.5. Correlation of CSF and serum concentrations of IL-2 and sIL-2R with the degree of blood-brain barrier damage in 110 patients with active multiple sclerosis. Values depict means + standard errors of the mean

Key to the degree of barrier damage: 1: none, 2: mild, 3: moderate, and 4: severe damage.

Calculation of CSF to serum cytokine ratios confirmed the above correlation (see p. 146).

Table 5.3. Mean values of IL-2 and sIL-2R in neurologic controls related to the degree of blood-brain barrier damage.

Degree of Barrier Damage	Interleukin-2		Soluble IL-2R	
	Serum	CSF	Serum	CSF
Mild	2.9	2.6	86.3	77.9
Moderate	2.1	2.7	76.1	62.4
Severe	1.4	1.3	65.8	63.1

5.4.5 Distribution of TNF-α in the test samples

Detectable TNF-α levels (mean±SD= 64.3±54.3 U/ml) were seen in the CSF of 68 patients (62%) with active MS and 11 (20%) neurologic controls (Fig. 5.6). As also shown in Fig. 5.6, high TNF-α levels (mean±SD= 44.1±39.2 U/ml) were detected in the serum of 92 patients (83%) with active MS. Detectable amounts of TNF-α (mean±SD= 29.4±18.7 U/ml) were found in serum but not CSF samples of 25 patients with active MS and 7 neurologic controls (mean±SD= 23.6±11.2 U/ml). However, CSF levels of TNF-α in patients with active MS were significantly higher than corresponding serum levels ($P< 0.005$).

Levels of TNF-α detected by ELISA correlated well with concentrations detected by bioassay ($r= 0.73$, $P< 0.001$). In particular, the bioassay detected similar TNF-α concentrations in MS patients who had high CSF TNF-α levels by ELISA.

5.4.6 Correlation of CSF TNF-α with pleocytosis

Cytological CSF examination in MS patients showed all cells to be mononuclear. The relationship of TNF-α to CSF leucocytes and reactive lymphocytes is shown in Table 5.4. Even when Pearson's correlation was applied, individual CSF concentrations of TNF-α in patients with active MS failed to correlate with CSF leucocyte count ($P=$ 0.12) or with the number of reactive lymphocytes ($P= 0.09$).

Table 5.4. CSF leucocyte count related to the detection of TNF-α in CSF from patients with active MS. Numbers in brackets represent patients with reactive lymphocytes.

CSF Leucocytes	TNF-α in CSF	
	Absent	Present
0-4 Cells/mm^3	27	30
5 or More Cells/mm^3	15 (12)	38 (28)

Corrected $\chi^2= 3.5$, $P= 0.08$
Corrected $\chi^2= 1.3$, $P= 0.26$ (for reactive lymphocytes)

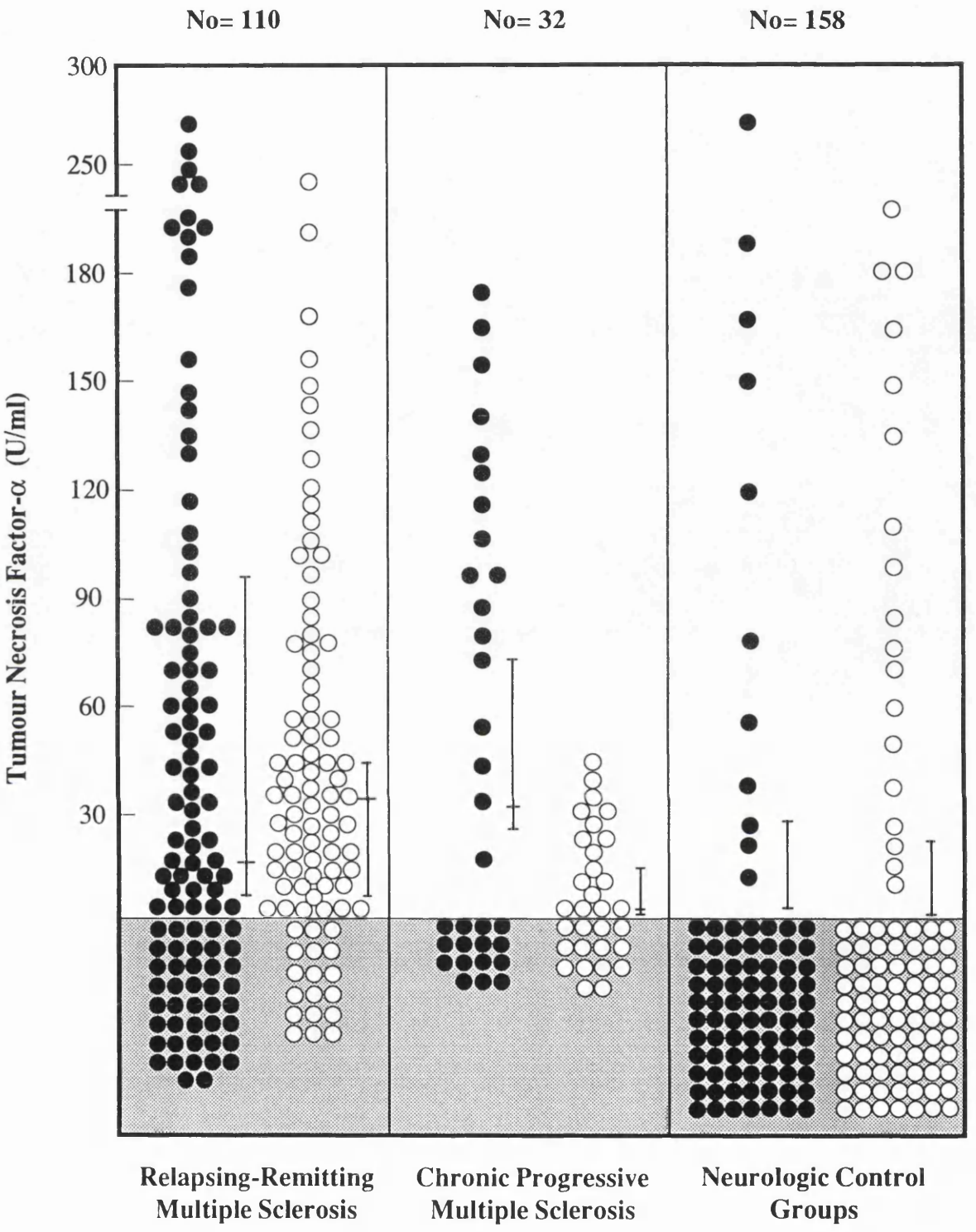


Fig. 5.6. Distribution of TNF-α in cerebrospinal fluid (●) and serum samples (○) from the study groups. Control patients who had detectable TNF-α levels in CSF include 2 patients with meningioma, 2 patients with gliomas, 3 patients with neurosarcoidosis, 2 patients with Neuro-Behçet's, and a patient with cerebral lupus.

5.4.7 Correlation of IL-2 with TNF-α

The relation between TNF-α and the IL-2 system was examined in MS patients. In patients with active MS, there was a significant relationship between the appearance of TNF-α in the CSF and the detection of IL-2 and sIL-2R (Table 5.5). In contrast, the presence of TNF-α in serum samples from these patients was not significantly relate to the serum levels of IL-2 or sIL-2R. Utilising Pearson's correlation, CSF levels of TNF-α in active MS positively correlated with CSF levels of IL-2 and sIL-2R (Fig. 5.7).

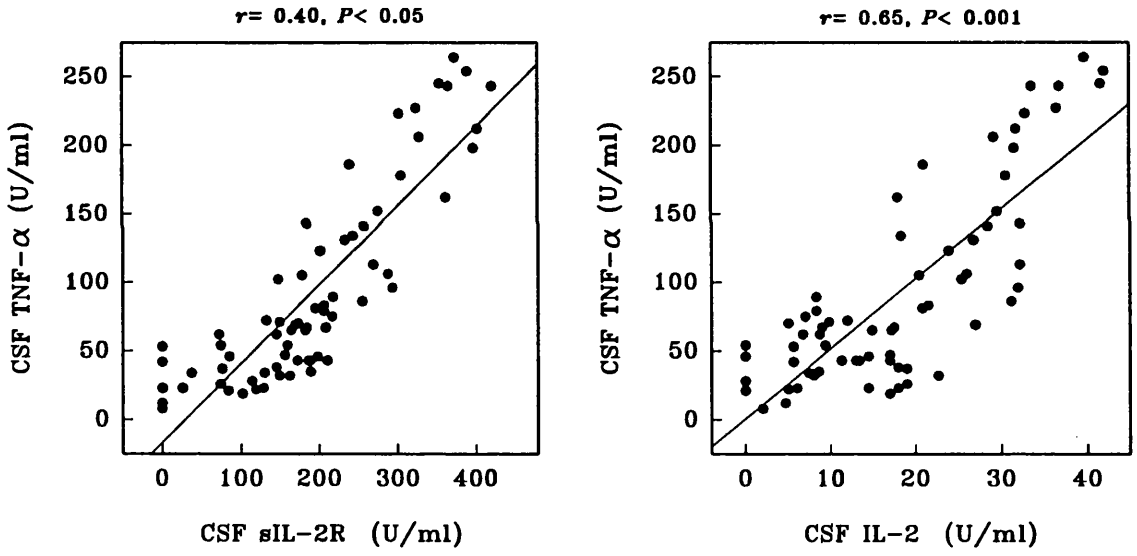


Fig. 5.7. The correlation between CSF concentrations of TNF-α and the concentrations of IL-2 and sIL-2R in CSF from patients with active multiple sclerosis. Patients who have no detectable TNF-α levels in the CSF are not included.

Table 5.5. Number of MS patients who had detectable TNF-α in CSF related to the presence of CSF IL-2 and sIL-2R.

CSF TNF-α	CSF IL-2[a]		CSF sIL-2R[b]	
	Absent	Present	Normal	High
Absent (no.= 42)	26	16	16	26
Present (no.= 68)	4	64	5	63

[a]*corrected* χ^2= *38.3, P< 0.0001.*
[b]*corrected* χ^2= *13.9, P< 0.001.*

5.4.8 Correlation of TNF-α with barrier impairment

High CSF levels of TNF-α in active MS significantly associated with higher incidence of barrier damage. Of the 68 patients with active MS who demonstrated high CSF levels of TNF-α, 54 (79%) had Q_{alb} values above $5.8x10^{-3}$, which suggest an abnormal barrier function. Of the 42 patients who had no detectable TNF-α in CSF, 27 (64%) showed no sign of barrier impairment (corrected χ^2= 19.4, $P< 0.0001$). Furthermore, individual concentrations of TNF-α in CSF of active MS positively correlated with Q_{alb} values (Fig. 5.8). On the other hand, the relationship between serum levels of TNF-α and Q_{alb} values barely reached statistical significance (Fig. 5.8).

Although CSF levels of TNF-α were significantly higher than corresponding serum levels in patients with active MS, CSF to serum ratios of TNF-α were calculated to correct for passive transudation across damaged blood-brain barriers. In patients with active MS who had detectable CSF levels of TNF-α, CSF to serum ratios of TNF-α significantly correlated with Q_{alb} (Fig. 5.9).

Having established a significant relationship between CSF TNF-α concentration and Q_{alb} value in active MS, the study sought to analyse the correlation between CSF TNF-α and the degree of barrier damage. As shown in Table 5.6, a significant association was detected between the presence of TNF-α and the degree of barrier damage. Moreover, low CSF to serum ratios of TNF-α were detected in patients with mild barrier impairment, while high ratios were associated with severe barrier damage (Fig. 5.10).

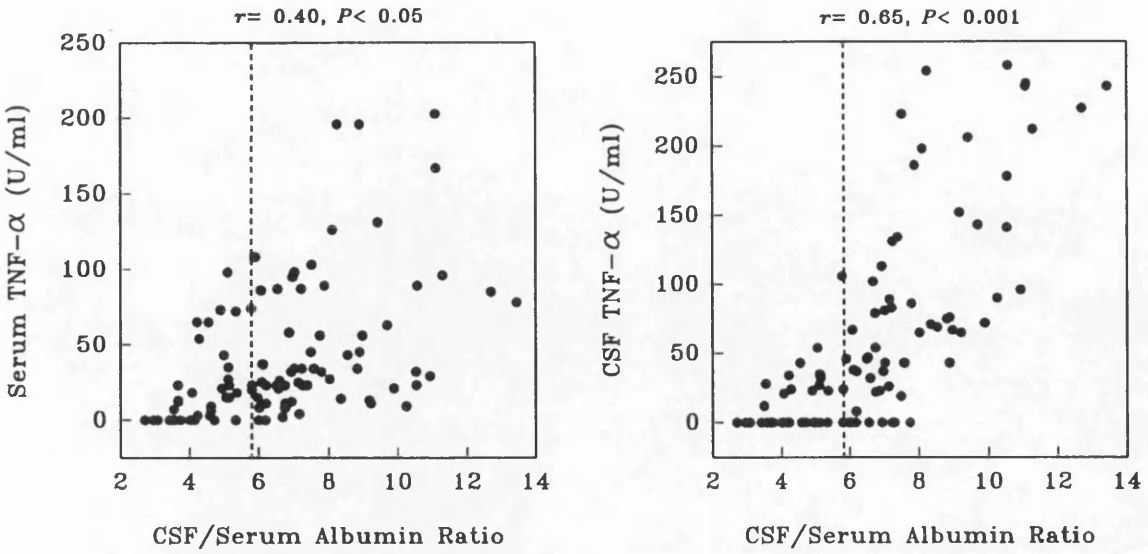


Fig. 5.8. Relation of TNF-α concentrations in the serum and cerebrospinal fluid with albumin ratio ($x10^{-3}$) in 110 patients with active MS. Vertical interrupted lines indicate the cut-off value of the albumin ratio in normal individuals.

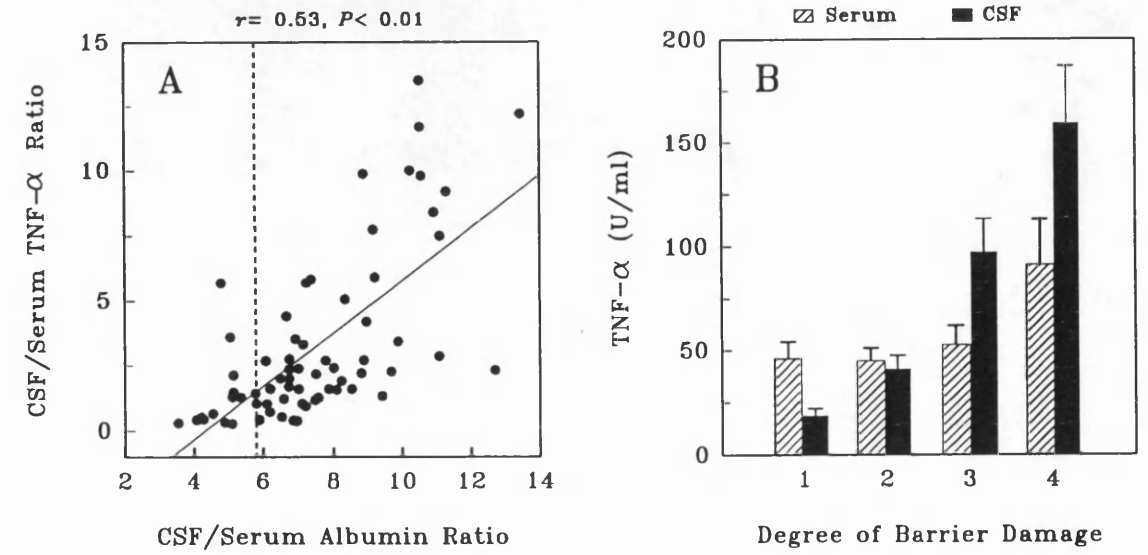


Fig. 5.9. **A**. Correlation between TNF-α and albumin ($x10^{-3}$) ratios in patients with active MS. Patients with no detectable TNF-α in CSF are not included. **B**. Correlation of CSF and serum concentrations of TNF-α with the degree of blood-brain barrier damage in 110 patients with active multiple sclerosis. Values depict means + SEM.

Key to the degrees of barrier damage: 1: none, 2: mild, 3: moderate, and 4: severe damage.

Fig. 5.10. Relation of intrathecal levels of IL-2, sIL-2R, and TNF-α with the degree of blood-brain barrier impairment in 110 patients with active MS. Values represent means+standard errors of the mean.

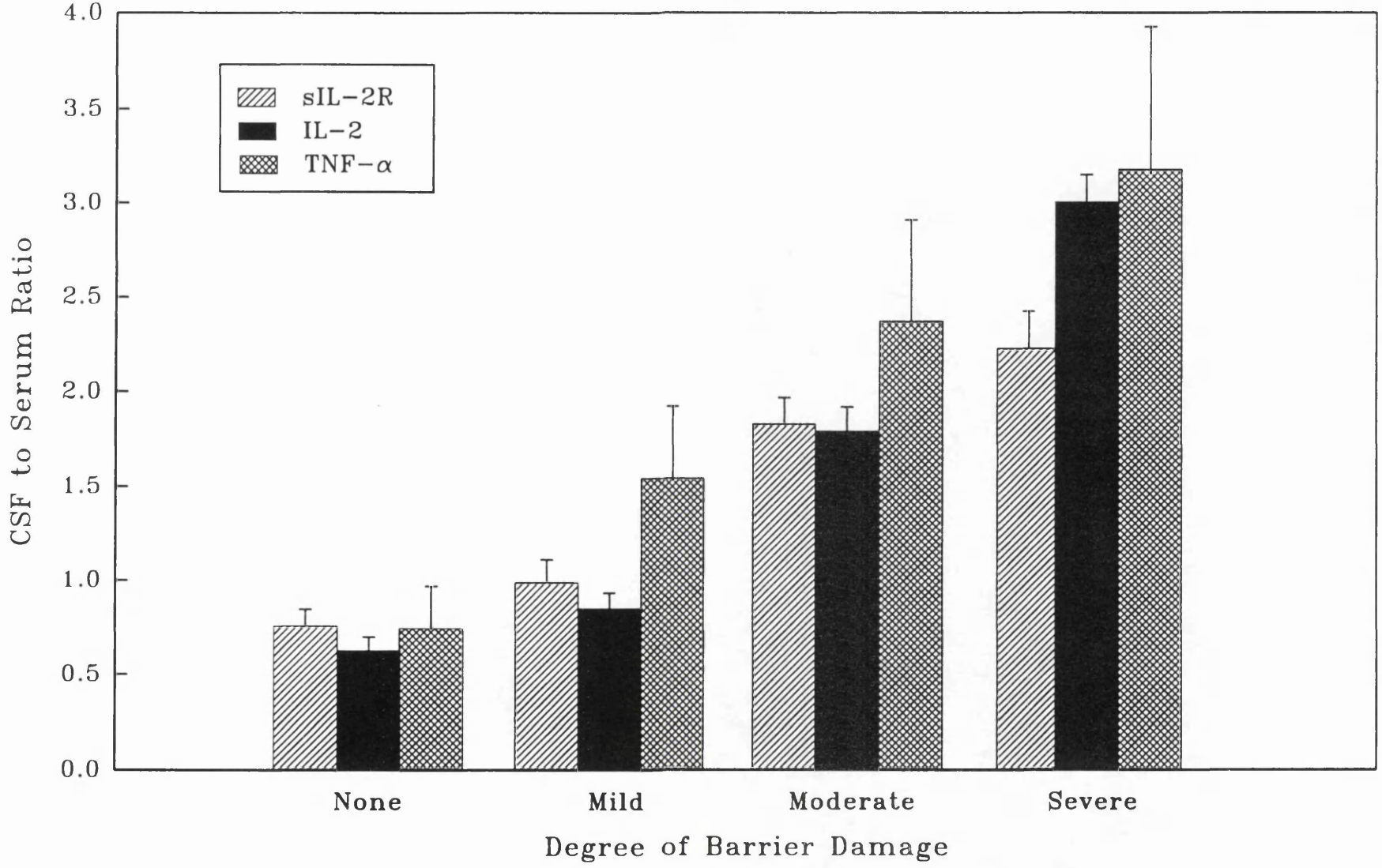

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CSF to Serum Ratio
sIL-2R
IL-2
TNF-α
None
Mild
Moderate
Severe
Degree of Barrier Damage

Table 5.6. Number of MS patients who had detectable TNF-α in CSF related to the degree of blood-brain barrier damage. See above for definition of individual grades.

CSF TNF-α	Extent of Barrier Damage			
	None	Mild	Moderate	Severe
Absent (no.= 42)	27	11	3	1
Present (no.= 68)	14	25	19	10

5.4.9 The dynamics of TNF-α and IL-2 in barrier damage

As could be seen in Fig. 5.10, intrathecal levels of TNF-α as well as those of IL-2 and sIL-2R correlated with the severity of barrier damage. However, intrathecal TNF-α levels in individual grades of barrier damage had a standard deviation much larger than that of IL-2 or sIL-2R. This may suggest that blood-brain barrier damage in active MS is more closely related to intrathecal levels of IL-2 and sIL-2R than to intrathecal levels of TNF-α.

The notion that blood-brain barrier impairment is more closely related to intrathecal levels of IL-2 than to TNF-α levels is further confirmed when the *in vivo* relationship of these cytokines to blood-brain barrier damage was precisely analysed. As already shown in Fig. 5.7, four patients with active MS who had no detectable IL-2 in CSF did not show signs of barrier impairment (mean Q_{alb}= $5.42x10^{-3}$) despite high CSF TNF-α concentration (mean= 37.3 U/ml). On the other hand, of the 16 MS patients who had detectable IL-2 but not TNF-α in CSF, 8 (50%) showed signs of blood-brain barrier impairment (Fig. 5.11). These patients also had elevated sIL-2R levels in CSF (mean±SD= 91.59±68.3 U/ml), confirming that high CSF levels of IL-2 are due to active release as a result of immune stimulation.

Since the precise relationship between barrier impairment and both IL-2 and TNF-α is crucial in understanding the pathophysiology of blood-brain barrier changes in MS, multivariate statistical procedures, including discriminant analysis, were employed to analyse cytokine levels together, not one at a time. Because IL-2, sIL-2R,

and TNF-α could be interrelated, considering them simultaneously in statistical analysis will incorporate important information about their relationships with each other and with blood-brain barrier impairment. Thus, the relative relations of intrathecal levels of IL-2, sIL-2R, and TNF-α to barrier impairment in MS were tested by multivariate analysis of variance combined with discriminant analysis. As shown in Table 5.7, the relation between barrier impairment and CSF concentration of IL-2 was stronger than the relation between barrier impairment and CSF TNF-α concentration. It will be discussed later that this finding is critical in investigating any causal relationship between high cytokine levels and blood-brain barrier impairment in MS.

Table 5.7. Summary table for multivariate analysis of variance and discriminant analysis of the relation between CSF cytokines and barrier impairment in 110 patients with active MS. Numbers of patients with detectable cytokines in CSF are presented in brackets.

CSF Variable	*F* Value	*P* Value
Interleukin-2 (80)	14.1	< 0.0001
Tumour Necrosis Factor-α (68)	11.9	< 0.001
Soluble IL-2 Receptor (89)	6.8	< 0.003

5.5 Discussion

As has been explained in previous chapters, higher CSF levels of IL-2 and sIL-2R compared to serum levels in MS patients suggest local release within the intrathecal compartment. It is unlikely that high CSF levels of IL-2 or sIL-2R are the result of passive leakage from the systemic circulation across impaired barriers since standardised levels in the two compartments, which correct for any transudation, showed a significant increase of intrathecal levels of these cytokines. Moreover, no significantly elevated CSF levels of IL-2 or sIL-2R were seen in patients from the neurologic control group despite severe barrier damage.

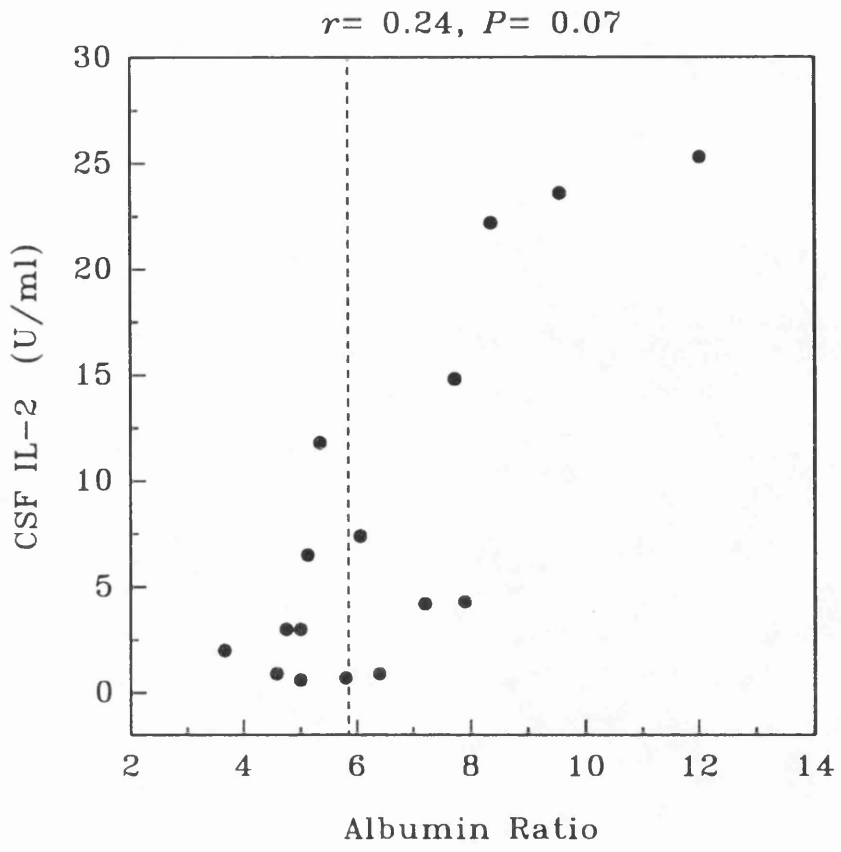


Fig. 5.11. The relation between CSF IL-2 concentration and CSF to serum albumin ratio ($x10^{-3}$) in 16 MS patients who had high levels of IL-2 but not TNF-α in the CSF. Vertical lines indicate cut-off value of the albumin ratio in normal control subjects.

5.5.1 Relationship of IL-2 and sIL-2R to barrier impairment

The main finding of this chapter was the higher incidence of barrier impairment in MS patients who had elevated CSF levels of IL-2 and sIL-2R. The degree of barrier damage in those patients was found to be proportional to the CSF levels of IL-2 and sIL-2R. In contrast, no significant correlation was detected between barrier impairment and serum levels of IL-2 or sIL-2R.

The blood-brain barrier represents a series of regulatory interfaces which determine the rate at which substances pass into the intrathecal compartment. In addition to the choroid plexus, the barrier includes the endothelial cells of the cerebral capillaries and venules. Results reported here are, therefore, relevant when considering the known effects of IL-2 on endothelial cells. Infusion of rIL-2 to animals has resulted in blood-brain barrier disruption in multiple brain regions, increase in

cerebral microvascular permeability, and alteration in cerebrovascular morphology and occasional demyelination (Ellison et al 1987 and 1990). In addition, an increased expression *in vivo* of endothelial activation antigens such as endothelial-leucocyte adhesion molecule-1, intercellular adhesion molecule-1 and HLA-DQ was observed on venular epithelium after systemic IL-2 administration (Cotran et al 1987).

Clinical studies have also shown specific effects of IL-2 on CNS endothelial cells that would be relevant to the pathologic changes in MS. Therapeutic administration of rIL-2 to patients with cancer has resulted in a "vascular leak syndrome" (Cotran et al 1987; Damel and Doyle 1989) and focal neurologic deficits similar to transient ischaemic attacks (Bernard et al 1990). Further, administration of IL-2 produced clinico-pathological features of acute encephalo-myelitis which was accompanied by multiple foci of perivascular demyelination (Vecht et al 1990).

5.5.2 Possible mechanisms of IL-2-related barrier damage

It is unclear from data reported here whether barrier impairment is directly related to the IL-2 system or is the result of other basic processes. High CSF IL-2 and sIL-2R levels and increased Q_{alb} could independently reflect the increased intrathecal immune activity and increased barrier permeability, which might both be expected during an active relapse. High levels of IL-2 and sIL-2R in the CSF may be secondary to barrier breakdown and inflammation in MS lesions, particularly those close to the meninges. They may be similar to abnormal immunoglobulin levels in the CSF in MS, which are usually the result of active inflammation.

Although results presented in this chapter are not sufficient to confirm a pathogenic role of IL-2 in blood-brain barrier breakdown, several mechanisms have been proposed to explain the effect of IL-2 on cerebral endothelial cells. First, cerebral endothelial damage may result from a direct effect of IL-2 on endothelial cells, which results in a local Shwartzman reaction (consisting of focal thrombosis, haemorrhage or inflammation). Second, endothelial damage could result from a direct cell-to-cell interaction between endothelial cells and IL-2-activated lymphocytes or vasoactive factors elaborated from IL-2-activated cells (Bechard et al 1987; Damel et al 1987). Separate studies have clearly demonstrated that IL-2-induced capillary leak is due to cell-mediated injury to the endothelium (Damel et al 1987), and a three-dimensional

ultrastructural model of target cell killing by IL-2-activated killer cells has already been presented (Iwasaka 1990). In addition, Watts and others (1989) attributed blood-brain barrier disruption to the presence of arachidonic acid and its oxidative products (such as prostaglandins, thromboxanes, and leukotrienes) which could be released by IL-2-activated lymphocytes.

A third mechanism to explain a putative IL-2 effect on blood-brain barrier may be an IL-2-enhanced production of other cytokines, particularly IL-1 and TNF-α (Cotran et al 1987; Mier et al 1988). Both IL-1 and TNF-α have profound pro-inflammatory effects on endothelial cell functions (Schleef et al 1988; Convay et al 1989) and could result in endothelial cell damage. This possibility, however, was not supported by *in vitro* studies on experimental animals, which reported that the ability of IL-2 to induce adhesion and subsequent damage to endothelial cells by natural killer cells *in vitro* was not blocked by a mixture of neutralising antisera raised against TNF-α and IL-1. In addition, TNF-α and IL-1 do not affect endothelial cell permeability when tested *in vitro* (Damel and Doyle 1989). Nonetheless, evidence will be presented later in this chapter to support a role of TNF-α in human endothelial damage *in vivo*. Furthermore, the relationship between TNF-α and barrier dysfunction in MS patients reported in this chapter suggests that TNF-α may be an important mediator of barrier impairment *in vivo*. The possible mechanisms of TNF-α-mediated blood-brain barrier damage will be discussed below.

5.5.3 Levels of TNF-α in multiple sclerosis

The finding of higher TNF-α levels in the CSF of patients with active MS is consistent with previous studies (Maimone et al 1991; Sharief and Hentges 1991; Tsukada et al 1991). Failure to detect TNF-α in the CSF of MS patients reported by some research groups (Gallo et al 1989; Franciotta et al 1989) is most probably the result of differences in selection of patients. The present study has included patients with severe disease, a fact that may explain the unusually high CSF levels of TNF-α compared to previous studies. This fact may also explain the relatively high number of patients with active MS who showed high Q_{alb} values. Another possible reason for the relatively higher CSF levels of TNF-α reported here may be the improved detectability following the addition of a protease inhibitor since like IL-2, the activity

of TNF-α is labile to various proteolytic enzymes, including trypsin, chymotrypsin, and *Staph aureus* V8 protease (Aggarwal and Pocsik 1992). In fact, separate studies have shown that the addition of the protease inhibitor improved detectability of TNF-α in CSF and serum sixfold (Sharief 1991).

The higher CSF levels of TNF-α in patients with active MS compared to serum levels reported here are also compatible with earlier reports (Maimone et al 1991; Tsukada et al 1991) and suggest local release of this cytokine within the intrathecal compartment (Sharief and Hentges 1991). In this regard, activated T lymphocytes and macrophages, which are abundant at the active edges of acute MS plaques, are potent sources of TNF-α. In support of this observation, Selmaj and others (1991a) demonstrated elevation of TNF-α-positive cells in MS brain lesions whereas no elevation of TNF-α-positive cells could be detected in the spleen or peripheral blood. In addition, astrocytes have been shown to produce TNF-α both in culture and in situ in MS brain lesions (Selmaj et al 1991a). The lack of correlation of CSF TNF-α with pleocytosis or reactive lymphocytes suggests that this cytokine may be released by CNS rather than CSF cells. The relation of TNF-α levels with CSF macrophages is currently unknown but would be the subject of future studies.

TNF-α is considered to be an important mediator of inflammatory responses within the CNS, and effects of this cytokine on neural cells are therefore widely studied. TNF-α-mediated responses within the CNS include *(1)* enhancement of class I MHC antigen expression on astrocytes and oligodendrocytes (Lavi et al 1988), *(2)* enhancement of class II MHC expression on astrocytes through its synergistic interaction with other cytokines (Massa et al 1987), *(3)* induction of ICAM-1 on human foetal astrocytes (Forhman et al 1989), *(4)* proliferation of adult astrocytes (Selmaj et al 190), *(5)* induction of other cytokine production by astrocytes (Frei et al 1989), and (7) lysis of oligodendrocytes, which produce myelin in the CNS (Robbins et al 1987), as well as direct damage to myelin sheaths (Selmaj and Raine 1988). Known effects of TNF-α on human endothelial cells will be discussed later.

5.5.4 Relationship between TNF-α and IL-2

Prominent correlation existed between TNF-α and IL-2 in the CSF of patients with active MS. This finding corroborates and extends previous reports, which detected

a correlation between serum levels of IL-2 and TNF-α in patients with progressive MS (Trotter et al 1991). Indeed, it is a known fact that IL-2 induces an increased production of TNF-α by lymphocytes *in vitro* (Nedwin et al 1985) and macrophages (Economou et al 1989). In addition, clinical studies have documented that adminstration of IL-2 to humans could result in secondary TNF-α secretion *in vivo* (Miles et al 1991).

The positive correlation between blood-brain barrier disruption and CSF levels of IL-2 and TNF-α may be relevant to the pathological endothelial abnormalities induced by TNF-α. Although the detection of elevated levels of IL-2 and TNF-α in the CSF may merely reflect the presence of activated T cells or macrophages , earlier pathologic evidence suggests that some IL-2-associated conditions of blood-brain barrier damage may be causally related to high CSF levels of TNF-α. It has been discussed in previous chapters that activated astrocytes, oligodendrocytes, and other microglial cells synthesise and secrete IL-2. Therefore, the intrathecal production of this cytokine can generate and potentiate inflammatory immune responses through the subsequent induction of other inflammatory mediators that include TNF-α. The following section will discuss the role of TNF-α in barrier damage based on the known effects of TNF-α on human endothelial cells.

5.5.5 Role of TNF-α in barrier damage

A strong correlation was detected between CSF levels of TNF-α and disruption of blood-brain barriers in active MS. Moreover, TNF-α levels correlated with the degree of barrier impairment suggesting that this cytokine may be related to the pathogenesis of barrier damage. Although endothelial cells immunoreactivity for TNF-α has recently been noted at the edge of acute lesions in MS (Selmaj et al 1991b), the current work represents the first report of a direct correlation between TNF-α and damage to the blood-brain barrier in patients with MS.

A putative TNF-α-induced disruption of blood-brain barriers could result from several mechanisms. TNF-α causes increased vascular permeability by inducing morphologic and structural changes of endothelial cells through a direct toxic effect. It also downregulates endothelial cell expression of thrombomodulin and causes enhanced procoagulant activity that promotes intravascular coagulation and capillary

thrombosis. In addition, TNF-α changes the propensity of endothelial cells to bind neutrophils, possibly contributing to the intense margination response that is observed *in vivo* following infusion of the cytokine (Beutler and Cerami 1990). Furthermore, leucocytes, particularly neutrophils, adherent to endothelial cells are stimulated by TNF-α to increase biosynthesis and release of reactive superoxide intermediates and arachidonic acid metabolites. Indeed, 100 U/ml of TNF-α, a concentration readily attainable in CSF of patients with active MS, stimulate eosinophils and other cells to damage human endothelial cells *in vitro* (Slungaard et al 1990). Hitherto, the interaction between TNF-α and cellular elements in MS brain lesions is not clearly identified.

5.5.6 Role of other mediators in barrier damage

It was clear form Fig. 5.11 that high concentrations of TNF-α are not always critical in mediating blood-brain barrier disruption in patients with active MS. The fact that IL-2-induced barrier disruption has developed in the absence of TNF-α reactivity suggests that other mediators may be involved in this process. Interleukin-1β has been suggested to cause disruption of blood-brain barrier in experimental animals (Quagliarello et al 1991). In addition, IL-1β was reported to initiate meningeal inflammation without a concomitant increase in TNF-α levels (Ramilo et al 1990). However, no IL-1β reactivity was detected in the CSF of MS patients included in the present study despite the presence of severe barrier disruption. Serum IL-1β was detected in only 4 (3.6%) patients with active MS included in this study (mean±SD= 84±49 ng/ml). Therefore, it is tempting to suggest that IL-1β is not associated with blood-brain barrier impairment in MS patients, although the influence of sub-clinical levels of IL-1β cannot be excluded.

This author believes that the influence of IL-1β in the generation of barrier disruption in humans is negligible. Evidence for this has emanated from studies of the kinetics of IL-1β and TNF-α in conspicuous models of blood-brain barrier damage, such as acute bacterial meningitis. As can been seen from Appendix A (page ?), clear evidence has been presented that a TNF-α-mediated endothelial damage can be dissociated from the presence of IL-1β.

5.5.7 An overview on the cytokine-mediated barrier damage

Although results presented above argue for a putative role of IL-2 and TNF-α in the disruption of blood-brain barrier in MS, pathological studies are required to provide a causal relationship between barrier damage and high levels of IL-2 and TNF-α. It must also be emphasized that cerebral endothelial damage should not be considered a result solely of overproduction of IL-2 and TNF-α, thereby ignoring the complex interaction between cytokines and other mediators such as prostaglandins and leukotrienes. Studies of these and other mediators will provide a further insight into the pathogenesis of barrier damage in patients with active MS. Moreover, the regulation of TNF-α expression is exceedingly complex, and can be controlled at both the transcriptional and posttranscriptional level. This regulation involves cooperative interaction between TNF-α promoter elements and the 3′ untranslated region (Han et al 1991). As a result, induction of TNF-α release should not be linked only to an IL-2 effect, thereby excluding the influence of other members of the cytokine network.

It should be pointed out that *in vivo* damage to blood-brain barrier could be more precisely detected by MRI with gadolinium-DTPA enhancement (Hawkins et al 1991). It remains to be determined whether intrathecal production of IL-2 or TNF-α correlates with MRI-detected blood-brain barrier damage. Similarly, disruption of blood-brain, and particularly blood-CSF, barriers could be better defined by throught polyacrylamide gel electrophoresis (PAGE) of the CSF (Reviewed by Thompson 1988). The correlation between intrathecal release of cytokines and CSF changes detected by PAGE is unknown at present but should be the subject of further investigation.

In conclusion, findings from this study provide a molecular basis for intrathecal inflammatory response in MS patients. Results presented here indicate that IL-2 and TNF-α are related to cerebral endothelial impairment in patients with active MS. In view of previous reports, it is likely that a direct cytokine effect mediated by IL-2 and TNF-α may underline not only the observed impairment of blood-brain barrier but other pathophysiological changes in MS. It is hoped that this and future related studies will add to the general understanding of the pathogenic effects of immunological alterations on CNS cells in patients with MS.

CHAPTER 6. CORRELATION OF IL-2 and sIL-2R WITH CLINICAL ACTIVITY OF MS

6.1 Introduction

It was clear form the data reported in Chapter 5 that intrathecal levels of IL-2 and sIL-2R are associated with impairment of the blood-brain barrier in active MS. Damage to the blood-brain barrier, in turn, is commonly associated with deteriorating clinical status of several neurologic diseases. As a result, intrathecal levels of IL-2 and its soluble receptor may correlate with MS diseases activity. There is indeed increasing evidence that elevated level of IL-2 and sIL-2R may reflect the clinical course of multiple sclerosis (Selmaj et al 1986; Calder et al 1987; Greenberg et al 1988; Trotter et al 1990). As discussed in previous chapters, several studies have reported increased levels of IL-2 in serum or CSF in patients with clinically active MS. Serum or CSF levels of sIL-2R were also shown to be significantly higher in patients with active MS when compared to patients in remission (Calder et al 1987; Adachi et al 1990; Kittur et al 1990).

One of the hallmarks of CNS lesions in MS is multifocal areas of primary demyelination (plaques). Extensive lymphoid infiltration is often found associated with plaques, although inflammatory cells are also found in normal appearing white matter, around venules, and in the leptomeninges. In these plaques, there is myelin phagocytosis, various degrees of axonal and oligodendroglial cell loss, and gliosis. These changes are a reflection of a dynamic multiphasic process , which places MS in the category of primary inflammatory demyelinating diseases (Adams 1983).

In view of the fact that T cells may be involved in the pathogenesis of MS (Waksman et al 1984; Hafler and Weiner 1989) and the fact that T cells regulate B-cell maturation and immunoglobulin synthesis, attempts have been made to identify T cells in brains of MS patients. A qualitative report by Traugott *et al* (1983a) described both helper-induced ($CD4^+$) and cytotoxic-suppressor ($CD8^+$) T cells at the margin of active chronic plaques, and mainly $CD4^+$ cells in adjacent normal-appearing white matter. A supplemental report (Traugott et al 1983b), using quantitative methods, showed the distribution of T cell subsets and macrophages to vary with lesion activity. In acute plaques, $CD4^+$ cells, $CD8^+$ cells, and Ia^+ macrophages were more numerous at the lesion centre, and no T cells or Ia^+ cells were seen in the normal

white matter. Further studies (McCallum et al 1984) found that both the $CD8^+$ and $CD4^+$ cells peaked at the plaque edge. Although the $CD8^+$ subset predominated, the ratio varied greatly.

Both the $CD4^+$ and $CD8^+$ subsets can secrete IL-2 (see Chapter 1). The correlation of these T cell subsets with active plaques, therefore, raises the question whether measurement of IL-2 and sIL-2R can reflect the degree of activity of MS. Indeed, serum samples from MS patients in stable clinical remission contained significantly lower concentrations of sIL-2R than from those with clinically active disease (Hartung et al 1990). However, the correlation of intrathecal IL-2 and sIL-2R levels with the extent of disease activity in patients with relapsing-remitting MS remains largely unknown.

Although moderns tests such as MRI can visualise lesion dissemination, assessment of disease activity in MS remains largely clinical (Poser et al 1982; Thompson et al 1987). Such assessment relies on a number of clinical features such as evidence of a relapse, number of relapses, degree of disability, and total duration of the disease process. Therefore, it would be of interest to examine whether clinical parameters of disease activity in MS correlate with intrathecal release of IL-2 and its soluble receptor.

Data presented in Chapter 5 have suggested that high CSF levels of IL-2 in patients with active MS may induce intrathecal release of TNF-α. Furthermore, evidence is accumulating that TNF-α is capable of inducing selective damage to oligodendrocytes and myelin sheaths *in vitro* (Selmaj and Raine 1988), a finding that may be relevant in the context of disease progression in patients with MS. However, the *in vivo* relation between TNF-α and signs of disease activity in patients with MS is currently poorly identified.

To determine whether the *in vivo* levels of IL-2, sIL-2R, and TNF-α in MS correlate with clinical features of activity, CSF and serum levels of these cytokines were measured in MS patients in relapse and those in clinical remission. These levels were compared with values of IgG index (Tibbling et al 1977), which estimates the intrathecal humoral immune response. Intrathecal IgM synthesis will not be evaluated in this chapter since it is not widely used by other laboratories to assess MS disease activity.

Hitherto, no known parameter has been demonstrated to predict the subsequent course of patients with chronic progressive MS. The second objective of this chapter therefore was to examine the *in vivo* correlation between TNF-α and MS disease progression. To achieve this objective, TNF-α levels in CSF and serum samples from patients with chronic progressive MS were correlated with clinical evidence of disease progression over a period of two years. Findings were compared to those from patients with clinically stable MS to establish whether TNF-α could be used as an independent marker of progression in chronic progressive MS.

6.2 Patients

6.2.1 Patients with relapsing-remitting MS

Paired serum and CSF samples from the 110 patients with active MS (see Table 3.4) were utilised in this study. These patients were in clinical relapse during sample collection. A relapse phase was defined as the interval within two weeks of worsening of existing symptoms provided that the course has been stationary or has improved during the previous month (see section 3.2). Paired samples from another 50 patients (29 females) with clinically definite MS were included in the present analyses. These patients were in clinical remission when CSF and homologous serum samples were collected. Clinical remission was defined as an improvement in symptoms or neurologic signs for at least one month (Poser et al 1983).

Elective lumbar punctures were performed on the MS patients in remission as part of the routine diagnostic work-up to investigate preceding neurologic complaints. No patient involved in the study had received immunosuppressive treatment for at least 8 months prior to CSF collection. Patients who had received total lymphoid irradiation at any stage of their illness were excluded from the study. Xanthochromic CSF samples or samples containing more that one erythrocyte per µl at the time of collection were excluded. All samples were filtered through a 0.45-µm disposable sterile filter and contained 1000 kallikrein inhibitor units of the protease inhibitor. Assay of IL-2, sIL-2R, and TNF-α were performed as described in previous chapters. Calculation of the IgG index was performed as described in Chapter 3.

6.2.2 Patients with chronic progressive MS

The study to assess the importance of TNF-α in disease progression utilised the 38 patients with chronic progressive MS described earlier (see Table 3.4). The study was planned prospectively with stringent selection criteria. Patients were selected for further study if they had a chronic progressive disease with clear adherence to the originally assigned disease type, were between 20 and 50 years of age, their CSF samples were collected on entry and at the same time of disability assessment, were able to attend hospital follow-up appointment two years after entry into the study, and had details of the duration of the disease clearly documented in their medical notes. Patients were excluded if they had disorders that compromised assessment of neurologic function, such as deforming arthritis, major amputations, or psychoses. The study also excluded patients who had other types of illnesses that could affect the outcome of the study; pregnant women or those planning to become pregnant within two years; and patients who had received treatment with corticosteroids for one year before entry, or immunosuppressive medications or total lymphoid irradiation at any time.

Of the 38 patients who were originally considered, 32 satisfied the pre-established selection criteria and were included in the study. They had a mean age±SD of 37.4±6.3 years and had steady progression of the disorder since its onset. Their degree of disability at the time of CSF collection was assessed using the expanded disability status scale as detailed below (see section 6.3.1). All patients with chronic progressive MS were required to have objective evidence of disease progression without remission or stabilisation over a minimum of one year before the study, with an increase of a 1.0 or more points on the disability score. At the beginning of the study they had disability scores of 3 to 6 points. On the initial visit, levels of TNF-α in CSF and corresponding serum samples were measured blind to clinical data but at the same time of disability rating. The patients were then followed regularly for two years. No samples obtained before the study began were included. At the end of the study, the degree of disability was determined for all patients by the same examiner who evaluated them at the beginning of the study to avoid inter-examiner variability (Noseworthy et al 1990).

6.3 Methods

6.3.1 Assessment of Disability

The degree of disability at the time of CSF collection was assessed by the expanded disability status scale (EDSS; Kurtzke 1983). The scale, which helps monitor the natural history of MS, uses a scoring system with 0.5-step increments to record objective neurologic impairment. The EDSS scores range from 0 (normal) to 10 (death due to MS) as summarised in Table 6.1. The EDSS scores of 98 MS patients with active relapsing-remitting MS were determined by this author within 48 hours of the lumbar puncture. Scores of the remaining 12 patients with active relapsing-remitting MS were calculated through examination of hospital notes. The EDSS scoring of patients with chronic progressive MS has been described above.

Certain drawbacks in the use of the EDSS system have been reported. The EDSS scores may not change linearly with time (Hughes 1991) and may have relatively wide inter-observer variability (Noseworthy et al 1990). However, the EDSS was designed to measure global impairment taking into account all the relevant functionally independent systems. It is widely recommended that until another scale has been developed and shown to be superior, the EDSS must remain the gold standard (Francis et al 1991; McFarland 1992).

The date of onset of MS and number of relapses were obtained from previous medical records since patients with MS are prone to some degree of cognitive impairment, which could lead to inability to provide a comprehensive medical history. As explained in section 6.4.6 below, accurate information regarding disease duration is important to calculate the progression index, which is the ratio of the EDSS score to the total duration of the disease in years.

6.3.2 Statistical analyses

Wilcoxon's, Spearman's, and two-sided Mann-Whitney tests, and Kruskal-Wallis one way analysis of variance were calculated, as appropriate, for statistical analysis. Relative relations of CSF TNF-α to clinical and biochemical indicators of progression in patients with chronic progressive MS were tested by forward stepwise multiple logistic regression analysis with CSF TNF-α as the dependent variable. Log transformation was necessary because of the skewed distribution. Backward stepwise logistic regressions were also performed and produced identical results in all cases.

Table 6.1. The Expanded Disability Status Scale.

Score	Definition
0	Normal neurologic examination
1.0	No disability, minimal signs in one FS[a]
1.5	No disability, minimal signs in more than one FS
2.0	Minimal disability in one FS
2.5	Minimal disability in two FS
3.0	Moderate disability in one FS, or mild disability in three or four FS though fully ambulatory
3.5	Fully ambulatory but with moderate disability in one FS
4.0	Fully ambulatory without weight, self-sufficient, up and about 12 h a day despite relatively severe disability. Able to walk 500 m without aid or rest
4.5	Fully ambulatory without aid, up and about much of the day, able to work a full day, may require minimal assistance. Has relatively severe disability. Able to walk 300 m without aid or rest
5.0	Ambulatory without aid or rest for about 200 m. Disability severe enough to impair full daily activities
5.5	Ambulatory without aid or rest for about 100 m. Disability severe enough to preclude full daily activities
6.0	Intermittent or unilateral constant assistance (cane, crutch, or brace) required to walk about 100 m with or without resting
6.5	Constant bilateral assistance (canes, crutches, or braces) required to walk about 20 m without resting
7.0	Unable to walk beyond about 5 m even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone. Up and about in wheelchair 12 h a day
7.5	Unable to walk more than a few steps; restricted to wheelchair; may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day. May require motorised wheelchair
8.0	Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms
8.5	Essentially restricted to bed much of the day; has some effective use of arms; retains some self-care functions
9.0	Helpless bed patient; can communicate and eat
9.5	Totally helpless bed patient; unable to communicate or eat/swallow
10	Death due to MS

[a]*FS denotes Functional Systems, which include pyramidal, cerebellar, brainstem, sensory, visual, cerebral, and sphincteric functions*

6.4 Results

6.4.1 Levels of IL-2 and sIL-2R

The distribution of IL-2 and sIL-2R in patients with active relapsing-remitting MS has been described in previous chapters. In brief, elevated CSF concentrations of IL-2 were detected in 80 (73%) patients, and elevated CSF sIL-2R in 89 (81%) patients. In contrast, abnormally high IL-2 concentrations were seen in the CSF of only 6 (12%) MS patients in remission (Fig. 6.1). Similarly, high levels of sIL-2R in CSF were detected in only 11 (22%) MS patients in clinical remission, indicating that IL-2 and sIL-2R levels in CSF could differentiate between MS patients in relapse and those in clinical remission. In contrast, high IgG index values were detected in MS patients in relapse and those in remission (a mean±SD of 1.13±0.53 versus 1.08±0.61 respectively, P= 0.21).

6.4.2 Correlation of cytokines with the degree of disability

Having established that CSF levels of IL-2 and sIL-2R clearly discriminate between patients with active MS and those in clinical remission, the next purpose of this chapter was to examine the correlation of these cytokines with the degree of MS disease activity. As has been presented earlier, clinical evaluation of disease activity depends on several factors, which include the EDSS score and number of relapses as well as total duration of the disease process.

The EDSS scores in patients with active MS ranged from 1 to 6.5 with a mean±SD of 3.4±1.4. The scores were significantly higher in MS patients who had detectable IL-2 in CSF when compared to MS patients with absent CSF IL-2 (Table 6.2). Similarly, MS patients with high CSF levels of sIL-2R had EDSS scores significantly higher than patients with normal CSF sIL-2R amounts (Table 6.3).

Table 6.2. Mean±SD of EDSS scores in patients with active relapsing-remitting MS related to the presence of IL-2 in the CSF.

IL-2 in Cerebrospinal Fluid	Mean±SD EDSS Score
Absent	1.97±0.8
Present	3.89±1.2

F value= 2.62, P< 0.005

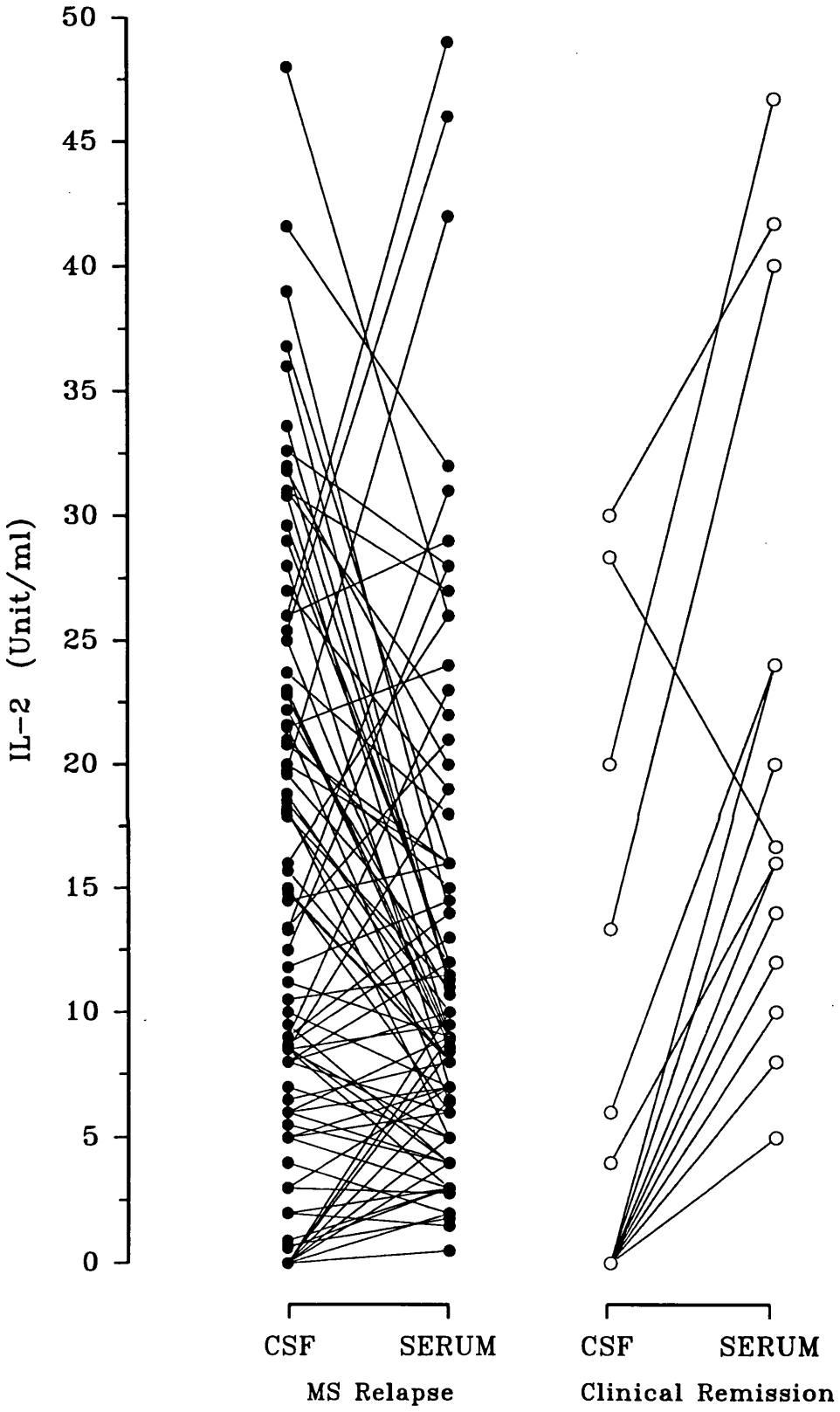


Fig. 6.1. Distribution of IL-2 in the CSF and serum of MS patients in relapse (total no.= 110) and those in clinical remission (total no.= 50). The diagram does not include patients who had no detectable IL-2 in CSF or serum (22 in relapse and 36 in remission).

Table 6.3. Mean±SD of EDSS scores in patients with active relapsing-remitting MS related to the presence of sIL-2R in the CSF.

sIL-2R in Cerebrospinal Fluid	Mean±SD EDSS Score
Absent	2.40±0.9
Present	3.60±1.4

F value= 2.12, P< 0.05

The significant relationship between the IL-2 system and the degree of disability was confirmed by Pearson's correlation matrix test. This test was also utilised to examine the correlation of serum cytokine levels with the degree of disability. CSF levels of IL-2 in patients with clinically active MS correlated with the degree of disability, as evaluated by EDSS, while serum levels of IL-2 in these patients failed to correlate with the degree of disability (Fig. 6.2). Similarly, CSF levels of sIL-2R in relapse correlated with the degree of disability, while serum levels of sIL-2R in these patients failed to correlate with EDSS score (Fig. 6.3).

CSF levels of IL-2 and sIL-2R in MS patients in remission did not correlate with the degree of disability. Although IgG index values did not discriminate between MS patients with active disease and those in clinical remission, IgG index in patients with active MS correlated with the degree of disability as assessed by the EDSS score (Table 6.4). CSF levels of IL-2 or sIL-2R did not correlate with the sex or age of MS patients included in the study.

Table 6.4. Mean±SD of EDSS scores in patients with active relapsing-remitting MS related to IgG Index, where normal value is < 0.85.

IgG Index Value	Mean±SD EDSS Score
Normal	2.78±0.9
High	3.48±1.5

F value= 2.03, P< 0.05

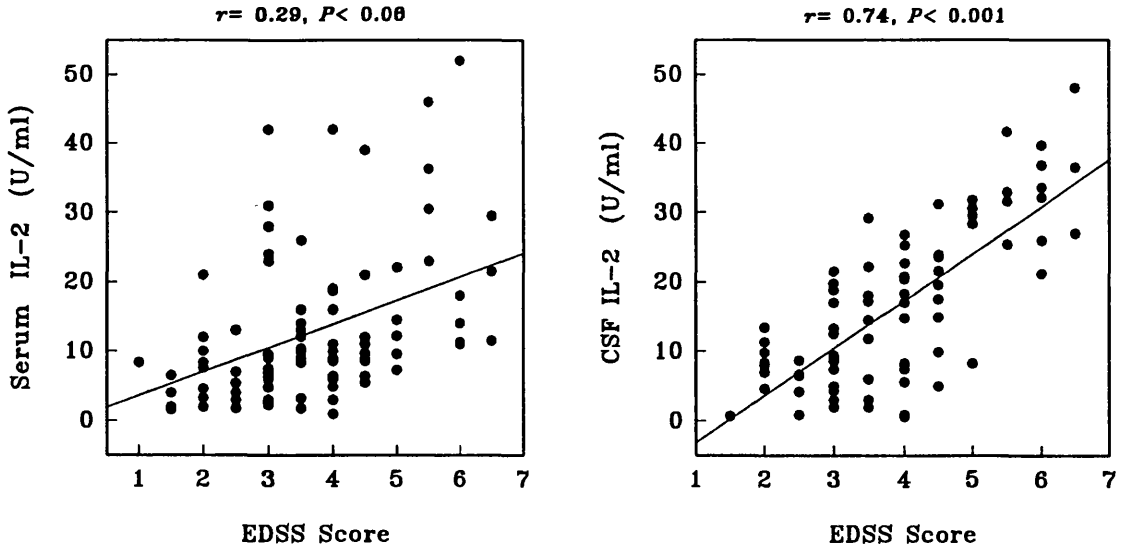


Fig. 6.2. Correlation of CSF and serum levels of IL-2 with the EDSS score in patients with active relapsing-remitting MS. Patients with no detectable IL-2 in CSF are not included.

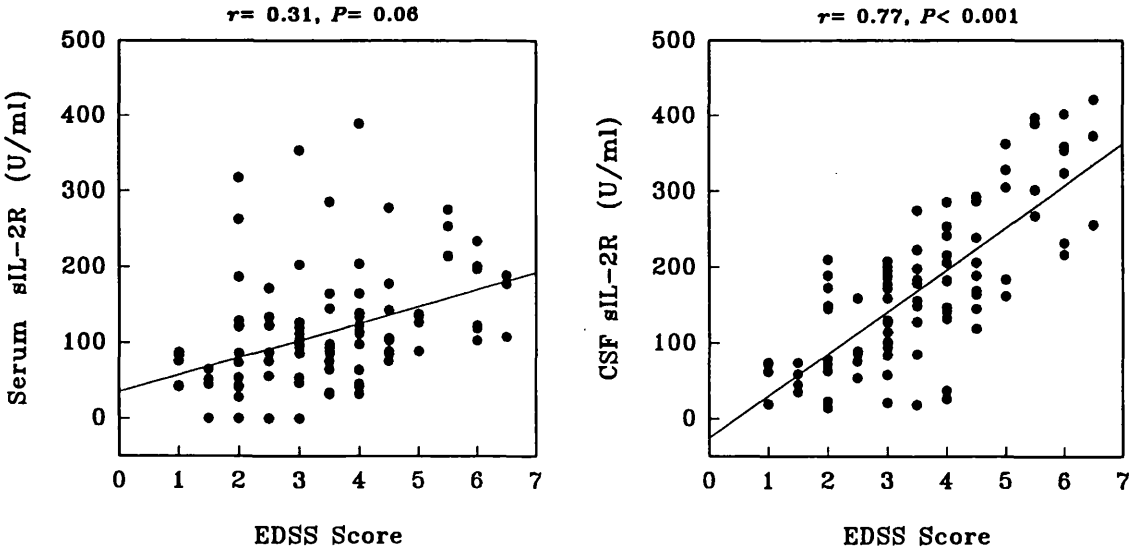


Fig. 6.3. Correlation of CSF and serum levels of sIL-2R with the EDSS score in patients with active relapsing-remitting MS. Patients with no detectable sIL-2R in CSF are not included.

Although it was obvious from Figures 6.2 and 6.3 that the degree of disability in patients with active MS correlated with CSF but not systemic levels of IL-2 and sIL-2R, correction for passive transudation of these cytokines from the systemic circulation was necessary. As can be seen from Fig. 6.4, intrathecal levels of IL-2 and sIL-2R, obtained through the calculation of CSF to serum ratios, correlated with the EDSS scores in patients with active MS ($P< 0.001$ using Kruskal-Wallis one way analysis of variance).

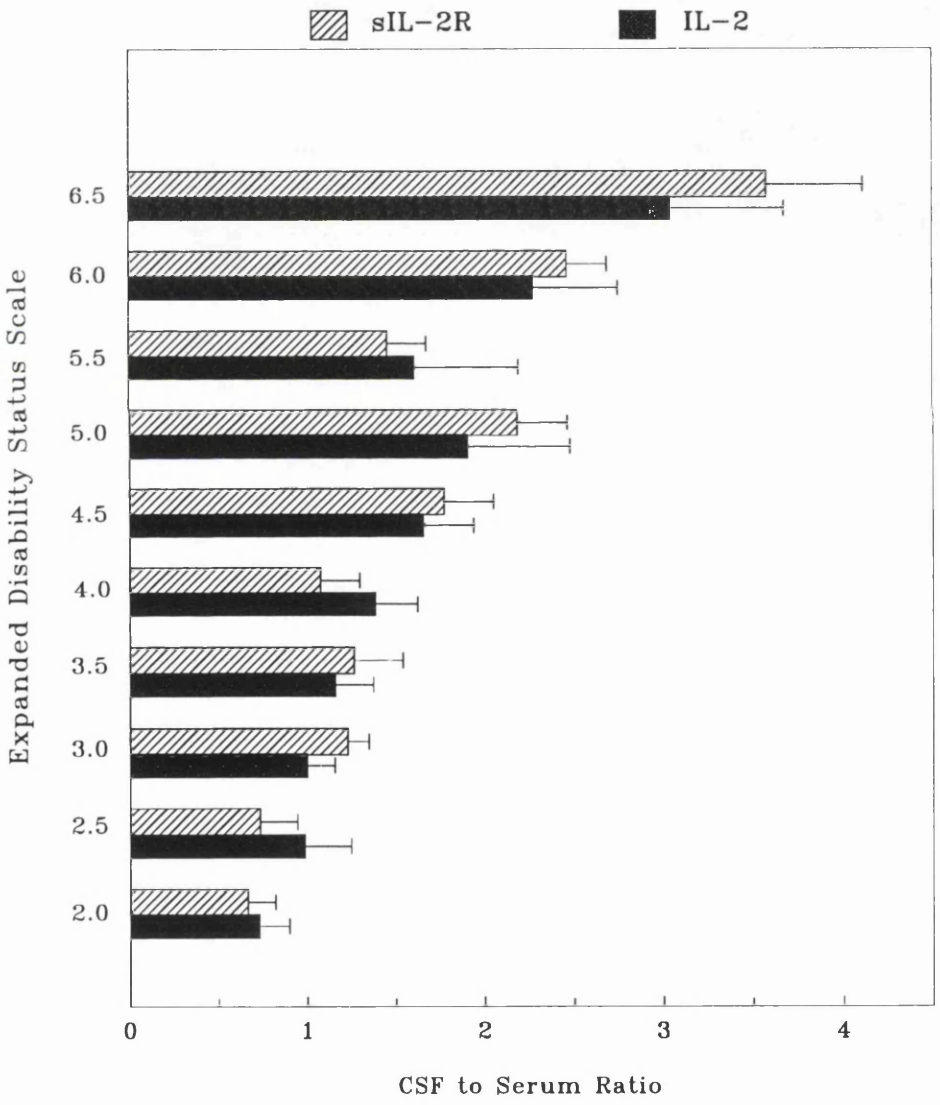


Fig. 6.4. Correlation of the EDSS score with CSF to serum ratios of IL-2 and sIL-2R in 110 patients with clinically active relapsing-remitting MS. Values depict means+SEM. See above for statistics.

6.4.3 Correlation of cytokines with other features of disease activity

In addition to their significant correlation with EDSS score, CSF levels of IL-2 and sIL-2R in active relapsing-remitting MS showed good correlation with other clinical manifestations of overall disease activity. In patients with active MS, disease duration ranged from 1.2 to 21.5 years with a mean±SD of 7.1±4.5 years. Total number of relapses in these patients ranges from 2 to 11 relapses (mean±SD= 4.6±2.1).

As shown in Fig. 6.5, CSF levels of IL-2 and sIL-2R negatively correlated with disease duration, while serum levels of IL-2 and sIL-2R in MS relapse failed to achieve significant correlation with total disease duration. Similarly, IgG index values failed to correlate with overall disease duration in patients with active MS. CSF levels of IL-2 and sIL-2R steadily decreased as the duration of MS became longer (see Fig. 6.9 below), confirming the earlier suggestion that high levels of these cytokines may reflect a relatively recent intrathecal immune response.

Table 6.5 describes the relation between CSF findings and other clinical manifestations of overall disease activity in patients with relapsing-remitting MS. As can be seen from the table, CSF levels of IL-2 and sIL-2R correlated directly with the total number of relapses during the course of the disease and inversely with the time period between a relapse and CSF collection. IgG index, on the other hand, failed to correlate with these features of overall disease activity.

Table 6.5. Correlation of CSF levels of IL-2 and sIL-2R and values of IgG index with some clinical features of activity in 110 patients with active MS. (*N.S.= Not Significant*).

Clinical Feature	Pearson's Coefficient	*P* Value
Time Interval from Last Relapse		
CSF IL-2	-0.67	< 0.001
CSF sIL-2R	-0.63	< 0.001
IgG Index	-0.27	N.S.
Total Number of Relapses		
CSF IL-2	0.60	< 0.005
CSF sIL-2R	0.47	< 0.01
IgG Index	0.11	N.S.

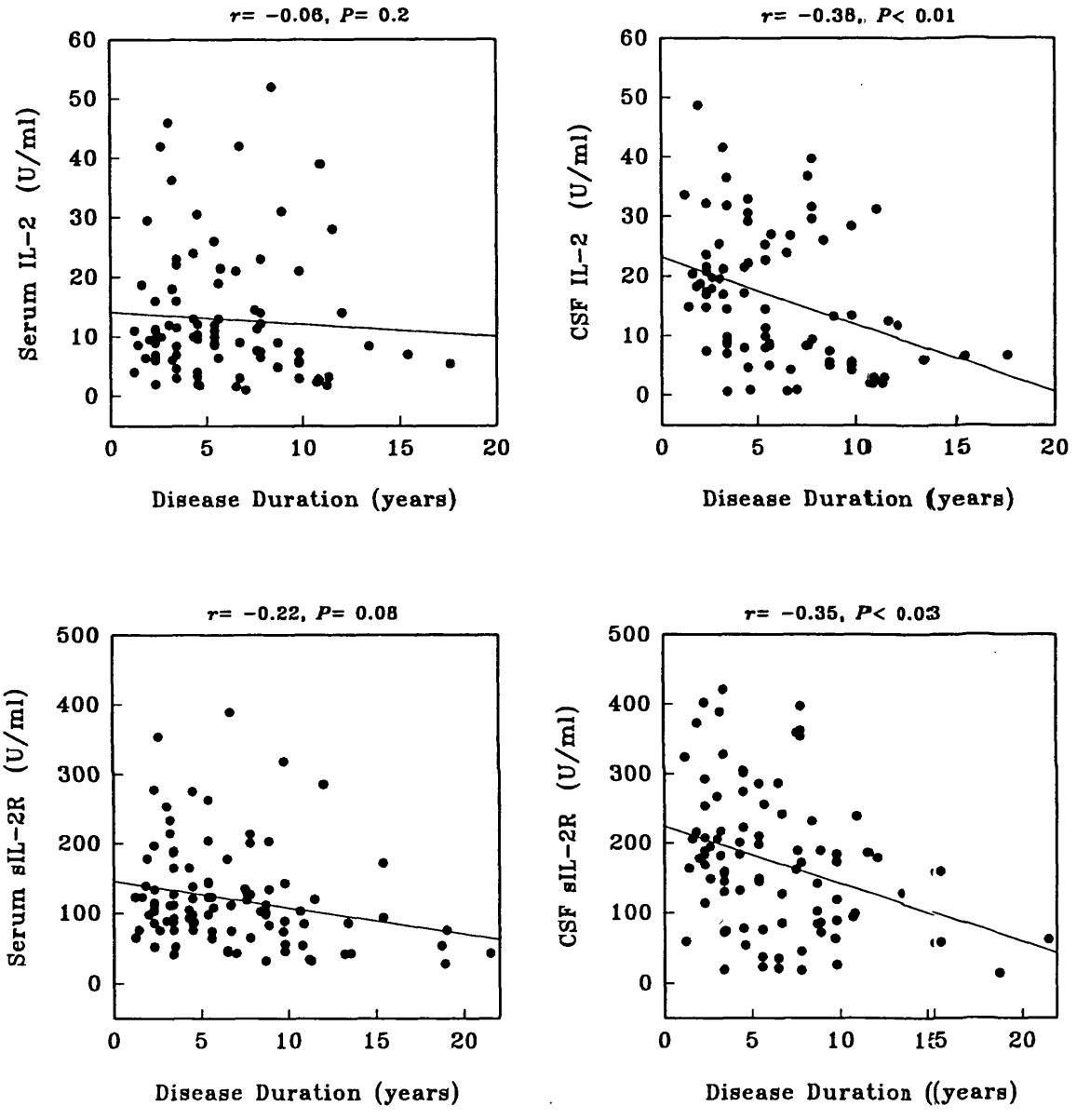


Fig. 6.5. Correlation of total disease duration with concentrations of IL-2 and sIL-2R in serum and CSF samples from patients with active relapsing-remitting MS. Patients with no detectable cytokine concentrations in serum and CSF are not included.

Calculation of CSF to serum cytokine ratios to correct for passive transudation accross the blood-brain barrier has confirmed a significant correlation between intrathecal cytokine levels and disease duration (data not shown).

6.4.4 Correlation of TNF-α with disease activity

The distribution of TNF-α in CSF and serum samples from the 110 patients with active MS has been presented in Chapter 5. Levels of TNF-α in CSF and serum in active MS were significantly higher than levels in MS patients in remission (Table 6.6), suggesting that TNF-α levels can discriminate between MS patients in relapse and those in clinical remission.

Table 6.6. Mean±SD of TNF-α concentrations in serum and CSF samples from 110 patients with active relapsing-remitting MS and 50 MS patients in clinical remission. Figures in brackets represent patients with abnormally high TNF-α levels.

Clinical Category	Mean±SD of TNF-α concentration	
	Serum[a]	CSF[b]
Active Relapsing-Remitting MS	44.1±36.2 (92)	54.3±38.9 (68)
Quiescent Relapsing-Remitting MS	5.1±1.6 (4)	4.5±2.8 (3)

[a] *F value= 43.7, P< 0.0001*
[b] *F value= 59.1, P< 0.0001*

In patients with active MS, CSF levels of TNF-α correlated with the degree of disability, as determined by the EDSS score (Fig. 6.6). As can also be seen in Fig. 6.6, serum levels of TNF-α in patients with active MS managed to achieve significant, albeit marginal, correlation with the EDSS score. However, when CSF to serum ratios of TNF-α were calculated, it was obvious that intrathecal levels of TNF-α in patients with active MS showed a significant correlation with the EDSS score (Fig. 6.7).

CSF levels of TNF-α in active relapsing-remitting MS correlated with other clinical features of overall disease activity, such as disease duration (Fig. 6.8), total number of relapses during the course of the disease, and the time interval separating the last relapse from the time of CSF collection (Table 6.7). Figure 6.9 depicts the dynamics of CSF cytokine levels during the duration of the MS disease process. CSF concentration of TNF-α in various time intervals mirrored the concentrations of IL-2 and sIL-2R — a fact that is not at all surprising, considering the relation between intrathecal levels of these cytokines (presented in section 5.4.7).

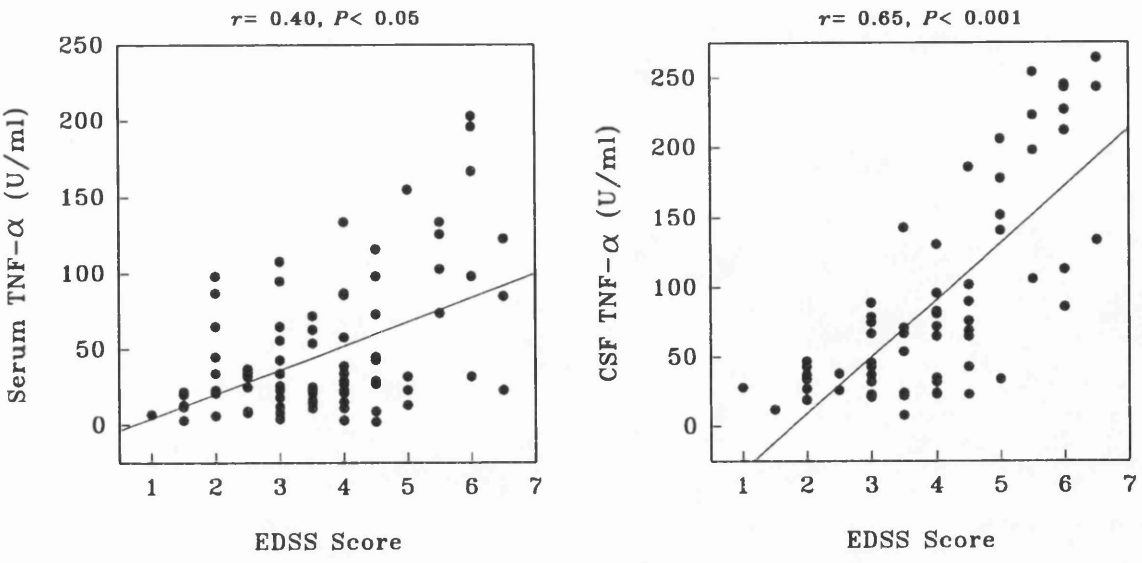


Fig. 6.6. Correlation of TNF-α concentrations in CSF and serum with EDSS score in patients with active relapsing-remitting MS. Patients with no detectable TNF-α are not included.

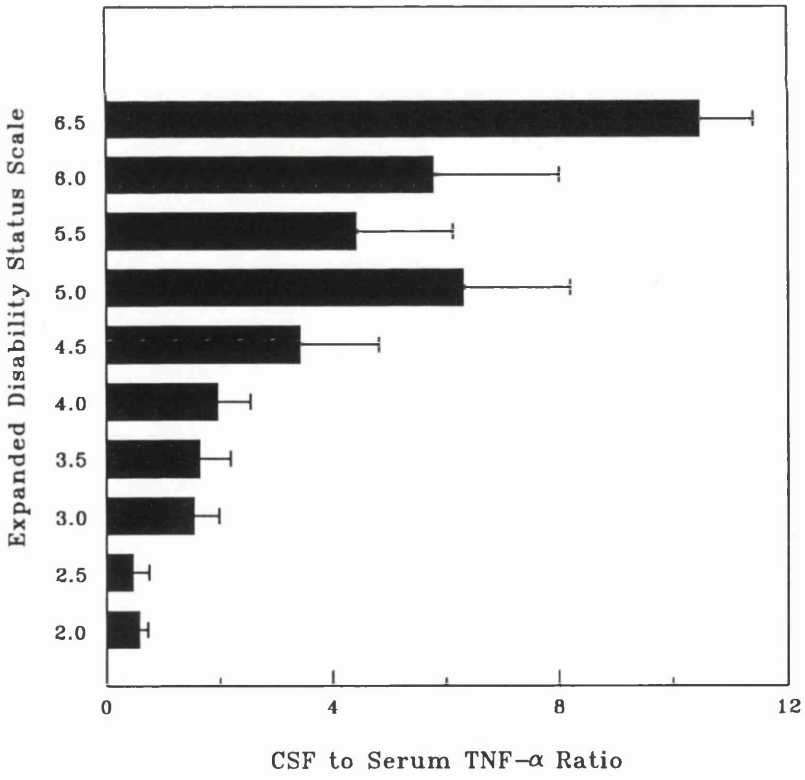


Fig. 6.7. Correlation of EDSS score with CSF to serum ratios of TNF-α in patients with active relapsing-remitting MS. Values represent means+SEM.

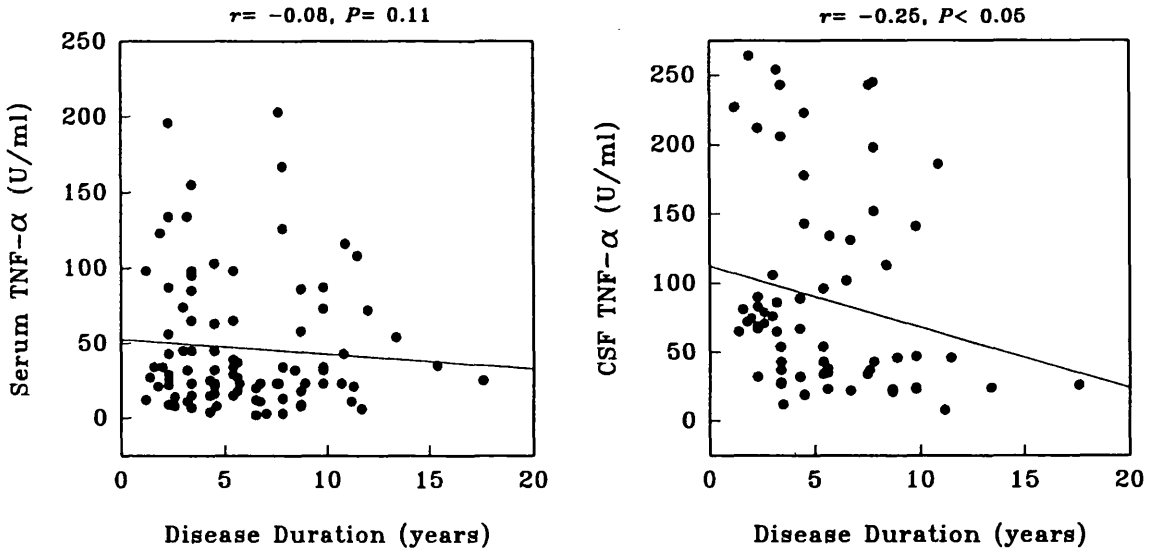


Fig. 6.8. Correlation of TNF-α concentrations in CSF and serum with total disease duration in patients with active relapsing-remitting MS. Patients with no detectable TNF-α are not included. Calculation of CSF : serum TNF-α ratio has confirmed a significant correlation between intrathecal TNF-α level and disease duration (data not shown).

Table 6.7. Correlation of serum and CSF levels of TNF-α with some clinical features of activity in 110 patients with active relapsing-remitting MS. Correlations of serum TNF-α were included in the table because of the significant relation of serum TNF-α with the EDSS score presented in Fig. 6.6.

Clinical Feature	Serum TNF-α		CSF TNF-α	
	r	*P*	*r*	*P*
Number of Relapses	0.22	NS	0.51	< 0.001
Time Interval from Last Relapse	-0.25	NS	-0.55	< 0.001

NS denotes Not Significant

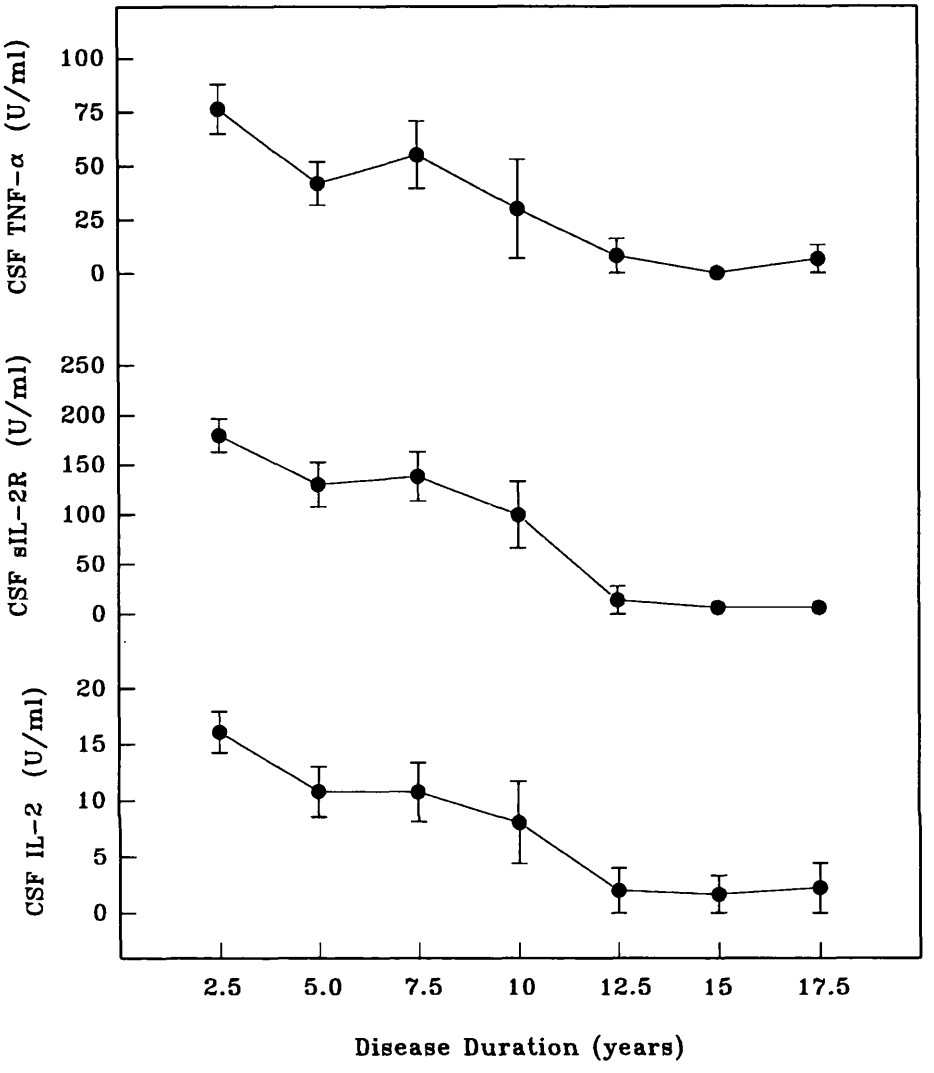


Fig. 6.9. Relationship of CSF cytokine levels with total disease duration in 110 patients with active relapsing-remitting MS. Values represent means + SEM of CSF cytokines in the periods between the intervals shown on the X axis. Calculation of CSF to serum cytokine ratios to correct for passive transudation across the blood-brain barrier has confirmed a significant correlation between intrathecal cytokine levels and disease duration (data not shown).

6.4.5 Cytokine levels in chronic progressive MS

Elevated levels of TNF-α were detected in the CSF of 17 (53%) patients with chronic progressive MS and in none of the stable MS group (Fig. 6.10). High CSF levels of TNF-α were detected in 2 chronic progressive MS patients in whom no serum levels were detected. Conversely, high TNF-α values (mean= 3.8 U/ml) were found in sera from 3 chronic progressive MS patients while CSF was negative for TNF-α. High CSF levels of IL-2 (mean= 4.3 U/ml) were detected in 5 patients with chronic progressive MS (see Fig. 3.1). These patients had a concomitant increase in serum IL-2 level (mean= 5.1 U/ml), and 4 of whom had elevated levels of sIL-2R in CSF and serum.

6.4.6 Relation between TNF-α and clinical features

In view of the relatively small number of patients with chronic progressive MS who had detectable amounts of IL-2 or sIL-2R, correlations between cytokine levels and clinical features of the disease will concentrate mainly on the CSF levels of TNF-α.

The rate of neurologic deterioration of patients with chronic progressive MS was evaluated by the progression index (Poser et al 1982), which is defined as the ratio of the disability status (i.e., the EDSS score) to the duration of the disease in years. This index is considered a reasonable estimate of the rate of deterioration (Poser et al 1982). A small quotient (< 0.2) means a benign course, whereas larger quotients indicate active disease (values above 1.5 indicate a malignant course). The duration of disease varied among the patients included in this study (range= 1 to 14 years), so that the EDSS and the progression index effectively measured two variables.

In patients with chronic progressive MS, CSF TNF-α levels correlated with the EDSS score at the beginning of the study and with the progression-index values (Fig. 6.11). Serum levels of TNF-α in these patients failed to correlate with the EDSS score or progression index.

In patients with chronic progressive MS, the relation of TNF-α levels in CSF with clinical and biochemical indicators of disease progression were analysed by multivariate regression analysis. TNF-α levels did not correlate with the presence of CSF oligoclonal IgG bands, intrathecal IgG synthesis, CSF total protein, or pleocytosis (Table 6.8). It is also clear from the table that the correlation between TNF-α levels and the EDSS score was more significant than the correlation with progression index.

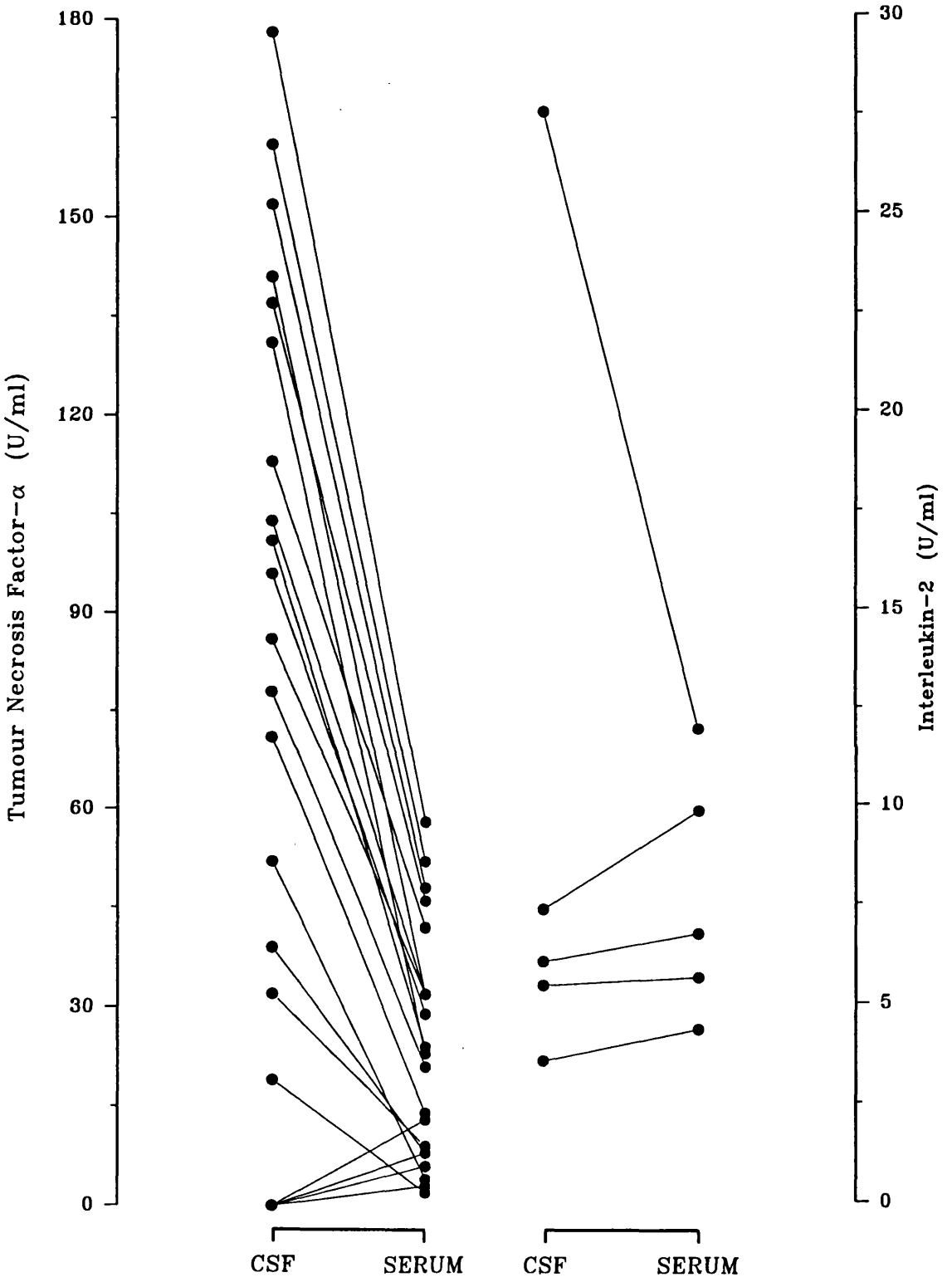


Fig. 6.10. Levels of TNF-α and IL-2 in samples from 32 patients with chronic progressive MS. Patients who had no detectable cytokine levels in both CSF and serum samples are not included. Two patients with chronic progressive MS who had elevated IL-2 in CSF are not included because they did not satisfy the inclusion criteria of this study.

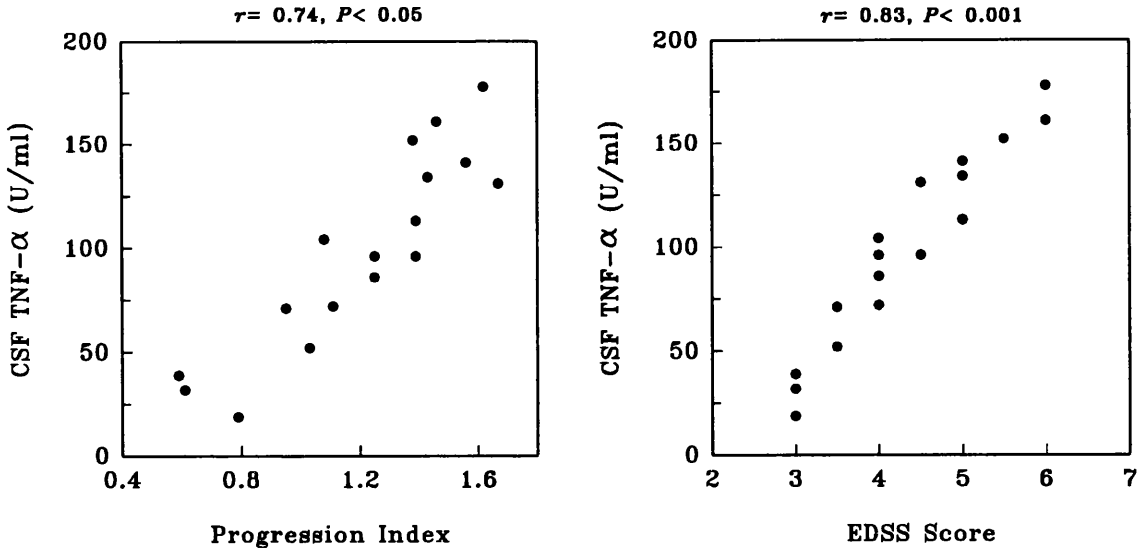


Fig. 6.11. Relation between the TNF-α level in CSF and the degree of disability (measured by EDSS) and the progression index in patients with chronic progressive MS on entry into the study. Patients with no detectable TNF-α in CSF are not included.

Table 6.8. Clinical and biochemical indicators in 32 patients with chronic progressive MS. *P* values are for multivariate regression analysis of the relations of TNF-α levels in CSF.

Variable	Mean ± SEM	*P* Value
EDSS at Entry	3.66±0.15	< 0.0005
Change in EDSS[a]	1.78±0.25	< 0.0001
Progression Index	0.99±0.07	0.027
Disease Duration (year)	3.86±0.41	NS
IgG Index	0.96±0.37	NS
CSF Total Protein (g/litre)	0.28±0.18	NS
Pleocytosis (no. of cells/mm^3)	8.27±0.91	NS

[a]Change in the EDSS score from the first to the 24-month visit.

NS denotes Not Significant

6.4.7 Disease progression after 24 months

In the majority of chronic progressive MS patients, symptoms continued to worsen during the subsequent two-year study period, whereas only two patients with stable MS had mild worsening of symptoms. There was a significant increase in disability during the 24-month period in chronic progressive MS patients who had elevated CSF TNF-α levels (Table 6.9). One patient, who had a TNF-α level of 176 U/ml in CSF and an EDSS score of 6.0 at entry to the study died after 22 months of follow-up. In contrast, there was no significant increase in disability in chronic progressive MS patients who showed no TNF-α reactivity on entry to the study (Table 6.9). Six chronic progressive MS patients who had a decline in the EDSS score of more than 3 points were treated with steroids during the follow-up period — an approach that did not significantly alter the rate of disease progression. No patient received azathioprine or cyclophosphamide during the follow-up period.

Table 6.9. Change in clinical indexes over a 24-month period in 32 patients with chronic progressive MS. Values represent means ± SEM.

Group	No. of Patients	Change in EDSS	Change in Progression Index
Elevated TNF-α in CSF	17	2.95±0.18[a]	0.18±0.07
No TNF-α in CSF	15	0.96±0.14	0.11±0.09

[a] $P = 0.004$ by the two-sided Mann-Whitney test.

In addition to their relation with the EDSS score, high CSF levels of TNF-α at the beginning of the study implied poor prognosis, since these levels correlated with an increase in the degree of disability over the next two years (Fig. 6.12). High CSF levels of TNF-α also correlated with increased EDSS scores at the 24-month visit ($r = 0.87$, $P < 0.001$). Although the EDSS score correlated with the value for the progression index at entry ($r = 0.71$, $P < 0.001$), the index values remained remarkably stable throughout the study period (Table 6.9). Nonetheless, high CSF levels of TNF-α correlated with the value for the progression index at the end of the study ($r = 0.85$, $P < 0.001$).

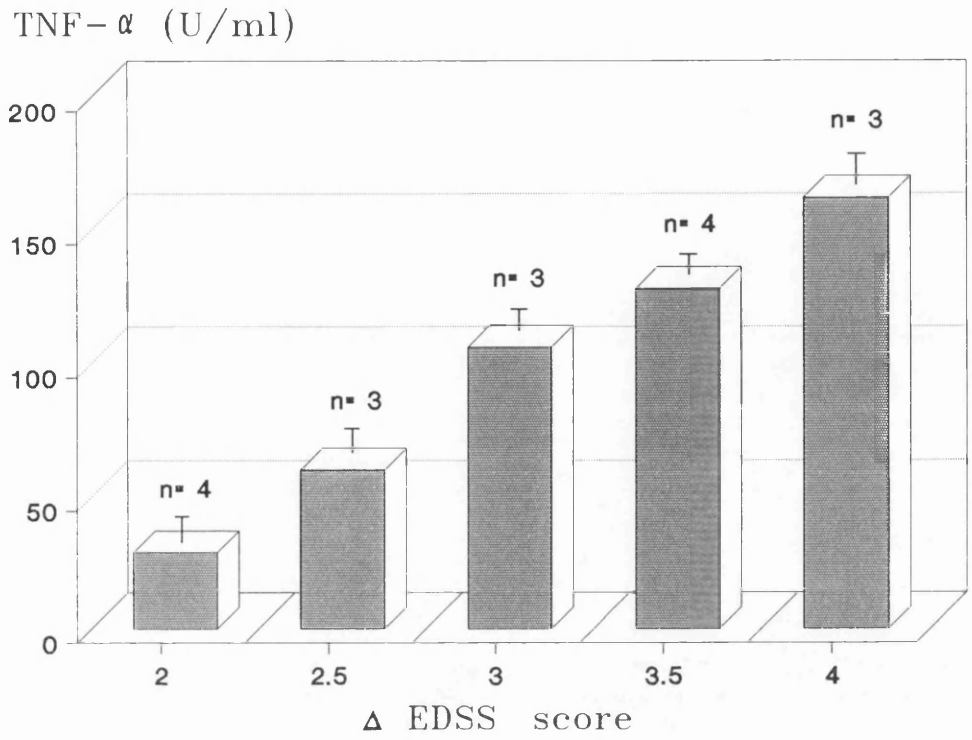


Fig. 6.12. Relation between the TNF-α level in CSF on the first visit and the change in the EDSS score (Δ EDSS) from the first visit to the 24-month visit. Values depict means + SEM. Patients with no detectable TNF-α in CSF were not included. Levels of TNF-α in CSF correlated with the difference between the two EDSS scores ($P < 0.001$).

6.5 Discussion

6.5.1 Relation between IL-2 system and the degree of disability

Results of this study clearly demonstrate that intrathecal synthesis of IL-2 and sIL-2R occurs mainly in MS patients in relapse. These results also show a significant correlation between the overall disease activity in MS patients and levels of IL-2 and sIL-2R in the CSF. Trotter and colleagues (1989) have already reported a significant correlation between serum IL-2 levels and the degree of disease disability in patients with progressive MS. They acknowledged, however, that serum levels of IL-2 may not necessarily be derived from peripheral lymphocytes, since cells staining for IL-2 may be found in plaque areas in brains of MS patients. Results presented here further extend findings of Trotter *et al* by reporting a significant correlation between levels of IL-2 and sIL-2R in the CSF and the degree of disability and relapse activity in patients with relapsing-remitting MS.

The mechanism by which intrathecal release of IL-2 and sIL-2R relate to the degree of disability in MS patients is unknown at present. However, T lymphocytes were suggested to be responsible for the progression of demyelinating plaques (Traugott et al 1983a) probably through elaboration of cytokines (Selmaj et al 1988). Moreover, a positive correlation was reported between the percentage of IL-2R-bearing lymphocytes and the degree of demyelination induced in vitro by supernatant from MS T lymphocytes (Selmaj et al 1988). Such reports indicate that IL-2 or sIL-2R are pathophysiologically important in the demyelination process. It is therefore tempting to suggest that intrathecal levels of IL-2 or sIL-2R may reflect histologic disease activity in patients with MS. Alternatively, intrathecal levels of IL-2 and sIL-2R may be a by-product of multifocal areas of white matter inflammation. This inflammation my be non-specific with demyelination occurring as a result of proteolytic enzymes liberated from cellular lysosomes (Tourtellotte 1985). Results presented here are insufficient to determine whether a cross-sectional measurement of increased CSF levels of IL-2 or sIL-2R could reflect an ongoing disease activity. Longitudinal studies involving serial testing are, therefore, necessary.

The finding of significantly higher intrathecal levels of IL-2 or sIL-2R in patients with MS relapse compared to patients in remission reported here is in full agreement with recent reports (Gall et al 1989; Adachi et al 1990). However, it is noteworthy that the clinical signs and symptoms of MS may only represent the "tip of the iceberg" (Tourtellotte 1985) of the active disease process. Signs and symptoms are a combination of residual effects of old lesions, as well as a direct expression of currently active lesions. Autopsy studies of MS brains often reveal extensive lesions that were without obvious clinical expression. There is also increasing neuroimaging evidence for the presence of subclinical lesions, either old and inactive or new and active. A classic example is that MRI scans may reveal an apparent change in the central nervous system in some MS patients without evidence of clinical change (Paty 1988) whereas, in other patients, extensive dissemination of plaques may be accompanied by no or only slight disability. Although preliminary results reported in Chapter 4 showed a satisfactory correlation between intrathecal release of IL-2 or sIL-2R and MRI-detected lesion burden, further studied are clearly required to evaluate the exact relation between cytokine levels and pathologic and MRI-detected brain lesions.

The inverse correlation between intrathecal synthesis of IL-2 or sIL-2R and total MS disease duration reported here confirms and extends earlier observation that local release of these cytokines in MS correlates with intrathecal synthesis of IgM and IgD (see Chapter 3). The process of immunoglobulin class switching from IgM to IgD or to other isotypes is important for the success of the humoral immune response. It has been established that intrathecal synthesis of IgM could indicate recent immune response within the central nervous system (Sharief and Thompson 1991 a&b), and that it is a reliable indicator of disease activity in MS (Sharief and Thompson 1989). Therefore, higher intrathecal levels of IL-2 and sIL-2R in MS patients with shorter disease duration may indicate a relatively recent immune process.

Results of this chapter suggest the presence of activated T lymphocytes within the central nervous system in MS and extend the notion that an activated cellular immune response parallels the evolution of the demyelinating process in this disease. Levels of IL-2 and sIL-2R in the CSF may be regarded as a reasonably good pathologic marker of MS disease activity. A laboratory test that reflects histologic activity is very useful not only for its diagnostic and prognostic significance but also to monitor response to treatment.

6.5.2 Therapeutic implications of IL-2-related clinical activity

The clinical and pathological heterogeneity of MS suggests that the aetiology or pathogenesis are also heterogenous. A single treatment modality, therefore, is not a reasonable goal. If an aetiologic factor has been established, it should be possible to produce specific and effective treatment regimen. However, as more and more research fails to demonstrate a single pathogenetic factor that causes MS, such a simple solution is increasingly untenable. What clinicians, and patients, require in such circumstances is some means of preventing the progression of the disease.

If intrathecal release of IL-2 and other cytokines is involved in MS progression, it might be expected that long-term reduction of intrathecal cytokines synthesis could be one aspect of an effective therapeutic intervention in this disease. Indeed, even if intrathecal cytokines synthesis is a by-product of CNS inflammation, an important goal of a putative treatment should be the eradication of this synthesis. This section will discuss therapeutic modalities that can reduce intrathecal IL-2 synthesis, whereas section 6.5.3 will discuss therapeutic approaches that may reduce TNF-α release.

6.5.2.1 Immunosuppressive drugs

Biologic and pharmacologic intervention designed to impair immunologic reactions have long been used in the treatment of immune diseases (Fahey et al 1987). Figure 6.13 represents a schematic diagram of the major cellular components that have been implicated in the pathogenesis of MS, along with an indication of the major sites of immune manipulation that may be utilised to treat patients with MS. This diagram emphasises the different functional effects of the individual compounds. Although this chapter was not primarily designed to discuss prospects for treatment in MS, a brief account of the commonly used compounds is given herein.

Glucocorticoids. Prednisone causes transitory redistribution of immune cells and numerous other metabolic changes including impaired release of destructive enzymes from inflammatory cells. It reduces the numbers of major T cell subsets and B cells, and suppresses IL-2 gene expression (Vacca et al 1992). Systemic use of ACTH and methylprednisolone has been reported to hasten recovery from an acute relapse (Milligan et al 1987).

Cyclophosphamide. This alkylating agent has profound effects on most components of the immune system. Pulse treatment with cyclophosphamide has been used with some success in MS patients (Hauser et al 1983). This drug has a selective effect that extends further within the major lymphoid subsets to include the subpopulation with several T-inducer functions. Treatment of MS patients with cyclophosphamide has resulted in temporary reduction of CD8 subsets and a progressive decline of CD4 subsets (Moodey et al 1987 a&b). Other lymphoid populations, including natural killer cells and B cells showed similar decline.

Azathioprine. It has a marginal effect on the EDSS sore and a small reduction in the number of relapses (British and Dutch trial 1988). The drug causes a preferential reduction in the natural killer lymphoid subpopulation, which is responsible for natural killer and antibody-dependent cellular cytotoxic activity. The drug has a lesser effect in reducing CD8 subsets.

Cyclosporine. It has been used with some success in MS (Trotter et al 1990). The main immunologic effect of cyclosporine is the inhibition of IL-2 release (Kermani-Arab et al 1985) and thereby inhibition of T cell-dependent proliferative reactions and some other functions, including T cell-specific cytotoxicity. It also reduces the activity of natural killer cells and induces changes in CD4 and CD8 subsets.

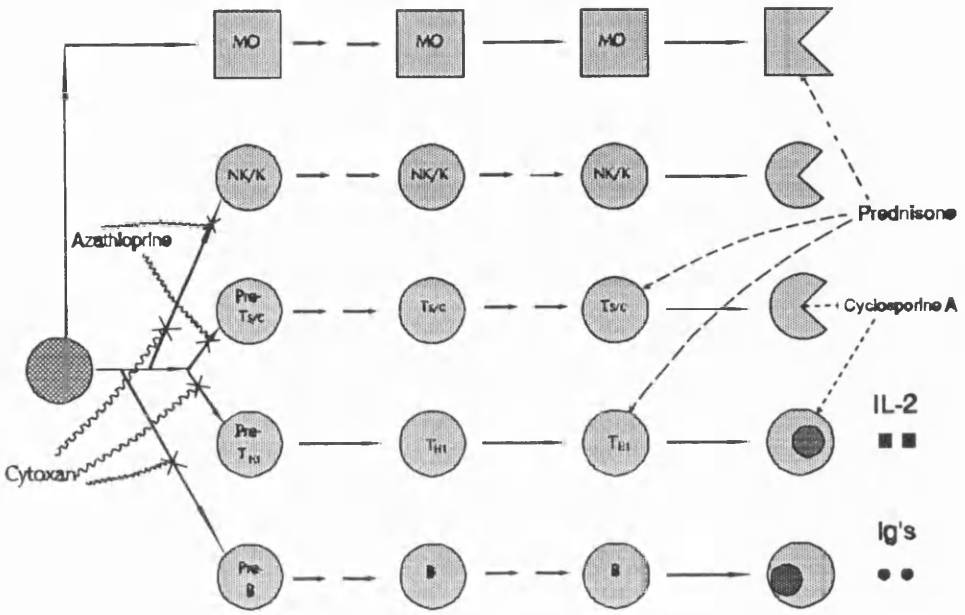


Fig. 6.13. The immune system has five major categories of cells. Maturation and activation of immune cells is indicated in the left-to-right flow of the diagram. Major sites of immunosuppressive drugs action are indicated in the diagram. Prednisone represents the glucocorticoids group, and cytoxan is a cyclophosphamide preparation.
MO, macrophages; NK, natural killer cells; K, killer cells acting in the antibody-dependent cytotoxicity reactions; Ts/c, suppressor/cytotoxic T cells; T_{H1}, T helper cells type-1; B, B lymphocytes.

6.5.2.2 IL-2 and sIL-2R as targets for immunotherapy

In view of the putative role of the IL-2 system in a variety of autoimmune disorders, monoclonal antibodies against IL-2R are being evaluated as potential therapeutic agents to eliminate IL-2R-expressing T cells in a variety of autoimmune conditions and in protocols involving organ allografts (Waldmann 1989). In addition, anti-IL-2R antibodies have been reported to suppress disease progression in experimental autoimmune encephalomyelitis, which is an experimental model of demyelination (Diamantstein 1986).

Monoclonal antibodies that recognised the IL-2-receptor have been used to inhibit organ allograft rejection. Anti-IL-2R was shown to inhibit the proliferation of T cells to foreign histocompatibility antigens expressed on the donor organ and to prevent the generation of cytotoxic T cells in allogenic cell cocultures. Furthermore, the survival of renal and cardiac allografts was prolonged in rodent recipients treated with an anti-IL-2R monoclonal antibody (Kirkman et al 1985).

In light of these encouraging results, human recipients of cadaver donor renal allografts are receiving different IL-2R monoclonal antibodies as adjunctive immunotherapy (Soulillow et al 1987). Antibody treatment has been well-tolerated, and 50 of 53 recipients treated retain a functioning allograft. Thus, the development of monoclonal antibodies and toxin-cytokine conjugates, directed toward the IL-2R expressed on autoreactive T cells of certain patients with autoimmune disorders and on host T cells responding to foreign histocompatibility antigens of organ allografts may lead to the development of rational, novel therapeutic approaches for these clinical conditions.

6.5.3 TNF-α and progression of MS

Results of this study demonstrated significantly higher levels of TNF-α in the CSF of patients with chronic progressive MS compared to patients with stable MS. Further, CSF levels of TNF-α correlated with the disability of patients with chronic progressive MS, and predicted poor prognosis after 24 months of observation. These results support a role for TNF-α in the progression of MS. It has already been suggested that TNF-α has a role in the pathogenesis of demyelination (Brosnan et al 1988). Cytotoxic T cells which can produce TNF-α, are involved in the events leading to acute demyelination (Hauser et al 1986), and TNF-α was reported to cause both disruption

of myelin and permanent damage to oligodendrocytes (Selmaj and Raine 1988). Furthermore, significantly higher amounts of TNF-α were released by mitogen-stimulated cells obtained from MS patients in clinical exacerbation than in those obtained from patients during remission (Beck et al 1988).

The degree of disease progression in patients with chronic progressive MS was evaluated by calculating the decline in the EDSS score during the study period. Although the EDSS score is an inherently ordinal scale and unit changes may not be of equal importance over its whole range, the average decline in the EDSS rating reported here is clinically important by any standard. Some natural history studies and clinical trials indicate that the EDSS scores change less than 1 point per year in patients with chronic progressive MS. To minimise the number of patients whose conditions stabilised spontaneously during the study period, more demanding selection criteria were adopted in this study compared to criteria used in previous studies (Hauser et al 1983; Khatri et al 1985). The strict entry requirements implemented here served to select patients with active disease, whose EDSS scores during the study period declined by an average of more than 2 points. Other studies that employed similarly stringent criteria (Goodkin et al 1987; Trotter et al 1989 also reported an increase in the EDSS score of 2 or more points (up to 4) during a follow-up period of 18 to 30 months.

It is important to note that each grade of disease disability in the study patients with MS was associated with a reasonable distribution of observed TNF-α concentrations, with no major overlap between individual grades. This fact, coupled with the significant correlations suggests that TNF-α may be a reliable indicator of disease progression in patients with MS. Whether TNF-α is directly involved in the progression of MS or merely reflects other basic processes is unclear. However, it has been discussed in the previous chapter that TNF-α causes severe damage to human endothelial cells (Slungaard et al 1990) and induces vascular leak syndrome — effects that may be relevant to the disease progression in MS. It has also been presented in that chapter that experimental, pathologic, and imaging data suggest that a fundamental feature of active MS lesions is a local breakdown of the endothelial barriers, particularly the blood-brain barrier (Hawkins et al 1991; Gay and Esiri 1991). The principal inflammatory process in MS usually occurs around blood vessels, with only scanty inflammatory activity in the brain parenchyma (Adams et al 1989).

Macrophages, which are abundant in all acute perivascular brain lesions, are the most potent producers of TNF-α. The perivascular infiltration in MS brain lesions also contains activated lymphocytes, which are able to produce TNF-α.

In addition to its effects on cerebral endothelial cells, TNF-α can mediate myelin destruction both *in vitro* and *in vivo*. The concept that TNF-α might be involved in myelin pathology emanated from studies showing that TNF-α was capable of inducing a delayed-onset lysis of myelin (Selmaj et al 1988) and oligodendrocyte necrosis (Robbins et al 1987). TNF-α also augments the proliferation of stimulated T and B lymphocytes (Kehrl et al 1987; Yokota et al 1988), and enhances the generation of cytotoxic T cells. Recent evidence has also been presented recently that TNF-α affects the differentiation and maturation of astrocytes, primarily through posttranscriptional alteration in gene expression of glial fibrillary acidic protein, possibly at the level of mRNA stability.

Chapter 5 has presented evidence that higher levels of TNF-α in CSF compared to serum levels indicate local release of TNF-α within the intrathecal compartment. CNS production of TNF-α may result from release by macrophages and T-lymphocytes, which are abundant in MS brain lesions, as well as astrocytes and microglial cells (Robbins et al 1987). In fact, TNF-α has been detected in astrocytes within MS brain tissues (Selmaj et al 1991b). The failure to detect TNF-α in the CSF of patients with MS reported in another study (Franciotta et al 1989) may have been due to differences in the way patients were selected or to the instability of TNF-α if not treated with a protease inhibitor. In addition, the ability to detect TNF-α depends on the particular assay used, since different procedures have been shown to vary significantly in their sensitivity and limits of detection (McLaughlin et al 1990). However, the demonstration of a significant increase in TNF-α levels in CSF relative to levels in serum has been corroborated by two recent independent studies (Maimone et al 1991; Tsukada et al 1991).

No correlation was found between neurologic impairment and serum TNF-α levels in MS patients. This finding raises the possibility that intrathecal concentrations of TNF-α are more important than systemic levels in the clinical progression, and perhaps histologic activity, of MS. Because this study was conducted in a blinded fashion, the clinical significance of TNF-α levels in the CSF during the follow-up period was not recognised. Thus, repeated collections of CSF samples were not

ethically justified. However, repeated measurements are indicated to evaluate how changes in TNF-α levels may be correlated with changes in the trajectory of disease. Similarly, further studies will be required to elucidate the interactions between TNF-α and other cytokines, such as lymphotoxin, in the progression of MS.

6.5.4 TNF-α as an independent marker of progression

It has been suggested recently (Liblau and Fugger 1992) that the elevated levels of TNF-α in patients with chronic progressive MS is not necessarily important as an independent marker of progression of this disease. It follows that because the genes encoding TNF-α are located within the major histocompatibility complex, high levels of this cytokine in MS may be due to an "abnormal" gene. The association between TNF-α levels and class II HLA molecules was thought to be the result of linkage disequilibrium between HLA genes and the closely located TNF-α genes. Alternatively, high levels of TNF-α may be due to the HLA molecules themselves, determining the intensity of the immune response.

In response to the above suggestion, this author presented several lines of evidence, which indicated that the above-mentioned mechanisms are not necessarily applicable to MS (Sharief 1992a). Such evidence was based on four main facts. First, TNF-α genes are more closely linked to class I genes, particularly the HLA-B locus (Müller et al 1987), than to class II genes, which include HLA-DR2 and HLA-DR4 loci. Yet, no correlation has been found between TNF-α and class I molecules. Second, there is no correlation between the production of TNF-β and HLA class I or II molecules, although TNF-β genes are also closely linked to the major histocompatibility complex (Spies et al 1986). Of interest is the finding that TNF-β has been identified in brain lesions due to MS (Selmaj et al 1991b). Third, a restriction-fragment-length polymorphism that distinguishes HLA-DR2-positive persons from most persons with additional class II molecules failed to correlate with the quantitative variation in the production of TNF-α (Jacob et al 1990). Finally, the quantitative variations in the production of TNF-α between HLA-DR2-positive and HLA-DR4-positive subjects are mutually exclusive in patients with chronic progressive MS because either haplotypes may be expressed in these patients.

The suggestion that HLA class II molecules could determine the intensity of the immune response has been refuted by a recent study which established that HLA types do not influence the immune response in multiple sclerosis (Kinnunin et al 1990). Moreover, the suggestion that there could be a linkage disequilibrium between HLA and tumour necrosis factor-α genes in MS patients is not supported by a recent report that failed to detect a significant difference in restriction-fragment-length polymorphism of TNF-α region between MS patients and healthy controls (Fuger et al 1990).

The findings presented here may have important prognostic and therapeutic implications. A test based on CSF analysis that can predict the progression of MS could be regarded as a marker of activity and would be of great importance in monitoring outcome. This author has suggested in August 1991 that the removal of TNF-α or neutralisation of its effect may have a therapeutic role in patients with chronic progressive MS (Sharief and Hentges 1991b). This was followed by an elegant study by Cedric Raine and colleagues, who reported in November 1991 that the therapeutic adminstration of a polyclonal antibody to TNF-α inhibits effectively the development of autoimmune demyelination (Selmaj et al 1991). Such an approach might be particularly relevant to the development of new treatment strategies for active MS to alleviate lesion progression.

CHAPTER 7. IMPORTANCE OF IL-2 and sIL-2R IN THE POST-POLIOMYELITIS SYNDROME

7.1 Introduction

It has been demonstrated in the previous four chapters that the IL-2 system has a legitimate role in the diagnosis and investigation of various inflammatory CNS disorder, particularly MS. The IL-2 system may be used to confirm the presence of an intrathecal immune response in certain neurologic diseases, to monitor the activity of established inflammatory diseases of CNS, and to predict outcome of early inflammatory syndromes. However, the value of IL-2 system in establishing a precise aetiology or providing a reliable diagnosis in poorly characterised illnesses of the nervous system is largely unknown. The purpose of this chapter, therefore, is to examine the importance of the IL-2 system in investigating diseases that are suspected to be inflammatory in nature but display no clear-cut evidence of immune activation. In this study, the poorly-characterised condition of progressive muscular atrophy following poliomyelitis (the post-polio syndrome) was chosen to represent this group of poorly understood neurologic diseases.

The post-polio syndrome is a distinct clinical condition that is characterized by new, slowly progressive muscle weakness affecting patients decades after reaching maximal recovery from acute paralytic poliomyelitis. The condition has distinctive clinical features that include muscle weakness and atrophy, muscle pain and fasciculations, weakness of bulbar or respiratory muscles, and sleep apnoea (Dalakas 1986; Lange et al 1989).

Although recrudescence of muscle weakness several years after paralytic poliomyelitis was described as early as 1875 (Raymond 1875; Cornil 1875), the precise pathogenesis of the post-polio syndrome has not yet been established. Current theory suggests that the illness is caused by attrition of surviving motor neurons with eventual loss of axonal terminals (Wiechers and Hubbell 1981; Dalakas 1986). However, immunological studies have demonstrated various lymphocytic abnormalities and the presence of oligoclonal bands in CSF from a substantial number of patients with the post-polio syndrome (Dalakas et al 1986; Ginsberg et al 1989), suggesting that immunopathogenic mechanisms may contribute to the pathogenesis of the disease process. Activation of poliovirus in post-polio patients may possibly

contribute to the development of post-polio syndrome although searches for poliovirus antibodies in the CSF have yielded negative results to date (Dalakas et al 1985).

The accurate diagnosis of the post-polio syndrome is imperative not only from a therapeutic point of view, but to plan a more effective preventive scheme. Clearly, if the pathogenesis of the post-polio syndrome is related to an aberrant immune response or to activation of a latent viral infection, a therapeutic approach designed to target the aetiologic mechanisms may substantially improve prospects of recovery and long-term prevention. If, on the other hand, the pathogenesis of the post-polio syndrome is related to attrition of surviving neurones, physiotherapy and measures designed to reduce neuronal work load would suffice to halt the progression of clinical signs and symptoms observed in this condition.

One way of assessing the role of the immune system in post-polio syndrome is to detect intrathecally produced specific immunoglobulins and soluble products of immune cells such as cytokines. An antigenic challenge of the central nervous system, as of any other organ, evokes T and B lymphocyte responses with production of immunoglobulins and cytokines. Amongst various immunoglobulin isotypes, IgM is of particular importance in evaluating viral infections, including those of the CNS (Chiodi et al 1986; Forsberg et al 1986). IgM is sensitive to minimal antigenic stimulation; has a relatively short half-life with very little, if any, memory; and has an important role in the clearance of viraemia. Furthermore, intrathecal IgM synthesis is an indicator of recent antigenic stimulation within the CNS and is useful in assessing disease activity of inflammatory CNS conditions (reviewed in previous chapters).

In addition to the analysis of specific immunoglobulins, the detection of IL-2 release in evaluating the immune response in patients with the post-polio syndrome would be important for a number of reasons. First, viral infections or natural immunity to an earlier infection commonly result in the activation of T lymphocytes with consequent secretion of IL-2 and release of sIL-2R (Hsia et al 1990; Kurane et al 1991). Second, certain viral infections induce virus-specific $CD4^+$ memory T cells, which are capable of secreting IL-2 (Kurane et al 1989). Third, evidence of IL-2 release and high levels of sIL-2R have been reported in several human viral infections, including measles (Griffin et al 1989), HTLV-1 (Yamaguchi et al 1989), and

HIV infections (Prince et al 1988). Fourth, a role of IL-2 in the pathogenesis of some viral infections has been suggested (Kurane et al 1991), which was primarily based on this cytokine's ability to induce the release of pro-inflammatory mediators, such as thromboxane A_2 and $C3_a$. Finally, the release of IL-2 may be causally linked to the production of IgM antibodies (see Chapter 3).

This chapter describes a study of 36 patients with post-polio syndrome and 67 controls in which the intrathecal synthesis of IL-2 and sIL-2R was analysed and correlated with intrathecal poliovirus immune response. The purpose of the study was to determine whether the measurement of IL-2 and its soluble receptor can be helpful in both understanding the pathogenesis and reaching a proper diagnosis of the post-polio syndrome.

7.2 Patients

Paired serum and CSF samples were obtained simultaneously from all patients after giving informed consent. Protease inhibitor (Aprotinin; 1000 kallikrein units/ml) were immediately added to the samples to prevent protein degradation, then samples were frozen in aliquots at -70°C and thawed just before use.

Strict criteria were adopted for the diagnosis of post-polio syndrome which include: (*1*) clear history of acute paralytic poliomyelitis in childhood or adolescence during a polio epidemic with functional stability or recovery for at least 15 years; (2) residual muscle atrophy, weakness, and areflexia in at least one limb with normal sensation and no sign of upper motor neurone weakness; (*3*) development of new neuromuscular symptoms in the form of progressive muscular atrophy or musculo-skeletal complaints or both; and (*4*) no clinical evidence of any known medical, neurological, orthopaedic, or psychiatric illness that can explain the new symptoms. Patients with diabetes, polyneuropathies, connective tissue diseases, back injuries, compression neuropathies, and those with positive family history of neuromuscular disorders were excluded. Only patients below the age of 60 years were included in the study to avoid non-specific changes associated with ageing.[29] Ultimately, 16 men and 20 women were included in the investigation.

7.3 Controls

Paired samples were collected from age and race matched 13 patients with history of antecedent paralytic poliomyelitis who had been stable for 19 to 41 years (mean= 29.6 years) after the original infection. Lumbar puncture in this group was performed to investigate unrelated complaints such as tension headache, blurring of vision, or mild psychoneurotic symptoms. Paired samples were also obtained from 18 patients with classic motor neurone disease (MND) and from 36 age, sex, and race matched patients with various neuromuscular diseases (6 chronic progressive multiple sclerosis, 4 Parkinson's disease, 6 Alzheimer's disease, 6 cerebrovascular diseases, 6 spinal cord compression, 5 muscular dystrophies, and 3 myasthenia gravis). Paired samples from 16 normal subjects (9 females), who presented with mild non-specific tension headache or neurotic syndromes, were used to determine reference ranges.

7.3.1 Clinical examples

The following is a brief clinical history of a representative example of the post-polio syndrome: a 49-year-old woman, who had paralytic poliomyelitis at the age of 6, presented with slowly progressive muscle weakness. Hospital records of the original attack documented the occurrence of an acute febrile illness followed by generalized asymmetrical muscle weakness. Two of her school friends were also affected at the same period. She improved after intensive rehabilitation but her right arm and leg remained weaker, thinner, and shorter. She functioned well for 37 years although she had to wear a leg brace. Six years ago, she noticed a slowly progressive weakness and wasting of the left arm and leg (she never before had any difficulty with this leg) which was accompanied by deep muscle pains and occasional fasciculations of the newly weakened muscles. Progression remained focal although she had to use a manual wheelchair for the past year. In addition to the pre-existing weakness and wasting, neurologic examination detected moderate weakness and atrophy of the left biceps, hamstrings and iliopsoas muscles with normal sensation. No upper motor neuron signs were noted and cranial nerves were not involved. Electromyographic studies revealed active denervation (fibrillation and positive sharp waves) in the newly involved muscles while nerve conduction studies were normal.

The following case history is representative of the control group: a 48-year-old woman had paralytic poliomyelitis at the age of 28 months. Her brother, aged 5 years, had died of pneumonia after developing a similar illness. The woman had residual muscle weakness and atrophy of both legs but managed well with leg braces and crutches. During the past 9 years, she complained of joint pain, unsteady gait, and easy fatiguability. However, she had no new muscle weakness or wasting, and examination did not detect new neurological signs. Her condition stabilised after she reduced her work demands and changed her leg braces.

7.4 Methods

7.4.1 Poliovirus antibody determination

All immunological and virological assays were performed in blinded fashion on coded samples. Sabin poliovirus types 1, 2, and 3 were grown on monolayers of fetal rhesus monkey kidney cells (FRhK-4T) then prepared as previously described (Ukkonen et al 1986). Viral antigen utilized for immunoassay was prepared from a mixture containing equal counts of the purified 3 poliovirus types. Intrathecal production of poliovirus specific IgM antibodies was determined by a sensitive capture enzyme linked immunosorbent assay (Sharief and Thompson 1990; see Appendix A for full details). In brief, suitably diluted CSF and homologous serum samples containing similar amount of IgM were examined, and a comparison of the photometric signals was utilised for the detection of intrathecal production of specific IgM antibodies. Polyvinyl chloride microtitre plates were activated by GA and then coated with the poliovirus antigen. Test samples were incubated with these "solid-phase" antigens, then the virus-specific IgM antibodies were detected using a peroxidase-conjugated $F(ab')_2$ fragment of human IgM antibody (Sigma, UK) to avoid interference with rheumatoid factors. The same ELISA method was utilised to detect IgM antibody levels to measles, mumps, herpes simplex type-1, and varicella-zoster viruses were also measured by the same immunoassay to serve as controls.

The clonal distribution of intrathecally produced anti-poliovirus IgM antibody was identified by the method of affinity immunoblot (Dorries and Ter Meulen 1984) with minor modifications. Unconcentrated CSF and adequately diluted homologous serum containing 20 ng IgM were electrophoresed in agarose gel then the separated proteins were passively transferred to polyvinyl difluoride membrane (PVFM) coated

with poliovirus antigen (500 μg/ml, 1 ml/10 cm^2 of PVFM area). The proteins were subsequently cross-linked to PVFM by glutaraldehyde (Sharief et al 1989) before the oligoclonal IgM bands against poliovirus were specifically immunostained.

7.4.2 Other assays

Levels of IL-2 in CSF and diluted serum were measured by the capture immunoassay described in Chapter 2. Levels of sIL-2R were measured by the commercial ELISA procedure described in Chapter 3. Oligoclonal IgG and IgM bands in the test samples were detected as already described. Agarose gel electrophoresis was used to detect oligoclonal IgA bands (Sharief et al 1991b). Total IgM concentration was measured by an enzyme immunoassay (Sharief et al 1990), then the IgM index was calculated as described in previous chapters.

Statistical analyses included nonparametric Wilcoxon sum rank, chi-square, and Spearman rank correlation tests. Analyses were performed with SPSS/PC+ software. All *P* values are two-tailed.

7.5 Results

7.5.1 Clinical observations

Muscle weakness in patients with the post-polio syndrome involved either muscles originally affected by polio (18 patients) or muscle groups that have been spared by the original disease (14 patients). Nine patients presented with weakness in previously normal muscle groups that were not segmentally contiguous to the already weak ones. New bulbar, respiratory, or sleep difficulties were only noticed in patients who already had residual bulbar or respiratory muscle weakness (4 patients). The mean age±SD of the patients at the time of acute poliomyelitis was 8.7±5.5 years (range 5 months to 21.5 years), whereas the mean±SD of onset of the post-polio syndrome was 40.2±9.1 (range 31.5 to 59) years.

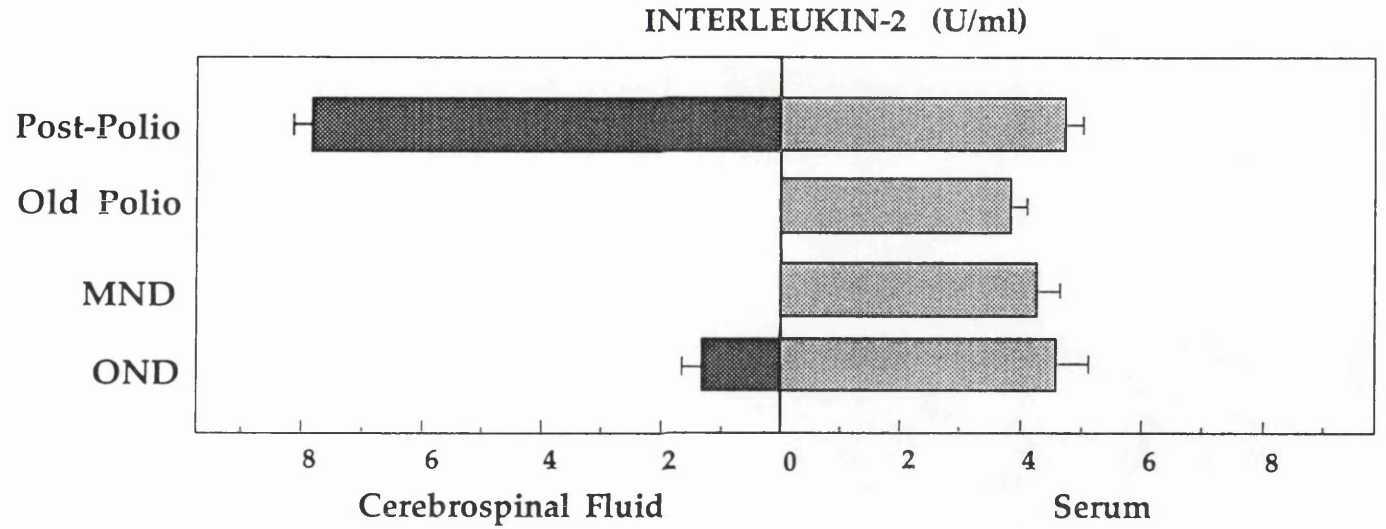
In most patients the onset of new symptomatology was insidious. Symptoms in 2 patients started after minor accidents involving a limb. Definite history of exposure to children with acute poliomyelitis or those who have recently received trivalent poliovirus vaccine was obtained from 4 patients a few months (mean= 6.8 months) prior to the development of the new symptoms.

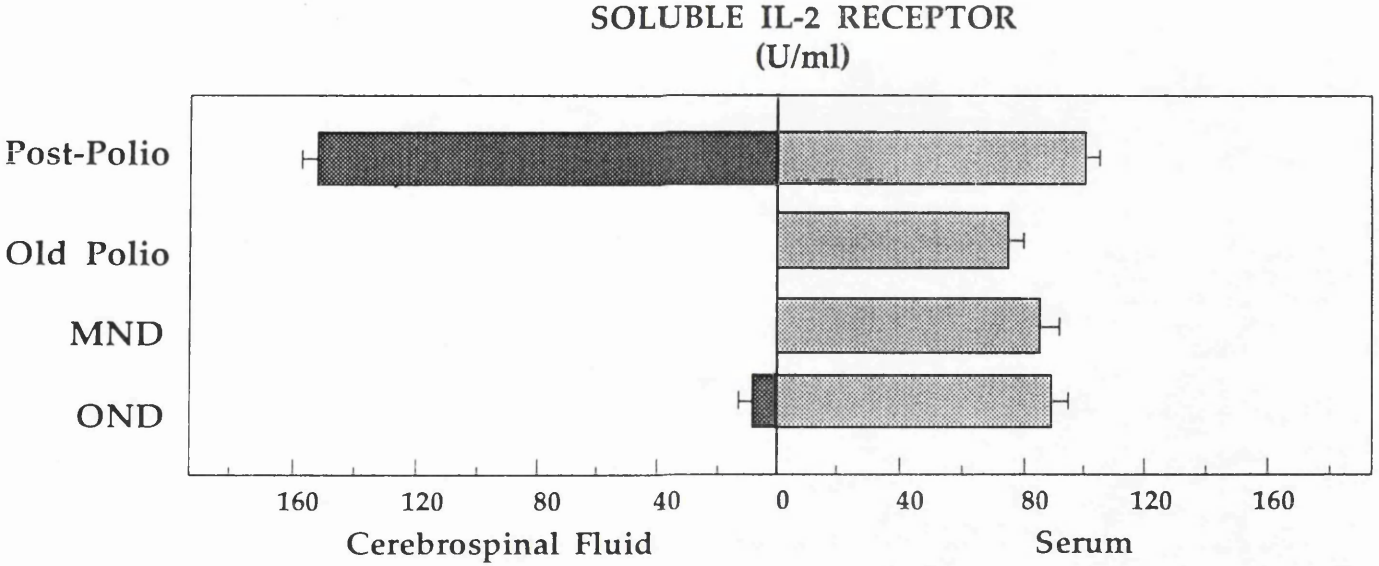
7.5.2 Intrathecal synthesis of IL-2 and sIL-2R

Detectable levels of IL-2 were found in CSF of 20 patients with the post-polio syndrome and 5 controls (3 with stroke; 1 with myasthenia gravis; and 1 with MS). Mean values of IL-2 in CSF and corresponding serum in the different clinical groups included in this study are depicted in Fig. 7.1. High CSF IL-2 levels in 18 patients with the post-polio syndrome were not associated with a concomitant increase in serum IL-2 levels, suggesting local CNS synthesis of IL-2. Similarly, intrathecal synthesis of sIL-2R was detected in 17 patients with the post-polio syndrome (see Fig. 7.1 for mean values).

The distribution of IL-2 and sIL-2R in patients with the post-polio syndrome the other clinical groups was studied using Kruskal-Wallis one way analysis of the variance. The intrathecal synthesis of both IL-2 and sIL-2R in the post-polio syndrome was significantly higher than the other groups, whereas serum levels were relatively similar in all groups (Fig. 7.1). Therefore, the significant increase in CSF levels of IL-2 and sIL-2R in the post-polio syndrome suggests that the detection of high intrathecal levels of IL-2 or sIL-2R may be helpful in establishing an accurate diagnosis.

Fig. 7.1. Mean levels of IL-2 and sIL-2R in CSF and serum in patients with the post-polio syndrome and control patients who had had poliomyelitis without the post-polio syndrome or who had motor neurone disease (MND) or other neurologic diseases (OND). Cerebrospinal fluid levels of IL-2 and sIL-2R were significantly higher in the patients with the post-polio syndrome than in other groups ($P< 0.001$ and $P< 0.0001$, respectively). T bars are standard errors.


INTERLEUKIN-2 (U/ml)
Post-Polio
Old Polio
MND
OND
8
6
4
2
0
2
4
6
8
Cerebrospinal Fluid
Serum


SOLUBLE IL-2 RECEPTOR
(U/ml)
Post-Polio
Old Polio
MND
OND
160
120
80
40
0
40
80
120
160
Cerebrospinal Fluid
Serum

7.5.3 Oligoclonal poliovirus antibodies

Oligoclonal IgM was the predominant immunoglobulin in the CSF samples from patients with the post-polio syndrome (Table 7.1). The oligoclonal IgM banding pattern in positive CSF samples either had no detectable counterpart in homologous serum (17 patients) or the number of CSF bands was substantially higher than the serum bands (4 patients), indicating that the synthesis of IgM of restricted heterogeneity in patients with the post-polio syndrome is predominantly intrathecal. In each positive case, qualitative characterization by immunoblotting identified the oligoclonal bands as poliovirus IgM antibody (Fig. 7.2).

Table 7.1. Distribution of oligoclonal immunoglobulin bands in samples of CSF from the study population.

Oligoclonal Bands	Patients with Post-Polio Syndrome (N= 36)	Control Patients		*P* Value[a]
		Old Polio (N= 13)	Neuro-Muscular Diseases (N= 54)	
IgM	21 (58%)	0	0	< 0.0001
IgA	10 (28%)	0	5 (9%)	0.026
IgG	12 (33%)	0	9 (17%)	NS

[a]The chi-square test was use to compare values for patients with the post-polio syndrome with those for control patients. NS denotes not significant.

To further confirm that the oligoclonal IgM bands are specific against the poliovirus, all positive samples underwent affinity immunoblotting with another two human enteroviruses: echovirus 14 and coxsackie virus B5. The oligoclonal bands in the post-polio syndrome did not cross-react with these enteroviruses (results not shown), indicating that the oligoclonal IgM bands in the CSF of patients with the post-polio

syndrome are specific for polioviruses. Similarly, all oligoclonal IgA bands were found to be virus specific, and IgG bands were specific for poliovirus in 7 (58%) of 12 patients. Oligoclonal IgG bands in the remaining 5 patients did not react with the echovirus 14 or the coxsackie virus B5.

Intrathecal synthesis of virus specific oligoclonal IgM bands was seen in the four patients who had a relatively recent exposure to the poliovirus prior to the development of new symptoms. Poliovirus-specific oligoclonal IgM bands were also detected in the CSF of a patient who developed new weakness after a minor accident.

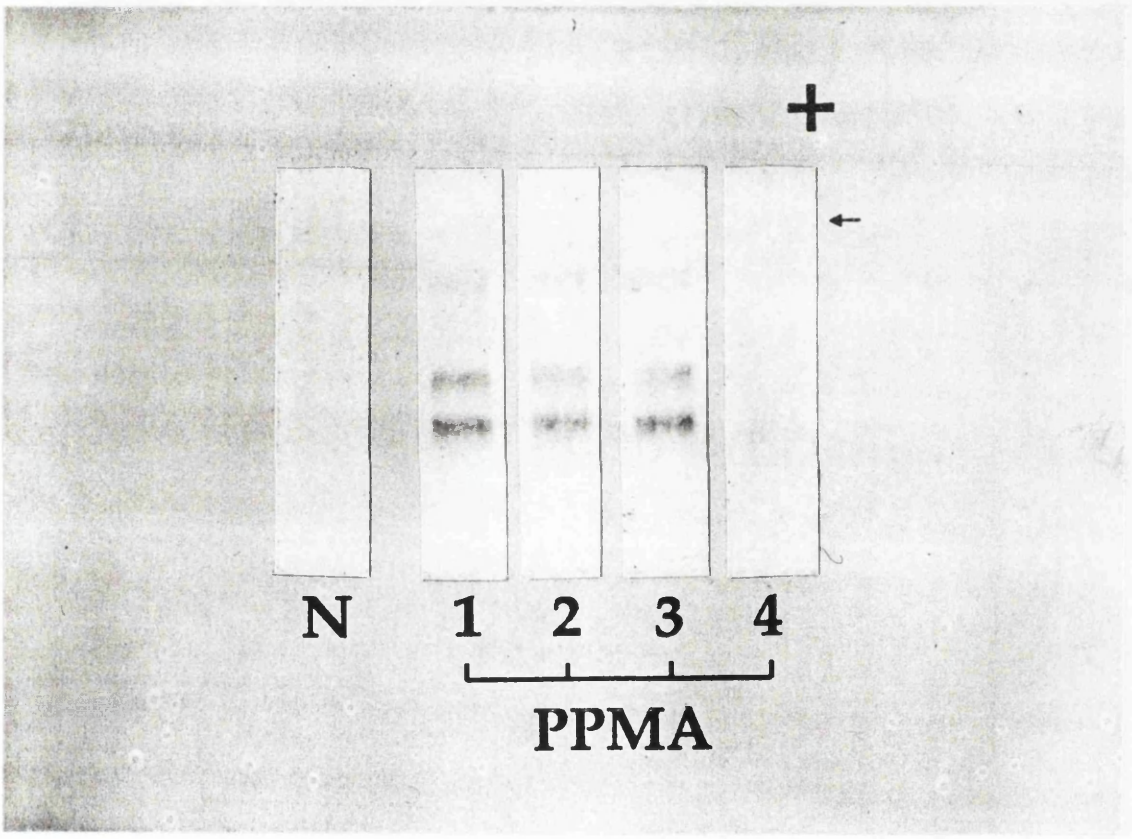


Fig. 7.2. Electrophoretic patterns in CSF from a patient with the post-polio syndrome (PPMA) (lanes 1 through 4) and a control patient (lane N). Lane 1 shows the total number of oligoclonal IgM bands. Lane 2 shows the number of bands that were found to be specific for poliovirus by affinity immunoblotting. The bands were not affected by passing the samples through Sepharose 6B (Pharmacia) immunoadsorbent gel containing uninfected FRhK-4T cells, on which the poliovirus was grown (lane 3), indicating that these bands are not artifacts or directed against non-viral material. These bands disappeared when the sample was preadsorbed with the poliovirus antigens (lane 4), confirming that these bands are specific for the poliovirus. Arrow marks application site, and the anode position is indicated.

7.5.4 Cerebrospinal fluid IgM antibody levels

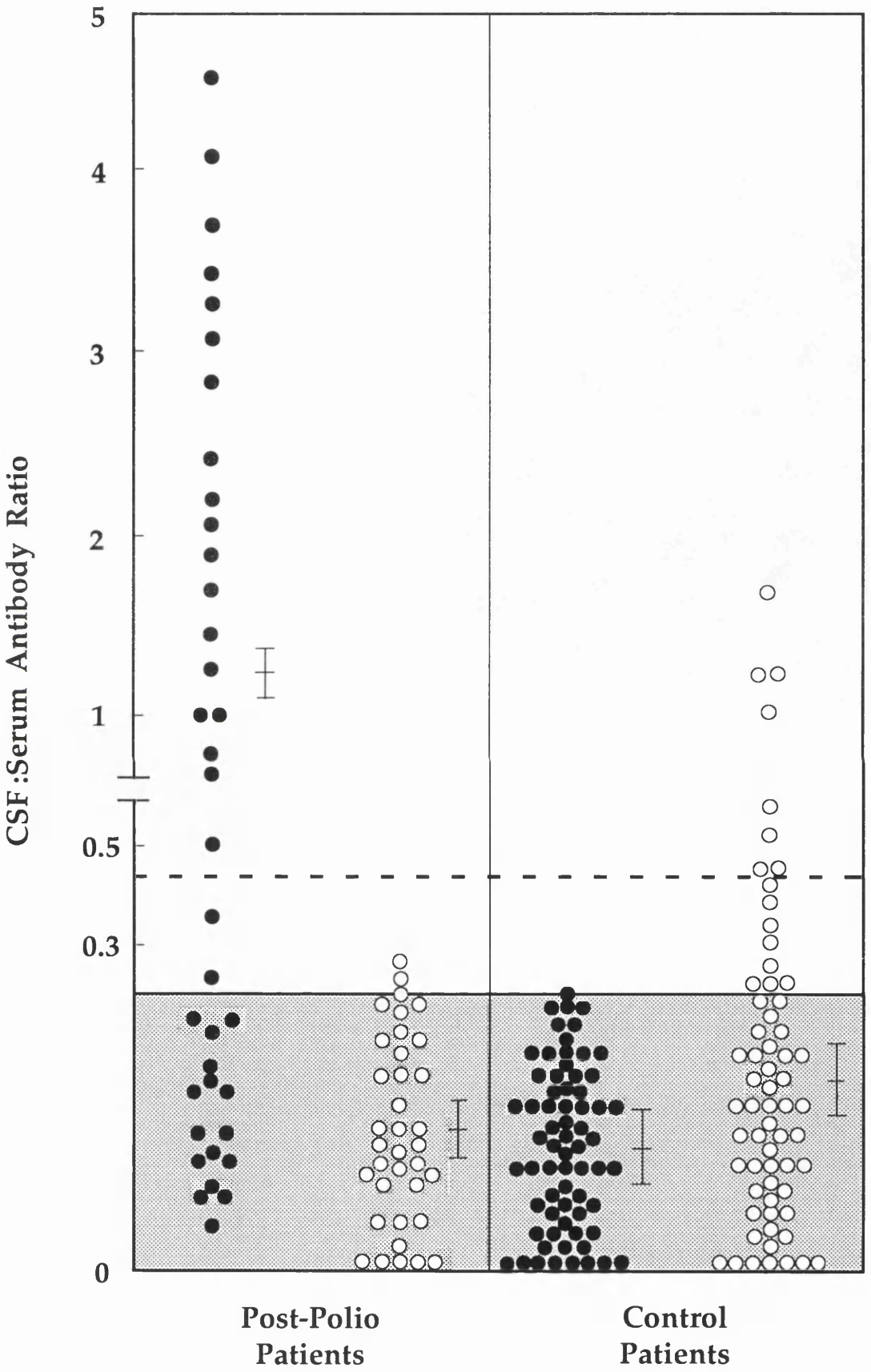
Total and virus specific IgM levels were determined to obtain quantitative information on intrathecal IgM synthesis. The cut-off (mean±3SD) value of IgM index in the reference normal population was 0.07. Abnormally high IgM index values were detected in 17 (49%) patients with the post-polio syndrome (all had CSF oligoclonal IgM bands) and in 6 controls (3 with MS; 1 with myasthenia gravis; and 2 with stroke; $p< 0.005$).

Table 7.2. Mean±SD values of immunoglobulin indices in patients with the post-polio syndrome and control groups. Figures in brackets represent number of patients who had abnormally high values.

Index	Patients with Post-Polio Syndrome (N= 36)	Control Patients	
		Old Polio (N= 13)	Neuro-Muscular Diseases (N= 54)
IgM Index	1.25±0.44 (17)	0.043±0.03	0.06±0.04 (4)
IgA Index	0.29±0.13 (9)	0.11±0.09	0.21±0.06 (5)
IgG Index	0.73±0.42 (13)	0.47±0.07	0.62±0.21 (9)

The extent of intrathecal synthesis of poliovirus IgM antibody was determined by measuring antibody optical density (OD) values per weight unit of IgM in serum and CSF to correct for blood-CSF barrier permeability. Intrathecal synthesis of poliovirus IgM antibody was seen in 21 (58%) patients with the post-polio syndrome and in none of the 67 control patients (Fig. 7.3).

Fig. 7.3. CSF to serum ratios of anti-poliovirus IgM antibody (•) as compared with CSF to serum ratios of anti-measles IgM antibody (o) (as a control) in patients with the post-polio syndrome and in the control group. The stippled area represents the normal range of the CSF to serum ratio of poliovirus antibody, and the dashed line represent the upper range of normal CSF to serum measles antibody. Bars are means±SEM.


CSF :Serum Antibody Ratio
5
4
3
2
1
0.5
0.3
0
Post-Polio Patients
Control Patients

High CSF to serum OD ratios of poliovirus IgM antibody were detected in 4 patients with the post-polio syndrome who had normal IgM index, providing further evidence that intrathecal IgM production could occur without concomitant increase in the index value (Sharief et al 1990). These 4 patients, however, had CSF virus specific oligoclonal IgM bands. IgM antibody ratios to the control viruses were within normal ranges. In contrast, high CSF to serum ratios of measles IgM antibody were seen in 6 controls (Fig. 7.3: 3 patients with MS; 2 patients who had a stroke during a course of a meningo-encephalitic illness; and a patient with muscular dystrophy who, on further epidemiological study, was found to have had measles during the time of CSF collection). As mentioned above, race-matched controls were selected for this study. These included 16 patients from developing countries which may explain the relatively wide range of CSF to serum measles antibody ratios detected in the control group.

To determine whether intrathecal synthesis of IL-2 and sIL-2R in the post-polio syndrome was related to poliovirus immune response, CSF levels of IL-2 and sIL-2R were correlated with the results of IgM immunoblot analysis. The association between CSF levels of IL-2 and sIL-2R and the presence of poliovirus specific oligoclonal IgM bands was significant (Fig. 7.4). Cerebrospinal fluid levels of IL-2 were significantly higher in patients with oligoclonal IgM bands than in those without IgM bands ($P< 0.0005$). Similarly, CSF levels of sIL-2R were significantly higher in the patients with oligoclonal IgM bands ($P< 0.0001$).

In addition to the significant correlation between IL-2 and oligoclonal IgM bands, CSF levels of IL-2 closely correlated with the amount of locally synthesized poliovirus IgM antibody ($r= 0.76$, $P< 0.0005$; Fig. 7.5). Similarly, the correlation between CSF levels of sIL-2R and intrathecal levels of poliovirus IgM antibody was statistically significant ($r= 0.63$, $P< 0.001$; Fig. 7.5).

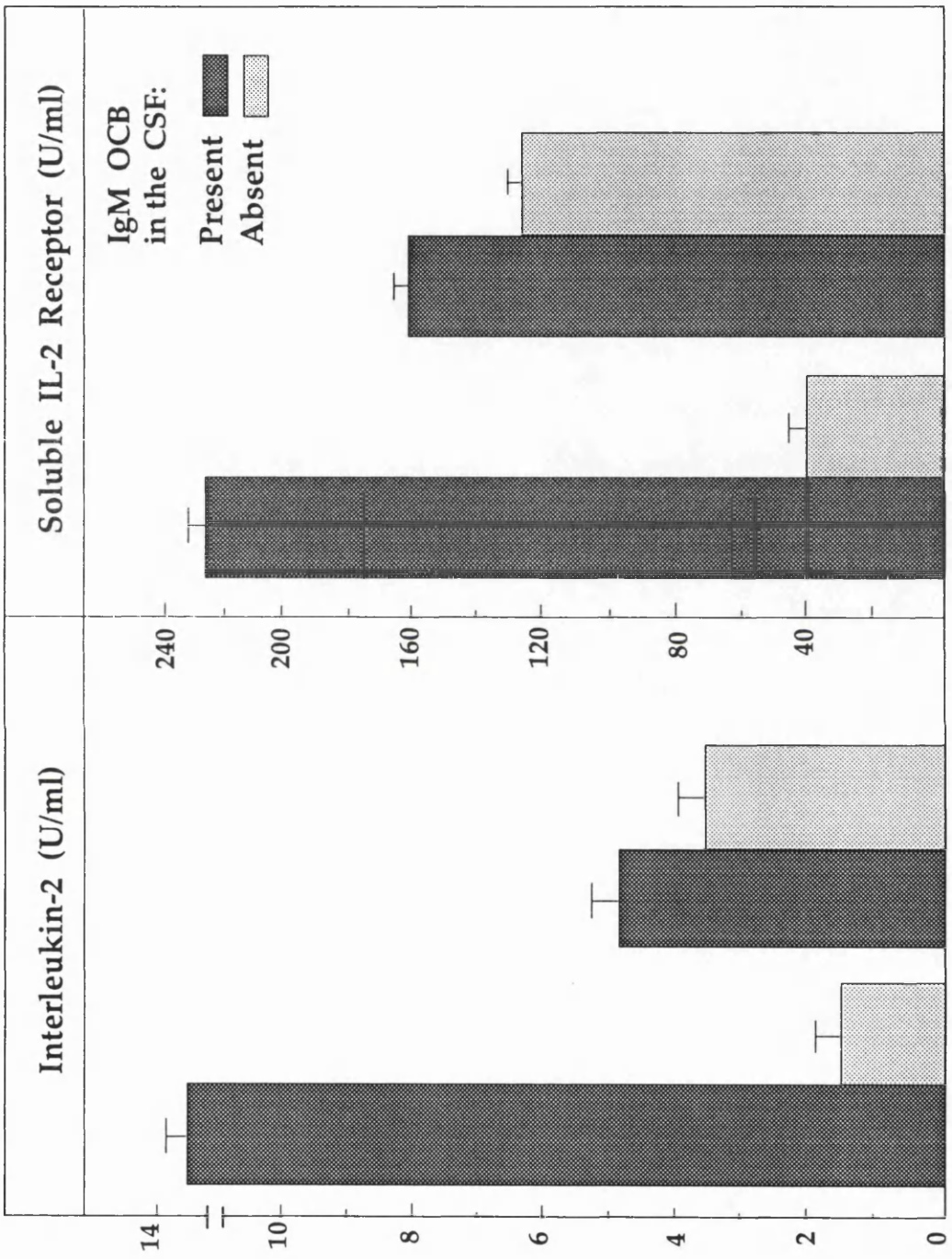


Fig. 7.4. Mean±SEM levels of IL-2 and sIL-2R in CSF and serum from patients with the post-polio syndrome, according to the presence of oligoclonal IgM bands in CSF. See text for statistical results.

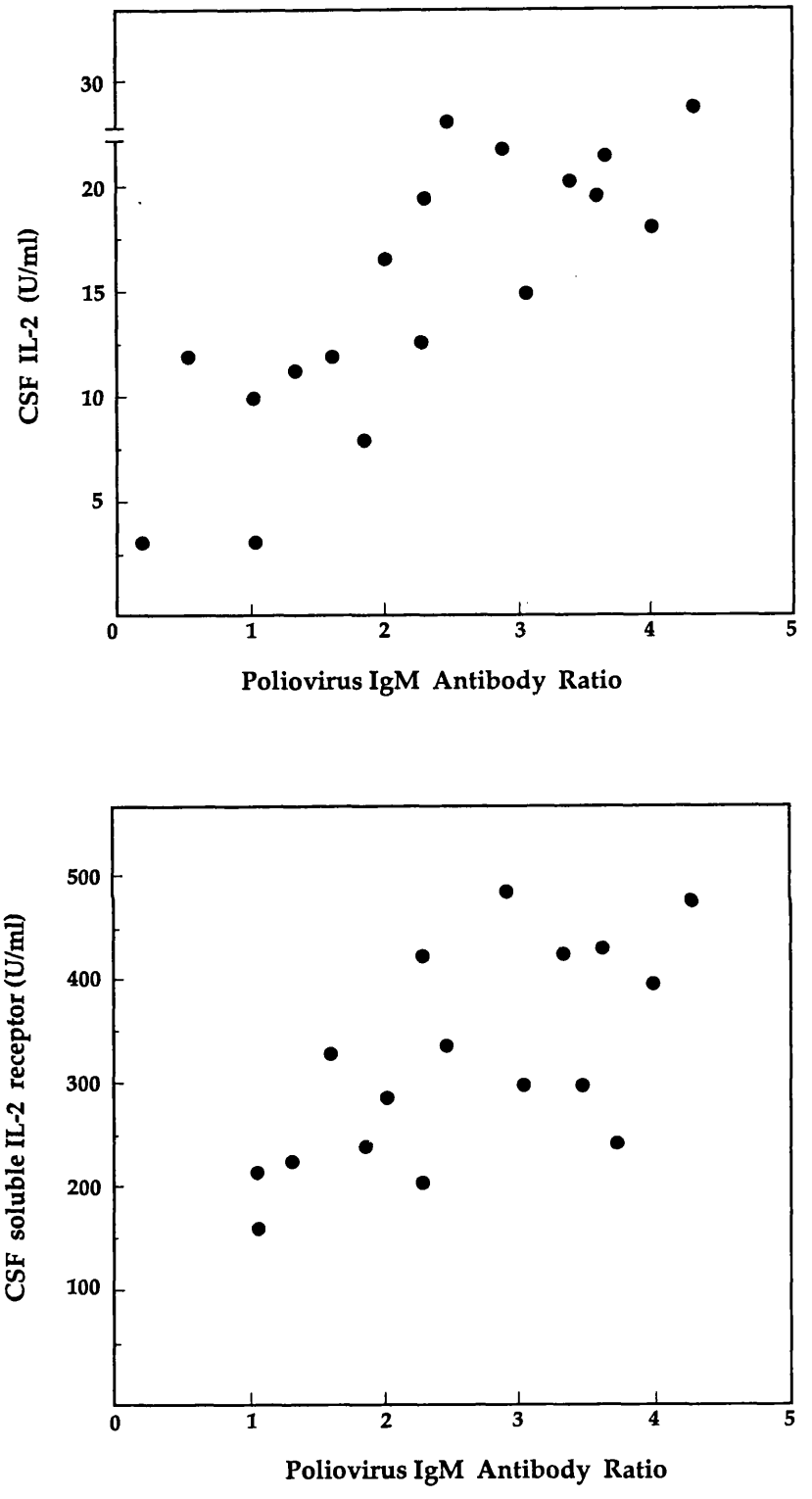


Fig. 7.5. Cerebrospinal fluid to serum ratios of poliovirus IgM antibody plotted against CSF levels of IL-2 and sIL-2R. See text for statistical results.

7.6 Discussion

The mechanism of new progressive muscle weakness in post-polio patients is important not only from a prognostic point of view, but also for preventive and therapeutic purposes. The finding that poliovirus-specific oligoclonal IgM bands were exclusively found in some patients with the post-polio syndrome suggests an immune response to an antigenic stimulation within the CNS, probably by poliovirus. The presence of poliovirus specific immune response may be regarded as an indirect, but reliable, evidence of a viral infection since polioviruses can not be isolated from the CSF even during the acute stage of paralytic poliomyelitis (Johnson 1984).

In CNS infections, immunoglobulin-secreting plasma cells differentiate from a limited number of B cells so that the immunoglobulins produced are of restricted heterogeneity, i.e. oligoclonal. Oligoclonal bands specific to the causal agent are frequently seen in patients with active viral infections of the CNS such as subacute sclerosing panencephalitis (Vandvik et al 1976) (caused by measles virus); herpes simplex encephalitis (Vandvik et al 1982); human immunodeficiency virus infection (Chiodi et al 1988); and enteroviruses-induced meningitis and encephalitis (Kaiser et al 1989). No poliovirus specific oligoclonal bands were detected in the control subjects with old stable poliomyelitis, suggesting that the intrathecal poliovirus immune response in the post-polio syndrome is due to a relatively recent or persistent antigenic stimulation. Salazar-Grueso and others (1989), using a silver-staining method, failed to detect oligoclonal bands in CSF of patients with the post-polio syndrome. Their negative results may be due to the relatively small sample size (only 9 patients with the post-polio syndrome) or to methodological differences (the disadvantages of silver-staining have been discussed elsewhere (Hames 1990). In support of the view that the silver-staining method is less sensitive than immunofixation, Kaiser (1991) has recently reported that an isoelectric focusing-immunoblotting method has detected oligoclonal bands in 5 out of 13 neurologic patients who were negative when a silver-staining method was used. The presence of CSF oligoclonal bands, however, is in agreement with previous observations reported by independent groups (Dalakas et al 1986).

The possibility that the intrathecal immune response in the post-polio syndrome is due to an active stimulation is further confirmed by the finding of an increased intrathecal synthesis of IL-2 and sIL-2R in the same group of patients. Local

CNS production of IL-2 or sIL-2R or both has been demonstrated during the course of several viral infections of the CNS (Greene et al 1986; Boutin et al 1987; Lang et al 1988). A successful immune response to viral infections involves a complex cascade of events which include the production of cytokines. The IL-2 system in particular, plays a major role in the immune responses against invading antigens (reviewed in Chapter 1). Interleukin-2 has an essential role in promoting replication and differentiation of T cells into effector cells, induction of B cell growth, and augmentation of immunoglobulin production. As has discussed in Chapter 1, cell proliferation after the binding of IL-2 to its membrane-bound receptor leads to the release of the sIL-2R.

The high intrathecal release of IL-2 and sIL-2R suggests an intense immunologic response within the CNS, which involves both T and B lymphocytes. These results therefore mitigate for a possible role of a new or persistent poliovirus CNS infection in the pathogenesis of the post-polio syndrome. The possibility that the post-polio syndrome is due to persistent infection by enterovirus is not unlikely. Poliomyelitis virus, an RNA virus, is usually cytolytic but can produce slowly progressive or persistent infections in both animals and immunosuppressed patients (Davis et al 1977; Miller 1981). Recent evidence has also been presented to support the notion that polioviruses can produce persistent infection in normal human neuronal cells (Colbère-G et al 1989; Pelletier et al 1991). In addition, Lipton (1975) reported a biphasic neurologic disease in mice infected with the Threiler's encephalomyelitis virus, a murine enterovirus, where the disease presents with acute encephalitic illness immediately after inoculation followed by a progressive demyelinating syndrome several months later. Moreover, the murine-adapted Lansing strain of type II poliovirus causes an acute and persistent infections of the CNS in mice (Jubelt et al 1980). In fact, even the human poliovirus has been shown to produce prolonged asymptomatic CNS infection (Davis et al 1977).

It is unclear form the data presented here whether patients the post-polio syndrome carry any risk of infectivity. Furthermore, the pattern of a possible reinfection needs to be determined, although recent exposure to poliovirus could be a plausible mechanism. Four of the patients included in this study developed new muscular weakness after relatively recent exposure to the virus. Similarly, Dalakas and collaborators (Dalakas et al 1984) reported a post-polio patient who developed

new muscular weakness accompanied by elevation of CSF poliovirus antibodies after exposure to children with acute poliomyelitis. In fact, neurologic abnormalities and elevated poliovirus antibody titres may also develop in normal subjects upon exposure to the virus (Ellis et al 1990).

Data presented in this chapter clearly demonstrate the diagnostic advantage of IL-2 and sIL-2R determination in patients with poorly characterised neurologic diseases. Although the detection of virus-specific antibodies is imperative in reaching a precise diagnosis, the presence of high levels of IL-2 or sIL-2R is an important step in the characterisation of the nature of the disease process. Table 7.2 clearly demonstrated that elevated immunoglobulin levels can be detected in neuro-muscular disease that do not have an inflammatory or infectious aetiology. In contrast, high CSF levels of IL-2 and sIL-2R seem to be specific for an inflammatory CNS diseases. A second advantage in favour of the detection of IL-2 or sIL-2R is the fact that their determination in body fluids is generally straightforward an could be performed in a routine laboratory setting, whereas the detection of virus-specific IgM antibodies, for instance, requires a certain amounts of proficiency and is rather labour-intensive.

The combination of high CSF levels of IL-2 and sIL-2R and elevated specific IgM antibody titres provide evidence of a marked intrathecal immune activation against poliovirus during the course of the post-polio syndrome. It is tempting to speculate that a reactivation of a latent or persistent poliovirus infection in the CNS may play a role in the pathogenesis of new muscle weakness in some patients with the post-polio syndrome. The unique lack of vascular permeability and absence of lymphatics or immunocompetent cells in the CNS as well as the static nature of neurons may encourage and promote viral persistence. A residual poliovirus could therefore cause persistent infection after the acute episode, possibly by escaping the immune system surveillance through antibody-induced antigen modulation, generation of suppresser T cells, or production of blocking factors (Johnson 1981). A persistent poliovirus infection may then cause gradual, but progressive, cytopathic effect which would eventually lead to either neuronal cell lysis or alteration of specialized cellular functions which potentially impairs physiologic activities. Longitudinal follow-up of patients with the post-polio syndrome, and therapeutic trials with antiviral agents will allow this hypothesis to be tested further.

CHAPTER 8. GENERAL DISCUSSION AND CONCLUSIONS

Interleukin-2 belongs to a series of mediators that are predominantly produced by T cells and exerts multiple, pleiotropic effects in an autocrine or paracrine fashion. IL-2 plays a fundamental role in the ontogeny of developing T cells in the thymus and supports the growth or effector function of a wide array of immunologically relevant cells, including macrophages and B and NK lymphocytes, as well as a variety of different T-cell subpopulations. Of all the cytokine-receptor systems in immunology, perhaps most is known about the structure, function, and binding properties of IL-2 and its cognate receptor. The importance of the IL-2 system in neurology, however, is less well-established. The primary reason for this is the lack of sensitive and standardised methods that are capable of detecting IL-2 or its soluble receptor in the cerebrospinal fluid.

The notion that the method of detecting IL-2 is critical in evaluating the dynamics of the IL-2 system is confirmed by the wide variation in IL-2 levels in neurologic patients, which have been reported in the literature. The variations in IL-2 or sIL-2R concentrations amongst different research laboratories illustrate the fact that each laboratory should estimate its own reference values before comparing results with those from other laboratories. As a result, the first objective of this study was to establish a sensitive, specific, and reproducible method for the detection of IL-2 in biological fluids from neurologic patients. Another factor that has proved important in determining cytokine levels in body fluids was the addition of a protease inhibitor, which seems to prevent *in vitro* degradation by several enzymes. Therefore, the addition of an enzyme inhibitor of a wide spectrum, such as aprotinin, seems to be important to improve cytokine detectability.

The establishment of a sensitive and specific method for the detection of IL-2 in the CSF has made it relatively easy to study the distribution of IL-2 and its soluble receptor in a mixed neurologic population. The study has clearly established that a common denominator of a number of inflammatory neurologic diseases is their association with high intrathecal levels of IL-2 and sIL-2R. Although elevated levels of IL-2 and sIL-2R by themselves do not discriminate between various inflammatory conditions of the CNS, it was obvious from the study that the diagnostic power of IL-2 and sIL-2R to identify an inflammatory neurologic process is quite impressive.

The ability of high CSF levels of IL-2 and sIL-2R to distinguish an intrathecal immune process may be employed in the routine diagnosis of inflammatory neurologic diseases, such as multiple sclerosis, in the same way as immunoglobulins synthesis is currently utilised. A point in favour of cytokine detection for diagnostic purposes is that increased levels of IL-2 and sIL-2R always indicate an active immune response, whereas elevated immunoglobulin levels may arguably be the result of an anamnestic response or due to the release of "nonsense" antibodies (see Goust et al 1982 for full references). This diagnostic specificity of the IL-2 system was clearly demonstrated in Chapter 7, which demonstrated that elevated levels of IL-2 and sIL-2R in post-polio patients not only help to diagnose an inflammatory immune process within the CNS, but also confirm that the high concentrations of antibodies are specific to the etiologic agent, namely poliovirus.

It could be argued that the determination of IL-2 and sIL-2R may be added to the rapidly expanding armamentarium available to physicians in their endeavours to diagnose inflammatory diseases in humans. However, the importance of studying the dynamics of IL-2 and its soluble receptor extends far beyond a mere diagnostic purpose. Indeed, this study has clearly demonstrated that IL-2 and sIL-2R occupy a central role in the analysis of the immunopathology and pathogenesis of various inflammatory neurologic diseases. The study has focused on multiple sclerosis because it is the commonest inflammatory neurologic condition seen in clinical practice. However, findings presented in the study could be extrapolated to other less common inflammatory CNS diseases. Elevated concentrations of IL-2 and its soluble receptor are usually encountered during an active disease process, suggesting that this cytokine participates in the original pathologic process.

Results presented in this study demonstrated a significant correlation of IL-2 and sIL-2R with intrathecal synthesis of IgM and IgD. The presence of IL-2 receptor on B cells has been demonstrated by several investigators, suggesting that the correlation between IL-2 and Ig release could be the result of the direct action of IL-2 on B cells. Indeed, several recent studies have provided convincing evidence that IL-2 may directly enhance the differentiation of Ig-secreting cells from antigen primed B cell. However, although it is becoming clearer that IL-2 may directly enhance some B cell responses, some questions remain concerning the physiologic relevance of these findings. It is not known at present whether IL-2 plays a central or auxiliary role in

B cell differentiation and Ig secretion *in vivo*, or perhaps is involved in only one of several separate pathways of B cell activation and differentiation. Further studies are clearly required to establish the mechanisms through which IL-2 regulates B cell functions.

Intrathecal synthesis of IL-2 was also found to be related to blood-brain barrier impairment in multiple sclerosis, and may be important in understanding some of the early pathological changes of this condition. Moreover, results presented in Chapter 6 have shown that levels of IL-2 and sIL-2R parallel the evolution of the pathologic process in MS and suggest that measurement of these cytokine may provide an objective marker of disease activity in patients with MS.

In assessing the role of IL-2 in immunopathology, it is important to consider the pleiotropic nature of humoral regulation, which allow many overlapping events to be directed by several factors. This author sought to study the distribution of TNF-α in patients with MS enroled in this study for several reasons that have been discussed in previous chapters. At present, little is known about the cascade of biochemical and physiological events that follow an augmented release of IL-2 in neurologic conditions. The study of the dynamics of other cytokines in such conditions will make it possible to gain further insights into the immunopathology and pathogenesis of several intractable diseases of the central nervous system.

An important issue that has to be addressed when discussing the immuno-pathology of IL-2 is the primary cause of hyperproduction of this cytokine. The function of IL-2 is usually highly controlled *in vivo* to avoid systemic effects that might endanger the specificity of an immune response and result in autoimmune reactions. Accordingly, various mechanisms guarantee compartmentalisation of IL-2, that is, chronological and spatial restriction of IL-2 production, bioavailability, and state of responsiveness. The secretion of IL-2, as well as the expression of the two components of the high-affinity IL-2 receptor, are developmentally controlled during ontogeny and, within the cellular immune system, are restricted to defined pre- and intrathymic stages of immature T cells or T-cell precursors. In the peripheral lymphoid organs, IL-2 is produced by a defined population of mature $CD4^+$ T lymphocytes in which the IL-2 gene is transcribed or silenced, depending on the combination of antigenic and nonspecific activation signals to which the cell is exposed. Thus, the absence of certain costimulatory signals leads to a long-lasting

inactivation of the IL-2 gene, a phenomenon that accompanies nondeletional T-cell tolerance. IL-2 has a short half-life and is secreted in apposition to the cell with which the T cell interacts. Expression of the high-affinity IL-2R is activation-dependent in most cell types. Therefore, different mechanisms, intervening in all compartments relevant for the action of IL-2, together contribute to a restriction of IL-2 effects, conferring a relative specificity to this pleiotropic mediator.

A defect in any of the above mechanisms could theoretically result in abnormally high levels of IL-2, and subsequently lead to local or systemic pathological changes. It is hoped that future research will identify the factors responsible for triggering an abnormal IL-2 release. Identification of these factor is pivotal in order to reach a comprehensive understanding of the pathological role of IL-2 in human diseases, and hence to plan more effective therapy.

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APPENDIX A

Selected publications related to methodology

Journal of Immunological Methods, 147 (1992) 51–56

JIM 06187

Determination of interleukin-2 in cerebrospinal fluid by a sensitive enzyme-linked immunosorbent assay

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A sensitive technique was developed for the quantitative detection of intrathecal production of interleukin-2 (IL-2). Concentrations of IL-2 in paired cerebrospinal fluid (CSF) and serum samples were measured by an enzyme-linked immunosorbent assay using a monoclonal antibody and an affinity purified polyclonal antibody. The assay produced a linear response with respect to IL-2 concentration, and could readily detect levels of IL-2 as low as 1.5 international units/ml. Concentrations of IL-2 in CSF and serum samples were standardised by calculating their ratio to albumin concentration in order to correct for passive transudation of IL-2 across blood-CSF barriers. CSF IL-2/albumin ratios higher than concomitant serum ratios were considered indicative of intrathecal IL-2 production. The technique provides a sensitive, specific, and reproducible method for the determination of in vivo synthesis of IL-2 within the central nervous system.

Key words: Interleukin-2; ELISA; Cerebrospinal fluid; Central nervous system infections; Intrathecal synthesis

Introduction

Interleukin-2 (IL-2), also called T cell growth factor, is a 15.5 kDa glycoprotein produced by mitogen or antigen-stimulated T lymphocytes (Smith, 1988). Its biological activities include growth activation of T lymphocytes, maintenance of cytotoxic and helper T cell, increase of natural killer cells, and induction of B cell growth factors and interferon-γ.

Several studies have recently addressed the importance of IL-2 in infectious and inflammatory diseases of the central nervous system (CNS) (De Micco, 1989; Fischer et al., 1989; Gallo et al., 1989; Trotter et al., 1990; Nisticò and De Sarro, 1991). Therefore, the detection of IL-2 production within the CNS compartment (i.e., intrathecal) is important not only for diagnostic purposes but to understand the molecular pathophysiology of different inflammatory neurologic disturbances. Levels of IL-2 in cerebrospinal fluid (CSF) are usually low and may be difficult to detect. Moreover, detectable levels of IL-2 in CSF may represent passive transudation from the systemic circulation across blood-CSF barriers (Saris et al.,

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Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; GA, glutaraldehyde; HRP, horseradish peroxidase; IL-2, interleukin-2; OD, optical density; PBS, phosphate-buffered saline; SD, standard deviation; SEM, standard error of the mean.

1988). We describe here a sensitive enzyme-linked immunosorbent assay (ELISA) which permits the detection of in vivo synthesis of IL-2 within the intrathecal compartment.

Materials and methods

Specimen preparation

1000 kallikrein inhibitory units of protease inhibitor (Aprotinin, Sigma) were added to each millilitre of CSF and serum samples immediately after collection to prevent protein degradation. Cells were then separated by cyto-centrifugation and all samples were filtered through a 0.22 μm disposable sterile filter (Millipore) to remove contaminating particulate materials. Samples were frozen in aliquots at −70°C shortly after collection and thawed just before use.

Preparation of horseradish peroxidase (HRP) anti-IL -2 conjugate

Affinity purified polyclonal rabbit anti-human IL-2 antibody (EP-100, Genzyme) was used to prepare the enzyme conjugate. Glutaraldehyde (GA) was utilised as a cross-linking agent in the HRP labelling of the anti-IL-2 antibody. Preliminary experiments using one- and two-step GA as well as periodate conjugation procedures showed that the two-step GA method was the most efficient technique for the coupling of HRP to anti-IL-2 antibody.

The coupling process was performed as described by Avrameas and Ternynk (1971). In brief, 10 mg peroxidase (type VI, Sigma, UK) in 0.2 ml 100 mM phosphate-buffered saline (PBS), pH 6.8, was activated by excess GA for 24 h at 24°C and then GA was removed by passage through a Sephadex G-25 column (Pharmacia, UK) equilibrated with 0.9% NaCl. Polyclonal anti-human IL-2 antibody equilibrated with 0.9% NaCl in 0.2 ml of 0.5 M sodium carbonate buffer, pH 9.5, was incubated with the activated peroxidase for 24 h at 4°C. The remaining activated groups were then blocked with 0.1 ml 1 M lysine, pH 7.0, for 4 h. The conjugate was subsequently dialysed overnight against PBS, filtered through a 0.22 μm Millipore membrane and separated from free peroxidase by passage on protein A (Pharmacia, UK) Sepharose column. Separation of free antibody from the HRP conjugate was achieved by affinity chromatography on ConA-Sepharose (Pharmacia LKB). The conjugate was finally desorbed with α-methyl-D-mannopyranoside at 100 mM in PBS, pH 7.2, and 100 mM NaCl and stored in the dark below 8°C.

Immunoassay for IL-2

All assays were performed without knowledge of the clinical data. Wells of polyvinylchloride microplates (Falcon MicroTest III) were coated with 100 μl of 1% gelatine in PBS containing 20 U/well monoclonal murine anti-human IL-2 antibody (DMS-1, Genzyme, Suffolk), incubated for 1 h at 37°C and left overnight at 4°C. Plates were washed four times with 200 μl of wash buffer (0.05% Tween 20 in PBS, pH 7.4) and incubated with 150 μl of 2% gelatin in PBS for 1 h at room temperature. The wells were emptied, filled with 150 μl of wash buffer and the plates stored at 4°C until use.

A standard calibration curve was generated on each assay by freshly prepared serial dilutions of the international standard for IL-2 (preparation code 86/504, NIBSC, UK) in PBS containing 2% gelatin. Results were expressed in international units (IU)/ml as approved by the Expert Committee on Biological Standards of the World Health Organization (Gearing and Thorpe, 1988).

100 μl of diluted standards, CSF and 1/100 diluted serum samples were added to the wells in duplicate. Control negative and control positive samples were added to each assay. Wells of the first row of each plate to which 100 μl of 2% gelatin in wash buffer were added served as blanks. Optical density values (OD) for the blanks were subtracted from all test values. Plates were incubated for 4 h at 37°C and washed four times. Then 80 μl of HRP-antibody conjugate (diluted 1/1000) were added to the wells. The plates were incubated for 1 h at 37°C and washed four times. 100 μl of chromogen solution were added to the wells and the reaction allowed to proceed for 20 min in the dark at room temperature. The reaction was stopped by 40 μl of 2 mol/l hydrochloric acid. Plates were read at 420 nm in a Titertek Multiskan reader.

IL-2 bioassay

Results of the IL-2 assay obtained by ELISA were compared with levels detected in an IL-2 bioassay. Bioassay of IL-2 was performed on all test samples as previously described (Gillis and Smith, 1977). In brief, IL-2-dependent mouse cytolytic T lymphocyte line cells were used as an indicator system for the determination of IL-2. Indicator cells (100 μl, 1×10^4) were placed in 96-well, round-bottomed microtitre plates (Microtest III) with serial dilutions of either the test samples or IL-2 international standard. IL-2 activity was assessed by [^{3}H]thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) uptake of cytolytic T lymphocyte line cells after a 6 h pulse at the end of a 24 h culture period.

Evaluation of intrathecal IL-2 synthesis

Levels of IL-2 in CSF and corresponding serum were standardised by calculating their ratios to albumin concentration (Adachi et al., 1989, 1990) to correct for passive transudation of IL-2 into the CSF from the systemic circulation. CSF IL-2 levels higher than concomitant serum levels would suggest intrathecal synthesis of this cytokine. Albumin concentration in CSF and serum specimens were assayed by electroimmunoassay (Ganrot and Laurell, 1974).

Results

Antibodies to IL-2

The DMS-1 monoclonal and EP-100 polyclonal antibodies used in this assay were shown to be neutralising to IL-2 bioactivity (Smith et al., 1983). Thus, the risk of detecting degradation products of IL-2 which are not biologically active was greatly minimised. We also found that these antibodies neutralised the biological effects of both recombinant IL-2 and natural IL-2 of various origins (data not shown).

Precision and specificity of the assay

Duplicate determination of 20 positive samples were run in 25 different microtitre plates in order to calculate the within-run variance. The coefficient of variation was less than 7.5% in 22 plates and less than 11% in the remaining three plates.

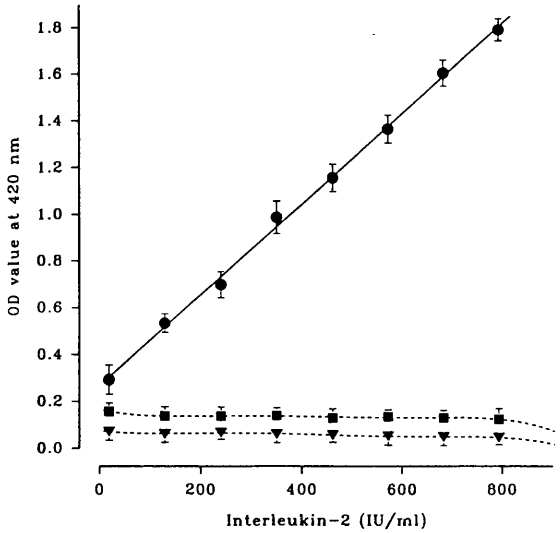

Fig. 1. Standard curve for the international standard human IL-2 (●———●) compared to standard curves of human IL-6 (■ — — — ■) and tumour necrosis factor α (▼ — — — ▼) used over the same concentration range. Values represent mean ± 3 SEM.

The between-run variance was estimated by repeated measurement of five positive dilutions of the positive samples, each assayed eight times in total. The coefficient of variation was less than 8.5%.

Standard calibration curves using serial dilutions of the international standard for IL-2 resulted in an excellent linear correlation (Fig. 1). A dose-response test was performed in serial dilutions on ten positive CSF and serum samples (Fig. 2*A*) in order to establish the minimal detectable concentration of IL-2. The assay was highly sensitive, detecting IL-2 amounts as low as 1.5 IU/ml. As also shown in Fig. 2*A*, negative samples yielded a constant OD less than 0.2 regardless of the dilution. The addition of protease inhibitor to prevent protein degradation improved the stability of IL-2 and consequently enhanced the detection limit of the assay (Fig. 2*B*).

Optimal conditions for the assay

The optimal dilution of the HRP-conjugated IL-2 antibody was found to be 1/1000. Lower dilutions increased the absorbance values of both the test and background. Satisfactory results were

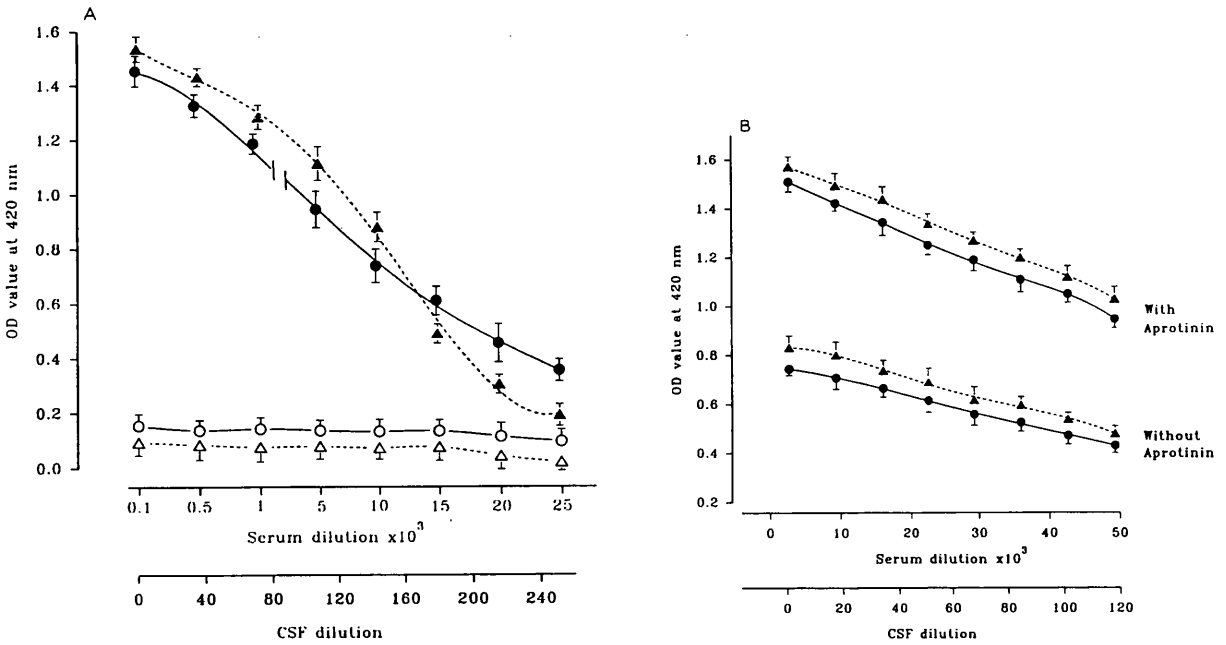

Fig. 2. *A*: Effect of serum (●———●) and CSF (▲— — —▲) dilution on absorbance values in ten positive samples compared to values from ten negative serum (○———○) and CSF (△— — —△) controls. *B*: Influence of protease inhibitor (Aprotinin) on absorbance values in ten positive serum (●———●) and CSF (▲— — —▲) controls. Values represent mean ± 3 SEM.

obtained when the HRP conjugate was incubated for 1 h. Longer incubation times (up to 3 h) did not result in any significant increase in background activity. Best results were produced when HRP conjugate was incubated at 37°C. Loss of activity was observed at 24°C, but slightly less activity was lost after incubation at 30°C.

Correlation between the ELISA and the IL-2 bioassay

Serum levels of IL-2 obtained by ELISA correlated remarkably well with biologically active IL-2 which was detected by bioassay (Fig. 3). As also shown in Fig. 3, there was a good correlation between the ELISA and the bioassay when detecting CSF levels of IL-2.

Intrathecal IL-2 production

We determined IL-2 concentrations in paired CSF and serum samples from 25 patients with acute untreated CNS infections. Levels of IL-2 were also measured in paired samples from 15 patients with active rheumatoid arthritis serving as controls. Control patients had no neurologic involvement and CSF was obtained as a result of investigations into vertebral complaints.

The absolute concentration of IL-2 in CSF (mean ± SD = 258.2 ± 93.7 IU/ml) was signifi-

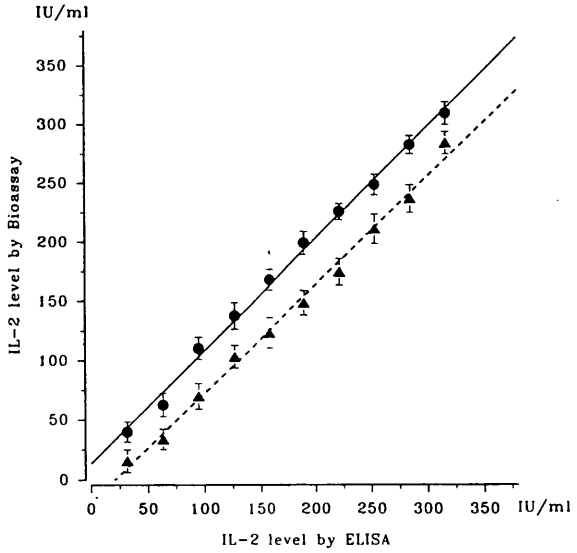

Fig. 3. Comparison of ELISA and bioassay in detecting IL-2 in 20 serum (●———●) and CSF (▲— — —▲) samples.

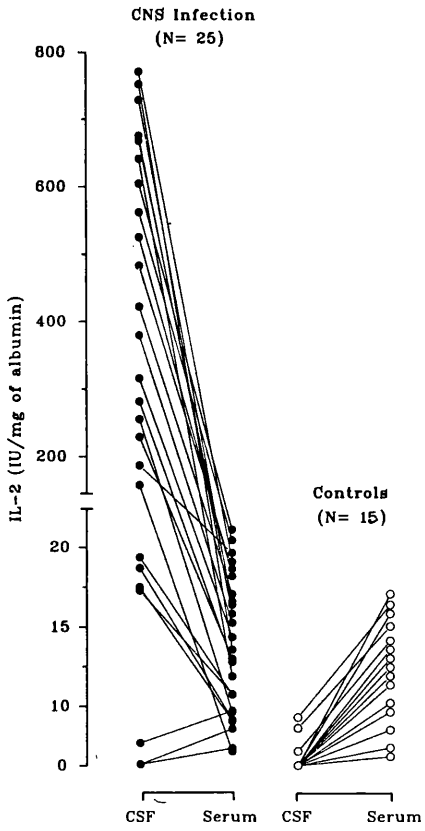

Fig. 4. Standardised CSF and serum levels of IL-2 in patients with acute CNS infections compared to those obtained from controls. Statistical analysis is presented in the text.

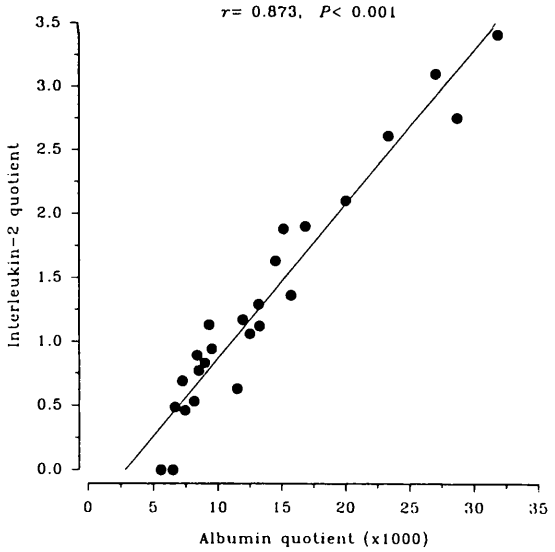

Fig. 5. Relationship between CSF to serum quotient of interleukin-2 and CSF to serum quotient of albumin in 25 patients with CNS infections.

cantly higher than the concomitant serum concentration (mean ± SD = 62.3 ± 44.1 IU/ml; $p < 0.01$). However, IL-2 concentrations in the CSF and serum samples were related to the albumin concentration. This was not unexpected since blood-CSF barrier dysfunction is known to occur in patients with CNS infections (Quagliarello et al., 1986; Tunkle and Scheld, 1989). Fig. 4 shows standardised values of IL-2 obtained in the study population. 22 (88%) of the 25 patients with CNS infections had significantly higher IL-2 levels in their CSF compared to their corresponding serum levels ($p < 0.001$) indicating intrathecal IL-2 production. CSF levels of IL-2 in CNS infections were also higher than those detected in the controls ($p < 0.0001$). A good correlation was detected between the CSF to serum ratios of IL-2 and albumin (Fig. 5).

Discussion

The ELISA technique described here was developed for the quantitative determination of IL-2 synthesis within the intrathecal compartment. The technique proved to be specific for IL-2 (Fig. 1) and reproducibility was found to be satisfactory. Moreover, the technique was found to be more sensitive than most commercially available ELISA kits, which usually detect IL-2 levels higher than 10 IU/ml.

Traditionally, IL-2 concentrations have been determined by bioassay using IL-2 dependent indicator cells which proliferate in the presence of IL-2 and become quiescent in its absence (Gillis et al., 1978). However, bioassay procedures may have some disadvantages when compared with immunoassays. The bioassay is both labour and material intensive and is critically dependent upon maintaining pathogen-free, IL-2 sensitive indicator cells. It is also possible that non-IL-2 components present in test supernatants, such as IL-4 and residual lectin or phorbol ester, may influence proliferation of the indicator cells (Grabstein et al., 1986, Hu Li et al., 1987; Gearing and Thorpe, 1988). The rapidity of the immunoassay procedure is another advantage since biological assays usually take 24–30 h.

The requirement of coupling anti-IL-2 antibody to peroxidase may be considered a disad-

vantage of the ELISA. However, no major problems were encountered in the preparation of HRP-labelled antibody. Furthermore, a large quantity of labelled antibody may be prepared at one time since the HRP conjugate can be stored for at least 6 months without loss of avidity.

Standardisation of IL-2 levels in CSF and serum by relating them to the albumin concentration is a crucial step in the objective determination of intrathecal IL-2 production. Albumin is synthesised only in the liver and its presence in CSF is dependent on the function of the blood-CSF barriers (Link and Tibbling, 1977; Tourtellotte and Ma, 1978). Fig. 5 suggests that blood and CSF levels of IL-2 are regulated in a manner similar to that of albumin. Therefore, taking into account the albumin and IL-2 concentrations in CSF and serum should detect significant elevation of CSF IL-2 levels in patients who produce this cytokine in the intrathecal compartment.

In conclusion, the ELISA method presented here is a sensitive and specific procedure that permits the routine detection of intrathecal synthesis of IL-2 in neurologic patients. Although anti-IL-2 antibodies were used to develop this method, antibodies against other cytokines, e.g., soluble IL-2 receptor and tumour necrosis factor, could be easily employed to detect their synthesis within the central nervous system.

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A sensitive ELISA system for the rapid detection of virus specific IgM antibodies in the cerebrospinal fluid

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The intrathecal production of IgM antibodies to different viral antigens was measured by a modification of ELISA that was both sensitive and specific. Suitably diluted CSF and homologous serum samples containing similar amount of IgM were examined, and a comparison of the photometric signals permitted the detection of specific antibodies secreted from activated lymphocytes into the CSF compartment during the course of viral infections of the central nervous system. Polyvinyl chloride (PVC) microtitre plates were activated by glutaraldehyde and then coated with different viral antigens. Test samples were incubated on these solid-phase antigens and virus-specific IgM antibodies were detected using a peroxidase-conjugated F $(ab')_2$ fragment of anti-human IgM antibody to avoid interference from rheumatoid factors.

Key words: Viral antibody; ELISA; IgM; Glutaraldehyde; Herpes simplex; Measles

Introduction

The detection of virus-specific immunoglobulin (Ig) M antibodies in the cerebrospinal fluid (CSF) is of particular importance in the early diagnosis of viral infections of the central nervous system (CNS). Until recent years detection of virus-specific IgM involved preliminary physical separation of IgM from IgG by a cumbersome sucrose-density gradient centrifugation (Vesikari and Vaheri, 1968; Cave, 1980) or by immunoglobulin fractionation by gel filtration (Pattison and Mace, 1975). The development of a solid-phase enzyme-linked immunosorbent assay (ELISA) for the detection of virus-specific IgM was a significant advance in the rapid diagnosis of viral infections (Yolken, 1980).

Recently, different ELISA systems have been developed to detect virus-specific IgM antibodies (Madeley and McQuillin, 1981; Jamnback et al., 1982; Bellamy et al., 1986; Chiodi et al., 1986; Mathiesen et al., 1988). However, the application of ELISA is limited by the fact that sensitivity and reproducibility depend on the antibody conjugate used. The anti-IgM peroxidase-conjugated antibody in most assays may give rise to false positive reactions, mainly due to rheumatoid factor (Duemeyer et al., 1979). Non-specific reactivity occurs even when antibodies are conjugated to alkaline phosphatase instead of peroxidase (Beyzavi et al., 1987). We have developed a sensitive, specific, and rapid assay for the detection of virus-specific IgM antibodies in unconcentrated CSF which overcomes a number of the problems encountered when other methods are employed. It

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Abbreviations: CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; GA, glutaraldehyde; HSV, herpes simplex virus; PVC, polyvinyl chloride; SSPE, subacute sclerosing panencephalitis.

utilizes a peroxidase-conjugated $F(ab')_2$ fragment of anti-IgM antibody and therefore does not suffer from interference of rheumatoid factor. The assay is sensitive enough to detect virus-specific IgM antibodies in CSF and serum samples diluted to an IgM level of 20 μg/100 ml. It is standardized according to the amount of IgM in the test sample in that CSF and serum containing the same amount of IgM are applied, thereby permitting direct comparison of the photometric signals. The assay utilizes commercially obtainable materials and is well-suited for routine laboratory use.

Materials and methods

Viral infections and control samples

Paired CSF and serum samples were taken from three patients with herpes simplex virus (HSV) encephalitis during the acute stage of the illness. Paired specimens from four patients with subacute sclerosing panencephalitis (SSPE) and from two patients with acute mumps encephalitis were also examined for specific IgM antibodies. CSF specimens positive for anti-cytomegalovirus (CMV) IgM antibody and others positive for anti-HSV-1 IgM antibody (gift from Dr. K. Shand) were utilized as control positive samples.

Paired CSF and serum samples from 20 normal control subjects and 30 neurological patients without inflammatory neurological diseases and who were devoid of any clinical or serological evidence of recent viral CNS infection were included as negative controls. A CSF pool containing samples positive for specific IgG antibodies was utilized as an IgG-positive control. Six paired CSF and serum samples with high CSF non-virus specific IgM content (more than 2 g/l) were included as non-specific IgM controls. These comprised four paired samples from patients with active neurosyphilis and two samples from patients with neuroborreliosis (Lyme disease).

Viral antigens and antisera

Freeze-dried measles, mumps, CMV, and HSV type 1 virus antigens (Behring Diagnostics, U.K.) were reconstituted with distilled water according to the manufacturer's instructions. These antigens were harvested from tissue cultures of human cells infected with the viruses and were lyophilized after addition of stabilizer.

Covalent attachment of antigens to solid phase

The method for covalent linkage of viral antigen to plastic is a modification of that described by Place and Schroeder (1982). Flat-bottomed wells of polyvinylchloride (PVC) microplates (Falcon MicroTest III, 3912) were pre-treated with 1% glutaraldehyde (GA) (BDH, England) in 100 mM sodium phosphate buffer, pH 5.0, for 4 h at room temperature. When plastic is activated at low pH, GA has no tendency to react or form polymers. The wells were washed twice with the same buffer and then incubated with a 1% dilution of the viral antigen in 100 mM sodium phosphate buffer, pH 8.0. This rise in pH increases the reactivity of GA and permits the formation of covalent linkage between viral antigens and the solid phase. After 2 h of incubation at room temperature, the wells were washed twice with distilled water before adding 100 mM Tris-glycine buffer, pH 8.0, for 30 min at room temperature to neutralize remaining aldehyde groups. The wells were finally washed with several changes of distilled water before use in the ELISA procedure.

ELISA for virus-specific IgM antibodies

Viral antigen-coated microtitre plates are incubated with 1% gelatine in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 1 h at room temperature to block any unoccupied binding sites and then washed twice with distilled water. Test CSF and serum samples were diluted in the gelatin-Tween solution to a total IgM content of 20 μg/100 ml and 100 μl incubated for 2 h at room temperature with vigorous shaking, by a Vari-Shaker (Dynatech). The method of measuring total CSF and serum IgM content is described below. It was essential that negative and positive control samples, diluted in the same way as test samples, were included in each plate so that the photometric signals obtained in each assay could be adjusted in an appropriate manner.

The wells were then washed repeatedly with PBS before adding 100 μl of 1/1000 dilution of the proxidase-conjugated $F(ab')_2$ fragment of anti-human IgM antibody (μ chain specific, Sigma, U.K., A-4290) in gelatin-Tween. Since conjugated

antibodies react more slowly than free antibodies (Tijssen et al., 1982), incubation was allowed to proceed for at least 2 h in a humid chamber at room temperature. The wells were washed twice in PBS and then 100 μl of substrate solution containing 34 mg of *o*-phenylenediamine dihydrochloride (OPD) (Sigma, P-1526) and 0.005% H_2O_2 in 100 ml of 0.02 M acetate buffer was added. Colour was allowed to develop for 30 min in a dark chamber at room temperature and the reaction terminated with 50 μl of 2 M HCl. The absorbance of each well was measured at 410 nm using a Titertek Multiskan ELISA plate reader.

Determination of total IgM level

The IgM content of unconcentrated CSF and serum samples was measured by a modified μ chain capture assay (Sharief et al., 1990). Briefly, wells of PVC microplates (Falcon Microtest III) were coated with 100 μl of 1/200 dilution of goat anti-human IgM (μ chain specific) antibody (ATAB, 013-11) then the unoccupied binding sites were blocked using 1% gelatin-Tween solution. Undiluted CSF and homologous serum diluted 1/10,000 were added and incubated for 2 h at room temperature. After washing, 100 μl of a 1/1000 dilution of peroxidase-conjugated $F(ab')_2$ fragment of anti-human IgM antibody were added. After incubation and washing, bound enzyme activity was measured using OPD and H_2O_2 and read in a dual wavelength ELISA plate reader.

Expression of results

Virus-specific IgM antibodies, as determined by ELISA, were expressed as absorbance at 410 nm (A_{410}) since optical density accurately reflects the actual amount of virus-specific antibodies present in the colorimetric assay (Felgenhauer et el., 1982). Absorbance of the negative control well was subtracted from the absorbance of the test well to give a corrected value. The minimal detectable concentration of CSF virus-specific IgM was established by serial dilutions of positive and negative samples.

Detection of intrathecally produced specific IgM

Intrathecally synthesized specific IgM was detected by calculating the difference between the CSF and serum A_{410} values (Felgenhauer, 1982). An increase in the A_{410} value above the cut-off level (mean + 2 SD) of the A_{410} values of CSF as compared to serum containing the same amount of IgM was considered to be indicative of intrathecal synthesis.

Results

The cut-off values were calculated from several examinations of positive control samples and mean values are shown in Table I.

Precision of the assay

Duplicate determination of 12 positive CSF samples were run in 25 different microtitre plates

TABLE I

MEAN OPTICAL DENSITY VALUES AT 410 nm (A_{410}) + 2 SD OF SPECIFIC IgM ANTIBODIES IN CSF AND HOMOLOGOUS SERUM IN DIFFERENT CLINICAL CATEGORIES

Diagnosis	Measles		HSV-1		CMV		Mumps	
	CSF	Serum	CSF	Serum	CSF	Serum	CSF	Serum
Acute HSV-1 encephalitis	N *	N **	1.073+0.26	0.524+0.21	N *	N **	N *	N **
SSPE	1.957+0.31	0.728+0.24	N *	N **	N *	N **	N *	N**
Acute mumps encephalitis	N *	N **	N *	N **	N *	N **	1.182+0.41	0.417+0.13
Neurosyphilis	N *	N **	N *	0.703+0.36	N *	0.636+0.28	N *	N **
Control IgG antibodies positve pool	N *	N **	N *	N **	N *	N **	N *	N **
Other neurological conditions	N *	N **	N *	N **	N *	N **	N *	N **
Normal controls	N *	N **	N *	N **	N *	N **	N *	N **

N * Negative results where A_{410} mean + 2 SD value was less than 0.250.

N ** Negative results where A_{410} mean + 2 SD value was less then 0.450.

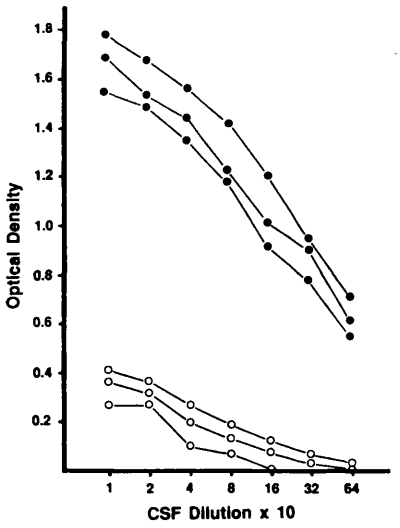

Fig. 1. Effect of glutaraldehyde (GA) on sensitivity. Three control positive specimens assayed in GA-activated plates (●———●) and in plates without GA activation (○———○).

to calculate the within-run variance. The coefficient of variation (standard deviation/mean) was between 2 and 4% in 18 plates and less than 6% in the remaining seven plates. The between-run variance was estimated by repeated measurement of five different dilutions of the positive controls, each assayed eight times in total. The coefficient of variation for measles, HSV-1, CMV, and mumps specific IgM antibodies were 5.4%, 6.1%, 4.8%, and 5.9% respectively.

Influence of glutaraldehyde on sensitivity

The detectability of virus-specific IgM antibodies was dependent on the pretreatment of plates with GA. Relatively low detectability was observed when the plates were not GA pre-treated (Fig. 1).

Specificity of the F(ab')$_2$ fragment

Fig. 2 compares results obtained using peroxidase-conjugated F(ab')$_2$ fragments of the anti-human IgM antibody and those obtained when peroxidase-conjugated anti-human IgM whole antibody molecule was used. The F(ab')$_2$ fragment conjugate was also specific for IgM class antibodies in that it did not show non-specific reactivity when an antigen-specific IgG-positive control sample pool was tested (Table I). Conversely, the whole molecule anti-human IgM conjugate showed marked cross-reactivity with the IgG positive sample (data not shown). Furthermore, the anti-IgM conjugate resulted in a considerably higher background reactivity whereas the F(ab')$_2$ fragment conjugate produced a lower background without any reduction in sensitivity.

The use of the F(ab')$_2$ fragment conjugate was also devoid of false-positive reactions due to rheumatoid factors. As shown in Fig. 3, the addition of rheumatoid factor-positive serum to virus specific IgM negative CSF samples did not increase the A_{410} values of the assay.

Type of plate

The type of plastic plate used for coating viral antigens was of crucial importance for the sensitivity and reproducibility of the assay. The best results were obtained with PVC microplates while signal detection was poor when rigid polystyrene plates (Flow Laboratories, 76-208-05) were used as the solid phase.

Specific IgM levels in neurological patients

Intrathecal production of specific IgM antibodies was detected in the 3 HSV-1 encephalitis pa-

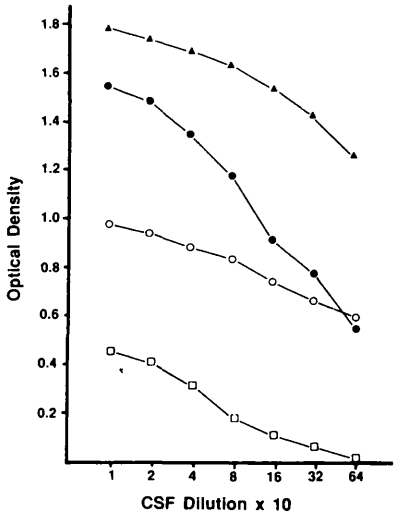

Fig. 2. Specificity of the F(ab')$_2$ fragment of anti-IgM antibody in detecting IgM antibodies. Antibodies in the HSV-1 positive control sample (▲———▲) and negative control sample (○———○) were assayed using HRP-conjugated whole anti-IgM antibody. Antibodies in the same HSV-1 positive sample (●———●) and control negative sample (□———□) were also evaluated using HRP-conjugated F(ab')$_2$ fragment.

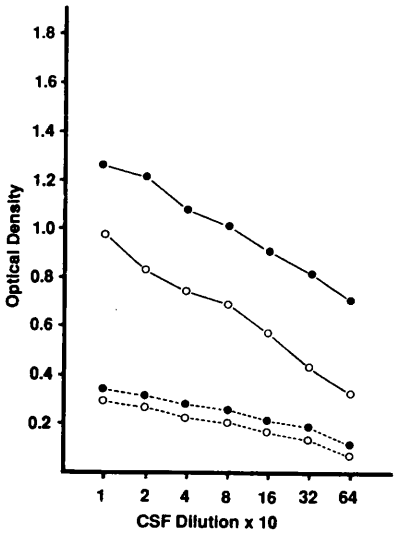

Fig. 3. Influence of rheumatoid factor (RF) on the assay. A negative control sample (○———○) and a negative control sample + RF (●———●) were assayed using HRP-conjugated intact anti-IgM. The same negative sample (○- - - - - ○) and negative sample + RF (●- - - - -●) were also evaluated using HRP-conjugated $F(ab')_2$ fragment.

tients and in the other two patients with mumps encephalitis. It was also noted in three of the four SSPE patients included in this study. Some patients with viral CNS infections showed high titres of specific IgM antibodies in serum as well as in CSF (Table I). However, the mean values ± 2 SD showed clear increase of antibody levels in CSF over homologous serum, implying that secretion of antibodies by activated B lymphocytes occurs into the CSF compartment.

Three patients with active neurosyphilis were found to have high serum levels of HSV-1 IgM antibody and two demonstrated high serum CMV IgM antibody titres. None, however, had high titres in the CSF suggesting that intrathecal production of virus-specific IgM antibodies is absent in uncomplicated neurosyphilis.

Discussion

The present method was devised because of the need to develop a highly sensitive and specific assay for the detection of virus-specific IgM antibodies in unconcentrated CSF. One of the advantages of the assay described here is the controlled fixation of viral antigens to the solid phase through covalent linkage. Viral antigens are poorly adsorbed to plastic, and commonly used methods of physical adsorption of viruses onto solid phases suffer from uncontrollable factors such as partial uncoupling of viral antigens by unspecified compounds in the sample, and unreliable hydrophobic binding to the solid phase. Covalent binding of the viruses to the solid phase, on the other hand, prevents antigen loss during washing and incubation steps and should provide free contact with antibodies present in the test sample.

The specificity of peroxidase-conjugated $F(ab')_2$ fragments is another advantage of the present assay. The presence of rheumatoid factor in test samples is a serious cause of interference in commonly used assays even when μ chain capture assays are employed (Kurtz and Malic, 1981). The use of $F(ab')_2$ fragment, on the other hand, eliminates interference by rheumatoid factor since the latter reacts only with the Fc fragment of antibodies. The other advantage of $F(ab')_2$ fragments is the absence of non-specific reactivity and thus a clearer background. Furthermore, $F(ab')_2$ fragments of the anti-IgM antibody used here was devoid of any cross-reactivity with non-IgM immunoglobulins (Sharief et al., 1989).

Previous reports (Schmitz, 1978) showed that human IgM without antiviral activity may result in non-specific reactions in IgM antibody assays. The assay reported here proved to be highly specific in that there was no evidence of false-positive results even when samples with very high IgM contents, e.g., those from patients with active neurosyphilis or Lyme disease, were used. Similarly, no false-positive results were noted in samples with high virus-specific IgG antibody titres.

The permeability of the blood-CSF barrier increases in many CNS infections allowing serum IgM antibody to leak into the intrathecal compartment causing increased, but diagnostically irrelevant, CSF titres. The present method, which compares CSF/serum ratios of viral antibody titres, accounts for the actual permeability state of the barriers. Applying CSF and serum samples containing identical amounts of IgM permits a direct comparison of their respective virus-specific antibody titres. Intrathecally synthesized antibod-

ies produce higher OD signals in CSF than homologous serum and thereby abolishes the need for quantification of reference antibodies. Application of standard dilutions of CSF and serum instead of constant IgM concentration, as advocated by some groups (Chiodi et al., 1986), should be discouraged since antibody titres in CSF and homologous serum vary widely amongst different clinical groups.

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Intrathecal synthesis of IgM in neurological diseases: a comparison between detection of oligoclonal bands and quantitative estimation

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SUMMARY

Immunoglobulin (Ig) M concentration in cerebrospinal fluid (CSF) was measured in 30 reference subjects to determine reference ranges which were up to 0.41 mg/l for CSF IgM, 0.06 for IgM index, and 9×10^{-4} mg/l for the Reiber empirical formula. Intrathecal IgM synthesis was then studied in 159 patients with different neurological diseases both by detecting CSF oligoclonal IgM bands and by determining the IgM index and Reiber's intrathecal IgM production values. At the same time, the state of blood–CSF barrier was evaluated in all patients by CSF polyacrylamide gel electrophoresis and by CSF/serum albumin ratio. Oligoclonal IgM bands were present mainly in patients with intrathecal humoral immune response, e.g. multiple sclerosis, other inflammatory nervous diseases, and infections of the central nervous system. Quantitative IgM ratios were, however, elevated in conditions not associated with local humoral immune response. Furthermore, quantitative ratios were elevated in patients with damaged blood–CSF barriers. Oligoclonal IgM bands are thus considered to be the most useful tool in detecting intrathecal synthesis of IgM.

Key words: IgM oligoclonal bands; IgM index; CSF IgM; Multiple sclerosis; Intrathecal synthesis

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INTRODUCTION

The protein fractions of cerebrospinal fluid (CSF) in normal subjects are mainly the same as those found in serum (Cutler et al. 1970), and their level in the CSF is limited by their diffusion across the blood-CSF barrier (Felgenhauer 1982). Thus, proteins with high molecular weight, e.g. immunoglobulin (Ig) M, cross intact barriers with great difficulty.

In pathological conditions 2 factors contribute to increased concentration of CSF IgM; an increased transudation through blood-CSF barriers due to changes in vascular and meningeal permeability, and a local intrathecal production by immunocompetent cells (lymphocytes and plasma cells). A combination of these two factors may operate in some cases. The purpose of laboratory investigations is to distinguish between these two situations, and hence to detect locally produced IgM.

The two approaches used to detect intrathecal synthesis of IgM are (1) empirical formulae based on physiologic principles governing transudation of albumin and IgM across the blood–CSF barrier that relies on quantitative determination of IgM and albumin levels in CSF and homologous serum, and (2) the qualitative detection of oligoclonal IgM bands in electrophoresed CSF which are absent in homologous serum, or the oligoclonal IgM bands are more numerous in CSF than in serum when equal quantities of CSF and serum IgM are analyzed by electrophoresis.

Until recently, intrathecal IgM synthesis was mainly determined through the empirical approach of referring CSF/serum IgM ratio to that of albumin to correct for transudation through blood–CSF barriers. This correction, however, could be insufficient since the passage of proteins is clearly dependent on their size. IgM, a macroglobulin with an extremely large hydrodynamic size, penetrates intact blood–CSF barriers with difficulty and is likely to give misleading results in the presence of barrier damage (Felgenhauer 1982).

Oligoclonal IgM bands are readily detected in unconcentrated CSF using a modified electrophoretic technique (Sharief et al. 1989). Hitherto, no detailed attempt has been made to compare the quantitative and qualitative methods of detecting intrathecal IgM synthesis. In this report a comparison has been made between agarose gel electrophoresis and quantitative estimation of IgM in CSF in order to assess intrathecal IgM response in various neurological conditions.

PATIENTS AND METHODS

Reference subjects

Paired CSF and blood samples of 30 subjects (18 females) were used to determine reference ranges. The ages of reference population ranged from 14 to 81 years with a mean of 44 years. Reference subjects had a variety of non-specific complaints such as headache, blurring of vision, or psychoneurotic features. Physical examination was normal as were routine blood tests and CSF parameters. Intrathecal synthesis of IgG was excluded by nitrocellulose immunoblotting after agarose isoelectric focusing

(Walker et al. 1983). The blood–CSF barrier was shown to be intact by determination of CSF total protein, CSF/serum albumin concentration ratio and by polyacrylamide gel electrophoresis (Thompson 1988).

Patients

The clinical groups of patients included in this study are shown in Table 1. The patient population is different from that already published (Sharief and Thompson 1989a) in order to avoid bias during data analysis. Paired CSF and serum samples from patients with inflammatory and infectious neurological diseases were obtained within 1 week of the clinical presentation. In patients with stroke, CSF and homologous serum samples were obtained within 24 h of the onset. Multiple sclerosis (MS) was diagnosed and classified according to the clinical criteria of McDonald and Halliday (1979). No MS patient included in this study was receiving corticosteroid therapy. Within the category of CNS infections were 4 patients with bacterial meningitis, 4 with viral encephalitis, 2 with active neurosyphilis, 1 with neuroborreliosis (Lyme disease), and one with HTLV-1 myelitis. Inflammatory diseases of the nervous system comprise various non-MS inflammatory conditions including cerebral lupus (2 patients), neurosarcoid (7 patients), encephalopathies (3 patients), 2 patients with acute necrotizing leukomyelitis, and a patient with post-infectious myelitis.

TABLE 1

FREQUENCY OF POSITIVE OLIGOCLONAL IgM BANDS IN VARIOUS DIAGNOSTIC CATEGORIES COMPARED TO THE FREQUENCIES OF ELEVATED IgM INDEX AND REIBER FORMULA VALUES

Diagnostic categories	Total No. of patients	No. of positive cases		
		Oligoclonal IgM bands	IgM index	Reiber formula
Multiple sclerosis	59	31	30	37
CNS infections	12	10	7	6
Inflammatory CNS conditions	24	10	12	15
Cerebrovascular diseases	5	0	4	4
CNS tumours	7	3	3	5
Degenerative CNS diseases	13	0	0	1
Vertebral lesions	10	0	5	5
Neuropathies	10	0	3	4
Other neurological conditions	19	0	3	2
Total	159	54 (34%)	67 (42%)	79 (50%)

Sixty four patients with non-inflammatory neurological diseases were also included. They are grouped under different categories listed in Table 1. Degenerative CNS conditions comprise 5 patients with Alzheimer's disease and other dementias, 3 motor neurone disease, 4 Parkinson's disease and one patient with system atrophy. Cerebrovascular diseases include 3 patients with stroke, a patient with transient ischaemic attacks, and a patient with moya moya disease. Other neurological conditions include diseases such as benign intracranial hypertension, epilepsy and myopathies. Care was taken not to include patients with possible intrathecal immune response in the above groups, e.g. stroke due to autoimmune diseases, para-neoplastic polyneuropathy, or neuropathies due to systemic autoimmune diseases. Cases with doubtful diagnoses were also excluded from the study.

Quantitative assays

CSF total protein was measured turbidimetrically using benzethonium chloride precipitation (Luxon et al. 1989). Albumin and IgG were determined on unconcentrated CSF and serum using electroimmunoassay. The blood–CSF barrier was evaluated by calculating CSF/serum albumin ratio (Ganrot and Laurell 1974; Lefvert and Link 1985) and by polyacrylamide gel electrophoresis (Thompson 1988).

CSF and serum IgM was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, goat anti-human IgM Fc (Atlantic Antibodies) is adsorbed to polyvinyl chloride microtitre plate wells (Falcon Micro-Test III). Unoccupied binding sites are blocked using 1% (w/v) gelatin. Undiluted CSF and serum diluted 1 : 10000 with 0.1% (w/v) gelatin in saline are added and incubated for 2 h at room temperature. After washing, peroxidase conjugated $F(ab')_2$ fragment of rabbit anti-human IgM Fc (Sigma, U.K.) are added. After incubation and washing, bound enzyme activity is measured using *o*-phenylenediamine and hydrogen peroxide and read in a dual wavelength ELISA plate reader (Dynateck Microplate Reader MR 700). The use of $F(ab')_2$ fragment as detector markedly reduced non-specific background. Reproducibility, particularly of the serum assay which uses a high dilution of sample, was aided by the use of a liquid handling station (Probus Quatro).

Intrathecally synthesized IgM was detected by determining the IgM index value to minimize the effect of alteration of blood–CSF barrier permeability on the absolute CSF IgM amount. The IgM index was calculated from CSF/serum ratios of IgM and albumin concentrations, by the following formula (Fryden et al. 1978):

$$\text{IgM index} = \frac{\text{CSF IgM} \times \text{serum albumin}}{\text{serum IgM} \times \text{CSF albumin}}$$

The proportion of CSF IgM that has been intrathecally synthesized was also quantitated by Reiber empirical formula (Reiber and Felgenhauer 1987) to represent empirical formulae (Tourtellotte 1970; Schuller and Sagar 1981) devised to calculate locally produced (loc) IgM in the intrathecal compartment. These formulae are based on the use of albumin as a quantitative marker of blood–CSF permeability and take into account proportional passage of albumin and IgM through an impaired barrier.

Reiber formula was calculated as follows:

$$\text{loc IgM} = [Q_{IgM} - a/b \sqrt{(Q_{alb})^2 + b^2} + c] \times \text{S-IgM}$$

Where Q_{IgM} represents CSF/serum concentration quotient of IgM, Q_{alb} represents CSF/serum concentration quotient of albumin, and S-IgM means serum concentration of IgM.

Qualitative assay

Oligoclonal IgM were detected in unconcentrated CSF and suitably diluted serum by electrophoresis in high electroendosmotic agarose followed by glutaraldehyde-enhanced nitrocellulose immunofixation using peroxidase-conjugated $F(ab')_2$ fragment against human IgM-Fc (Sharief et al. 1989). The method is standardized according to the amount of IgM in that suitable volumes of CSF and diluted serum containing 10 ng IgM are used for electrophoresis to achieve balanced staining for comparative purposes.

RESULTS

The IgM ELISA assay showed coefficient of variation (CV) of 3.8% within and 5.4% between batches. The CV was 4.4% at IgM concentration of 0.1 mg/l and 3.2% at 0.5 mg/l. In the 30 reference subjects, the mean normal value for CSF IgM was 0.41 mg/l with a logarithmic normal distribution. The IgM index also showed a log-normal distribution, with a mean of 0.027 and 95% population limits of 0.01–0.068.

We slightly modified Reiber formula so that constants a/b, b^2, and c are 0.64, 145×10^{-6}, and 7.3×10^{-3}, respectively rather than 0.65, 150×10^{-6}, and 7.5×10^{-3} described in Reiber's original formula. Our values for these constants were obtained by plotting all quotients available in a double linear diagram as originally described by Reiber and Felgenhauer (1987). The upper reference limit of intrathecally produced IgM calculated by Reiber empirical formula was found to be 9×10^{-4} mg/l.

There was a statistically significant correlation between serum and CSF IgM values in reference population ($r = 0.28$, $P < 0.01$). No significant correlation was detected between serum and CSF IgM when all patients, including those with intrathecal humoral immune response, were analyzed together. There was, however, significant correlation when only patients with damaged blood–CSF barrier ($n = 53$) were included in the statistical calculation ($r = 0.235$, $P < 0.05$). Similarly, there was a significant correlation when patients with non-inflammatory CNS diseases were analyzed separately ($r = 0.198$, $P < 0.05$). The role of barrier damage in individual clinical groups will be assessed later (see below).

Of the 159 patients included in this study, 54 (40%) showed evidence of intrathecal synthesis of IgM in the form of CSF oligoclonal bands. Fifty-two patients showed distinctive bands in CSF with no bands in corresponding serum and 2 other patients had multiple bands in CSF and one band in corresponding serum. The latter

pattern represents intrathecal synthesis of IgM as well as leakage of other oligoclonal bands from serum and was regarded as positive for local IgM synthesis. Five patients had similar multiple bands in CSF and corresponding serum implying leakage of bands from serum and were therefore regarded as negative. One patient had an IgM paraprotein band in CSF and serum and was also regarded as negative for intrathecal IgM production.

Table 1 summarizes frequencies of intrathecal IgM synthesis as detected by qualitative (oligoclonal bands) and quantitative (IgM index and Reiber formula) methods. Oligoclonal IgM bands were mainly detected in patients with infectious and inflammatory nervous conditions. Of the 64 patients with no apparent inflammatory disease of the CNS, oligoclonal IgM bands were detected in only 3 (5%), all of whom were cases of CNS tumours. In contrast, the IgM index value was increased in 18/64 (28%) patients with non-inflammatory CNS diseases, and Reiber's empirical formula showed intrathecal IgM production in 21/64 (33%) patients with non-inflammatory diseases.

Table 2 compares quantitative ratios to CSF oligoclonal IgM bands. Of the 54 patients with oligoclonal IgM bands, 26 (48%) had normal index value and 20 (37%) patients showed no abnormal IgM synthesis by Reiber's formula. Another 38 (36%) and 40 (43%) of the 105 patients with no oligoclonal bands had elevated index and Reiber formula values, respectively. Furthermore, the IgM index and Reiber formula failed to discriminate between patients who have leakage of bands from serum and the patient with IgM paraprotein from those who do not show any banding pattern.

Table 3 shows the relationship of different CSF parameters to IgM index and oligoclonal IgM bands. Some 76% with normal CSF cell count (less than 5 cells/mm^3) did not show oligoclonal bands and 68% had normal IgM index. Despite the significant relationship, however, there was no correlation between the number of cells and the value of IgM index ($r = 0.107$).

TABLE 2

NUMBER OF PATIENTS DIVIDED ACCORDING TO CSF OLIGOCLONAL IgM PATTERN RELATED TO IgM INDEX AND INTRATHECAL IgM SYNTHESIS (REIBER)

Oligoclonal IgM pattern	IgM index		Reiber formula	
	≤ 0.068	> 0.068	$\leq 9 \times 10^{-4}$	$> 9 \times 10^{-4}$
Positive				
Local synthesis	24	28	19	33
Local synthesis + serum leakage	2	0	1	1
Negative				
No CSF bands	67	32	58	41
Serum leakage	0	5	2	3
Paraprotein	0	1	0	1
Total	93	66	80	79

TABLE 3

RELATIONSHIP BETWEEN CSF CELL COUNT, FREE LIGHT CHAINS, AND IgG INDEX AND BOTH IgM INDEX AND OLIGOCLONAL IgM BANDS

Method	Leukocyte count*		Free lambda light chain**		Free kappa light chain +		IgG index + +	
	<5	⩾5	⩽2	>2	⩽2	>2	⩽0.85	>0.85
IgM index								
⩽0.068	76	22	90	8	88	10	72	26
>0.068	35	26	44	17	55	6	33	30
Oligoclonal IgM bands								
Negative	84	21	98	7	100	5	74	31
Positive	27	27	36	18	43	11	28	26

* $\chi^2 = 5.11$ ($P = 0.024$) for IgM index; $\chi^2 = 9.59$ ($P = 0.002$) for oligoclonal bands.
** $\chi^2 = 9.57$ ($P = 0.002$) for IgM index; $\chi^2 = 17.18$ ($P < 0.0001$) for oligoclonal bands.
\+ $\chi^2 = 0.00$ ($P = 1.000$) for IgM index; $\chi^2 = 7.95$ ($P < 0.01$) for oligoclonal bands.
\+ \+ $\chi^2 = 6.07$ ($P = 0.013$) for IgM index; $\chi^2 = 4.47$ ($P = 0.034$) for oligoclonal bands.

About 72% of patients with elevated CSF free lambda light chain bands (2 or more bands) had oligoclonal IgM bands and another 68% had elevated IgM index, both of which were statistically significant (Table 3). In contrast, no significant relationship was found between free kappa light chain bands and IgM index or Reiber formula values, while 69% of patients who had oligoclonal IgM bands showed 2 or more free kappa light chain bands in CSF ($P < 0.01$).

Individual Q_{IgM} values were plotted against Q_{alb} values. These findings, together with different oligoclonal IgM patterns, will be discussed separately according to major clinical groups, taking into account the function of blood–CSF barriers.

MS patients

Signs of blood-CSF barrier damage were detected in 19 (32%) of the 59 patients with MS (Fig. 1). Among the 23 patients with normal index, 13 (57%) showed intrathecal synthesis of IgM in the form of oligoclonal bands in CSF. Conversely, 12 patients with normal CSF electrophoretic pattern had elevated index values, 7 of whom had damaged blood–CSF barriers. In fact 3 of these patients have normal Q_{alb} values despite high index implying that CSF IgM level could increase in blood–CSF barrier dysfunction without concomitant increase in albumin level.

Patients with CNS infection and non-MS inflammatory diseases

The frequency of oligoclonal IgM bands and elevated quantitative ratios has already been described (Table 1). A patient with aseptic meningitis had a borderline IgM index value but showed oligoclonal IgM bands in CSF. Two patients were positive for oligoclonal IgM bands but IgM index and Reiber formula values were within the normal range; one of them had HTLV-1 myelitis while the other was suffering from chronic

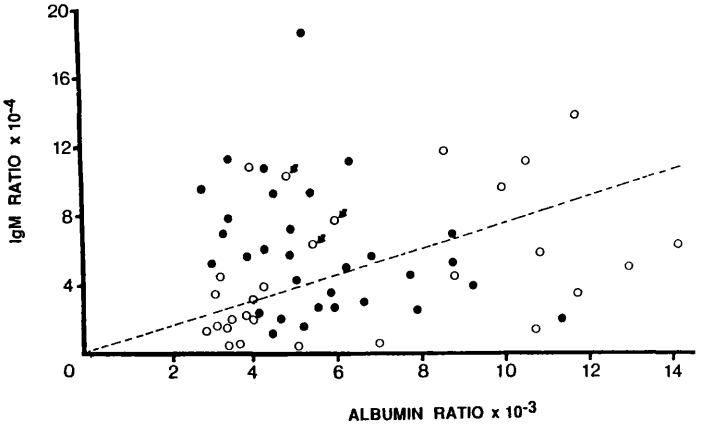

Fig. 1. Correlation between IgM and albumin ratios in patients with multiple sclerosis (n = 59). o, patients without CSF oligoclonal IgM bands; •, patients positive for oligoclonal bands. Arrows point to patients who had damaged blood–CSF barriers (detected by polyacrylamide gel electrophoresis) despite a normal albumin ratio. Broken line represents upper limit of normal for IgM index.

tuberculous meningitis. Two patients suffering from meningococcal meningitis were negative for oligoclonal IgM and had normal index values.

Eight patients with inflammatory neurological conditions (MS excluded) who had elevated index value were negative for oligoclonal IgM, 6 of whom had evidence of damage to blood-CSF barriers (Fig. 2). Another 5 patients with normal index values showed evidence of intrathecal synthesis of IgM manifested as oligoclonal bands in CSF.

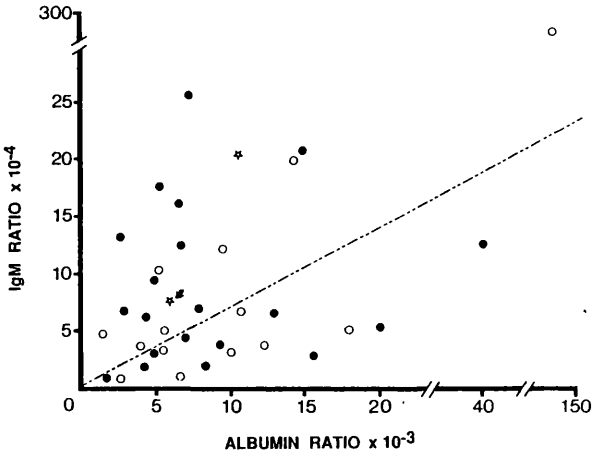

Fig. 2. Correlation between IgM and albumin ratios in patients with CNS infections (n = 12) and patients with non-MS inflammatory CNS diseases (n = 24). o, patients with no CSF oligoclonal IgM bands; •, patients positive for oligoclonal bands, and stars represent patients who have leakage of oligoclonal IgM bands from serum. Arrow points to a patient who had damaged blood–CSF barriers (detected by polyacrylamide gel electrophoresis) despite normal albumin ratio. Interrupted line represents upper limit of normal for IgM index.

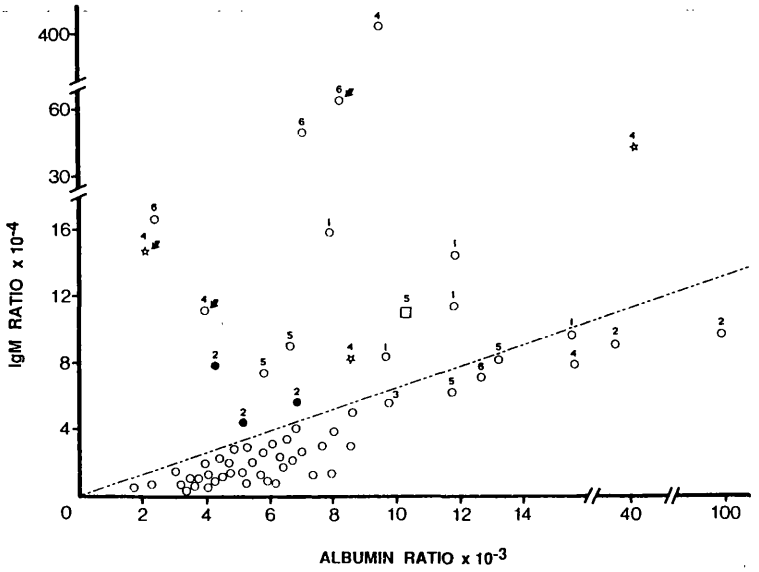

Fig. 3. Correlation between IgM and albumin ratios in patients with non-inflammatory neurological diseases (n = 64). o, patients with no oligoclonal IgM bands; •, patients with oligoclonal bands; asterisks represent patients with leakage of IgM bands from serum; □, patient with IgM para-proteinaemia. Arrows point to patients with damaged blood–CSF barriers (detected by polyacrylamide gel electrophoresis) despite normal albumin ratio. Broken line represents upper limit of normal for IgM index. Patients with high IgM index or raised albumin ratio are identified by the following numbers: (1) cerebrovascular diseases, (2) tumours, (3) degenerative diseases, (4) vertebral lesions, (5) neuropathies, and (6) other neurological diseases.

Patients with other neurological conditions

Of the 64 patients with non-inflammatory and non-infectious neurological conditions, oligoclonal IgM bands were detected in only 3 (5%), all of whom had CNS tumours. One patient had malignant meningoendotheliosis while the other 2 were suffering from brain and spinal cord lymphoma, respectively. IgM index was elevated in 18 (29%), 10 (56%) of whom showed evidence of blood–CSF barrier damage (Fig. 3). The frequency of raised IgM index and Reiber formula values in individual clinical groups has already been mentioned (Table 1). The index and Reiber formula values were elevated in all 3 patients with stroke and the index value was borderline in the patient with moya moya disease. These four patients had evidence of damaged blood–CSF barrier and none had oligoclonal IgM in CSF. Another 5/10 patients with vertebral lesions showed elevated index and Reiber formula values, 4 of whom had damaged blood–CSF barriers but none had oligoclonal bands in CSF.

DISCUSSION

The upper reference limit for IgM index varies according to heterogeneous control groups included and different methods of analysis. It was first arbitrary set as 1.0 (Link et al. 1979; Strandberg et al. 1982), probably because of lack of sensitive methods for

IgM estimation. The reference limit was estimated by other group of researchers as 0.071 (Sindic et al. 1982), 0.08 (Brouwer and Leeuwen-Herberts 1983), and later as 0.061 (Forsberg et al. 1984). This variation in reference limits illustrates the fact that each laboratory should estimate its own reference values before comparing results with those from other laboratories.

The reference value of IgM index (0.068) agrees well with that of 0.061 already reported (Forsberg et al. 1984, 1986). It also agrees well with that of 0.071 reported by Sindic et al. (1982). Similarly, the reference value of local IgM synthesis (Reiber) of 9×10^{-4} is similar to that originally reported by Reiber and Felgenhauer (1987). It is, therefore, feasible to perform inter-laboratory comparisons. Similarly, the method of glutaraldehyde-enhanced immunofixation is standardized according to the IgM content of CSF. Thus, it is quite acceptable to compare qualitative results obtained through this method with results obtained from quantitative ratios.

The significant correlation between CSF IgM and serum IgM in reference group is in accordance with previous reports (Sindic et al. 1982; Forsberg et al. 1984). The significant correlation between CSF and serum IgM in patients with damaged blood–CSF barrier suggests that CSF IgM is derived from serum in patients with altered barrier functions.

IgM index has been shown to increase with progressive dysfunction of blood–CSF barrier (Felgenhauer 1982). The present study, by corroborating such finding and by extending further previous reports (Reiber 1980) provides good evidence that the discriminatory functions of intact and impaired blood–CSF barriers are different. Thus, referencing CSF IgM to CSF albumin or to albumin quotient may lead to erroneous results in patients with barrier dysfunction.

It is not the aim of this study to discuss the importance of oligoclonal IgM in neurological diseases as this has already been reported (Sharief and Thompson 1989a,b). However, we managed to corroborate our previous findings that oligoclonal IgM is mainly detected in neurological diseases that display intrathecal humoral immune response. Boeer et al. (1988) found no local IgM production by quantitative studies in a patient with neuroborreliosis while CSF showed oligoclonal IgM pattern. It seems clear from our study that both IgM index and Reiber formula could be normal in patients with intrathecal IgM synthesis. Conversely, not all patients with elevated IgM index or Reiber formula values produce IgM locally.

Sindic and collaborators (1982) found in a group of 21 patients with stroke 5 with elevated IgM index. No details about the state of the blood–CSF barriers were given in that particular study but one must assume that the raised index values are related to barriers dysfunction. The local IgM response may otherwise be due to secondary infection or arteritis. Rostrom and Link (1981) detected oligoclonal IgG patterns after stroke. We did not detect any oligoclonal pattern in stroke patients perhaps because of the brief time period between lumbar puncture and onset. Sindic et al. (1982) also reported a slightly increased IgM index value in one patient with sciatica whom CSF IgM was thought to be of serum origin, emphasizing the importance of interpreting index values in the context of blood–CSF barriers function.

The present study provides good evidence that quantitative formulae may yield

abnormal results in neurological conditions not associated with a local humoral immune response. They should, therefore, be interpreted in light of other CSF parameters, particularly the state of blood–CSF barrier. The qualitative detection of oligoclonal bands represents a better way of detecting intrathecal IgM response in such cases. The importance of detecting oligoclonal bands is not only to confirm the presence of intrathecal production of IgM in patients who have elevated quantitative ratios, but particularly to detect local IgM synthesis when these ratios are normal.

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APPENDIX B

Selected publications related to clinical findings

The New England
Journal of Medicine

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INTRATHECAL IMMUNE RESPONSE IN PATIENTS WITH THE POST-POLIO SYNDROME

MOHAMMAD K. SHARIEF, M.B., CH.B., M.PHIL., ROMAIN HENTGES, M.D., AND MARIA CIARDI, M.D.

Abstract *Background.* The syndrome of progressive muscular atrophy decades after acute paralytic poliomyelitis (post-polio syndrome) is not well understood. The theory that physiologic changes and aging cause the new weakness does not explain the immunologic abnormalities reported in some patients. An alternative explanation is persistent or recurrent poliovirus infection.

Methods. We assessed the intrathecal antibody response to poliovirus and intrathecal production of interleukin-2 and soluble interleukin-2 receptors in 36 patients with the post-polio syndrome and 67 controls (including 13 who had had poliomyelitis but had no new symptoms and 18 with amyotrophic lateral sclerosis). Intrathecal antibody responses to measles, mumps, herpes simplex, and varicella–zoster viruses were also determined.

Results. Oligoclonal IgM bands specific to poliovirus were detected in the cerebrospinal fluid of 21 of the 36 patients with the post-polio syndrome (58 percent) but in none of the control group ($P<0.0001$). In quantitative studies there was evidence of increased intrathecal synthesis of IgM antibodies to poliovirus only among the patients with the post-polio syndrome; there was no increased synthesis of IgM to measles, mumps, herpes simplex, or varicella–zoster viruses. The patients with post-polio syndrome had significantly higher mean (±SD) cerebrospinal fluid levels of interleukin-2 and soluble interleukin-2 receptors than the controls (8.1±5.3 vs. 1.4±0.8 U per milliliter and 159.6±102.9 vs. 10.7±6.2 U per milliliter, respectively). The intrathecal synthesis of IgM antibodies to poliovirus correlated with the cerebrospinal fluid concentrations of interleukin-2 ($P<0.0005$) and soluble interleukin-2 receptors ($P<0.001$).

Conclusions. An intrathecal immune response against poliovirus is present in many patients with the post-polio syndrome. In some of these patients the recrudescence of muscle weakness may be caused by persistent or recurrent infection of neural cells with the poliovirus. (N Engl J Med 1991; 325:749-55.)

POST-POLIOMYELITIS progressive muscular atrophy (post-polio syndrome)[1-3] is characterized by new, slowly progressive muscle weakness affecting patients decades after there has been maximal recovery from acute paralytic poliomyelitis. The condition has distinctive clinical features that include muscle weakness and atrophy,[3-5] muscle pain[6] and fasciculations,[1,2] weakness of bulbar[3] or respiratory[7] muscles, and sleep apnea.[8]

Although recurrence of muscle weakness several years after the original attack of acute paralytic poliomyelitis was described as early as 1875,[9,10] the pathogenesis of post-polio syndrome has not yet been established. Current theory suggests that the illness is caused by attrition of surviving motor neurons, with eventual loss of axonal terminals.[3,11,12] However, immunologic studies have demonstrated various lymphocytic abnormalities and the presence of oligoclonal bands in the cerebrospinal fluid of a substantial number of patients with the post-polio syndrome,[1,13,14] suggesting that immunopathogenic mechanisms may contribute to the pathogenesis of the disease process. Activation of poliovirus may contribute to the development of the syndrome, although searches for poliovirus antibodies in the cerebrospinal fluid have yielded negative results to date.[1,15,16]

One way of assessing the role of the immune system in the post-polio syndrome is to detect intrathecally produced specific immunoglobulins and soluble products of immune cells, such as cytokines. An antigenic challenge of the central nervous system, as of any other organ, evokes responses of T and B lymphocytes,[17-20] with production of immunoglobulins and cytokines. Among the various isotypes of immunoglobulins, IgM is of particular importance in the evaluation of viral infections, including those of the central nervous system.[21-23] IgM is sensitive to minimal antigenic stimulation; has a relatively short half-life, with little if any memory; and has an important role in the clearance of viremia.[24] Furthermore, we have demonstrated that intrathecal synthesis of IgM is an indicator of recent antigenic stimulation in the central nervous system[25,26] and that it is useful in assessing disease activity of inflammatory conditions of the central nervous system.[27,28] In this study, we examined the intrathecal immune response to poliovirus

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and local synthesis of interleukin-2 and soluble interleukin-2 receptors by the central nervous system in 36 patients with the post-polio syndrome and 67 control patients.

Methods

Patients

Paired samples of serum and cerebrospinal fluid were obtained simultaneously from all patients after informed consent had been given. Protease inhibitor (aprotinin; 1000 kallikrein units per milliliter) was immediately added to the samples to prevent protein degradation, and the samples were then frozen in aliquots at −70°C and thawed just before use.

We have adopted strict criteria for diagnosing the post-polio syndrome[2,3]: there must be a clear history of acute paralytic poliomyelitis in childhood or adolescence during a polio epidemic, with functional stability or recovery for at least 15 years; residual muscle atrophy, weakness, and areflexia in at least one limb, with normal sensation and no sign of upper-motor-neuron weakness; new neuromuscular symptoms in the form of progressive muscular atrophy or musculoskeletal symptoms or both; and no clinical evidence of any known medical, neurologic, orthopedic, or psychiatric illness that could account for the new symptoms. Patients with diabetes, polyneuropathies, connective-tissue diseases, back injuries, or compression neuropathies and those with a family history of neuromuscular disorders were excluded. Only patients below the age of 60 years were studied, to avoid nonspecific changes associated with aging.[29] Ultimately, 16 men and 20 women were included in the investigation.

Controls

Paired samples of serum and cerebrospinal fluid were collected from 13 control patients matched for age and race who had a history of paralytic poliomyelitis and whose condition had been stable for 19 to 41 years (mean, 29.6) after the original infection. Lumbar puncture was performed in this group to investigate unrelated symptoms, such as tension headache, blurring of vision, or mild psychoneurotic symptoms. Paired samples were also obtained from 18 patients with classic amyotrophic lateral sclerosis and from 36 age-, sex-, and race-matched patients with various other neuromuscular diseases (6 with chronic progressive multiple sclerosis, 4 with Parkinson's disease, 6 with Alzheimer's disease, 6 with cerebrovascular diseases, 6 with spinal-cord compression, 5 with muscular dystrophy, and 3 with myasthenia gravis). Paired samples from 16 normal subjects (9 of whom were female), who presented with mild, nonspecific tension headache or other nonspecific syndromes, were used to determine reference ranges.

Clinical Examples

The following is a brief clinical history of a representative case of post-polio syndrome: a 49-year-old woman who had had paralytic poliomyelitis at the age of 6 presented with slowly progressive muscle weakness. Hospital records of the original attack documented the occurrence of an acute febrile illness followed by generalized asymmetric muscle weakness. Two of her school friends were also affected. The patient improved after intensive rehabilitation, but her right arm and leg remained weaker, thinner, and shorter than her left arm and leg. She functioned well for 37 years, although she had to wear a leg brace. Six years ago, she noticed slowly progressive weakness and wasting of the left arm and leg (she had not had earlier difficulty with this leg) that was accompanied by deep muscle pains and occasional fasciculations of the newly weakened muscles. Progression remained focal, although she had had to use a manual wheelchair for the past year. In addition to the preexisting weakness and wasting, neurologic examination detected moderate weakness and atrophy of the left biceps, hamstring, and iliopsoas muscles, with normal sensation. No upper-motor-neuron signs were noted, and cranial nerves were not involved. Electromyographic studies revealed active denervation (fibrillation and positive sharp waves) in the newly involved muscles, whereas nerve-conduction studies were normal.

The following case history is representative of the control group: a 48-year-old woman had paralytic poliomyelitis at the age of 28 months. Her brother had died of pneumonia after a similar illness at the age of five years. The woman had residual muscle weakness and atrophy of both legs but managed well with leg braces and crutches. During the past nine years, she had had joint pain, unsteady gait, and easy fatigability. She had no new muscle weakness or wasting, however, and examination did not detect new neurologic signs. Her condition stabilized after she reduced her work demands and changed her leg braces.

Determination of Poliovirus Antibodies

All immunologic and virologic assays were performed on coded samples. Sabin poliovirus types 1, 2, and 3 were grown on monolayers of fetal rhesus monkey–kidney cells (FRhK-4T) and then prepared as described previously.[30] Viral antigen for the immunoassay was prepared from a mixture containing equal numbers of the three types of poliovirus. Intrathecal production of poliovirus-specific IgM antibodies was determined by a sensitive capture enzyme-linked immunosorbent assay.[31] IgM antibody levels to measles, mumps, herpes simplex type 1, and varicella–zoster viruses were also measured by the same immunoassay to serve as controls.

The clonal distribution of intrathecally produced antipoliovirus IgM antibody was identified by affinity immunoblotting[32] with minor modifications. Unconcentrated cerebrospinal fluid and adequately diluted homologous serum containing 20 ng of IgM were electrophoresed in agarose gel and the separated proteins were then passively transferred to a polyvinyl difluoride membrane coated with 500 μg of poliovirus antigen per milliliter (1 ml per 10 cm^2 of membrane area). The proteins were subsequently cross-linked to the polyvinyl difluoride membrane by glutaraldehyde[33] before the oligoclonal IgM bands against poliovirus were specifically stained.[33]

Assays

Levels of interleukin-2 in cerebrospinal fluid and diluted serum were measured by a capture immunoassay,[34] and levels of soluble interleukin-2 receptors were measured by an indirect sandwich immunoassay.[35] Oligoclonal IgM bands in the test samples were detected as described previously.[33] Isoelectric focusing was used to detect oligoclonal IgG bands, whereas IgA bands were detected by agarose electrophoresis.[28] The total IgM concentration was measured by an enzyme immunoassay,[36] and then the IgM index[37] was calculated to determine the amount of intrathecally synthesized IgM as follows: IgM index = (cerebrospinal fluid IgM × serum albumin)/(serum IgM × cerebrospinal fluid albumin).

Statistical Analysis

Nonparametric Wilcoxon rank-sum, chi-square, and Spearman rank-correlation tests were used, as appropriate, for statistical analysis. Analyses were performed with SPSS/PC+ software. All P values were two-tailed.

Results

Clinical Observations

Muscle weakness in patients with the post-polio syndrome involved either muscles originally affected by polio (18 patients) or muscle groups that had been spared by the original disease (14 patients). Nine patients presented with weakness in previously normal muscle groups that were not segmentally contiguous to the already weak ones. New bulbar, respiratory, or sleep difficulties were noticed only in the four patients who already had residual bulbar or respiratory muscle weakness. The mean (±SD) age of the patients at the time of the acute attack of poliomyelitis was 8.7±5.5 years (range, 5 months to 21.5 years), whereas the mean age at the onset of

the post-polio syndrome was 40.2±9.1 years (range, 31.5 to 59).

In most patients the onset of new symptoms was insidious. Symptoms in two patients started after minor accidents involving a limb. Four patients had a definite history of exposure to children with acute poliomyelitis or those who had recently received trivalent poliovirus vaccine a few months (mean, 6.8) before the development of the new symptoms.

Oligoclonal Poliovirus Antibodies

Oligoclonal IgM was the predominant immunoglobulin in the cerebrospinal fluid of patients with the post-polio syndrome (Table 1). Either the IgM bands in positive samples of cerebrospinal fluid had no detectable counterpart in homologous serum (17 patients) or the number of bands in cerebrospinal fluid was substantially higher than the number in serum (4 patients), indicating that the synthesis of IgM of restricted heterogeneity in the syndrome was predominantly intrathecal. In each case that was positive, qualitative characterization by immunoblotting identified the oligoclonal bands as IgM antibody against poliovirus (Fig. 1). Similarly, all oligoclonal IgA bands were found to be virus-specific, and IgG bands were specific for poliovirus in 7 of 12 patients.

Intrathecal synthesis of virus-specific oligoclonal IgM bands was seen in all four patients who had been exposed to poliovirus relatively recently before the development of new symptoms and in one patient in whom new weakness developed after a minor accident.

IgM Antibody Levels in Cerebrospinal Fluid

Total and virus-specific IgM levels were determined to obtain quantitative information on intrathecal synthesis of IgM. The mean cutoff value (+3 SD) of the IgM index in the normal reference population was 0.07. Abnormally high values were detected in 17 patients (47 percent) with the post-polio syndrome (all had oligoclonal IgM bands in cerebrospinal fluid) and in 6 control patients (3 with multiple sclerosis, 1 with myasthenia gravis, and 2 with stroke) ($P<0.005$).

The extent of intrathecal synthesis of poliovirus IgM antibody was determined by measuring antibody optical-density values per unit of weight of IgM in serum and cerebrospinal fluid to correct for permeability of the blood–cerebrospinal fluid barrier and expressing the results as the ratio of cerebrospinal fluid values to serum values.[31] Intrathecal synthesis of poliovirus IgM antibody was found in 21 patients (58 percent) with the post-polio syndrome and in none of the 67 control patients (Fig. 2). High cerebrospinal fluid:serum ratios of poliovirus IgM antibody were detected in four patients with the post-polio syndrome who had normal IgM indexes, providing further evidence that intrathecal production of IgM could occur without concomitant increase in the index value.[36] These four patients, however, had virus-specific oligoclonal IgM bands in cerebrospinal fluid.

Table 1. Distribution of Oligoclonal Immunoglobulin Bands in Samples of Cerebrospinal Fluid from the Study Population.

Oligoclonal Band	Patients with Post-Polio Syndrome (N = 36)	Control Patients		P Value*
		Childhood polio (N = 13)	Neuromuscular disease (N = 54)	
IgM	21	0	0	<0.0001
IgA	10	0	5	0.026
IgG	12	0	9	NS

*The chi-square test was used to compare values for patients with the post-polio syndrome with those for control patients. NS denotes not significant.

Cerebrospinal fluid:serum ratios of IgM antibody to the control viruses were within the normal ranges. In contrast, clearly elevated cerebrospinal fluid:serum ratios of antimeasles IgM antibody were found in six controls (three patients with multiple sclerosis, two patients who had a stroke during a meningoencephalitic illness, and one patient with muscular dystrophy who, on further epidemiologic study, was found to have had measles during the time of cerebrospinal fluid collection) (Fig. 2). As mentioned above, race-matched controls were selected for this study. They included 16 patients from developing countries, which may explain the relatively wide range

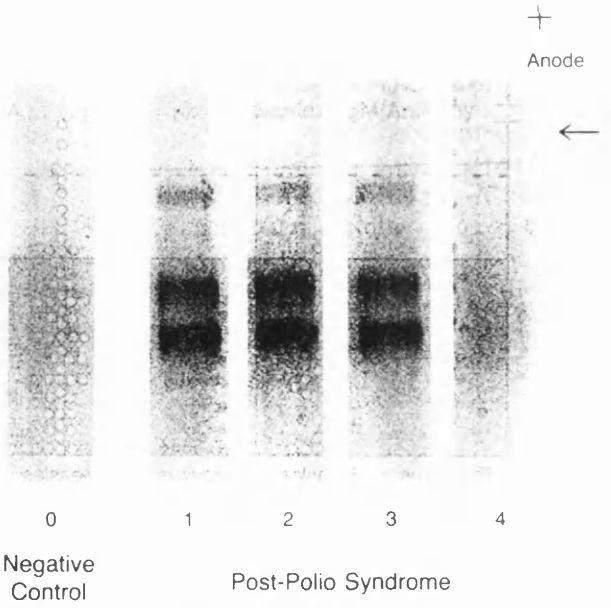

Figure 1. Electrophoretic Patterns in Cerebrospinal Fluid from a Patient with the Post-Polio Syndrome (Lanes 1 through 4) and a Control Patient (Lane 0).

Lane 1 shows the total number of oligoclonal IgM bands. Lane 2 shows the number of bands that were found to be specific for poliovirus by affinity immunoblotting. The bands were not affected by passing the sample through Sepharose 6B (Pharmacia) immunoadsorbent gel containing uninfected FRhK-4T cells, on which the poliovirus was grown (lane 3), but disappeared when the sample was preadsorbed with the poliovirus antigens (lane 4). Arrow marks application site.

of cerebrospinal fluid:serum ratios of measles antibody in the control group.

None of the patients with the post-polio syndrome had evidence of intrathecal synthesis of antibodies to the other control viruses. The median cerebrospinal fluid:serum IgM antibody ratios (and ranges) were 0.08 (0 to 0.25) for mumps virus, 0.15 (0 to 0.31) for herpes simplex virus, and 0.14 (0 to 0.27) for varicella zoster virus.

Intrathecal Synthesis of Interleukin-2 and Soluble Interleukin-2 Receptors

Detectable levels of interleukin-2 were found in the cerebrospinal fluid of 20 patients with the post-polio syndrome and 5 control patients (3 with stroke, 1 with myasthenia gravis, and 1 with multiple sclerosis). High cerebrospinal fluid levels of interleukin-2 in 18 patients with the post-polio syndrome were not associated with a concomitant increase in serum levels, suggesting local central nervous system synthesis of interleukin-2.[38,39] Similarly, local central nervous system synthesis of soluble interleukin-2 receptors was detected in 17 patients with the syndrome. The increase in intrathecal synthesis of both interleukin-2 and soluble interleukin-2 receptors in the post-polio syndrome was statistically significant (Fig. 3).

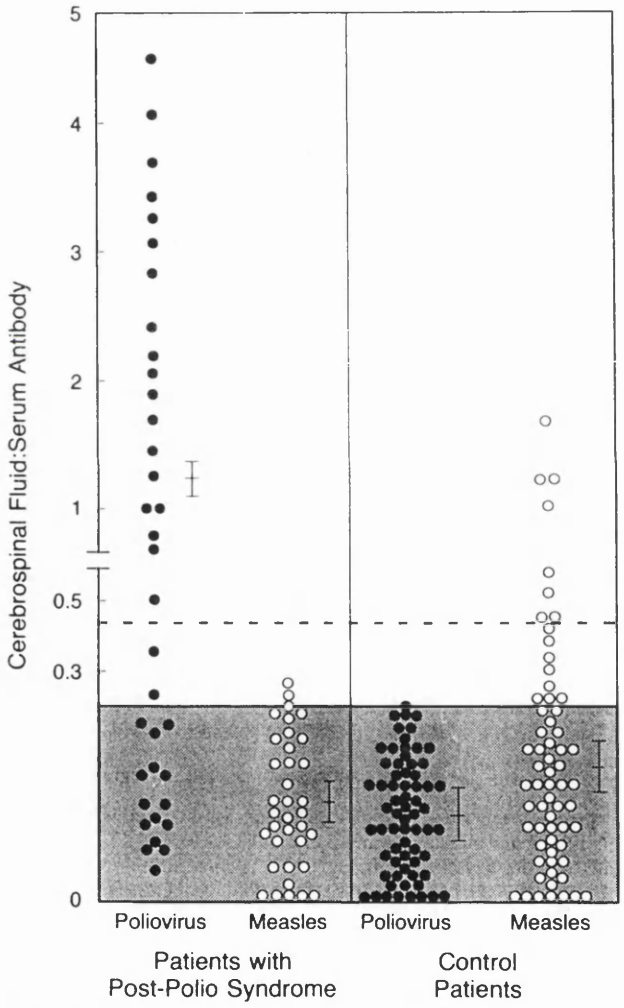

Figure 2. Cerebrospinal Fluid:Serum Ratios of Antipoliovirus IgM Antibody (●) as Compared with Cerebrospinal Fluid:Serum Ratios of Antimeasles IgM Antibody (○) (as a Control) in Patients with the Post-Polio Syndrome and in the Control Group.

The stippled area represents the normal range of the cerebrospinal fluid:serum ratio of poliovirus antibody, and the dashed line represents the upper range of normal for the cerebrospinal fluid:serum ratio of measles antibody. Bars are means ± SE. The ratios of poliovirus antibody were significantly higher in patients with the post-polio syndrome than in the control patients ($P<0.0001$). Antibody ratios against poliovirus were significantly different from those against measles virus in patients with the post-polio syndrome ($P<0.0005$).

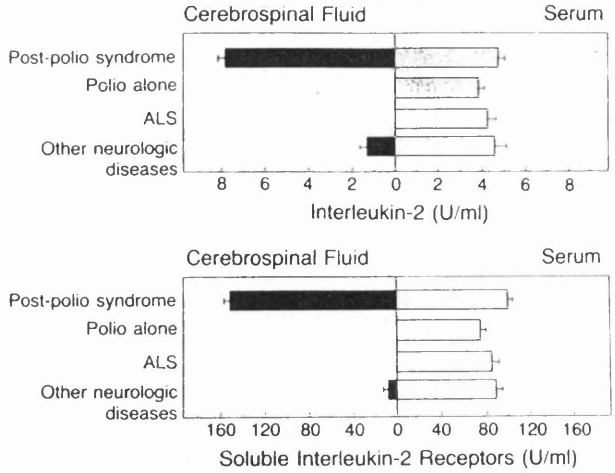

Figure 3. Mean Levels of Interleukin-2 and Soluble Interleukin-2 Receptors in Cerebrospinal Fluid and Serum in Patients with the Post-Polio Syndrome and Control Patients Who Had Had Poliomyelitis without the Post-Polio Syndrome or Who Had Amyotrophic Lateral Sclerosis (ALS) or Other Neurologic Diseases.

Cerebrospinal fluid levels of interleukin-2 and soluble interleukin-2 receptors were significantly higher in the patients with the post-polio syndrome than in the other groups ($P<0.001$ and $P<0.0001$, respectively). T bars are standard errors.

To determine whether intrathecal synthesis of interleukin-2 and soluble interleukin-2 receptors in the post-polio syndrome was related to the immune response to poliovirus, cerebrospinal fluid levels of both factors were correlated with the results of IgM immunoblot analysis. The association between cerebrospinal fluid levels of interleukin-2 and soluble interleukin-2 receptors and the presence of poliovirus-specific oligoclonal IgM bands was significant (Fig. 4). Furthermore, cerebrospinal fluid levels of interleukin-2 closely correlated with the amount of locally synthesized poliovirus IgM antibody (Fig. 5). The correlation between cerebrospinal fluid levels of soluble interleukin-2 receptors and locally synthesized poliovirus IgM antibody was also significant (Fig. 5).

Discussion

The mechanism of new progressive muscle weakness in patients with the post-polio syndrome is important not only for prognosis, but also for preventive and therapeutic purposes.[40] Our finding that poliovirus-specific oligoclonal IgM bands were found only in some patients with the post-polio syndrome suggests

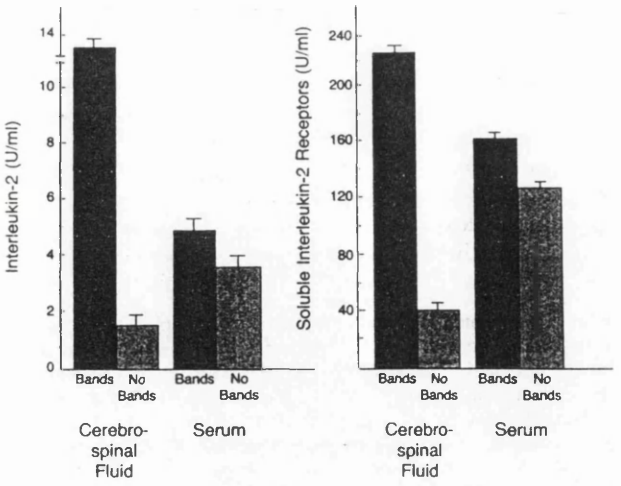

Figure 4. Mean Levels of Interleukin-2 and Soluble Interleukin-2 Receptors in Cerebrospinal Fluid and Serum from Patients with the Post-Polio Syndrome, According to the Presence of Oligoclonal IgM Bands in Cerebrospinal Fluid.

Cerebrospinal fluid levels of interleukin-2 were significantly higher in patients with oligoclonal IgM bands than in those without IgM bands ($P<0.0005$). Similarly, cerebrospinal fluid levels of soluble interleukin-2 receptors were significantly higher in the patients with oligoclonal IgM bands ($P<0.001$). T bars are standard errors.

that there is an immune response to antigenic stimulation in the central nervous system, probably by poliovirus. The presence of a poliovirus-specific immune response may be regarded as indirect evidence of a viral infection, since poliovirus cannot be isolated from the cerebrospinal fluid[41] even during the acute stage of paralytic poliomyelitis.[42]

In central nervous system infections, immunoglobulin-secreting plasma cells differentiate from a limited number of B cells, so the immunoglobulins produced are of restricted heterogeneity — that is, they are oligoclonal.[43,44] Oligoclonal bands specific to the causal agent are frequently found in patients with active viral infections of the central nervous system, such as subacute sclerosing panencephalitis[45] (caused by measles virus), herpes simplex encephalitis,[43] human immunodeficiency virus infection,[46] and enterovirus-induced meningitis and encephalitis.[47] We failed to detect poliovirus-specific oligoclonal bands in the control patients who had had polio but not the post-polio syndrome, suggesting that the intrathecal immune response to poliovirus in the post-polio syndrome was due to relatively recent or persistent antigenic stimulation. Salazar-Grueso et al.,[48] using a silver staining method, failed to detect oligoclonal bands in the cerebrospinal fluid of patients with the post-polio syndrome. Their negative results may be due to the relatively small sample (only nine patients) or to methodologic differences (the disadvantages of silver staining have been discussed elsewhere[49]). The presence of oligoclonal bands in the cerebrospinal fluid, however, is in agreement with other observations.[1,13]

The possibility that the intrathecal immune response in the post-polio syndrome is due to active stimulation is further confirmed by our finding of increased intrathecal synthesis of interleukin-2 and soluble interleukin-2 receptors in patients with the syndrome. Local central nervous system production of interleukin-2, the soluble interleukin-2 receptors, or both have been demonstrated during the course of several viral infections of the central nervous system.[50-53] A successful immune response to viral infections involves a complex cascade of events that includes the production of cytokines. The system comprising interleukin-2 and interleukin-2 receptors in particular plays a major part in the immune responses against invading antigens.[54-56] Interleukin-2, a pluripotent cytokine secreted by antigen-activated T cells,[57] has an essential role in promoting replication

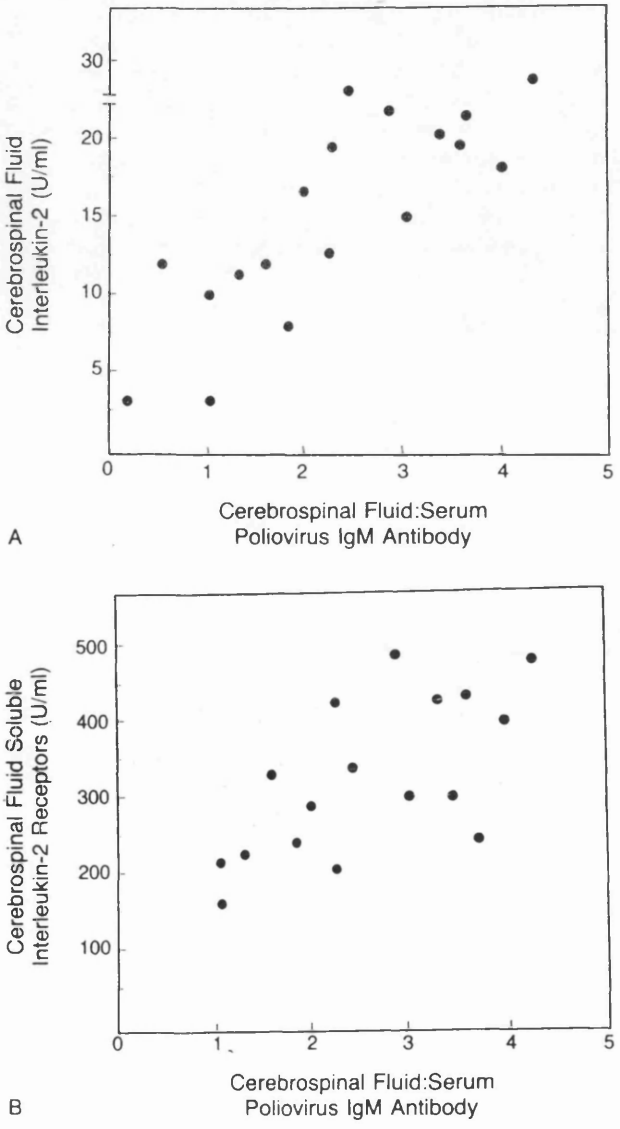

Figure 5. Cerebrospinal Fluid:Serum Ratios of Poliovirus IgM Antibody Plotted against Cerebrospinal Fluid Levels of Interleukin-2 (Panel A) and Soluble Interleukin-2 Receptors (Panel B).

The cerebrospinal fluid:serum ratio of poliovirus antibody correlated with cerebrospinal fluid levels of both interleukin-2 ($r = 0.764$, $P<0.0005$) and soluble interleukin-2 receptors ($r= 0.625$, $P<0.001$).

of T cells and their differentiation into effector cells, induction of B-cell growth, and augmentation of immunoglobulin production. It exerts a pleotropic effect through its specific high-affinity receptor,[58] which is present on activated T and B cells, natural killer cells, monocytes, and thymocytes as well as oligodendrocytes[59] and endothelial cells.[60] This interleukin-2 receptor is formed by the association of a 55-kd α chain (Tac antigen) and a 70-to-75-kd β chain. Cell proliferation after the binding of interleukin-2 leads to the release of a smaller (45 kd) form of α chain, the soluble interleukin-2 receptor.

Our findings therefore support the possible role of a new or persistent poliovirus central nervous system infection in the pathogenesis of the post-polio syndrome. The possibility that the syndrome is due to persistent infection by an enterovirus is not unlikely. Poliomyelitis virus, an RNA virus, is usually cytolytic but can produce slowly progressive or persistent infections in both animals and immunosuppressed patients.[61-63] Lipton[64] reported a biphasic neurologic disease in mice infected with Theiler's encephalomyelitis virus, a murine enterovirus; the mice developed an acute encephalitic illness immediately after inoculation, followed by a progressive demyelinating syndrome several months later. Moreover, the murine-adapted Lansing strain of type 2 poliovirus causes acute and persistent infections of the central nervous system in mice.[65] In fact, even the human poliovirus has been shown to produce prolonged asymptomatic infection of the central nervous system.[63]

It is unclear from our data whether patients with the post-polio syndrome carry any risk of infectivity. Furthermore, the pattern of possible reinfection needs to be determined, although recent exposure to poliovirus could be a plausible mechanism. Four of our patients experienced new muscular weakness after relatively recent exposure to the virus. Similarly, Dalakas and collaborators[1] reported a patient with the syndrome in whom new muscular weakness developed accompanied by an elevation in the cerebrospinal fluid levels of poliovirus antibodies after exposure to children with acute poliomyelitis. In fact, neurologic abnormalities and elevated poliovirus antibody titers may also develop in normal subjects on exposure to the virus.[66]

Our data provide evidence of intrathecal immune activation against poliovirus during the course of the post-polio syndrome. It is tempting to speculate that reactivation of latent or persistent poliovirus infection in the central nervous system may have a role in the pathogenesis of new muscle weakness in some patients with the syndrome. The unique lack of vascular permeability and absence of a lymphatic system or immunocompetent cells in the central nervous system as well as the static nature of neurons may encourage and promote viral persistence.[24] Residual poliovirus could therefore cause persistent infection after the acute episode, possibly by escaping surveillance of the immune system through antibody-induced antigen modulation, generation of suppressor T cells, or production of blocking factors.[67,68] Persistent poliovirus infection might then have a gradual but progressive cytopathic effect, which would eventually lead to either neuronal-cell lysis or alteration of specialized cellular functions[69] and thus potentially impair physiologic function. Longitudinal follow-up of patients with the post-polio syndrome and therapeutic trials with antiviral agents[40,70] will allow our hypothesis to be tested further.

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scroll-like profile on electron microscopy.[7] In contrast, the mast cells that predominate in the submucosa of the intestine and airways, the skin, breast parenchyma, and lymph nodes (designated MC_{TC}) express the epitopes for a tryptase, a chymotryptic serine protease (termed chymase), and carboxypeptidase A and lack the scroll-like appearance. Of the two types, only the MC_{TC} is present in the skin and bone marrow of patients with indolent systemic mastocytosis, in a pattern consistent with hyperplasia or a controlled clonal expansion.[1]

Because mast cells can elaborate and respond to cytokines,[8,9] it is quite possible that cytokines regulate the activity of the mast cell in systemic mastocytosis. The in vitro studies with the atypical bone marrow mast cells of the patient described in this issue revealed that interferon alfa-2b was not cytotoxic but did reduce spontaneous and induced degranulation. One could thus speculate that the cytokine production that controlled cell activation and degranulation was down-regulated by the intervention. Since the life expectancy of two or three years for patients with aggressive systemic mastocytosis without treatment has improved to only three to five years with current treatment,[2] these encouraging results after one year for a patient with this uncommon form of the disease require follow-up studies, most likely with historical controls. Indeed, the authors recognize that the patient was not in remission at any time, inasmuch as symptoms and signs of systemic mastocytosis persisted and required continued medication. Furthermore, there is no a priori reason to conclude that the cytokine providing counterregulation with some efficacy in aggressive systemic mastocytosis with atypical mast cells will be the same as the cytokine that controls the hyperplasia of mature mast cells in indolent systemic mastocytosis. Clearly, much more needs to be done to elucidate and manage this complex set of disorders.

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CORRESPONDENCE

IMMUNE RESPONSES IN THE POST-POLIO SYNDROME

To the Editor: We suggest that the conclusions reached by Sharief et al. (Sept. 12 issue)[1] are insufficiently supported by their data. They have convincingly shown that an oligoclonal IgM antibody population that reacts with a mixture of types 1, 2, and 3 poliovirus is present in the spinal fluid of patients with the post-polio syndrome. They have also shown that this antibody population does not react with measles, mumps, herpes simplex, or varicella–zoster virus. Furthermore, control patients, some of whom had poliomyelitis but no evidence of the post-polio syndrome and others of whom had other neurologic diseases, did not manifest the antibody response.

It was shown many years ago that there are common antigenic determinants among many human picornaviruses, including the polioviruses, coxsackieviruses, echoviruses, and even the rhinoviruses.[2-5] The antibodies for these determinants are not neutralizing, and they presumably recognize antigenic determinants shared by these viruses. In the article by Sharief et al.,[1] there is no evidence to exclude the possibility that the IgM antibodies demonstrated were directed against any of a number of antigenically related viruses that could be causally related to the post-polio syndrome.

We believe that the idea that persistent poliovirus infection or even reexposure to poliovirus is related to the post-polio syndrome is premature until appropriately designed studies demonstrate that the IgM response is at least poliovirus-specific, if not also poliovirus-neutralizing. In other words, it must be demonstrated that the oligoclonal bands seen do not recognize other viruses known to contain common picornavirus determinants. In short, additional controls consisting of human enteroviruses other than polioviruses would have been more relevant than those used in the study.

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To the Editor: The study by Sharief et al. reporting oligoclonal IgM bands in patients with the post-polio syndrome is intriguing and, in the light of our previous report,[1] prompts us to raise several questions and to comment on their conclusions.

In our investigation,[1] we were unable to detect silver-stained oligoclonal IgG bands in 21 patients with a history of poliomyelitis, including 13 with the post-polio syndrome, but we did find the bands in a patient with multiple sclerosis who had had polio. The specificity of these bands for IgG was confirmed by immunofixation. We did not test for the presence of IgM antibody. We also found viral-specific IgG antibody with an overlay method that used radiolabeled poliovirus in samples of cerebrospinal fluid and serum from three patients who had had polio, two of whom had had post-polio muscular atrophy. We do not believe that our failure to detect oligoclonal IgG bands in patients with a history of polio was due to limitations in the isoelectric-focusing–silver-staining method[2] or to the small number of patients. In a companion study using a poliovirus enzyme-linked immunosorbent assay,[3] we found polyclonal antipoliovirus antibody in samples of cerebrospinal fluid and serum from all 19 patients with a history of polio, but only 1 of these patients had evidence of intrathecal production of poliovirus antibody.

Table 1 of the article by Sharief et al. shows that 12 of the 36 patients with the post-polio syndrome had IgG oligoclonal bands, and these bands were found to be virus-specific in 7 of 12 patients. What was the antipoliovirus IgG response in samples of serum and cerebrospinal fluid? What were the cerebrospinal fluid:serum ratios of antipoliovirus IgG in these seven patients as well as in the remaining patients with the post-polio syndrome and in the control patients? If the data supporting the presence of a poliovirus-specific IgM response (found in 21 of 36 patients) are compared with those supporting an IgG response (found in 7 of 12 patients), it appears that several patients with a history of polio had a poliovirus-specific IgM response without an accompanying IgG response. We found this result unusual, and we would like to know whether Sharief and coworkers know of any precedent for the presence of an IgM antibody with no accompanying elevated IgG response in chronic viral infections.

We were surprised that four patients had a "definite" history of exposure to children with acute poliomyelitis vaccine or to those who had recently received trivalent poliovirus vaccine. It is also noted that 16 race-matched control patients were from developing countries. Is there something that we should know about the epidemiology of the patients with a history of polio? Were they also from developing countries? Were there additional patients with possible (rather than "definite") recent exposure to the poliovirus, as might be the case in a developing country, perhaps leading to an increased IgM antipoliovirus-antibody response?

It is clear that some picornaviruses can cause persistent infections. This is true, for example, of Theiler's virus. However, it is now accepted among picornavirologists that this virus is most closely related to the cardiovirus genus and should not be classified as a member of the enterovirus genus, as is poliovirus.[4,5] It is also true that poliovirus can persist in immunocompromised humans[6] and in immunosuppressed animals.[7] However, there is currently no evidence of persistent poliovirus infection in immunocompetent persons.

Finally, although the Sabin poliovirus vaccine can certainly cause poliomyelitis, this is a rare event. Patients with the post-polio syndrome should be at no greater risk for a neurologic complication from the vaccine than normal age-matched persons. The results of Sharief et al. need confirmation (and further study) before a change in the recommendations regarding poliovirus vaccination can be considered.

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To the Editor: The report by Sharief et al. deserves an alternative interpretation or hypothesis for the cause of the authors' findings. The occurrence of a new or primary immune response to the poliovirus is obvious from the findings of IgM antibodies to the virus and on the basis of the interleukin-2 and receptor levels. However, the question must be raised whether the immune response is to live virus (the authors' assumption) or to viral material, such as genome segments, that may have been sequestered in host lymphocytes or other memory cells decades before. Clearly, the new response is that of B lymphocytes. But is it a response to live virus or to retained, noninfectious viral segments recently released from some reproducing storage genome?

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Dr. Sharief replies:

To the Editor: Drs. Rosenberg and Hamparian express a legitimate concern about the specificity of our findings. The aim of our report was to describe a recent poliovirus immune response rather than to discuss immunologic techniques. We have demonstrated that the IgM antibodies do not cross-react with common antigenic determinants of antigenically related viruses.[1] Moreover, the entire population of oligoclonal IgM antibodies in our study failed to react with two human enteroviruses, echovirus 14 and coxsackievirus B5.

Despite the comments of Dr. Salazar-Grueso and colleagues, limitations of the silver-staining method could still be used to explain the discrepancy between their findings and ours. In support of this view, Kaiser[2] has recently reported that an isoelectric-focusing–immunoblotting method detected oligoclonal IgG bands in 5 of 13 patients with neurologic disease for whom results were negative when the silver-staining method was used.

We detected evidence of the intrathecal production of poliovirus IgG antibody only in the seven patients with the post-polio syndrome who had virus-specific IgG bands. An additional 22 patients with the post-polio syndrome and 34 control patients had elevations of polyclonal poliovirus IgG antibody in serum or cerebrospinal fluid but no evidence of intrathecal release.

The predominance of the IgM response in our patients may be due to the cross-sectional nature of the study, which did not analyze the temporal relation of IgM to IgG. During experimental poliovirus infection,[3] IgM antibodies appear first, and their levels peak earlier than those of IgG antibodies. Brain lymphocytes bear IgM almost exclusively in mice with recent intracerebral viral infections.[4] Moreover, IgM antibodies without an accompanying IgG response have been detected in chronic viral infections of the nervous system.[5]

Sixteen of our patients with the post-polio syndrome were from developing countries. Thus, 16 race-matched controls were included to correct for any racial or epidemiologic factors that might modify the immune response. Some of these control patients had potentially been recently exposed to poliovirus, with no subsequent increase in the IgM response.

Salazar-Grueso and colleagues inaccurately state that persistent poliovirus infection occurs only in immunocompromised humans or immunosuppressed animals. Colbère-Garapin et al.[6] have provided clear evidence that the three serotypes of poliovirus can induce persistent infection of normal human neurons. Indeed, these researchers have elegantly characterized and discussed patterns of poliovirus persistence in normal human neurons that may help to explain the post-polio syndrome.[7,8] I agree with Salazar-Grueso and colleagues that further studies are needed, and this was emphasized in our concluding remarks.

The alternative explanation put forward by Dr. Dawson is plausible. However, it does not explain how a retained noninfectious viral segment could lead to weakness and wasting in muscle groups that had been spared by the original disease. The seroepidemiologic and clinical results in our patients suggest an immune response against a live virus. Yet, molecular biologic techniques that can detect viral genome in body fluids or tissue preparations may be required to provide a definite answer.

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POSTNATAL TRANSMISSION OF HIV INFECTION

To the Editor: In the article of Van de Perre et al. (Aug. 29 issue)[1] there are some important definitions concerning the transmission of the human immunodeficiency virus (HIV) that are not proved. The authors assume that a negative result on polymerase-chain-reaction (PCR) analysis is a demonstration that there is no infection at that time. We think that a negative result can never be a proof, even with such techniques as the nested PCR. Seroconversion three months after delivery does not prove that infection occurred postnatally. Although seronegative,[2] the mothers were most probably infected before delivery, but no virologic or immunologic data from pregnancy are shown that indicate the time of infection.

In an infection by a retrovirus such as HIV type 1 that has a long incubation period[3] even after the transfusion of contaminated blood,[4] it is very difficult to accept such quick seroconversion as due to breast-feeding: five of the nine infants seroconverted three months after delivery. It is true that milk cells can be infected, but there is no demonstration that the virus can be recovered from the stomach and infect blood cells, except in rare cases[5] after HIV-positive transfusion of the mother at delivery. If milk cells were so infectious, many more children who were born to seropositive mothers and breast-fed would be infected. The experience of other groups shows no statistical differences in the rates of transmission of HIV infection from positive mothers to breast-fed infants and bottle-fed infants.[6] (The title of the article should be "postnatal seroconversion" instead of "postnatal transmission.")

The editorial by Pizzo and Butler[7] in the same issue is very cautious about the number of children presumably infected postnatally by breast-feeding. The editorial is correct to emphasize that breast-feeding from an infected mother can increase viral burden and so should be discouraged if sanitary conditions are acceptable, as in most of Europe. But this report does not demonstrate that breast-feeding by itself is a transmission route of HIV infection.

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To the Editor: We question the conclusions of the investigation of Western blot seropositivity reported as occurring in 9 of 16 breast-

The Predictive Value of Intrathecal Immunoglobulin Synthesis and Magnetic Resonance Imaging in Acute Isolated Syndromes for Subsequent Development of Multiple Sclerosis

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The intrathecal synthesis of IgM in patients presenting with acute monosymptomatic syndromes may be relevant in predicting eventual progression to multiple sclerosis but has not been previously evaluated. We undertook a prospective 18-month combined clinical, cerebrospinal fluid, and magnetic resonance imaging study of 45 patients who had presented with acute isolated lesions of brainstem and spinal cord to evaluate the predicive value of intrathecal synthesis of IgM and IgG (through the detection of cerebrospinal fluid oligoclonal bands) and magnetic resonance imaging brain lesions at presentation, for the subsequent progression to multiple sclerosis. Results indicate that the highest risk of developing multiple sclerosis is seen in patients who are positive for oligoclonal IgM and IgG bands and who have disseminated cerebral white matter magnetic resonance imaging lesions at the initial presentation. Oligoclonal IgM bands predict progression to multiple sclerosis more frequently than oligoclonal IgG or magnetic resonance imaging alone. Our results confirm and further extend previous reports, and indicate that detection of cerebrospinal fluid oligoclonal IgM bands at presentation is a valuable prognostic indicator in patients presenting with acute isolated brainstem or spinal cord syndromes.

Sharief MK, Thompson EJ. The predictive value of intrathecal immunoglobulin synthesis and magnetic resonance imaging in acute isolated syndromes for subsequent development of multiple sclerosis. Ann Neurol 1991;29:147–151

Isolated lesions of brainstem or spinal cord are frequently seen in patients with multiple sclerosis (MS) and may be the presenting feature in about one-third of them [1, 2]. From a clinical point of view, particularly for patient management purposes, it would be helpful if the future development of MS could be predicted during the early stages of disease process.

MS has definite aberrations of immune functions, most consistent of which is the exaggerated intrathecal immunoglobulins synthesis [3–7]. The study of cerebrospinal fluid (CSF) immunoglobulin changes may therefore be particularly important in determining the early risk of MS. A recent combined clinical, CSF oligoclonal IgG bands, and magnetic resonance imaging (MRI) study [8] reported that progression to MS occurs in about one-half of patients who presented with an isolated brainstem or spinal cord lesion. The predictive value of oligoclonal IgM for subsequent development of MS, however, is still unknown.

We have established that CSF oligoclonal IgM has considerable clinical relevance in monitoring disease activity in MS [9], and that it is an indicator of recent immunological stimulation [10]. We therefore undertook an 18-month prospective combined CSF and MRI study to determine whether the presence of oligoclonal IgM bands in the CSF at presentation is a prognostic indicator for progression to MS.

Patients and Methods

Patients

Paired CSF and serum samples were obtained from 60 patients who presented with clinically monofocal acute lesion of either the brainstem or spinal cord. The study was restricted to patients younger than the age of 50 years who

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exhibited unequivocal objective evidence of unifocal white matter disease and who had adequate follow-up for at least 18 months. Patients with evidence of vascular disease were excluded. Nine patients did not fulfill such criteria and were excluded from the study. Four patients were dropped from the study because a non-MS cause of the presenting symptoms was determined on follow-up. Another 2 patients became claustrophobic and were unable to continue the MRI follow-up. No patients had received immunosuppressive or glucocorticoid treatment within the study period.

The study population had a mean age of 37.3 years (range, 19–47 years; ± SD, 8.2). Twenty patients had isolated brainstem lesions and complained of diplopia, ataxia, or vertigo. Clinical signs included nystagmus, various gaze palsies, and trigeminal and facial nerve involvement with concomitant long tract signs. The 25 patients who presented with clinically isolated acute spinal cord lesions developed maximal neurological deficit within 2 weeks of the onset of symptoms. All underwent myelography to exclude compressive lesion, and no patient was suffering from tranverse myelitis.

On initial presentation, full history was documented and findings of neurological examination were recorded in the patient's clinical notes. Clinical and MRI follow-up data were obtained during subsquent assessments at regular intervals. MS was diagnosed at follow-up according to the criteria of Poser and colleagues [11]. Clinically definite MS requires evidence of further lesions involving different parts of the central nervous system (CNS) after an interval of at least 1 month. Paraclinical evidence of new CNS lesions allows the diagnosis of probable MS. Cerebrospinal fluid data were evaluated blind, and patients were divided into two main groups according to the state of CSF oligoclonal IgM banding pattern. Clinical, pathological, and imaging features in oligoclonal IgM-positive and oligoclonal-negative patients were then compared to test the hypothesis that intrathecal IgM synthesis predicts progression to MS.

Cerebrospinal Fluid Studies

Cerebrospinal fluid was obtained during the acute episode within 7 days of the MRI study. We have already established that oligoclonal IgM is a more useful clinical tool than quantitative changes in relating to intrathecal immune disturbance [12]. Oligoclonal IgM bands were detected by the method of glutaraldehyde-enhanced immunofixation previously described by us [13]. In the present study population, the presence of two or more separate IgM bands in the CSF that have no counterpart in homologous serum was regarded as evidence of intrathecal IgM production [12, 13]. Intrathecal IgG synthesis was assessed by isoelectric focusing of CSF and homologous serum on agarose gel and subsequent immunoblotting as previously reported [14]. CSF white cell count was performed using Fuchs-Rosenthal chambers, and cytological examination was performed using Giemsa stain from a Shandon cytocentrifuge preparation [15].

Neuroimaging

All patients underwent MRI studies of brain and spinal cord at presentation as part of the routine diagnostic investigations, and they also had follow-up scans 4 to 19 months after the initial presentation. MRI was performed on a Picker 0.5-T superconducting imager (Picker Intl, Cleveland, OH). A spin-echo encephaolographic sequence ($SE_{2,000/60}$) with 5 to 10-mm thick contiguous slices was obtained in all patients. The scans were reviewed by neuroradiologists who reported the number, anatomical location, and extent of MRI lesions while blind to the CSF data. The presence of three or more asymmetrical cerebral white matter (i.e., multifocal) lesions, at least one of which is periventricular in location, was considered strongly suggestive of MS. Lesions explaining the presenting syndrome, if detected by MRI, were excluded from the analysis of MRI findings.

Statistics

The significance of differences in negative and positive groups of patients were compared by χ^2 analysis. The predictive values of oligoclonal bands and MRI for progression to MS were calculated by the multivariate technique of discriminant analysis [16], using SPSS/PC$^+$ software (SPSS Inc., Chicago, IL). The probability that a patient belonged to the MS group at follow-up (predictive value) was estimated according to Bayes's theorem [17] by using the following formula:

$$\text{Relative predictive value} = \frac{p}{p + (1 - p)/C} \times 100$$

where p, prior probability, is estimated from observed proportion of patients who subsequently develop MS, and C, conditional probability, is calculated from the assumption that a patient belongs to the group of patients with MS, and the probability of the observed score given membership in the group is estimated.

Results

Twenty-six (58%) patients with isolated brainstem or spinal cord syndromes demonstrated oligoclonal IgM bands in CSF at presentation (Table 1). No significant relation was detected between patients' age or sex and the presence of oligoclonal IgM bands. Among the

Table 1. Clinical and Pathological Features at Presentation in Patients with Isolated Brainstem or Spinal Cord Syndromes

	CSF Oligoclonal IgM	
Characteristics	Positive	Negative
No. of patients (females)	26 (16)	19 (12)
Mean age ± SD (yr)	32.6 ± 7.5	34.7 ± 8.1
Isolated brainstem lesions (total = 20)	12	8
Isolated spinal cord lesions (total = 25)	14	11
CSF white cell count (mean ± SD)	3.1 ± 1.6	2.9 ± 1.2
Presence of oligoclonal IgG in cerebrospinal fluid	18	4
MRI-detected disseminated cerebral lesions	18	6

CSF = cerebrospinal fluid; MRI = magnetic resonance imaging.

Table 2. Number of Patients Who Progressed to Multiple Sclerosis at Follow-Up Compared With the Statistical Significance and Predictive Value of CSF Oligoclonal Bands and MRI Brain Lesions at Presentation

Parameter	Findings at Presentation	Outcome after Mean Follow-Up of 18 Months: Multiple Sclerosis	Outcome after Mean Follow-Up of 18 Months: No Progression to Multiple Sclerosis	F^a	p	Relative Predictive Value (%)
CSF-OC IgM bands	Positive	22	4	109.2	< 0.0001	89
	Negative	0	19			
CSF-OC IgG bands	Positive	18	4	24.3	< 0.001	78
	Negative	4	19			
MRI of brain	Multifocal lesions	17	7	11.3	< 0.01	70
	No lesions	5	16			

[a] Calculated from one-way analysis of variance with multiple sclerosis as the grouping variable.

CSF = cerebrospinal fluid; MRI = magnetic resonance imaging; OC = oligoclonal.

parameters listed in Table 1, only three (oligoclonal IgM, oligoclonal IgG, and MRI brain lesions) significantly correlated with the risk of developing MS. Results of an 18-month follow-up study are reported herein.

Oligoclonal IgM and Progression to MS

As judged from both clinical and MRI criteria, 22 of the 26 (85%) patients who had oligoclonal IgM bands at the original episode progressed to MS after a mean interval of 12.8 months (corrected $\chi^2 = 31.6$, $p < 0.0001$) (Table 2). It is noteworthy that no patient negative for oligoclonal IgM at presentation progressed to MS within a mean follow-up period of 18.3 months. Ten patients who progressed to MS presented initially with an isolated brainstem syndrome, whereas the remaining patients had an isolated spinal cord syndrome on presentation.

After a mean period of 13.6 months, 6 of the 10 patients who presented with an isolated brainstem syndrome developed clinical relapses outside the brainstem and were therefore classified as clinically definite MS; another 2 patients had a brainstem relapse; and MRI follow-up revealed new cerebral white matter lesions in the remaining 2 patients (see Table 2). Similarly, 7 patients who had presented with an isolated spinal cord syndrome had clinical relapses outside the spinal cord within a mean interval of 15.2 months (i.e., clinically definite MS). Three other patients developed new relapses in the spinal cord at a level different from the presenting lesions, and 2 patients had new cerebral white matter lesions after a mean MRI follow-up of 14.5 months.

Oligoclonal IgG Bands and the Risk of MS

Eleven of the 22 (50%) patients who demonstrated CSF oligoclonal IgG bands presented with an isolated brainstem syndrome, and the other 11 had isolated spinal cord syndrome. Table 2 shows the relation between the presence of oligoclonal IgG bands in CSF at presentation and progression to MS. Eighteen of the 22 (82%) patients progressed to MS within a mean interval of 15.1 months (corrected $\chi^2 = 13.5$, $p < 0.001$). Four patients (2 with isolated spinal cord syndrome) who had no detectable oligoclonal IgG bands in the original episode progressed to MS within a follow-up period of 13.7 months. Two of the 4 patients progressed to clinically definite MS within a mean of 16.2 months; 1 patient progressed to develop new multifocal MRI brain lesions; and another patient had a new relapse at a different spinal cord level.

MRI and the Risk of MS

MRI demonstrated disseminated cerebral white matter lesions in 24 patients at initial presentation; 7 of these patients were without oligoclonal IgG bands, and 6 did not demonstrate oligoclonal IgM bands. As judged from clinical and paraclinical criteria, 17 of the 24 (71%) patients progressed to MS within a mean interval of 14.3 months (corrected $\chi^2 = 13.1$, $p < 0.01$).

Progression to MS occurred in 17 (7 with isolated brainstem syndrome) of 18 patients with, and in none of the 6 patients without, oligoclonal IgM bands. Eleven patients who initially had disseminated brain lesions progressed to clinically definite MS; 4 patients subsequently developed clinical relapses at a level different from that of the original lesion; and 2 patients demonstrated new periventricular MRI lesions at follow-up.

Clinical multiphasic disease developed in 2 patients who had normal brain MRI scan at presentation, but were positive for CSF oligoclonal IgM and IgG bands, after follow-up intervals of 15 and 16.5 months, respectively. Another 3 patients who did not have multifocal MRI cerebral lesions at presentation developed new brain white matter lesions within a mean MRI

follow-up period of 15.4 months. All 5 patients who progressed to MS despite normal MRI brain scans at presentation had oligoclonal IgM bands detected in CSF during the original episode. No evidence of MS was detected in 7 patients who had MRI brain lesions at presentation (see Table 2) after a mean follow-up interval of 18.7 months.

Predictive Value of CSF and MRI Studies

The highest rate of progression to MS (95%) was detected in patients who demonstrate both oligoclonal IgM and IgG bands as well as multifocal MRI brain lesions at presentation. Findings depicted in Table 2 suggest that the predictive value of oligoclonal IgM bands at initial presentation is higher than that of either oligoclonal IgG bands or MRI brain lesions. Two of the 5 patients in whom oligoclonal IgG bands and MRI were negative, but who had oligoclonal IgM bands at presentation, subsequently developed several periventricular MRI lesions after a mean interval of 16.5 months. Another 1 of those 5 patients developed a single new cerebral white matter lesion after 16 months, but the diagnosis of MS was not made because of the strict criteria in MRI interpretation (see Discussion).

Discussion

An important prognostic issue in patients with monofocal syndromes concerns progression to MS. We demonstrate in this study that the detection of oligoclonal IgM bands at the initial presentation is an important prognostic factor in determining subsequent development of MS. In our study population, CSF oligoclonal IgG and MRI findings at presentation had less predictive value than oligoclonal IgM. This is perhaps expected because IgM is recognized as an indicator of early immunological stimulation in MS [9, 10, 18]. Furthermore, a number of immunological abnormalities similar to those found in clinically definite MS are common in patients with isolated CNS lesions [8, 19–21]. Although MRI is undoubtedly the best test for lesion dissemination in space, the study of immunological alteration in early acute episodes may be more appropriate when evaluating recent relapse or disease activity. Early MS lesions could be below the detection threshold of the technique or the particular MRI machine used while still manifesting intense immunological activity.

Recent criteria [11] allow the use of paraclinical evidence to demonstrate lesion dissemination in space for research protocols. Caution should be exercised when evaluating multifocal cerebral white matter MRI lesions for diagnosis of MS because MRI techniques at their present status lack specificity, and lesions similar to those found in MS are seen in many disorders such as vasculitis; lacunar infarcts; Binswanger's, Alexander's, and Fabry's diseases; systemic lupus erythematosus; leukodystrophies; postinfection syndromes; and radiation and chemotherapy reactions (reviewed in [22]) as well as in healthy individuals [23]. In this study, our MRI interpretation was very conservative in that only lesions very strongly suggestive of MS (i.e., three or more lesions, at least one periventricular in location [20, 21]) were considered as evidence of dissemination in space.

Our findings of higher risk of progression to MS in patients with intrathecal IgG synthesis at presentation are in full agreement with several previous longitudinal studies of monosymptomatic presentation [8, 24–26]. Furthermore, Miller and colleagues [8], using one new MRI lesion as evidence of dissemination in space, failed to detect significant risk of MRI brain lesion at presentation for the progression to MS, in patients with acute isolated brainstem syndromes. They reported, however, that CSF oligoclonal bands significantly predicted subsequent development of MS in these patients. CSF oligoclonal bands are not only of better prognostic value than MRI, but their diagnostic importance in MS is well established. Paty [21], in his review of the literature, found that MRI was positive in 86% of patients with clinically definite MS and in 59% of patients with suspected MS (73% overall). CSF oligoclonal bands are usually positive in 95% of patients with clinically definite MS and in 86% of patients with probable MS, with 90% positivity overall [27].

There has not been a study on the predictive value of oligoclonal IgM bands in MS. Thus, we are unable to compare our results of research on oligoclonal IgM bands with those of other research groups. Clearly, further prospective studies are needed to determine the significance of oligoclonal IgM bands in other presenting syndromes of MS, for example, optic neuritis and chronic progressive myelopathy. Miller and collaborators [8] reported that none of 13 patients presenting with acute isolated syndromes who were negative for oligoclonal IgG and MRI brain scan had progressed to MS within 16 months. We extend their findings by demonstrating that oligoclonal IgM is a valuable predictive indicator of progression to MS in acute presenting syndromes even when oligoclonal IgG bands in CSF or MRI findings are negative. Although our results demonstrate the advantage of CSF oligoclonal IgM bands in predicting subsequent progression to MS, the follow-up period is relatively short, and MS is likely to develop in more patients as time progresses. Whether patients negative for oligoclonal IgM bands will ultimately develop MS is undetermined.

Hartard and colleagues [28] failed to detect significant correlation between CSF oligoclonal bands and progression of MS. Their findings are difficult to ex-

plain as the method of detection of oligoclonal bands has not been forward reported. CSF methodology is particularly important especially when prospective studies are undertaken. The notion that intrathecal production of immunoglobulins does not fluctuate with disease activity in any meaningful way has recently been disputed [29, 30]. MS is a dynamic process, and intrathecally synthesized immunoglobulins are affected by varying metabolic activities in different stages of the disease. Binding of CSF immunoglobulins to target structures can also lead to varying CSF levels in different clinical stages of the disease process. The electrophoretic or focusing process for the detection of oligoclonal bands should therefore be standardized according to the amount of immunoglobulin present in the test sample.

Intrathecal production of IgM is not only a parameter of disease activity in patients with MS [9, 10] but may also permit a prospective assessment of the subsequent evolution of the disease process. Further advantage of the predictive value of intrathecal IgM synthesis is best illustrated in patients who have negative CSF oligoclonal IgG bands or normal MRI findings at presentation.

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ASSOCIATION BETWEEN TUMOR NECROSIS FACTOR-α AND DISEASE PROGRESSION IN PATIENTS WITH MULTIPLE SCLEROSIS

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Abstract *Background.* Cachectin, or tumor necrosis factor-α (TNF-α), is a principal mediator of the inflammatory response and may be important in the pathogenesis and progression of multiple sclerosis, an inflammatory disease of the central nervous system.

Methods. In a 24-month prospective study, we used a sensitive enzyme-linked immunosorbent assay to determine levels of TNF-α in cerebrospinal fluid and serum in 32 patients with chronic progressive multiple sclerosis and in 20 with stable multiple sclerosis and 85 with other neurologic diseases. An attempt was made to relate TNF-α levels with the degree of disability of the patients with multiple sclerosis and with their neurologic deterioration during the 24 months of observation.

Results. High levels of TNF-α were found in the cerebrospinal fluid of 53 percent of the patients with chronic progressive multiple sclerosis and in none of those with stable multiple sclerosis ($P<0.001$). TNF-α was detected in the cerebrospinal fluid of 7 percent of the controls ($P<0.01$) with other neurologic disease. In patients with chronic progressive multiple sclerosis, mean TNF-α levels were significantly higher in the cerebrospinal fluid than in corresponding serum samples (52.41 vs. 11.88 U per milliliter; range, 2 to 178 vs. 2 to 39; $P<0.001$). In these patients, cerebrospinal fluid levels of TNF-α correlated with the degree of disability ($r = 0.834$, $P<0.001$) and the rate of neurologic deterioration ($r = 0.741$, $P<0.001$) before the start of the study. Cerebrospinal fluid levels also correlated with the increase in neurologic disability after 24 months of observation ($r = 0.873$, $P<0.001$).

Conclusions. These data provide evidence of intrathecal synthesis of TNF-α in multiple sclerosis and suggest that the level of TNF-α in cerebrospinal fluid correlates with the severity and progression of the disease. Our results suggest that TNF-α may reflect histologic disease activity in multiple sclerosis and could be used to monitor outcomes or responses to therapy. (N Engl J Med 1991; 325:467-72.)

MULTIPLE sclerosis, an inflammatory and demyelinating disease of the central nervous system, is regarded as a major cause of neurologic disability among young adults.[1] The disease follows a chronic, recurrent course and is associated with well-established immunologic aberrations that involve both B lymphocytes[2] and T lymphocytes.[3] Newly formed plaques in the brains of patients with multiple sclerosis have been reported to contain T lymphocytes and macrophages at their active edges.[4] Both types of cell secrete cachectin, also termed tumor necrosis factor-α (TNF-α), a cytokine that has been suggested as an important mediator in several inflammatory disorders, including those affecting the central nervous system.[5-7] TNF-α has also been shown to be capable of selectively damaging oligodendrocytes and myelin sheaths in vitro,[8] a finding that may be relevant in the context of disease progression in patients with multiple sclerosis.

In an attempt to identify an in vivo correlation of TNF-α levels to the severity of disease in multiple sclerosis, we measured free TNF-α levels in samples of cerebrospinal fluid and serum from 32 patients with chronic progressive multiple sclerosis and correlated them with clinical evidence of disease progression over a period of two years. Findings were compared with those from patients with clinically stable multiple sclerosis as well as from patients with various other inflammatory and noninflammatory neurologic diseases.

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METHODS

Selection of Patients

The study was planned prospectively with stringent selection criteria. Patients were selected for further study if they had clinically established multiple sclerosis,[9] had a chronic progressive disease with clear adherence to the originally assigned disease type, were between 20 and 50 years of age, had cerebrospinal fluid samples collected at entry and at the time of the disability assessment, were able to attend a follow-up appointment 2 years after entry into the study, and had details of the duration of the disease clearly documented in their medical notes. We excluded patients with disorders that compromised assessment of neurologic function, such as arthritis deformans, major amputations, or psychoses; patients who had other types of illnesses that could affect the outcome of the study; pregnant women or those planning to become pregnant within two years; and patients who had received treatment with steroids for one year before entry, or immunosuppressive medications or total lymphoid irradiation at any time.

Of 265 patients with multiple sclerosis who were originally considered, 32 patients with chronic progressive multiple sclerosis satisfied the preestablished selection criteria and were included in the study. They had a mean age (±SD) of 37.4±6.3 years and had had steady progression of the disorder since its onset. Their degree of disability at the time of the collection of cerebrospinal fluid was assessed with the expanded disability status scale (EDSS).[10] This scale, which helps monitor the natural history of multiple sclerosis and the response to treatment, uses a scoring system with 0.5-step increments to record objective neurologic impairment; scores range from 0 (normal) to 10 (death due to multiple sclerosis).

All patients with chronic progressive multiple sclerosis were required to have objective evidence of disease progression without remission or stabilization for a minimum of one year before the study, with an increase of 1.0 or more points on the EDSS. At the beginning of the study they had EDSS scores of 3 to 6. The rate of neurologic deterioration was evaluated by the progression index,[11,12] which is defined as the ratio of the disability status (i.e., the EDSS score) to the duration of the disease in years. This index is considered a reasonable estimate of the rate of deterioration.[12] A small quotient (<0.2) means a benign course, whereas larger quotients indicate active disease (values above 1.5 indicate a malignant course). The duration of disease varied among the patients (range,

1 to 14 years), so that the EDSS and the progression index effectively measured two variables.

On the initial visit, levels of TNF-α in cerebrospinal fluid and serum were measured by an investigator who was unaware of the patients' clinical status and the degree of disability was assessed with the EDSS. The patients were then followed regularly for two years. No samples obtained before the study began were included. At the end of the study, progression-index scores and EDSS scores were determined for all patients by the same examiners who evaluated them at the beginning of the study to avoid interexaminer variability.[13] The investigator who measured EDSS at the end of the study did not know the patients' TNF-α levels.

Controls

Paired samples of cerebrospinal fluid and serum were obtained from 20 patients with stable multiple sclerosis who were matched for age, sex, and the extent of disability to serve as disease controls. Paired samples were also obtained from 85 age-matched and sex-matched patients with various neurologic diseases to serve as a neurologic control group. This group consisted of 6 patients with bacterial meningitis, 3 with the acquired immunodeficiency syndrome (AIDS)–dementia complex, 7 with acute Guillain–Barré syndrome, 5 with neurosarcoidosis, 6 with cerebral lupus, 5 with myasthenia gravis, 10 with cerebrovascular accident, 8 with motor neuron disease, 5 with hereditary sensorimotor neuropathy, 10 with benign intracranial hypertension, 10 with Alzheimer's disease, 6 with Parkinson's disease, and 4 with Huntington's disease.

Assays

All assays were performed in a blinded fashion on coded sterile samples. To prevent the degradation of protein, 1000 kallikrein inhibitory units of protease inhibitor (aprotinin, Sigma) was added to each milliliter of cerebrospinal fluid and serum at the time of the sample collections. The cells were then separated by cytocentrifugation, and all samples were filtered through a 0.22-μm disposable sterile filter (Millipore) to remove contaminating particulate materials. Samples were subsequently frozen in aliquots at −70°C and thawed just before use.

Levels of TNF-α in native cerebrospinal fluid and diluted serum samples were measured by a sensitive sandwich-type enzyme-linked immunosorbent assay[14] with a purified monoclonal antibody to human TNF-α (Chiron) and rabbit polyclonal antihuman TNF-α antibody (Genzyme). A standard curve was generated on each assay with freshly diluted standard concentrations of purified recombinant human TNF-α (Genzyme). This method can detect levels in excess of 0.01 U per milliliter, and reproducible positive results were obtained on repeated testing. One unit of TNF-α was defined as the amount of TNF-α required to mediate half-maximal cytotoxicity of L929 cells in the presence of dactinomycin.

Oligoclonal IgG bands were detected in cerebrospinal fluid by agarose isoelectric focusing.[15] The amount of IgG synthesized intrathecally was calculated according to the formula of Tourtellotte and Ma,[16] and the total protein content of cerebrospinal fluid was determined by the benzethonium chloride precipitation technique.[17] The cerebrospinal fluid cell count was performed within six hours of the lumbar puncture; after preparation in a Shandon cytocentrifuge, cells were stained with Giemsa stain and examined.

Statistical Analysis

Confidence intervals for nonparametric data were calculated,[18] and the Wilcoxon rank-sum test, Spearman rank correlation, and two-sided Mann–Whitney test were used as appropriate for statistical analysis. The relative relations of levels of TNF-α in cerebrospinal fluid to clinical and biochemical indicators in all patients with chronic progressive multiple sclerosis were tested by forward stepwise multiple logistic-regression analysis,[19] with TNF-α levels in cerebrospinal fluid as the dependent variable. Log transformation was necessary because of the skewed distribution. Backward stepwise logistic-regression analyses were also performed and produced identical results in all cases. Statistical analyses were performed with SPSS/PC+ software. All P values in the study were two-tailed.

Results

Separate studies of samples not treated with aprotinin showed that the addition of the protease inhibitor improved the detectability of TNF-α in cerebrospinal fluid and serum sixfold. TNF-α was stable provided repeated thawing and refreezing of the samples was avoided.

Elevated levels of TNF-α were detected in the cerebrospinal fluid of 17 patients with chronic progressive multiple sclerosis (53 percent) and in none of the group with stable multiple sclerosis (Fig. 1). High cerebrospinal fluid levels of TNF-α were detected in two patients with chronic progressive multiple sclerosis in whom no serum levels were detected. Conversely, high TNF-α levels (mean, 3.8 U per milliliter) were found in serum samples from three patients with progressive disease who had no detectable levels of TNF-α in cerebrospinal fluid. In the control groups, high levels of TNF-α in cerebrospinal fluid and serum were detected in six patients (7 percent): three with bacterial menin-

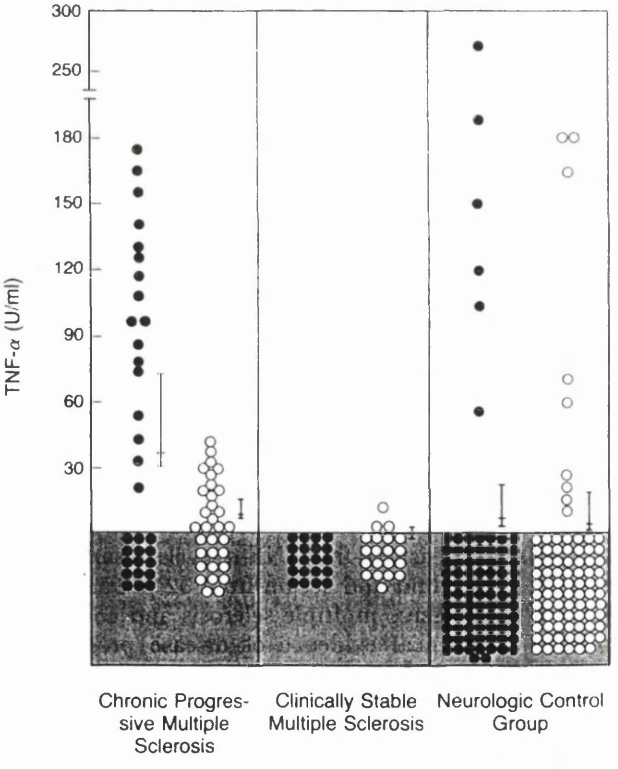

Figure 1. Levels of TNF-α in Cerebrospinal Fluid (●) and Serum (○) in the Study Groups.

Bars indicate median values and 95 percent confidence intervals. The shaded area represents the limit of detection of the assay. Cerebrospinal fluid levels of TNF-α in patients with chronic progressive multiple sclerosis were significantly higher than in those with stable multiple sclerosis ($P<0.001$) or in the control group ($P<0.01$).

gitis, two with AIDS–dementia complex, and one with Guillain–Barré syndrome (Fig. 1). Three other patients in the control group (two with Guillain–Barré syndrome and one with AIDS–dementia complex) had detectable levels of TNF-α (mean, 14.2 U per milliliter) only in serum.

In 17 patients with chronic progressive multiple sclerosis who had detectable levels of TNF-α, the mean ($\pm$SD) levels in cerebrospinal fluid were significantly higher than those in corresponding samples of serum (97.88$\pm$47.84 vs. 20.94$\pm$10.92 U per milliliter; 95 percent confidence interval, 74.42 to 122.87 vs. 15.32 to 26.55). Cerebrospinal fluid levels of TNF-α in these patients correlated with the EDSS scores at the beginning of the study (Fig. 2) and with the progression-index values (Fig. 3).

In the patients with chronic progressive multiple sclerosis, the relation of TNF-α levels in cerebrospinal fluid to clinical and biochemical indicators of disease progression were analyzed by multivariate regression analysis. TNF-α levels did not correlate with the presence of oligoclonal IgG bands in cerebrospinal fluid, intrathecal IgG synthesis, cerebrospinal fluid total protein, or pleocytosis (Table 1). TNF-α levels were correlated with the EDSS score ($P<0.0005$), the change in the EDSS score from the first visit to the 24-month visit ($P<0.0001$), and the progression-index value ($P = 0.027$).

In the majority of patients with chronic progressive multiple sclerosis, symptoms continued to worsen during the two-year study period, whereas only two

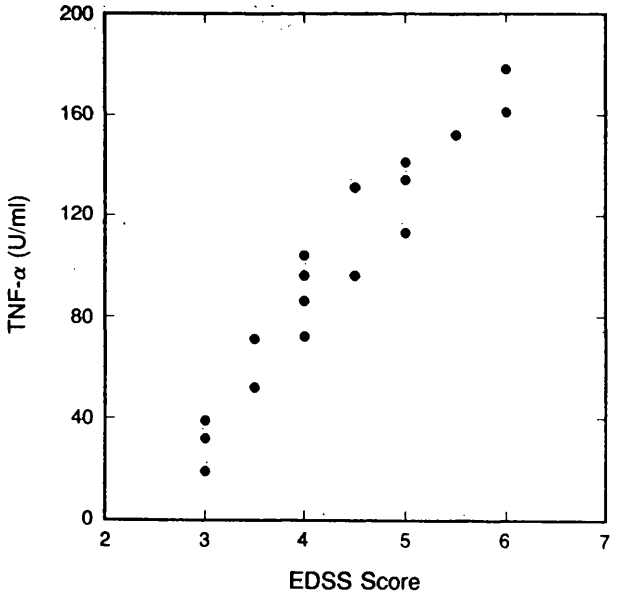

Figure 2. Relation between the TNF-α Level in Cerebrospinal Fluid and the Degree of Disability on Entry into the Study in Patients with Chronic Progressive Multiple Sclerosis.

The degree of disability was evaluated by the EDSS. TNF-α levels were significantly correlated with the EDSS score ($r = 0.834$, $P<0.001$) in patients who had detectable levels of TNF-α in cerebrospinal fluid.

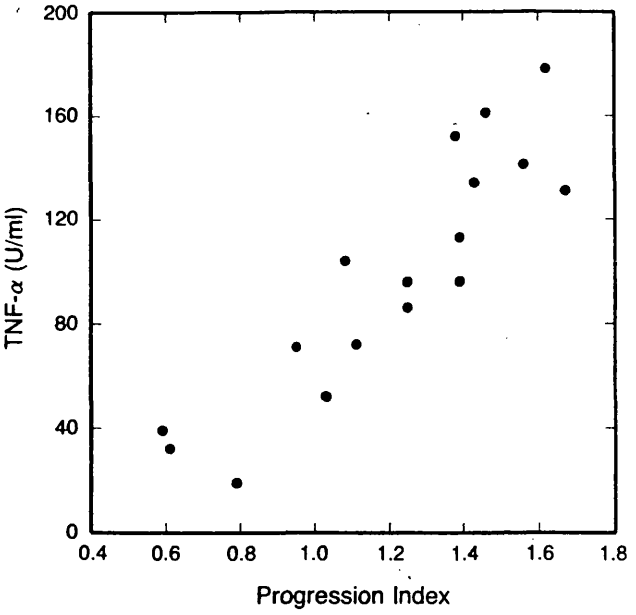

Figure 3. Relation between the TNF-α Level in Cerebrospinal Fluid and the Progression Index in Patients with Chronic Progressive Multiple Sclerosis on Entry into the Study.

TNF-α levels were significantly correlated with the progression index ($r = 0.741$, $P<0.001$) in patients who had detectable TNF-α in cerebrospinal fluid.

patients with stable disease had mild worsening of symptoms. There was a significant increase in the degree of disability during the 24-month period in patients with chronic progressive multiple sclerosis who had elevated cerebrospinal fluid TNF-α levels at entry (Table 2). One patient, who had a TNF-α level of 176 U per milliliter in cerebrospinal fluid and an EDSS score of 6.0 on entry into the study, died after 22 months of follow-up. In contrast, there was no significant increase in the degree of disability in patients with progressive disease who showed no TNF-α reactivity on entry into the study (Table 2). Six patients with chronic progressive multiple sclerosis who had a decline in the EDSS score of more than 3 points were treated with steroids during the follow-up period — an approach that did not significantly alter the rate of disease progression. No patient received azathioprine or cyclophosphamide during the follow-up period.

In addition to their relation to the EDSS score, high cerebrospinal fluid levels of TNF-α at the beginning of the study implied a poor prognosis, since these levels correlated with an increase in the degree of disability over the next two years (Fig. 4). High cerebrospinal fluid levels of TNF-α also correlated with increased EDSS scores at the 24-month visit ($r = 0.873$, $P<0.001$). Although the EDSS score correlated with the value for the progression index at entry ($r = 0.741$, $P<0.001$), the index values remained remarkably stable throughout the study period (Table 2). Nonetheless, high cerebrospinal fluid

Table 1. Clinical and Biochemical Indicators in 32 Patients with Chronic Progressive Multiple Sclerosis.

VARIABLE	MEAN ±SEM	P VALUE*
EDSS at entry	3.66±0.15	<0.0005
Change in EDSS†	1.78±0.25	<0.0001
Progression index	0.99±0.07	0.027
Disease duration (yr)	3.86±0.41	NS
Intrathecal IgG synthesis (mg/day)	18.46±2.65	NS
Cerebrospinal fluid total protein (g/liter)	0.58±0.18	NS
Pleocytosis (no. of cells/mm^3)	8.27±0.91	NS

*P values are for multivariate regression analysis of the relations of TNF-α levels in cerebrospinal fluid. NS denotes not significant.

†Change in the EDSS score from the first visit to the 24-month visit.

levels of TNF-α correlated with the value for the progression index at the end of the study ($r = 0.851$, $P<0.001$).

DISCUSSION

We found significantly higher levels of TNF-α in the cerebrospinal fluid of patients with chronic progressive multiple sclerosis than in patients with stable multiple sclerosis or with other neurologic diseases. Cerebrospinal fluid levels of TNF-α were also correlated with the degree of disability in patients with progressive disease, and high levels predicted a poor prognosis during a 24-month period of observation. These results support a role for TNF-α in the progression of multiple sclerosis. It has already been suggested that TNF-α has a role in the pathogenesis of demyelination.[20] Cytotoxic T lymphocytes, which can produce TNF-α, are involved in the events leading to acute demyelination,[4] and TNF-α was reported to cause both disruption of myelin and permanent damage to oligodendrocytes.[8] Furthermore, significantly higher amounts of TNF-α were released by mitogen-stimulated cells obtained from patients with multiple sclerosis during clinical exacerbation than in those obtained from patients during remission.[21]

We evaluated the degree of disease progression in patients with chronic progressive multiple sclerosis by calculating the decline in the EDSS score during the study period. Although the EDSS is an inherently ordinal scale and unit changes may not be of equal importance over its entire range, the average decline in the scores reported here is clinically important by any standard. Some natural-history studies and clinical trials[22,23] indicate that the EDSS scores change less than 1 point per year in patients with chronic progressive multiple sclerosis. To minimize the number of patients whose conditions stabilized spontaneously during the study period, we adopted more demanding selection criteria than those used in previous studies.[24,25] These strict entry requirements served to select patients with active disease, whose EDSS scores during the study period declined by an average of more than 2 points. Other studies that employed similarly stringent criteria[26-28] also reported an increase in the EDSS score of 2 or more points (up to 4) during a follow-up period of 18 to 30 months.

It is important to note that each grade of disease disability in the study patients with multiple sclerosis was associated with a reasonable distribution of observed TNF-α concentrations, with no major overlap between individual grades. This fact, coupled with the significant correlations, suggests that TNF-α may be an indicator of disease progression in patients with multiple sclerosis. Whether TNF-α is directly involved in the progression of multiple sclerosis or merely reflects other basic processes is unclear. However, TNF-α causes severe damage to human endothelial cells[29] and induces vascular "leak" syndrome[30] — effects that may be relevant to disease progression in multiple sclerosis. Experimental, pathological, and imaging data suggest that a fundamental feature of lesions in active multiple sclerosis is local breakdown of the endothelial barriers, particularly the blood–brain barrier.[31-33] The principal inflammatory

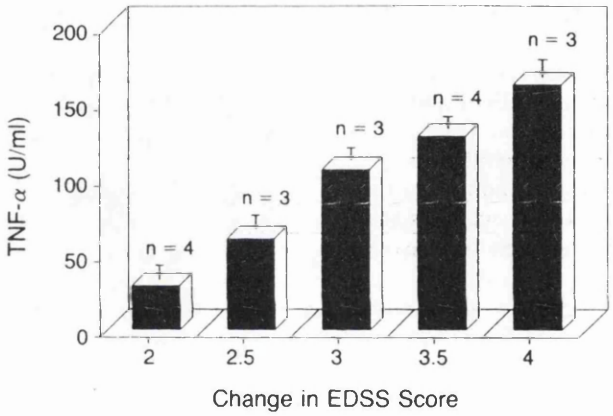

Figure 4. Relation between the TNF-α Level in Cerebrospinal Fluid on the First Visit and the Change in the EDSS Score from the First Visit to the 24-Month Visit.

Values are means ±SEM. The number of patients in each category is also shown. Fifteen patients with no detectable TNF-α in cerebrospinal fluid were not included. Levels of TNF-α in cerebrospinal fluid were significantly correlated with the difference between the two EDSS scores ($P<0.001$).

Table 2. Change in Clinical Indexes over a 24-Month Period in 32 Patients with Chronic Progressive Multiple Sclerosis.*

GROUP	NO. OF PATIENTS	CHANGE IN EDSS	CHANGE IN PROGRESSION INDEX
Elevated TNF-α in CSF	17	2.94±0.18†	0.18±0.07
No TNF-α in CSF	15	0.96±0.14	0.11±0.09

*Plus–minus values are means ±SEM. CSF denotes cerebrospinal fluid.

†$P = 0.004$ by the two-sided Mann–Whitney test.

process in this disease usually occurs around blood vessels, with only scanty inflammatory activity in the brain parenchyma.[34] Macrophages, which are abundant in all acute perivascular brain lesions,[34] are the most potent producers of TNF-α. The perivascular infiltrate in brain lesions in multiple sclerosis also contains activated lymphocytes, which can produce TNF-α.

Our finding that the cerebrospinal fluid of patients with chronic progressive multiple sclerosis had significantly higher levels of TNF-α than did corresponding serum samples suggests that this cytokine is released locally in the intrathecal compartment. The lack of correlation between the presence of pleocytosis and TNF-α levels suggests that TNF-α may be derived from the central nervous system and not cerebrospinal fluid cells. Production of TNF-α by the central nervous system may result from its release by macrophages and T lymphocytes, which are abundant in brain lesions due to multiple sclerosis, as well as by astrocytes and microglial cells.[35,36] TNF-α has been detected in astrocytes in brain tissues of patients with multiple sclerosis.[37] The failure to detect TNF-α in the cerebrospinal fluid of patients with multiple sclerosis in another study[38] may have been due to differences in the way patients were selected or to the instability of TNF-α if it is not treated with a protease inhibitor. In addition, the ability to detect TNF-α depends on the particular assay used, since different procedures have been shown to vary significantly in their sensitivity and limits of detection.[39] Our demonstration of a significant increase in TNF-α levels in cerebrospinal fluid relative to levels in serum has been corroborated by a recent independent study.[40]

We found no correlation of neurologic impairment with serum TNF-α levels in patients with multiple sclerosis. This finding raises the possibility that intrathecal concentrations of TNF-α are more important than systemic levels in the clinical progression, and perhaps histologic activity, of multiple sclerosis. Because this study was blinded, we were unaware of the clinical importance of TNF-α levels in cerebrospinal fluid during the follow-up period. Thus, repeated collections of cerebrospinal fluid samples were not ethically justified. However, repeated measurements are indicated to evaluate how changes in TNF-α levels may be correlated with changes in the trajectory of the disease. Similarly, further studies will be required to elucidate the interactions of TNF-α and other cytokines, such as lymphotoxin, in the progression of multiple sclerosis.

The findings presented here may have important prognostic and therapeutic implications. A test based on cerebrospinal fluid analysis that can predict the progression of multiple sclerosis could be regarded as a marker of activity and would be of great importance in monitoring outcome. The removal of TNF-α[41] or neutralization of its effect[42,43] might be of benefit in patients with chronic progressive multiple sclerosis.

We are indebted to June Smalley, who tirelessly developed and appended computer data-base files while blinded to clinical and pathologic data; to Naghat Lakdawala for preparing and coding the samples while blinded to clinical information; to Magenlal Chowhan for detecting oligoclonal IgG bands; and to the neurologists who allowed us to obtain clinical data on their patients.

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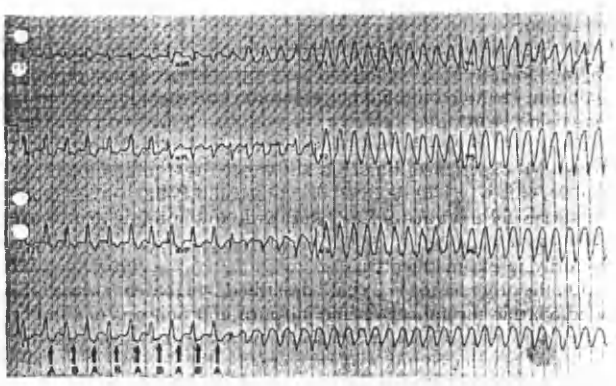

Figure 1. Twelve-Lead Electrocardiogram Recorded at 8:01 p.m. in Patient 1, Showing Atrial Fibrillation with the Onset of Sustained Ventricular Tachycardia.

The arrows labeled A and B indicate the different QRST forms.

the heart in a pericardial effusion, affecting the entire P-QRST wave form.[9] Alternation of intrinsic ventricular conduction patterns may elicit QRST alternans, which may be a marker of ventricular electrical instability.[3-5] We report on two patients in whom electrical alternans appeared immediately before sudden cardiac death.

Patient 1 was a 68-year-old man with an acute lateral myocardial infarction without pericardial effusion. Three electrocardiograms were recorded at 7:57, 8:00, and 8:01 p.m. The first electrocardiogram did not show electrical alternans, but the last two showed QRST alternans, followed by ventricular tachycardia, fibrillation, and death (Fig. 1).

Patient 2 was a 54-year-old woman who had an episode of ventricular fibrillation in the emergency room after experiencing cardiac arrest outside the hospital. There was no evidence of myocardial infarction, congestive heart failure, or pericardial tamponade. The patient received no antiarrhythmic drugs and had normal electrolyte levels. One day later, an electrocardiogram revealed T-wave alternans in multiple leads. On day 3, ventricular fibrillation recurred, preceded by T-wave alternans (Fig. 2), and the patient died.

We analyzed the electrical alternans in these patients by autocorrelation of the amplitudes of successive QRST wave forms. In Patient 1, a statistically significant negative correlation was not present at 7:57 p.m. ($r = -0.34$), but was present at 8:00 p.m. ($r = -0.65$) and 8:01 p.m. ($r = -0.89$), demonstrating two populations of QRST complexes in alternating sequence. This time course suggests that QRST alternans may emerge just before a life-threatening arrhythmia. In Patient 2, the correlation coefficient was also statistically significant ($r = -0.75$). These findings are in accord with recent reports of an association of electrical alternans with increased susceptibility to ventricular fibrillation. Adam et al.,[4] Smith et al.,[3] and Nearing et al.[5] found that hypothermia or coronary occlusion elicits QRS and ST-T alternans, with a concomitant reduction in the threshold for ventricular fibrillation.

Only sparse data are available on the importance of spontaneous electrical alternans in patients. Smith et al.[3] studied 19 patients and found a correlation between alternating electrocardiographic morphology and the inducibility of ventricular tachycardia or fibrillation. The present report provides additional evidence of a link between spontaneous electrical alternans and sudden cardiac death.

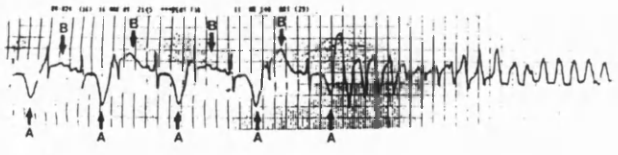

Figure 2. Telemetry Strip Showing the Inception of Polymorphic Ventricular Tachycardia in Patient 2.

Note the striking alternation of T-wave forms (indicated by the arrows labeled A and B).

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TUMOR NECROSIS FACTOR-α AND DISEASE PROGRESSION IN MULTIPLE SCLEROSIS

To the Editor: The study by Sharief and Hentges (Aug. 15 issue)[1] provides evidence that cerebrospinal fluid levels of tumor necrosis factor-α are higher in patients with chronic progressive multiple sclerosis than in patients with stable disease and that cerebrospinal fluid levels of tumor necrosis factor-α are correlated with disease progression in chronic progressive multiple sclerosis. On the basis of these results the authors suggest that tumor necrosis factor-α has a role in the progression of multiple sclerosis. We believe that the authors should provide additional information concerning the HLA Class II typing of the patients studied, for the following reasons.

The gene encoding tumor necrosis factor-α is located in the HLA region between the HLA-DR and HLA-B loci. Recent studies have shown that HLA-DR2–positive subjects secrete low levels of tumor necrosis factor-α, whereas HLA-DR4–positive subjects secrete high levels.[2,3] Furthermore, the HLA-DR2 DQw6 haplotype is associated with susceptibility to both chronic progressive and relapsing or remitting multiple sclerosis, whereas the HLA-DR4 DQw8 haplotype has been associated with chronic progressive multiple sclerosis.[4]

This HLA association may be due to the HLA Class II molecules themselves, determining the intensity of the immune response to the pathogenic foreign or self antigen, or to linkage disequilibrium between HLA genes and closely located genes, such as the tumor necrosis factor-α gene, or it may be due to both. Therefore, the high level of tumor necrosis factor-α in patients with both chronic progressive disease and a high progression index could merely reflect a higher frequency of the HLA-DR4 DQw8 haplotype in these patients. HLA-typing data for the patients studied by Sharief and Hentges could help resolve the question of whether tumor necrosis factor-α acts as an independent risk factor.

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Dr. Sharief replies:

To the Editor: Drs. Liblau and Fugger make an interesting suggestion. Because the genes encoding tumor necrosis factor-α are located in the major histocompatibility complex, the possibility that elevated levels of this cytokine in autoimmune disease are due to an "abnormal" gene is an attractive hypothesis. However, several lines of evidence suggest that this mechanism is not applicable to multiple sclerosis. First, tumor necrosis factor-α genes are more closely linked to Class I genes, particularly the HLA-B locus,[1] than to Class II genes, which include HLA-DR2 and HLA-DR4 loci. Yet no correlation has been found between tumor necrosis factor-α and Class I molecules. Second, there is no correlation between the production of tumor necrosis factor-β and HLA Class I or II molecules, although tumor necrosis factor-β genes are also closely linked to the major histocompatibility complex.[2] Of interest is the finding that tumor necrosis factor-β has been identified in brain lesions due to multiple sclerosis.[3] Third, a restriction-fragment–length polymorphism that distinguishes HLA-DR2–positive persons from most persons with additional Class II molecules failed to correlate with the quantitative variation in the production of tumor necrosis factor-α.[4] Finally, the quantitative variations in the production of tumor necrosis factor-α between HLA-DR2–positive and HLA-DR4–positive subjects are mutually exclusive in patients with progressive multiple sclerosis because either haplotype may be expressed in these patients.

Liblau and Fugger's suggestion that HLA Class II molecules could determine the intensity of the immune response has been refuted by a recent study that established that HLA types do not influence the immune response in multiple sclerosis.[5] Moreover, their suggestion that there could be a linkage disequilibrium between HLA and tumor necrosis factor-α genes in multiple sclerosis is rather surprising in view of the failure of Fugger et al. to detect a significant difference in the restriction-fragment–length polymorphism of the tumor necrosis factor-α region between patients with multiple sclerosis and healthy controls.[6]

Liblau and Fugger refer to a quantitative polymorphism that is detected in vitro on peripheral-blood lymphocytes or monocytes from healthy persons or patients with lupus erythematosus. Extrapolation of such a polymorphism to patients with multiple sclerosis requires in vivo studies with proper disease controls. It is noteworthy that there is still considerable doubt as to whether HLA molecules are the ones that predispose patients to multiple sclerosis or are markers of a closely linked gene or genes.

To determine whether tumor necrosis factor-α acts as an independent risk factor in the progression of multiple sclerosis, correlations with other indicators of disease progression should be examined. My colleagues and I have already described basic immunologic differences between relapsing or remitting and chronic progressive multiple sclerosis.[7,8] The correlation of these immunologic aberrations with the production of tumor necrosis factor-α is currently under investigation.

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PERIOPERATIVE TOTAL PARENTERAL NUTRITION IN SURGICAL PATIENTS

To the Editor: The study by Buzby et al. (Aug. 22 issue)[1] should not be interpreted as casting doubt on the efficacy of total parenteral nutrition. Instead, it points out the difficulty in determining which, if any, surgical patients might benefit.

Since the studies by Dudrick et al.[2] of patients who were unable to obtain adequate nutrition enterally, many investigators have clearly shown that total parenteral nutrition can produce a positive energy and nitrogen balance. The problem arises in trying to use simple anthropometric and biochemical variables, such as weight and serum albumin and prealbumin concentrations, to diagnose malnutrition. Short-term weight loss is usually due to changes in hydration, whereas most long-term weight loss is due to changes in fat mass (often excessive at base line in the United States). Hence weight, weight change, and even changes in body composition poorly reflect nutritional status in patients in this country. On the other hand, serum albumin and prealbumin levels, and most other biochemical indicators of malnutrition, are negative acute-phase reactants, and their levels decrease independently of food intake in patients with inflammatory conditions, sepsis, or cancer. Although many surgical patients have anthropometric and biochemical findings suggesting malnutrition and are often subjected to fasting in the hospital, it is an open question whether they are in the same state as a person deprived of energy and protein intake by famine or by a medical condition such as a short bowel.

Many years ago it was proposed[3] that nutritional deficiency should be defined by the response to treatment rather than by secondary phenomena, such as weight loss or hypoalbuminemia. Since most of the patients in the Veterans Affairs study[1] did not benefit from total parenteral nutrition, one can argue that either they were not malnourished or nutrition does not affect the end points measured. According to the definition of Yudkin,[3] they were not malnourished.

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To the Editor: The investigators of the Veterans Affairs Total Parenteral Nutrition Cooperative Study Group are to be commended for their well-designed and clearly reported study. Some comments, however, are pertinent.

Significance of CSF immunoglobulins in monitoring neurologic disease activity in Behçet's disease

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Article abstract—We examined the intrathecal production of immunoglobulins (Ig) G, A, and M in 16 patients with Behçet's disease, 13 of whom have CNS involvement, and in 40 neurologic controls. Oligoclonal IgA and IgM bands were mainly detected in CSF samples from patients with active neuro-Behçet's disease and were documented to disappear when neurologic manifestations remit. Oligoclonal IgG bands, however, were not related to disease activity and were also found in some neurologic controls. High immunoglobulin index values were detected in both active and quiescent diseases and were high in some patients with impaired blood-CSF barriers. The study presented here demonstrates that CSF oligoclonal IgA and IgM may be helpful in monitoring CNS disease activity in neuro-Behçet's and could be useful in understanding the pathogenesis of this disease.

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CNS involvement in Behçet's disease, known as neuro-Behçet's, is one of the most serious complications of the disease and occurs in 10 to 49% of patients.[1-4] Although the neurologic manifestations in Behçet's disease vary considerably, a prominent feature is exacerbations and remissions that can be confused with multiple sclerosis.[5] Another characteristic is the specific involvement of the central rather than the peripheral nervous system. Therefore, CSF examination is of particular importance in the diagnosis of neuro-Behcet's disease. Several CSF findings have been reported, such as elevated total protein content,[6] lymphocytic pleocytosis,[2] high C3 level,[7] and elevated levels of immunoglobulins (Ig) G, A, and M.[7,8] The presence of CSF immunoglobulins of restricted heterogeneity (ie, oligoclonal), however, was not thoroughly evaluated.

We investigated the distribution of oligoclonal IgG, IgA, and IgM bands in the CSF of 16 patients with Behçet's disease and compared such findings with other CSF findings that include immunoglobulin indices and the state of the blood-CSF barriers. Results were compared with those obtained from 40 neurologic control patients in an effort to determine changes that may distinguish active from quiescent disease.

Methods. *Patients.* Diagnosis of Behçet's disease was made according to the criteria of the International Study Group for Behçet's disease.[9] Paired CSF and serum samples from 16 patients with Behçet's disease were examined. Thirteen patients had neuro-Behçet's disease as defined earlier,[3,4] while the other three patients had Behçet's disease without CNS manifestation (table 1). Two separate CSF specimens were taken from the same patient (patient 1): the first during a period of active CNS involvement and the second about 8 months later during a period of remission following immunosuppressive therapy. Samples from patients with active neuro-Behçet's disease were collected within 10 days of onset. Patients without neurologic involvement had lumbar puncture to investigate unrelated vertebral complaints. All patients had brain CTs with enhancement or MRIs. Clinical signs of disease activity were associated with radiologic evidence of multiple cerebral or brainstem lesions. Clinical remission was always associated with complete resolution of the CT or MRI abnormalities.

Paired CSF and serum specimens from 40 patients with inflammatory and noninflammatory CNS diseases were used as neurologic controls (table 2). Paired samples were also obtained from 20 normal subjects to determine normal reference ranges of immunoglobulin levels. Those subjects presented with nonspecific headache or neurotic syndromes, but neurologic examination and investigations such as MRI, evoked potentials, and CSF analysis were negative.

Quantification of immunoglobulins. All assays were done blindly on coded samples. Total IgG amount in CSF and homologous serum samples was determined by electroimmunoassay. The IgA content of the test samples was determined by an enzyme-linked immunosorbent assay (ELISA)[10] using peroxidase-conjugated $F(ab')_2$ fragment of anti-human IgA antibody (Sigma, UK). The total IgM content in CSF and serum samples was also established by an ELISA technique.[11] CSF and homologous serum samples were run in triplicate in the same batch. Coefficient of variation between batches was less than 5%.

Detection of oligoclonal bands. CSF and homologous serum samples were run in parallel; control positive and negative samples were included in each batch as quality control. Oligoclonal IgG bands were detected by isoelectric focusing on agarose gel.[12] Oligoclonal IgM reactivity was detected by the

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Table 1. Clinical and CSF data on patients with Behçet's disease included in the study

Pt no.	Age/Sex	Clinical state	Blood-CSF barrier	TP (mg%)	WBC		IgG		IgA		IgM	
					N	L	OCB	Index	OCB	Index	OCB	Index
1	28/F	Active	Damaged	53	17	4	+	High	+	High	+	High
2	54/F	Active	Damaged	89	37	14	−	High	−	N*	+	High
3	62/F	Active	Damaged	110	3	1	−	N*	+	N*	+	High
4	42/M	Active	Damaged	63	2	0	−	N*	+	N*	−	N*
5	31/M	Active	Damaged	37	11	4	+	High	+	High	+	High
6	23/M	Active	Damaged	41	3	0	−	N*	+	N*	+	High
7	44/F	Active	Intact	56	7	3	−	N*	−	N*	+	N*
8	37/M	Active	Intact	32	6	2	+	High	+	High	+	High
1	29/F	Remission	Damaged	47	8	2	+	High	−	High	−	High
9	52/F	Remission	Damaged	51	0	2	−	N*	−	High	−	N*
10	46/M	Remission	Damaged	54	4	0	−	High	−	N*	−	N*
11	20/F	Remission	Damaged	32	4	2	+	High	−	High	−	N*
12	32/M	Remission	Intact	43	1	3	−	N*	−	N*	−	N*
13	27/M	Remission	Intact	42	2	0	−	N*	−	N*	−	N*
14	40/F	No CNS involvement	Intact	29	2	2	−	N*	−	N*	−	N*
15	36/M	No CNS involvement	Intact	36	0	1	−	N*	−	N*	−	N*
16	26/M	No CNS involvement	Intact	28	0	3	−	N*	−	N*	−	N*

TP Total protein (normal, 15 to 40 mg%).
WBC White blood cell count (normal, <5/mm³).
N Neutrophils.
L Lymphocytes.
OCB Oligoclonal bands.
− Absent.
+ Present.
* Within normal range (IgG index <0.85; IgA index <0.34; and IgM index <0.068).

method of glutaraldehyde (GA)-enhanced immunofixation,[13] which utilizes agarose gel electrophoresis, GA cross-linkage, and subsequent immunostaining. Oligoclonal IgA bands were also detected essentially by the method of GA-enhanced immunofixation.[13] In brief, native CSF and diluted serum samples containing 15 ng of IgA were electrophoresed on agarose gel and then the separated proteins were covalently linked to nitrocellulose membrane using GA. IgA bands were immunostained by a peroxidase-conjugated $F(ab')_2$ fragment of anti-human IgA antibody.

Routine CSF tests. CSF specimens were examined for pleocytosis within 6 hours of the lumbar puncture and then frozen at −20 °C. Cytologic examination was performed using Giemsa stain from a Shandon cytocentrifuge preparation. CSF total protein was measured using benzethonium chloride precipitation technique.[14] CSF and serum albumin levels were determined by electroimmunoassay.

The integrity of blood-CSF barriers was assessed by the quotient of CSF/serum albumin.[15] Intrathecal immunoglobulins synthesis was estimated qualitatively by the presence of CSF oligoclonal bands that were absent in homologous serum.[11] Quantitative evaluation of intrathecally synthesized immunoglobulin was done by calculating the index value[15,16] according to the following formula:

$$\frac{\text{CSF immunoglobulin} \times \text{serum albumin}}{\text{serum immunoglobulin} \times \text{CSF albumin.}}$$

Statistical methods. Comparison of values in different patient groups was performed by Wilcoxon's rank sum test. Oligoclonal immunoglobulin bands in the study groups were compared by Fisher's test of exact probability.

Results. *Oligoclonal bands in the CSF.* In patients with Behçet's disease, CSF oligoclonal IgG bands were detected in five patients, IgA bands in six, and IgM bands in seven patients (table 1). Oligoclonal IgA and IgM bands were significantly more prevalent in active neuro-Behçet's disease when compared with neuro-Behçet's in remission ($p < 0.01$) or with the neurologic control group ($p < 0.05$). Furthermore, oligoclonal IgA and IgM bands disappeared after immunosuppressive therapy in one patient.

Oligoclonal IgG bands did not correlate with activity of neuro-Behçet's disease (table 1) and were also detected in some patients with noninflammatory neurologic diseases (table 2).

Immunoglobulin indices. In the normal subjects, mean ± SD of IgG index value was 0.53 ± 0.16, IgA index was 0.14 ± 0.10, and IgM index was 0.028 ± 0.02. High immunoglobulin indices were found in some patients with quiescent neuro-Behçet's disease who had impaired blood-CSF barrier (table 1). IgM index, however, was significantly higher in patients with active neuro-Behçet's disease when compared with patients in remission or those from the neurologic controls (figure).

Other CSF assays. Routine CSF analyses were less valuable than oligoclonal bands in detecting active neuro-Behçet's disease (table 1). However, a significant increase in CSF neutrophil count was detected in neuro-Behçet's patients when compared with the neurologic control group ($p < 0.05$). Similarly, nine of 13 patients (69%) with neuro-Behçet's disease had signs of

Table 2. CSF data on patients of the neurologic control group

Clinical group	No. pts	Mean TP value	Mean cell count/mm³	Patients positive for oligoclonal bands			Mean Ig index values*		
				IgG	IgA	IgM	IgG	IgA	IgM
Multiple sclerosis	7	48	6	6	3	2	1.23	0.48	0.121
TB meningitis	4	57	12	2	3	3	0.82	0.41	0.082
Myasthenia gravis	4	26	2	1	0	0	0.65	0.29	0.060
Dementia	5	29	2	0	0	0	0.53	0.19	0.041
Parkinson's disease	4	34	3	0	0	0	0.58	0.22	0.056
Neuropathies and nerve injury	5	47	4	1	0	0	0.76	0.31	0.071
Myopathy	3	23	2	0	0	0	0.51	0.27	0.052
Cervical spondylosis	4	42	5	0	0	0	0.73	0.30	0.059
Stroke	4	49	8	2	0	0	0.87	0.36	0.066
Total	40	39	6	12	6	5	0.84	0.31	0.072

TP Total protein (normal range, 15 to 40 mg%).
* Reference Ig index values = IgG index <0.85; IgA index <0.34; and IgM index <0.068.

blood-CSF barrier damage compared with only eight (20%) of the neurologic controls ($p < 0.03$).

Discussion. Results of this study represent the first detailed study of oligoclonal immunoglobulins in patients with neuro-Behçet's disease. Our data suggest that oligoclonal IgM and IgA are valuable indicators of activity in this disease. Such finding corroborates and extends previous reports[7,8] that utilized quantitative immunoglobulin values in assessing disease activity.

Immunoregulatory disturbances within the CNS play an important role in the pathogenesis and progression of neuro-Behçet's disease.[17-20] Furthermore, increased intrathecal production of immunoglobulins is a recognized feature of active disease.[7,8] To evaluate intrathecal synthesis of immunoglobulins, it is necessary to take into account the high levels of serum immunoglobulins that are frequently observed in Behçet's disease.[21-25] Earlier estimation of ratios of CSF immunoglobulin to total protein[7] or immunoglobulin indices[8] could result in high values when blood-CSF barriers are impaired. In contrast, oligoclonal immunoglobulin bands in the CSF are unlikely to be affected by alterations in barrier functions.[11] Moreover, CSF oligoclonal bands could be detected in some patients with normal immunoglobulin index values.[11,26,27]

The observation that oligoclonal IgM and IgA bands in neuro-Behçet's are more prevalent than IgG bands is somewhat unexpected. However, an association between Behçet's disease and infections,[28,29] particularly those caused by herpes simplex virus,[30,31] was suggested. Increased intrathecal synthesis of IgA or IgM is commonly detected in various CNS infections such as aseptic meningitis,[16] bacterial and viral meningoencephalitis,[32] neuroborreliosis,[26,33] and neurosyphilis.[26,34] Thus, our finding of increased intrathecal immunoglobulin synthesis associated with the high incidence of blood-CSF barrier damage and the elevated CSF neutrophils in neuro-Behçet's disease is consistent with an infectious meningoencephalitic illness.

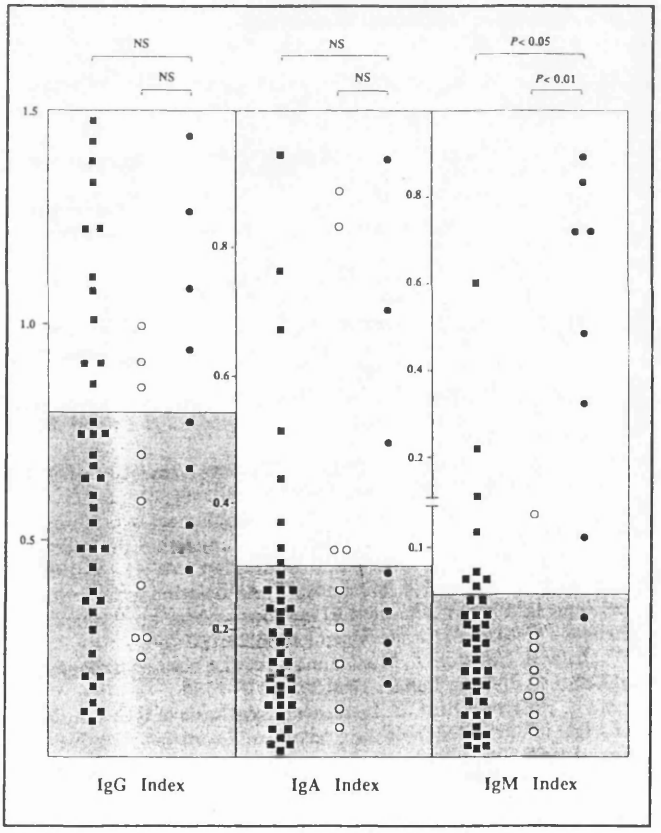

Figure. Cerebrospinal fluid IgG, IgA, and IgM indices in active neuro-Behçet's disease (●); quiescent neuro-Behçet's (○) including Behçet's disease with no CNS involvement; and neurologic control group (■). Shaded areas indicate normal reference values of immunoglobulin indices. (NS = not significant.)

Oligoclonal immunoglobulin bands are not pathognomonic for neuro-Behçet's disease as they are detected in other immunologically mediated diseases such as multiple sclerosis. Therefore, CSF immunoglobulin changes, particularly those involving IgG isotype, may

not distinguish neuro-Behçet's disease from multiple sclerosis. This diagnostic difficulty is compounded by the overlapping clinical[5] and neuroradiologic[35,36] features of both conditions. However, our data justify the use of CSF oligoclonal IgM and IgA bands to monitor CNS disease activity of neuro-Behçet's disease. Longitudinal studies with serial CSF evaluations at various stages of disease activity would be useful in further characterizing the nature and frequency of immunoglobulin abnormalities in neuro-Behçet's disease.

Although we are unable to elucidate at this stage the antigen specificity of intrathecally synthesized immunoglobulins, the detection of IgM and IgA in the CSF could be valuable in monitoring neuro-Behçet's disease activity. Further studies are required to establish whether intrathecal alterations of humoral immunity have a role in the pathogenesis of neuro-Behçet's disease.

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Importance of Intrathecal Synthesis of IgD in Multiple Sclerosis

A Combined Clinical, Immunologic, and Magnetic Resonance Imaging Study

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• **There is increasing evidence that soluble IgD has a certain role in the humoral immune response within the central nervous system. We report herein the results of a combined clinical, magnetic resonance imaging, and immunopathologic study to determine the clinical importance of intrathecal IgD synthesis. Intrathecal synthesis of IgD (detected through the calculation of index values) was studied in 64 patients with multiple sclerosis and in 50 neurologic control patients and normal subjects. Locally secreted IgD was detected in 30% of patients with clinically active multiple sclerosis, including two in whom magnetic resonance images of brain and spinal cord were normal and who had no evidence of intrathecal IgG synthesis. No intrathecal IgD production was detected in patients with clinically stable multiple sclerosis or those suffering from chronic progressive multiple sclerosis, while it significantly correlated with the interval from the last relapse and with the total duration of the disease process in patients with relapsing, remitting multiple sclerosis. Intrathecal IgD synthesis also correlated with the degree of cerebrospinal fluid pleocytosis and with the presence of free κ and λ light chain bands in cerebrospinal fluid. Present results supplement and expand earlier data and suggest that intrathecally secreted IgD is a putatively important part of the immune response in clinically active relapsing, remitting multiple sclerosis.**

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IgD is expressed on greater than 90% of mature B lymphocytes[1] and is considered to be an indicator of B-cell maturity. Evidence suggests that soluble (ie, secreted) IgD has a legitimate antibody function[2] that makes it particularly helpful in the study of early immune response, including that of the central nervous system.[3] The level of soluble IgD in cerebrospinal fluid (CSF), however, is extremely low compared with levels of IgG, IgA, and IgM and is technically difficult to detect. Thus, although IgD levels in the normal reference population[4] and in various neurologic disorders[5] have been previously described, intrathecal production of IgD, through the calculation of the IgD index, has only recently been reported.[6,7]

So far, the clinical importance of intrathecal IgD synthesis in patients with multiple sclerosis (MS) has not been evaluated. Similarly, immunopathologic correlation of this finding with other CSF features of MS has not been properly investigated. We report herein a combined study in which we evaluated the relationship of intrathecal production of IgD to both clinical and paraclinical manifestations of MS.

PATIENTS AND METHODS

Patients

Paired CSF and serum samples from 64 patients with clinically definite MS[8] were utilized in our study (Table 1). Patients with clinically active disease had not received immunomodulatory treatment within 9 months of CSF collection. Patients with chronic progressive MS had progressed steadily since the onset of their disease,[9] while patients with clinically stable MS had relapsing, remitting disease and were in clinical remission at the time of the lumbar puncture.

The neurologic control group (Table 1) contained patients with various neurologic disorders in whom immunologic mechanisms were unlikely to participate. This control group included patients with Alzheimer's disease (four patients), benign intracranial hypertension (four patients), cervical spondylosis without myelopathy (three patients), epilepsy (three patients), amyotrophic lateral sclerosis (five patients), cerebrovascular diseases (four patients), Parkinson's disease (four patients), and peripheral neuropathy (five patients). Normal control subjects presented with tension headache or neurosis and detailed clinical and laboratory investigations showed no sign of neurologic disease. These normal subjects were included to calculate normal reference ranges of IgD and IgG in CSF and serum samples.

Preparation of Samples

Paired CSF and serum samples were collected simultaneously, and 1000 kallikrein inactivator units of affinity purified protease inhibitor, aprotinin (A4529, Sigma Chemical Company, St Louis, Mo), were immediately added to each millimeter of test samples to prevent IgD degradation. Cerebrospinal fluid samples were selected to exclude xanthochromic specimens and specimens containing more than one erythrocyte per field at the time of collection. The CSF samples were centrifuged for 20 minutes at 200*g*, and then both CSF and serum specimens were frozen in aliquots at −70°C immediately after centrifugation and thawed just before use.

Quantitative Determinations

IgD concentration in CSF and serum samples was determined by enzyme-linked immunosorbent assay, as recently described.[7] In brief, wells of polyvinyl chloride microtiter plates were coated with 100 mL of 1:4000 dilution of anti-human δ chain antiserum (A-093, Dakopatts, Dako, Copenhagen, Denmark), blocked by 2% bovine serum albumin and 0.1% polysorbate (Tween, BDH Ltd, Poole, England) in phosphate-buffered saline, then incubated with test samples. After overnight incubation, IgD reactivity in the test samples was detected by affinity

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purified rabbit peroxidase-conjugated anti-human δ chain (A-6281, Sigma). Known amounts of human IgD standard (OTRD 03, Behring, Marburg, Germany), as well as control CSF and serum, and background control wells were included in each plate. Optical densities of test samples were averaged, read from the standard curve, and corrected by the original dilution factor. Albumin and IgG concentrations in CSF and serum specimens were assayed by electroimmunoassay.[10] Total protein content in CSF was measured turbidometrically with the benzethonium chloride precipitation technique.[11]

Other CSF Tests

The CSF specimens were examined for pleocytosis within 6 hours of the lumbar puncture. Cell count was performed with the use of Fuchs-Rosenthal chambers (Hemocytometer, Aimer Products, London, England), and cytologic examination was performed with a Giemsa stain from a cytocentrifuge (Cytospin 2, Shandon Products Ltd, Cheshire, England) preparation. Oligoclonal κ and λ light chain bands in the CSF were detected by replicate immunoblotting technique.[12]

Evaluation of Intrathecal Immunoglobulin Synthesis

Intrathecal production of immunoglobulins was determined by calculating the immunoglobulin index value,[13] which compensates for the influence of leakage of immunoglobulin from the plasma into CSF. The immunoglobulin index was determined according to the following formula: CSF immunoglobulin × serum albumin/serum immunoglobulin × CSF albumin.

Magnetic Resonance Imaging (MRI)

All patients with clinically active MS underwent MRI as part of their routine investigations, and the images were reviewed by experienced radiologists who were "blinded" to the CSF data. Magnetic resonance imaging was performed on a 0.27-T superconducting imager (Picker International, Cleveland, Ohio). The same spin-echo sequence ($SE_{2000/50}$ [numbers are milliseconds]) was performed in all patients who underwent MRI.

Statistical Methods

Values were evaluated, as appropriate, by Spearman's Rank Correlation Coefficient, linear correlation coefficient, Student's *t* test, and the Wilcoxon Rank-Sum Test. Statistical analyses were performed with SPSS/PC+ software (SPSS Inc, Chicago, Ill).

RESULTS

CSF IgD Estimation

The IgD enzyme-linked immunosorbent assay showed a coefficient of variation of 5.1% within and 5.8% between batches and had a detection limit of 0.2 ng/L. Separate studies on samples without aprotinin showed that addition of the protease inhibitor has improved detectability of CSF and serum IgD by a factor of 5. Detectability of IgG, however, was not altered by the addition of aprotinin.

One normal control subject was a high outlier for CSF IgD concentration, and the IgD amount was undetectable in three of the 18 normal control subjects and in two patients of the 32 subjects in the neurologic control group (one with Alzheimer's disease and another with epilepsy). These patients were excluded from the calculations of CSF IgD results.

In the reference control group, the CSF IgD showed a log normal distribution. Logarithmic transformation resulted in a mean ± SD reference CSF IgD of 1.86 ± 0.62 mg/L (range, 0.7 to 459 mg/L) and serum IgD of 1.54 ± 0.78 mg/L (range, 0.1 to 128 mg/L). The IgD index in the reference group was 0.12 ± 0.085, while the mean IgG index was 0.53 ± 0.16.

Table 1.—Clinical Features of the Study Population

Clinical Group	Total No. (Female)	Mean ± SD Age, y	Mean ± SD Disease Duration, y
Clinically active multiple sclerosis			
Relapsing remitting	34 (20)	33.2 ± 5.7	7.9 ± 4.2
Chronic progressive	14 (9)	37.1 ± 7.6	9.4 ± 5.1
Clinically stable multiple sclerosis	16 (10)	35.6 ± 6.4	7.1 ± 5.6
Neurologic controls	32 (17)	40.2 ± 11.3	...
Normal subjects	18 (10)	31.5 ± 8.2	None
Total	**114 (66)**	35.5 ± 7.8	...

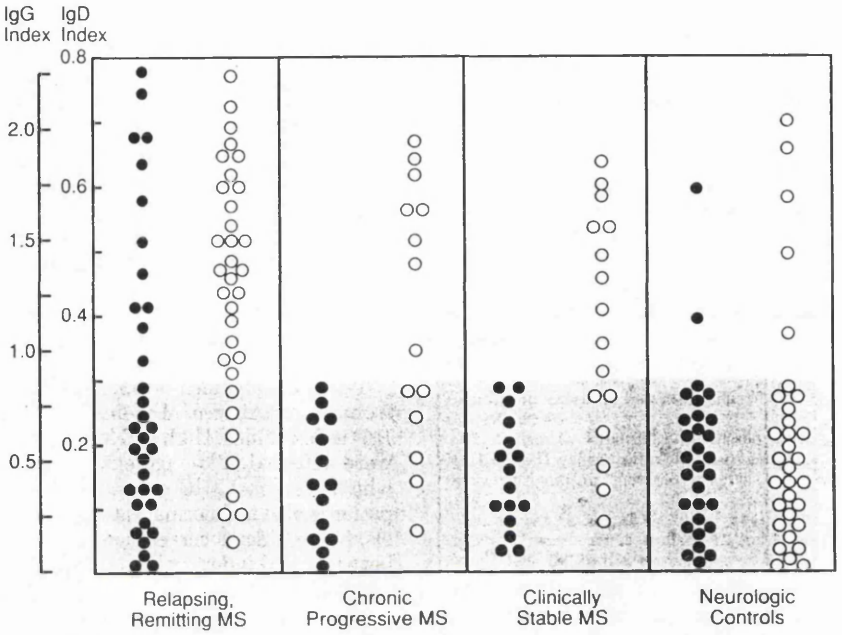

Fig 1.—Individual levels of IgD (closed circles) and IgG (open circles) indexes in the various study groups of patients with multiple sclerosis (MS). Shaded area represents the cutoff value of both indexes in the normal reference population. Statistical analyses are presented in the text.

IgD Index in MS

High levels of IgD index were detected in the CSF of 12 patients (30%) with clinically active MS but was not detected in any patient with stable MS ($P<.01$; Fig 1). None of the patients with chronic progressive MS demonstrated a high IgD index value. Significantly higher IgD index values were seen in patients with active relapsing, remitting MS compared with the neurologic control group ($P<.02$). Only two neurologic control patients (6%) showed an abnormally high IgD index; one was suffering from type 1 hereditary sensory and motor neuropathy, while the other sustained a pontine infarct. Both also demonstrated high IgG index levels.

In patients with relapsing, remitting

MS, high IgD index levels correlated with the interval that separates the onset of a clinical relapse and the lumbar puncture. Of 21 patients who had the lumbar puncture within 1 week of a relapse, 11 (52%) demonstrated high IgD index values, while only one of 13 patients from whom CSF was collected more than a week (mean, 3.6 weeks) after the onset of relapse had a high IgD index ($P<.01$). High IgD index values, however, did not significantly correlate with the number of relapses in patients with relapsing, remitting disease. A negative correlation was found between IgD index and the total disease duration in patients with active MS (Fig 2).

IgD Index and CSF Features in MS

The relationship between IgD and IgG indexes in different groups of patients with MS has already been shown in Fig 1. Abnormally high IgD index levels were detected in two patients

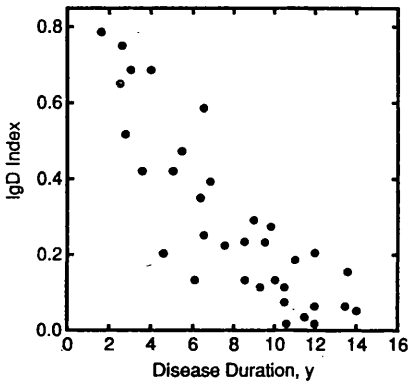

Fig 2.—Correlation of IgD index to total disease duration in patients with clinically active relapsing, remitting multiple sclerosis ($r=.857$, $P<.001$).

with active MS who had a normal IgG index. IgD index values in patients with MS failed to correlate with IgG index values ($r=.426$, $P=.08$). Furthermore, high IgG index values were detected in both clinically active and stable MS groups. Five patients (16%) in the neurologic control group had abnormally high IgG index levels: two presented with stroke, one with cervical spondylosis, and two with a high IgD index (see above).

IgD index significantly correlated with CSF pleocytosis ($P<.005$) and with the detection of CSF reactive lymphocytes, ie, cells with altered staining characteristics due to increased cytoplasmic contents of immunoglobulins ($P<.01$; Table 2). A significant correlation was also found between high IgD index and the presence of free κ ($P<.005$) and free λ ($P<.001$) light chain bands in the CSF (Table 2). There was no significant correlation between IgD index and CSF-bound κ or λ light chain bands. Similarly, there was no correlation with CSF total protein content.

IgD Index and MRI Findings in MS

Table 3 summarizes data obtained from a comparison of MRI findings with IgD index values. High IgD index levels were detected in two patients with MS who had normal MRI scans. These two patients also had normal IgG index levels. No significant correlation was found between IgD index and either the total number of MRI lesions or their anatomic distribution.

COMMENT

Human IgD is different from other immunoglobulins in structure, exon arrangement, and physiologic location (reviewed by Blattner and Tucker[14]). The secreted form is a monomer, with a sedimentation rate of 7 *s*, which consists of two heavy (κ) and two light chains. Unlike other immunoglobulins, IgD has a relatively large hinge region that is sometimes present between the first and second region domains, probably to introduce flexibility into the structure to allow the antigen binding site to achieve better contact with antigens.[15] The suggestion that IgD has no function or that it simply serves as an auxiliary antigen receptor on B cells[16] seems a trivial interpretation of the evolution of such a complex system.[14] Despite its discovery two decades ago,[17] the biologic function(s) of soluble IgD is not fully understood because of its very low serum concentration. The levels of soluble IgD in human CSF are even lower, so when relatively insensitive methods of detection are utilized, none could be detected.[18,19] Furthermore, secreted IgD is susceptible to rapid proteolysis, probably by plasmine, although the addition of ε-aminocaproic acid, which is a relatively specific inhibitor of plasmine activation, failed to stop spontaneous protein degradation in some cases.[20] We found that the addition of enzyme inhibitor of a wider spectrum, eg, aprotinin, prevents protein fragmentation in all patients studied so far.

Results obtained in our reference group showed CSF and plasma levels of IgD and IgD index values relatively similar to those previously reported.[6,7] The data reported herein extend these reports and provide good evidence that intrathecal synthesis of IgD could be detected in a substantial number of patients with clinically active MS. The significant correlation of intrathecally synthesized IgD with clinical and

Table 2.—Correlation Between IgD Index and Other Cerebrospinal Fluid Features in Patients With Clinically Active Multiple Sclerosis, Including Those With Chronic Progressive Disease

	White Blood Cell Count, ×10⁶/L			κ Light Chain Bands		λ Light Chain Bands		Total Protein, g/L	
IgD Index*	<0.005	≥0.005	Reactive Lymphocytes	Free	Bound	Free	Bound	≤0.4	>0.4
High	1	11	8	7	5	11	7	5	7
Normal	28	8	1	0	4	3	6	22	14

*Normal index value is below 0.3.

Table 3.—Correlation of Intrathecal IgD Synthesis to Magnetic Resonance Imaging (MRI) Lesions (Locations of MRI Lesions Overlap) in Patients With Clinically Active Multiple Sclerosis, Including Those With Chronic Progressive Disease

IgD Index Value (No. of Patients)	No. of MRI-Positive Patients	Location of MRI Lesions					Mean No. of MRI Lesions
		Periventricular	Cerebral	Cerebellar	Brain Stem	Cord	
High (12)	10	8	7	7	3	2	21.9
Normal (36)	31	26	17	9	8	3	16.7
Total (48)	41	34	24	16	11	5	19.3

paraclinical variables in MS suggests that secreted IgD is not merely the result of cell surface shedding, as previously suggested.[21] Although systematic studies are warranted to establish the IgD profile in relation to clinical variables in greater detail, the detection of high IgD index values might have a place in routine characterization of the humoral immune response in patients with MS. Moreover, the demonstration that high IgD index values are predominantly detected in MS relapse may imply that they could be regarded as a reasonable indicator of relatively active disease. In contrast, no significant difference in intrathecal IgG synthesis was detected between clinically active and stable disease processes.

The absence of intrathecal synthesis of IgD in patients with chronic progressive MS is interesting. There have been several reports suggesting basic differences between chronic progressive and relapsing, remitting MS, which were based on genetic[22] and epidemiologic[23] studies. Furthermore, fundamental differences in clinical[23,24] and neuroimaging[25] features were noticed between the two MS groups. The data described in our study show a basic difference in the intrathecal IgD pattern between the two groups of patients with MS. In contrast, previous studies of CSF IgG, IgM, and IgA changes failed to detect any change in immunoglobulin pattern. Lolli and collaborators,[7] however, reported lower plasma IgD levels in patients with chronic progressive MS compared with those with relapsing, remitting disease.

The absence of a significant correlation between intrathecal IgD synthesis and MRI-detected lesion burden is not surprising. Muller and collaborators[26] failed to establish any correlation between CSF absolute γ-globulin production and quantified MRI changes in patients with MS. It is quite difficult to test for the relative number of new plaques in routine MRI, as chronic lesions are also visible.[27] Until the correlation between the measured factors in acute states is standardized, one should not draw conclusions as to the precise relationship among new plaques, intrathecal IgD synthesis, and the number of MRI-detected high signals.

The intrathecal production of IgD in 30% of patients with MS and the fact that this IgD production is mainly detected in patients with recent relapse indicates that IgD may play a role in the regulation of intrathecal humoral immune response in patients with MS.

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In vivo relationship of tumor necrosis factor-α to blood–brain barrier damage in patients with active multiple sclerosis

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Summary

Tumor necrosis factor-α (TNF-α) has well recognized effects on cerebral endothelial cells and, therefore, may mediate disruption of blood–brain barrier in patients with multiple sclerosis (MS). To evaluate the in vivo relationship of TNF-α to blood–brain barrier impairment in MS, levels of this cytokine in cerebrospinal fluid (CSF) and serum samples from 38 patients with active MS and 48 controls were correlated with CSF to serum albumin ratios. TNF-α was detected in the serum of 74% and the CSF of 66% of patients with active MS. CSF levels of TNF-α were significantly higher in active MS compared to stable MS or other controls, and were significantly higher than corresponding serum levels. In patients with active MS, only those with detectable TNF-α showed signs of blood–brain barrier damage. Moreover, intrathecal levels of TNF-α in active MS correlated with albumin ratios and with the degree of barrier damage. Our findings are important in understanding some of the pathological changes in active multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system that is a major cause of neurological disability in young adults (reviewed by Ebers, 1986). The disease follows a chronic recurrent course and has several well-established immunological aberrations that involve B-lymphocytes (Walsh and Tourtellotte, 1986) and various subsets of T-lymphocytes (Waksman and Reynolds, 1984).

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There is considerable evidence that alterations of the blood–brain barrier functions and cerebral endothelial damage are important in the pathogenesis of MS (James, 1989; Koopmans et al., 1989; Kermode et al., 1990). There is also increasing realization that tumor necrosis factor-α (TNF-α) is capable of causing vascular endothelial damage both in experimental animals and humans. Effects of TNF-α that are relevant to MS include modulation of endothelial cell functions (Nawroth and Stern, 1986; Kahaleh et al., 1988), resulting in vascular endothelial damage, and an increase in vascular endothelial permeability leading to vascular leak syndrome (Tracey et al., 1987).

TNF-α has already been identified in MS brain lesions (Hofman et al., 1989; Selmaj et al., 1991a). Moreover, independent research groups have provided evidence that TNF-α levels in cerebrospinal fluid (CSF) reflect disease activity in MS (Sharief and Hentges, 1991; Tsukada et al., 1991). However, the pathogenic mechanisms of central nervous system damage caused by TNF-α remain incompletely defined. In addition, some research groups (Gallo et al., 1989; Franciotta et al., 1989; Peter et al., 1991) have failed to detect TNF-α in the CSF of MS patients. The aim of this study was to analyse the in vivo relationship of TNF-α to impairment of blood–brain barriers in acute relapsing-remitting MS and to review the distribution of TNF-α in CSF of MS patients.

Patients and methods

Patients

Paired CSF and serum samples were collected from 38 patients with clinically definite MS (Poser et al., 1983) who had the relapsing–remitting form of the disease and were in clinical relapse during CSF collection. Their clinical features are listed in Table 1. The degree of disability at the time of CSF collection was assessed using the expanded disability status scale (Kurtzke, 1983). No patient involved in the study had received immuno-suppressive treatment for at least 1 year before CSF collection. Lumbar CSF was obtained from all patients and none had a reduced CSF flux rate, e.g., due to immobilization or vertebral disc protrusion.

CSF samples were selected to exclude those containing more that 1 erythrocyte per μl at the time of collection. 1000 kallikrein inhibitory units of protease inhibitor (Aprotinin, Sigma, U.K.) were added to each ml of CSF and serum samples to prevent protein degradation. All samples were filtered through a 0.45-μm disposable sterile filter (Millipore) to remove contaminating particulate materials. samples were then frozen in aliquots at -70°C and thawed just before use.

Controls

Paired CSF and serum samples were obtained from 20 patients with stable MS to serve as disease controls. Paired samples were also obtained from 28 patients with various non-inflammatory neurological diseases, in whom blood–brain barrier damage was detected, to serve as neurological controls. They included eight patients with meningioma, three with craniopharyngioma, four with intracranial arteriovenous malformation, eight with cerebrovascular diseases, three with obstructive hydrocephalus, and two with benign intracranial hypertension.

Assays

All assays were performed in a blinded fashion on coded sterile samples. Levels of TNF-α in CSF and homologous serum samples were determined by a sandwich-type enzyme-linked immunosorbent assay (ELISA) described previously (Mitchie et al., 1988). The ELISA had a coefficient of variation of 4.8% and a lower limit of detection of 0.01 U/ml. A standard curve was run on each ELISA plate using recombinant human TNF-α in serial dilutions. A bioassay utilizing highly sensitive WEHI cells (Espevik and Nissen-Meyer, 1986) was employed to verify results obtained by the ELISA. Albumin concentrations in CSF and serum samples were assayed by electroimmunoassay (Ganrot and Laurell, 1974). CSF leukocyte count was done using Fuchs-Rosenthal chambers. CSF cytological examination was performed after centrifugation using a Shandon cytocentrifuge. The presence of reactive

TABLE 1

CLINICAL CHARACTERISTICS OF PATIENTS WITH MS INCLUDED IN THE STUDY.

Clinical group	Total No. (Females)	Age (years)		Duration of the illness (years)		Degree of disability (EDSS [a] score)	
		Mean	SD	Mean	Range	Mean	Range
Active MS	38 (24)	31.6	8.1	6.4	1–14.5	5.1	2–8
Stable MS	20 (13)	32.2	8.7	8.9	1–16.2	2.3	0–3.5

[a] EDSS, expanded disability status scale and SD denotes standard deviation.

lymphocytes in CSF was noted. These cells were distinguished from normal lymphocytes by their larger size, increased amount of cytoplasm, and darker nuclear staining.

Evaluation of blood–brain barrier

The term blood–brain barrier describes the overall exclusionary interfaces that include the epithelium of the choroid plexus, the endothelial cells of cerebral capillaries, and the layer of cells lining the arachnoid membrane (Felgenhauer, 1986). The integrity of the blood–brain barrier was evaluated by calculating the CSF to serum albumin quotient (Tibbling et al., 1977) (Q_{alb}) which is the best chemical indicator of barrier damage (Tibbling et al., 1977; Schliep and Felgenhauer, 1978; Reiber, 1980). It is noteworthy, however, that measurement of Q_{alb} represents an approximation to blood–brain barrier breakdown as it commonly measures breakdown of blood–CSF barrier. The choroid plexus, in particular, has no significant blood–tissue barrier function.

Statistics

Non-parametric Wilcoxon sum rank and Pearson's correlation matrix tests were used, as appropriate, for statistical analysis. Distribution of TNF-α in the study population was evaluated by confidence intervals for non-parametric data (Campbell and Gardener, 1988).

Results

TNF-α levels

Detectable TNF-α levels (mean ± SD = 84.3 ± 46.7 U/ml) were seen in the CSF of 25 (66%) patients with active MS and three (11%) neurological controls (one patient with craniopharyngioma and two patients with stroke) (Fig. 1). No TNF-α was detected in CSF from patients with stable MS. As also shown in Fig. 1, high TNF-α levels (mean ± SD = 29.6 ± 13.5 U/ml) were detected in the serum of 28 (74%) patients with active MS. Detectable amounts of TNF-α (mean ± SD = 13.4 ± 4.2 U/ml) were found in serum but not CSF samples of three patients with active MS and three neurological controls (one with neurinoma and two with meningioma; mean ± SD = 23.6 ± 11.2 U/ml). However, CSF levels of TNF-α in patients with active MS were significantly higher than corresponding serum levels ($P < 0.005$).

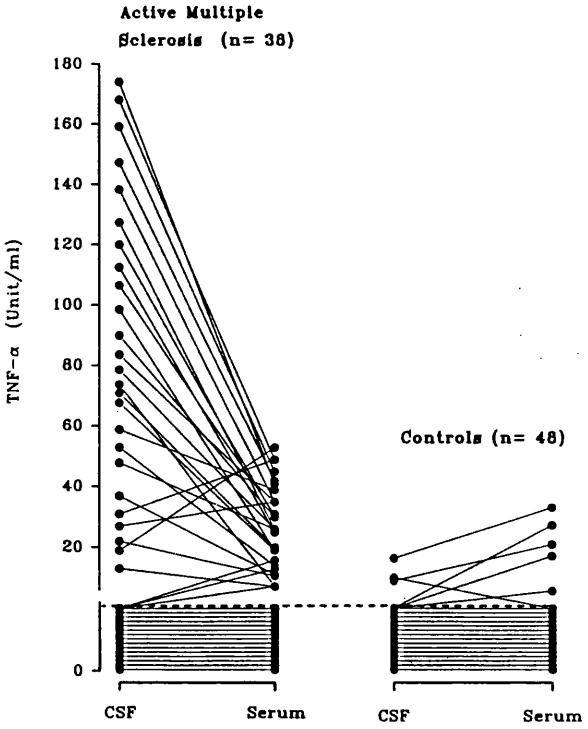

Fig. 1. Levels of TNF-α in CSF and serum samples from patients with active multiple sclerosis and controls (including 20 patients with stable multiple sclerosis). Interrupted line represents the detection limit of the TNF-α assay.

Levels of TNF-α detected by ELISA correlated well with concentrations detected by bioassay ($r = 0.73$, $P < 0.001$). In particular, the bioassay detected similar TNF-α concentrations in MS patients who had high CSF TNF-α levels by ELISA.

Correlation of TNF-α with CSF leukocytes

Cytological examination of the CSF in MS patients showed all cells to be mononuclear. No polymorphonuclear cells were detected in CSF of patients with acute MS. The relationship of TNF-α to CSF leukocytes is shown in Table 2. TNF-α in the CSF of patients with active MS failed to correlate with CSF leukocyte count ($P = 0.12$) or with the number of reactive lymphocytes ($P = 0.09$).

TABLE 2

RELATIONSHIP OF TNF-α TO LEUKOCYTES IN CSF OF PATIENTS WITH ACTIVE MULTIPLE SCLEROSIS.

TNF-α in CSF (no. of patients)	CSF mononuclear cell count ($\times 10^6$/l)		
	≤ 5	6–14	≥ 15
Present (25)	12	8	5
Absent (13)	7	4	2

Correlation of TNF-α with barrier damage

We have already established (Sharief et al., 1990) that mean CSF albumin in normal reference subjects was 198 mg/l (range 132 to 295) and the mean Q_{alb} was $2.1 \pm 1.6 \times 10^{-3}$. Twenty-eight (74%) patients with active MS had abnormally high Q_{alb} suggestive of barrier impairment. Although CSF levels of TNF-α were significantly higher than corresponding serum in patients with active MS, we calculated CSF to serum ratios of TNF-α to correct for passive transudation across damaged blood–brain barriers. In patients with active MS who had detectable TNF-α levels in CSF or serum, CSF to serum ratios of TNF-α significantly correlated with Q_{alb} (Fig. 2). In contrast, patients with active MS who had no detectable TNF-α showed no sign of barrier impairment (mean $Q_{alb} = 5.02 \times 10^{-3}$; $P < 0.001$).

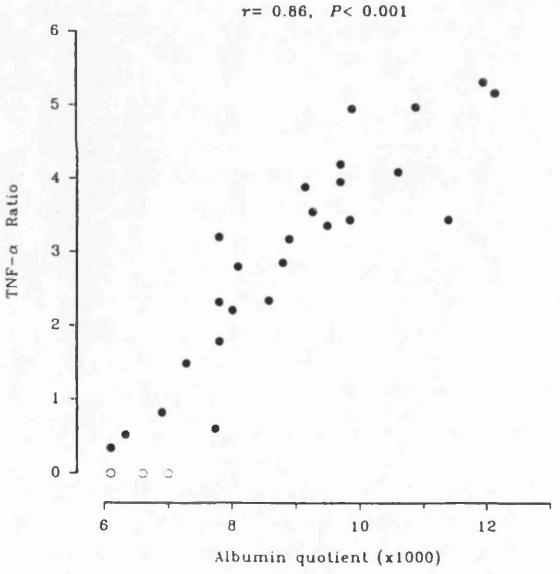

Fig. 2. Correlation of CSF/serum ratio of TNF-α with CSF/serum albumin ratio in patients with active multiple sclerosis. Open circles depict patients who had detectable TNF-α levels in serum but not CSF samples. Patients with undetectable TNF-α in serum or CSF ($n = 10$) are not included (see text).

TABLE 3

CONDITION OF BLOOD–BRAIN BARRIER IN THE STUDY POPULATION.

Clinical group (total no.)	Extent of blood–brain barrier damage [a]			
	No damage	Mild	Moderate	Severe
Active multiple sclerosis (38)	10	11	12	5
Stable multiple sclerosis (20)	12	6	2	0
Neurologic controls (28)	0	7	11	10

[a] According to CSF:serum albumin quotient $\times 10^3$ where values below 6 indicate no barrier damage, 6–8 = mild; 8.1–10 = moderate; and values above 10 = severe barrier damage.

The degree of barrier disruption in the study population was graduated according to Q_{alb} values, as described earlier (Schliep and Felgenhauer, 1978), which produced four separate groups of barrier condition (Table 3). Low CSF to serum ratios of TNF-α were detected in patients with mild barrier impairment, while high ratios were associated with severe barrier damage (Fig. 3; $P < 0.005$).

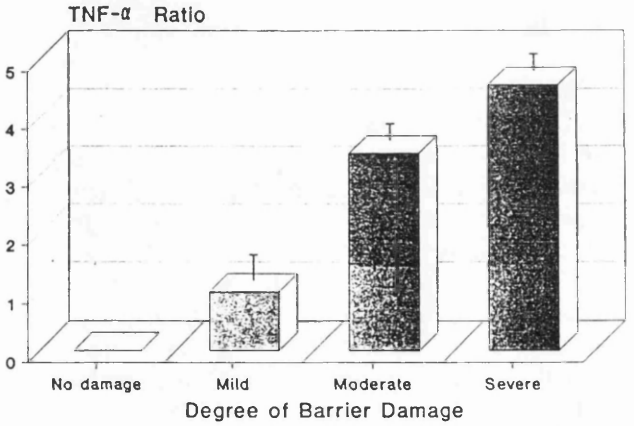

Fig. 3. Correlation of CSF to serum ratio of TNF-α with the degree of blood–brain barrier damage in patients with active multiple sclerosis. Values represent means ± SEM.

Discussion

The finding of higher TNF-α levels in the CSF of patients with active, but not in stable, MS is consistent with previous studies (Maimone et al., 1991; Sharief and Hentges, 1991; Tsukada et al., 1991). Failure to detect TNF-α in CSF of MS patients reported earlier (Gallo et al., 1989; Franciotta et al., 1989) is most probably due to differences in patients selection. We have included patients with severe disease, as shown in Table 1, a fact that might explain the unusually high CSF levels of TNF-α compared to previous studies. This fact may also explain the relatively high number of patients with active MS who showed high Q_{alb} values.

Higher CSF levels of TNF-α in patients with active MS compared to serum levels reported here are also compatible with earlier reports (Maimone et al., 1991; Tsukada et al., 1991) and suggest local release of this cytokine within the intrathecal compartment (Sharief and Hentges, 1991). In support of this observation, Selmaj et al. (1991a) demonstrated elevation of TNF-α-positive cells in MS brain lesions while no elevation of TNF-α-positive cells was detected in the spleen or peripheral blood. In this regard, astrocytes have been shown to produce TNF-α both in culture (Lieberman et al., 1989; Chung and Benveniste, 1990) and in situ in MS brain lesions (Selmaj et al., 1991a). The lack of correlation of CSF TNF-α with pleocytosis or reactive lymphocytes suggests that this cytokine may be released by CNS rather than CSF cells.

We detected a strong correlation between CSF levels of TNF-α and disruption of blood–brain barriers in active MS. Moreover, TNF-α levels correlated with the degree of barrier impairment suggesting that this cytokine may be related to the pathogenesis of barrier damage. Although immunoreactivity for TNF-α in endothelial cells has recently been noted at the edge of acute lesions in MS (Selmaj et al., 1991b), this is the first time to our knowledge that TNF-α is directly related to blood–brain barrier damage in patients with MS. However, our results are not sufficient to confirm a pathogenic role of TNF-α in blood–brain barrier breakdown. High TNF-α levels in the CSF may be secondary to barrier breakdown and inflammation in MS lesions, particularly those close to the meninges. They may be similar to abnormal immunoglobulin levels in the CSF in MS, which are usually the result of chronic inflammation.

A putative TNF-α-induced disruption of blood–brain barriers could result from several mechanisms. TNF-α causes increased vascular permeability by inducing morphological and structural changes of endothelial cells through a direct toxic effect (Sato et al., 1986). It also down-regulates endothelial cell expression of thrombomodulin and causes enhanced procoagulant activity that promotes intravascular coagulation and capillary thrombosis (Nawroth and Stern, 1986). Furthermore, leukocytes, particularly neutrophils, adherent to endothelial cells are stimulated by TNF-α to increase biosynthesis and release of reactive superoxide intermediates and arachidonic acid metabolites (Nathan, 1987). Indeed, 100 U/ml of TNF-α, a concentration readily attainable in CSF of patients with active MS, stimulate eosinophils and other cells to damage human endothelial cells in vitro (Slungaard et al., 1990). Hitherto, the interaction between TNF-α and cellular elements in MS brain lesions is not clearly identified.

It must be emphasized that endothelial damage should not be considered a result solely of overproduction of TNF-α, thereby ignoring the complex interaction between cytokines and other mediators such as prostaglandins and leukotrienes. Studies of these and other mediators will provide a further insight into the pathogenesis of barrier damage in patients with active MS. We could not detect any correlation between interleukin (IL)-1β or IL-6 and blood–brain barrier damage in MS (Sharief and Thompson; unpublished data). Such finding indicates that a TNF-α-mediated endothelial damage can be dissociated from the presence of IL-1β or IL-6. Nonetheless, further pathological studies are necessary to elucidate any pathological role of TNF-α in barrier breakdown. It should also be pointed out that in vivo damage to blood–brain barrier could be more precisely detected by magnetic resonance imaging (MRI) with gadolinium-DTPA enhancement (Hawkins et al., 1991). It remains to be shown whether intrathecal production of

TNF-α correlates with MRI-detected blood–brain barrier damage.

Findings from this study provide a molecular basis for intrathecal inflammatory response in MS patients, and implicate TNF-α in the pathogenesis of cerebral endothelial damage in MS. These findings may have important diagnostic and prognostic implications in patients with MS. They could also be therapeutically significant as the biosynthesis of TNF-α or TNF-α-dependent endothelial toxicity could be inhibited by several therapeutic manoeuvres (Tracey et al., 1989; Slungaard et al., 1990).

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The relationship of interleukin-2 and soluble interleukin-2 receptors to intrathecal immunoglobulin synthesis in patients with multiple sclerosis

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Summary

The in vivo relationship of interleukin-2 (IL-2) to the local humoral immune response within the central nervous system (CNS) in patients with multiple sclerosis (MS) is hitherto largely unknown. Intrathecal levels of IL-2 and soluble IL-2 receptors (sIL-2R) were correlated to the local CNS synthesis of immunoglobulin G, A, D, and M isotypes in 70 patients with clinically definite MS. Levels were also determined in 19 normal control subjects to establish normal reference limits. High cerebrospinal fluid levels of IL-2 and sIL-2R were detected mainly in patients with acute relapsing-remitting MS and were significantly higher than corresponding serum levels. Intrathecal levels of IL-2 significantly correlated with local CNS synthesis of IgD and IgM, while no correlation was found with either IgG or IgA. Similarly, intrathecal sIL-2R levels significantly correlated with local CNS production of IgD and IgM, but not IgG or IgA. These findings further extend previous reports and also suggest that IL-2 and sIL-2R are involved in the early intrathecal humoral immune response in MS.

Introduction

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS) that has well-established abnormal humoral immune responses (reviewed by Tourtellotte et al., 1988). Interleukin (IL)-2, a pluripotent lymphokine secreted by antigen-activated T cells (Fletcher and Goldstein, 1987), exerts biological effects on a variety of cells that include T cells, natural killer, cytotoxic, and B cells. The proliferation of activated, antigen-reactive T cell populations is dependent on the sequential expression of IL-2 and the IL-2 receptor and the subsequent binding of IL-2 to its membrane receptor.

The in vitro relationship of IL-2 to B cells is well established: IL-2 has been shown to induce

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both proliferation (Tsudo et al., 1984) and differentiation (Nakanishi et al., 1984) of B cells, and high-affinity IL-2 receptor sites were demonstrated on cloned, activated B cell lines (Waldmann et al., 1984). Moreover, these receptors were upregulated on that cell line in response to exogenous IL-2. However, the in vivo relationship of IL-2 and soluble IL-2 receptors (sIL-2R) to B cells, especially in CNS diseases, is not clear. Similarly, the relationship of these cytokines to the intrathecal production of immunoglobulins in inflammatory conditions of the CNS, such as MS, has not hitherto been reported. We report here the results of a 2-year study where we determined intrathecal levels of IL-2 and sIL-2R in 70 patients with clinically definite MS and correlated them with local synthesis of immunoglobulin G, A, D and M isotypes.

Patients and methods

Patients and controls

Paired CSF and serum samples from 70 patients (41 females) with clinically definite MS (Poser et al., 1983) were utilized for the study. Forty-six patients had relapsing remitting disease while the remaining 24 patients had chronic primary progressive MS in whom the disease process progressed steadily since onset. Samples from patients with relapsing-remitting disease were obtained within 96 h of acute exacerbation. No patient involved in the study had received immunosuppressive treatment for at least 8 months before the lumbar puncture.

Paired CSF and serum samples from 19 normal subjects (11 females) were also analyzed to establish normal reference levels. Those normal subjects presented with mild non-specific tension headache or neurotic syndromes, and neurological examination as well as detailed clinical investigations (magnetic resonance imaging, computerized tomography, evoked potentials, and cerebrospinal fluid (CSF) analysis) had excluded a possible organic origin for their symptoms.

Assays

Levels of IL-2 in unconcentrated CSF and 1 : 100 diluted serum were detected by a capture enzyme-linked immunosorbent assay (ELISA) as previously described (Gallo et al., 1988). In brief, the IL-2 standard curve and test specimens were assayed in triplicate using commercial ELISA kits (Interest 2, Genzyme Co.) according to the manufacturer's instructions. A standard curve of recombinant human IL-2 (Koch-Light, U.K.) ranging from 0.02 units (U)/ml to 120 U/ml was included in each trial, and native CSF and 1 : 100 diluted sera were used in the assay. One unit of IL-2 is defined by the manufacturer as the amount of IL-2 which is required to support half-maximal tritiated thymidine ([^{3}H]TdR) incorporation by CTLL-2 cells. Levels of sIL-2R were also measured by a sandwich enzyme immunoassay according to Zucchelli et al. (1989) using two different anti-IL-2R monoclonal antibodies; the first is adsorbed onto polystyrene microtitre wells (Falcon, Micro-Test III) and the other is conjugated to horseradish peroxidase. Standard curve was achieved by using recombinant human IL-2R (Genzyme, Boston, MA, U.S.A.).

Albumin and IgG concentrations in the test samples were analyzed by electroimmunoassay (Tourtellotte et al., 1971). Levels of IgM were measured by a modified capture enzyme-linked immunosorbent assay (Sharief et al., 1990). IgA levels were determined by an enzyme immunoassay according to Lolli et al. (1990). Cerebrospinal fluid and serum amounts of IgD were also detected by the enzyme immunoassay reported by Lolli et al. (1990) with only minor modifications. In brief, affinity purified peroxidase-conjugated anti-human δ-chain antibody (1 : 5000 dilution) was used instead of the biotin-conjugated anti-human IgD antibody originally described. Intrathecal synthesis of immunoglobulins (Ig) was measured through the calculation of the index values (Tibbling et al., 1977) according to the following formula: Ig index = (CSF Ig $\times$ serum albumin)/(serum Ig $\times$ CSF albumin).

Statistics

Non-parametric Wilcoxon sum rank test and Pearson correlation matrix tests were used, as appropriate, for statistical analysis. Analyses were performed using the SPSS/PC$^+$ software program (SPSS, Chicago, IL, U.S.A.).

Results

The assays were able to detect levels of IL-2 and sIL-2R as low as 0.1 and 1 U/ml respectively, and reproducible results were obtained on repeated testing. Results obtained from the normal control as well as MS patients groups are presented in Table 1.

Levels of IL-2 and sIL-2R in MS

Interleukin-2 levels were considerably elevated in the CSF of 25 patients with relapsing remitting MS and in two patients with chronic progressive MS ($p < 0.01$) compared to normal control group. Mean ± SD level of CSF IL-2 in relapsing-remitting MS patients was 8.32 ± 1.7 U/ml, while the mean serum IL-2 level in the same group of patients was 2.16 ± 1.2 U/ml ($p < 0.01$). In contrast, mean IL-2 level in the CSF of patients with chronic progressive MS (3.58 ± 1.6 U/ml) was not significantly different from the mean serum IL-2 level (2.6 ± 1.3 U/ml).

Compared to the normal control group, soluble IL-2 receptor levels were elevated in the CSF of 21 patients with relapsing-remitting MS and in four chronic progressive MS ($p < 0.05$). The CSF levels of sIL-2R (mean ± SD = 164.7 ± 30.2 U/ml) were significantly higher than serum levels (mean ± SD = 68.7 ± 24.3, $p < 0.01$) in patients with relapsing-remitting MS. In contrast, CSF levels of sIL-2R in patients with chronic progressive MS (mean ± SD = 140.3 ± 37.6) were not significantly different from levels in corresponding serum (mean ± SD = 74.4 ± 26.8, $p = 0.086$).

In MS patients who had detectable levels of IL-2 in CSF, significant correlation was found between CSF levels of IL-2 and sIL-2R ($r = 0.81$, $p < 0.001$). Increased levels of both IL-2 and sIL-2R in CSF were found simultaneously in 21 (30%) MS patients. Only four patients with high CSF levels of IL-2 had low sIL-2R levels (mean ± SD = 57.6 ± 28.1 U/ml) in the CSF. Similarly, only two patients demonstrated high CSF levels of sIL-2R while IL-2 was undetectable in the CSF.

TABLE 1

MEAN ± SD OF VARIABLES IN 19 NORMAL CONTROL SUBJECTS AND 70 PATIENTS WITH CLINICALLY DEFINITE MS

Values in the MS group include number (and percentage) of patients who show values above the cut-off levels of the normal controls [a].

Variable	Normal controls	Multiple sclerosis
CSF IL-2 (U/ml)	0 [b]	5.95 ± 1.1 27 (39%)
Serum IL-2 (U/ml)	2.07 ± 0.6	2.38 ± 0.9
CSF sIL-2R (U/ml)	39.52 ± 26.7	152.5 ± 32.9 25 (36%)
Serum sIL-2R (U/ml)	58.16 ± 31.4	71.53 ± 25.6
IgG index	0.53 ± 0.14	1.40 ± 0.68 55 (79%)
IgA index	0.19 ± 0.07	0.49 ± 0.28 29 (41%)
IgD index	0.13 ± 0.10	0.50 ± 0.26 17 (24%)
IgM index	0.031 ± 0.022	0.137 ± 0.089 32 (46%)

[a] 0.81 for IgG index, 0.34 for IgA index, 0.33 for IgD index, and 0.075 for IgM index.

[b] Below the detection limit of the assay.

Comparison of serum and CSF levels

Individual serum and CSF levels of IL-2 and sIL-2R in MS patients were compared to determine if these was a correlation between levels in the two compartments. No significant correlation of individual serum and CSF IL-2 levels was found ($r = 0.34$, $p = 0.081$). Similarly, there was no correlation between serum and CSF sIL-2R levels ($r = 0.33$, $p = 0.094$).

Kinetics of IL-2 and sIL-2R levels

The levels of IL-2 and sIL-2R in CSF and corresponding serum samples from MS patients were correlated with values of IgG, IgA, IgD, and IgM indices. Two subgroups were analyzed for each immunoglobulin index: first group, those with abnormally high index value, i.e. more than the cut-off level (mean ± 2 SD) of the group of 19 normal control subjects; and the second group, those with normal index value.

Cerebrospinal fluid levels of IL-2 were found to be significantly higher in patients with abnormally high IgD and IgM index values compared to levels seen in patients with normal index values, while no such relationship was observed for either IgG or IgA index (Fig. 1*A*). Similarly, levels of sIL-2R in the CSF were significantly higher in patients who had abnormally high IgD and IgM index

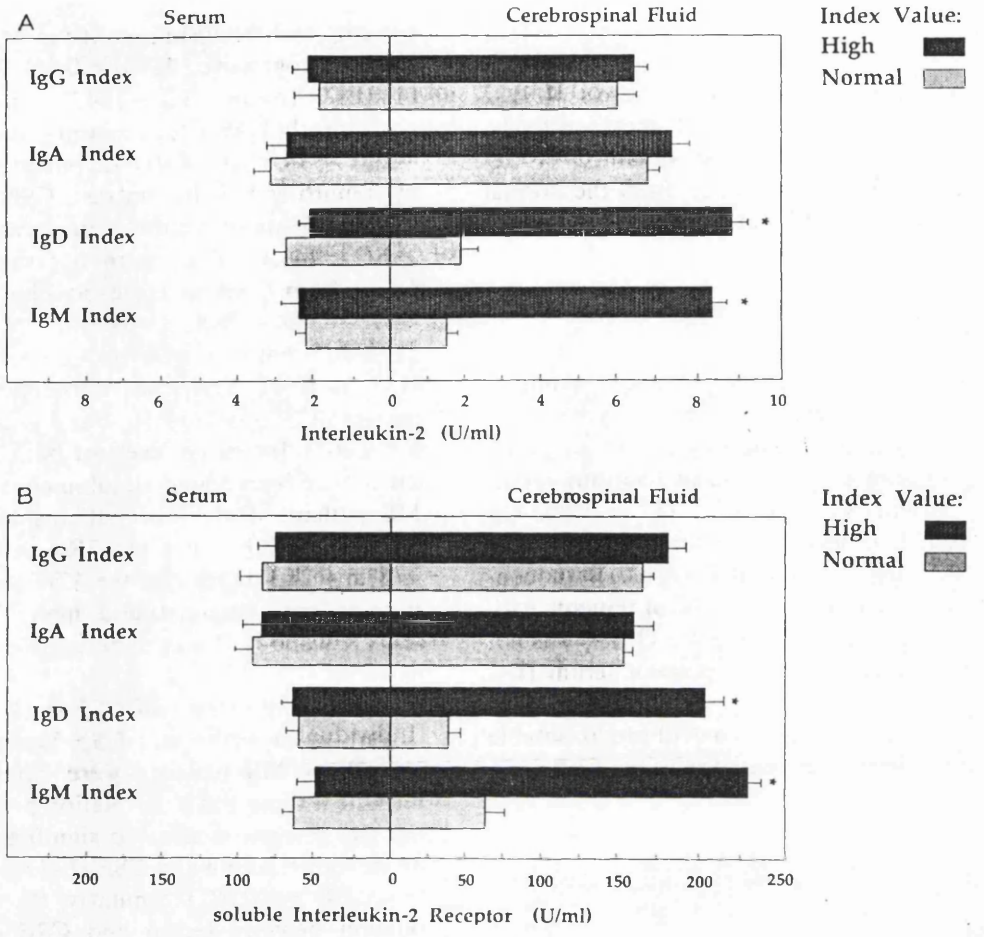

Fig. 1. Mean (±SEM) levels of serum and CSF IL-2 (*A*) and soluble IL-2 receptors (*B*) in relation to values of immunoglobulin indices in patients with multiple sclerosis. Normal index values are defined in the text. * $p < 0.01$ relative to cytokine levels in patients with normal index values.

values (Fig. 1*B*), whereas patients with high IgG and IgA index values did not demonstrate significant elevation of sIL-2R in the CSF. The serum levels of IL-2 and sIL-2R were not observed to be significantly related to any immunoglobulin index values (Fig. 1*A* and *B*).

We then analyzed the correlation between index values of individual immunoglobulins and the intrathecal amounts of IL-2 and sIL-2R. Only patients with detectable CSF levels of IL-2 or sIL-2R were included in this correlation. Cerebrospinal fluid to serum ratios of IL-2 and sIL-2R were calculated to correct for passive transudation through blood–CSF barriers (Keir and Thompson, 1986). There was a significant correlation between intrathecal levels of IL-2 and both IgD and IgM index values, while no such correlation was found with either IgG or IgA (Fig. 2). Similarly, intrathecal sIL-2R levels correlated with IgD and IgM index values, while failed to correlate with values of IgG or IgA indices (Fig. 3).

Discussion

Increased levels of IL-2 or sIL-2R or both have been demonstrated in the serum (Selmaj et al., 1986; Trotter et al., 1988; Brajczewska et al., 1989) and CSF (Gallo et al., 1988, 1989; Adachi et al., 1989) samples of MS patients. Our finding of significantly higher levels of IL-2 and sIL-2R in the CSF of MS patients relative to corresponding

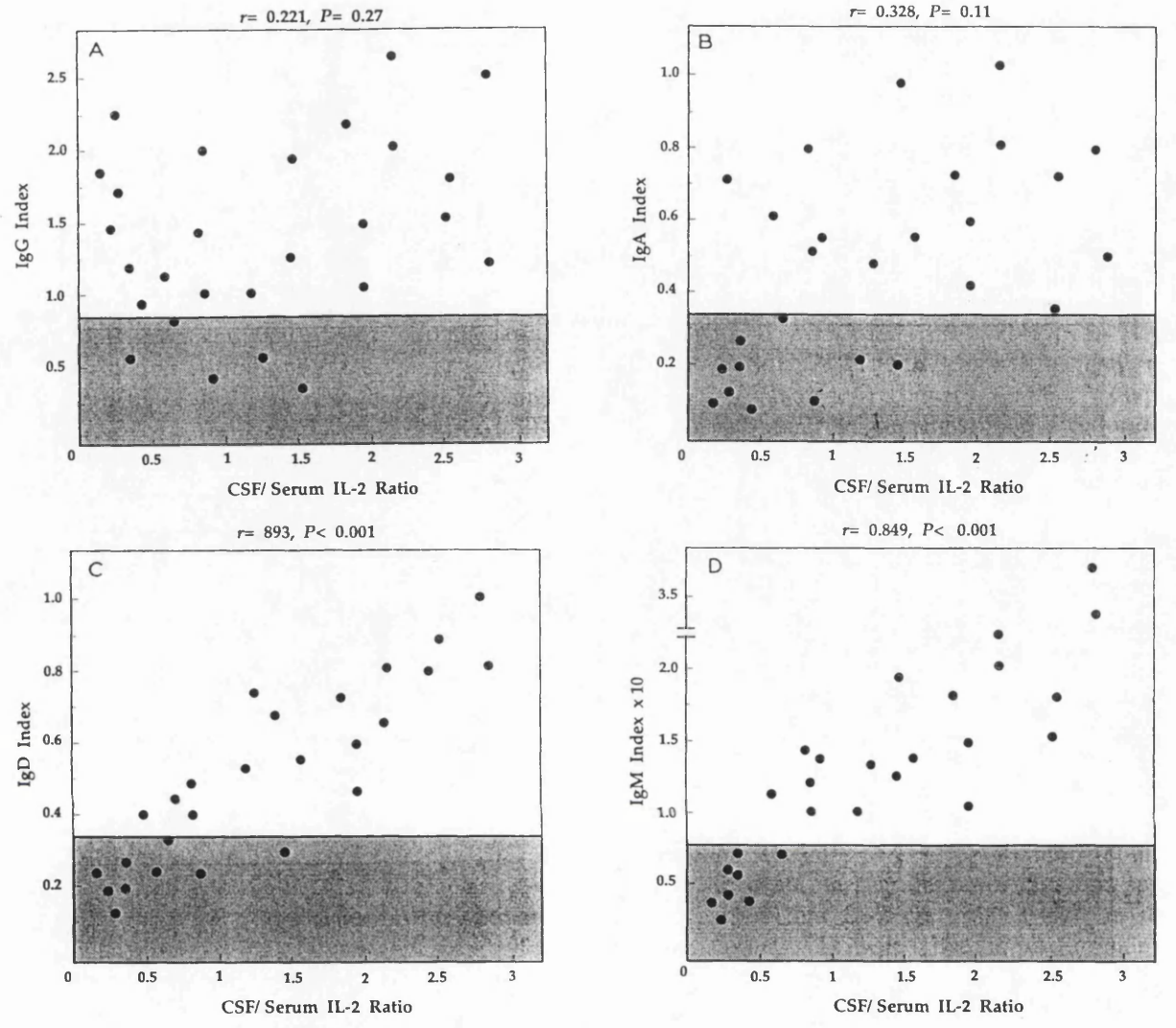

Fig. 2. Correlation between CSF/serum ratio of IL-2 and individual values of IgG index (*A*), IgA index (*B*), IgD index (*C*), and IgM index (*D*) in patients with multiple sclerosis. Patients with undetectable IL-2 levels were excluded from this correlation. Shaded areas represent reference ranges of immunoglobulin indices in normal subjects.

levels in serum suggests that IL-2 and sIL-2R are locally produced in the intrathecal compartment (Boutin et al., 1987; Kittur et al., 1990). Some previous reports (Gallo et al., 1989; Trotter et al., 1990) suggested that the increased synthesis of IL-2 and sIL-2R in MS is a systemic, rather than local CNS immune response. The difference between our findings and these reports may have been the degree of disease activity, total disease duration, degree of disability, or a combination of these or other factors in different MS groups. There are definite aberrations of intrathecal immune response in MS patients that involve both T (Waksman and Reynolds, 1984) as well as B lymphocytes (Link et al., 1987). However, the disease process is a dynamic one and MS patients are therefore heterogeneous regarding intrathecal synthesis of immunoglobulins or other soluble products of the immune cells.

Gallo et al. (1989) failed to detect sIL-2R activity in MS CSF, probably because of differences in patient selection or the use of less sensitive meth-

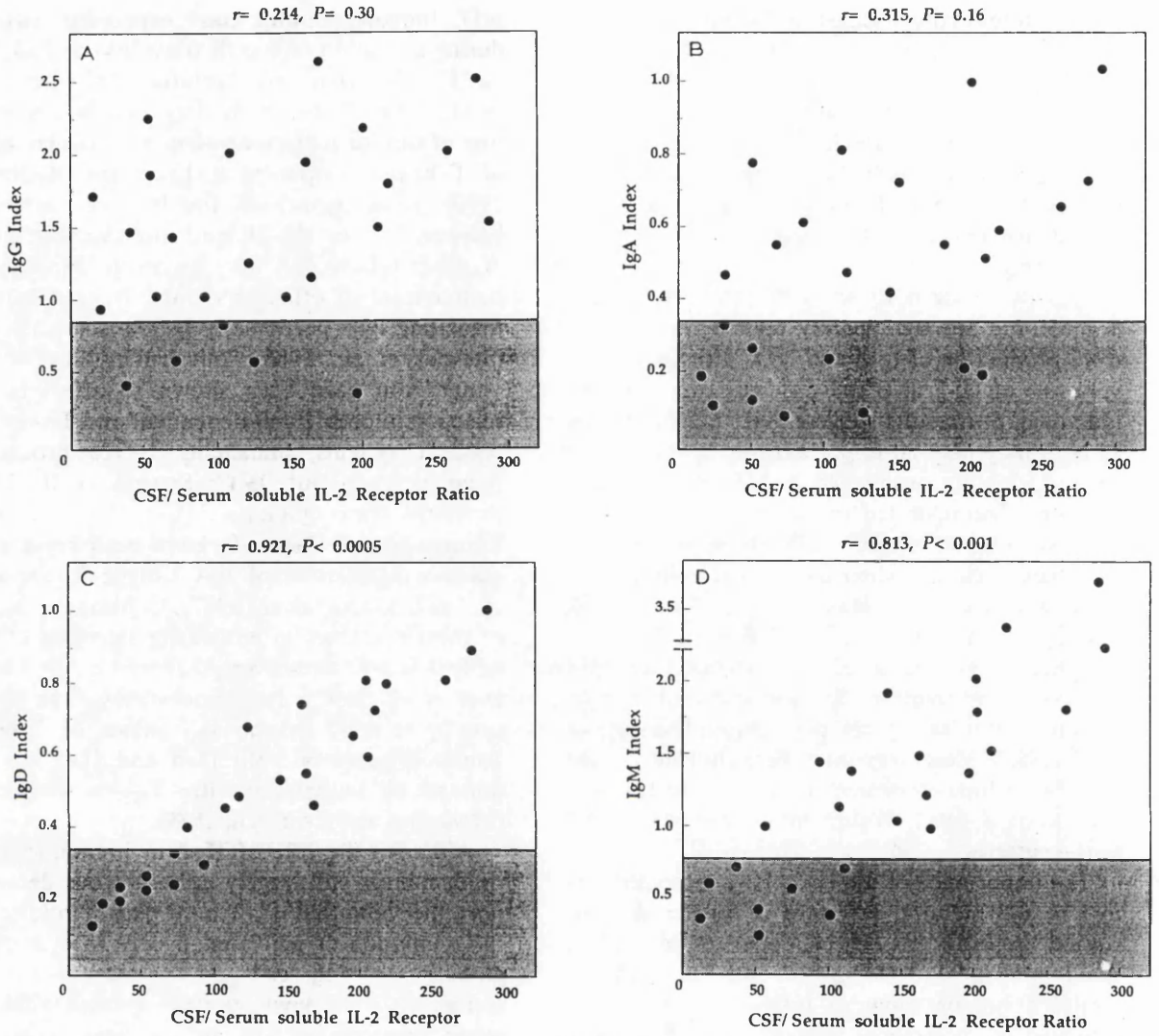

Fig. 3. Correlation between CSF/serum ratio of sIL-2R (×100) and individual values of IgG index (*A*), IgA index (*B*), IgD index (*C*), and IgM index (*D*) in patients with multiple sclerosis. Patients with undetectable sIL-2R levels in the CSF were excluded from this correlation. Shaded areas represent normal reference ranges of immunoglobulin indices.

ods. The detection of high levels of sIL-2R in the CSF of MS patients reported here, however, is in full agreement with the findings of Adachi et al. (1989) and Kittur et al. (1990). The elevated IL-2 levels in CSF from patients with relapsing-remitting MS compared to chronic progressive disease corroborate similar results reported by Gallo et al. (1989). Such finding suggests basic immunological differences between relapsing-remitting and chronic progressive MS. In fact, the two disease processes have already been reported to have differences in genetic (Olerup et al., 1987), epidemiological (Larsen et al., 1985), clinical (Confavreux et al., 1980; Larsen et al., 1985), as well as radiological (Thompson et al., 1989) features.

The increased local CNS synthesis of IL-2 in MS patients reported here emphasizes intense activation of intrathecal immune cells and further extends previous reports of IL-2 detection in active plaques and white matter in MS brain (Hofman et al., 1986; Cuzner et al., 1988). The finding of increased sIL-2R levels in CSF of MS patients

is also noteworthy because it further stresses an intrathecal immune activation in MS. High levels of sIL-2R are usually detected in conditions associated with lymphocyte activation, such as infectious (Boutin et al., 1987) or autoimmune states (Lawrence et al., 1988). In fact, increased level of sIL-2R was suggested to parallel the evolution of the demyelinating pathologic process in MS (Greenberg et al., 1988).

Results of our study show that intrathecal IL-2 and sIL-2R are significantly related to the local CNS production of IgM and IgD. Although the in vivo role of IL-2 in B cell differentiation remains unknown, our results suggest that IL-2 or sIL-2R or both may play an important role in intrathecal immunoglobulin production in MS. In vitro, IL-2 has been demonstrated to cause an 8- to 16-fold increase in levels of mRNA for the secretory form of μ heavy chains when used on B cells co-cultured with anti-IgM (Hamaoka and Ono, 1986), although high concentration (100 U/ml) of this lymphokine was required for induction of IgM synthesis. Furthermore, IL-2 induced proliferation of anti-μ-stimulated B cell populations (Nakagawa et al., 1985). These responses were abolished when anti-Tac, which recognizes IL-2 receptor (Greene and Leonard, 1986; Waldmann, 1986), was added to the cultures.

The importance of the IL-2/IL-2 receptors system in the humoral response is further demonstrated in other reports (Depper et al., 1983; Waldmann et al., 1984) when anti-Tac antibody inhibited immunoglobulin production by B cell activated by polyclonal activators. Because IL-2 stimulates T cells as well as B cells, our findings could theoretically reflect either a synthesis of T cell-derived cytokines that act on B cells, or a direct IL-2 action on B cells themselves, or both. The data do not allow us to clearly distinguish these two possibilities but suggest the relationship of the intrathecal synthesis of IgM to IL-2 is similar to that of IgD.

We did not find a significant correlation between IL-2 or sIL-2R and the intrathecal synthesis of IgG or IgA. In B cell ontogeny, the process of immunoglobulin class switching is important for the success of the humoral immune response. IgM is the first isotype expressed on the surface of B cells followed by the subsequent coexpression of IgD. Immunoglobulin class expression switches during activation of B cells from IgM to IgG, IgA, or IgE (Flanagan and Rabbitts, 1982; Takahashi et al., 1982). It has been suggested that modulation of isotype pattern involves a particular subset of T helper lymphocytes (Esser and Radbruch, 1990). As a hypothesis the lack of correlation between IL-2 or sIL-2R and intrathecal synthesis of either IgG or IgA may be due to the concomitant release of other cytokines. A candidate for regulating IgG, particularly IgG2a isotype, is IL-4 (Stevens et al., 1988). Interleukin-4 as well as γ-interferon have been shown to affect isotype selection in both T cell-dependent and T cell-independent systems, enhancing IgG2a production (Snapper and Paul, 1987; Stevens et al., 1988). Similarly, three cytokines: IL-4, IL-5 and transforming growth factor-β, have been reported to enhance production of IgA isotype (Coffman et al., 1987; Murray et al., 1987). Conversely, the role of these cytokines in enhancing secretion of IgM or IgD is still controversial (reviewed by Finkelman et al., 1990). It is noteworthy that IL-2 is usually released by the T_{H1} subset of T helper lymphocytes while both IL-4 and IL-5 are predominantly secreted by the T_{H2} subpopulation (Mosmann and Coffman, 1989).

Although the role of IL-2 in immunoglobulin production is still largely unknown our data support the view that IL-2 may play a role in the differentiation of activated B cells into immunoglobulin-secreting cells in patients with MS. Longitudinal in vivo studies of intrathecal immune response in MS are required to further elucidate the mechanisms by which IL-2 and its receptor contribute to the generation of local CNS immune response and to the control of immunoglobulin isotype selection.

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