STUDIES OF

IMMUNOREGULATION IN PATIENTS

WITH JUVENILE DERMATOMYOSITIS

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for examination for the degree of
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Juvenile dermatomyositis (JDM) is a rare, inflammatory disease of children. The aetiology and pathogenesis of the disease are unknown, although it is believed to be autoimmune in origin.

The target antigens on affected tissues have not been identified and initial experiments failed to demonstrate significant differences between the proliferative responses of control and patient peripheral blood lymphocytes (PBL) to skeletal muscle antigens. In addition, autoantibodies with specificity for skeletal muscle or endothelial cell antigens were not detected by immunofluorescence. In the absence of detectable antigen specific responses to tissues in these patients, studies of immunoregulation were undertaken to determine whether defective lymphocyte function was contributing to the persistence of tissue damage. Such studies have not previously been undertaken in JDM patients.

In vitro experiments compared cooperation between mononuclear cells from the peripheral blood of 14 JDM and 8 adult polymyositis (PM) patients with appropriate normal controls. Mitogen stimulation experiments showed anergic proliferative responses (30-50% of controls) by adult patients' cells in response to T cell mitogens. In contrast, JDM patients' cells showed no significant differences in proliferative response to T cell mitogens but did show significantly elevated ($p<0.01$) spontaneous IgM production and significantly reduced ($p<0.01$) IgM production in response to pokeweed mitogen, compared to controls. Coculture experiments indicated a defect at the level of the T cell in JDM patients.

Analysis of T cell function in JDM patients was further investigated employing autologous stimulation as a model system. Significantly reduced proliferation, compared to controls, was measured ($p<0.01$) and, more significantly, four patients, in contrast to controls, were unable to generate suppressor T cells. These results could partially be explained by inability of JDM T cell populations to produce and respond to IL-2. These results were independent of corticosteroid therapy. A JDM serum factor, identified as an IgG antibody, was observed to inhibit autologous stimulation. Further study indicated that this effect was due to inhibition of responses to IL-2, although the antibody did not appear to directly bind IL-2 or the IL-2 receptor. Although experiments indicated that the antibody might have specificity for Human Lymphocyte Antigen class II determinants, this could not be confirmed.

The activity of NK cells in the peripheral blood of 18 JDM patients was found to be significantly less than that of controls ($p<0.01$). Sequential measurements showed that this was a consistent finding, not associated with corticosteroid therapy, disease severity or lymphopaenia. The activity of LAK cells from 15 JDM patients was also significantly less than controls ($p<0.01$). This was principally due to the low frequency of LAK precursors in patient PBL. The lymphocyte population exhibiting the major share of LAK activity in normals was shown to be the CD16+ NK population. However, T cells could be induced to express LAK activity, but, this was dependent on an NK cell intermediary. NK cells from JDM patients were unable to provide this cooperation.

These results demonstrate a number of defects of immunoregulation in PBL

populations from JDM patients. Since JDM has many features of systemic disease, these abnormalities may reflect immunopathological processes at disease sites. Defective interactions and cooperation between T, B and NK cells may be associated with tolerance abrogation and development and/or maintenance of autoimmune pathology.

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ABBREVIATIONS

ADDC	Antibody-dependent-cellular-cytotoxicity
AMLR	Autologous mixed lymphocyte reaction
ANA	Anti-nuclear antibody
CAH	Chronic active hepatitis
CCM	Complete culture medium
CF	Complement fixing
CM	Culture medium
ConA	Concanavalin A
CPM	Counts/minute
CPK	Creatine phosphokinase
CVB	Coxsackievirus B
CsA	Cyclosporin A
DM	Dermatomyositis
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
EAM	Experimental allergic myositis
EBV	Epstein-Barr Virus
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked-immunosorbent-assay
FCS	Fetal calf serum
GVHD	Graft-versus-host-disease
HI	Heat inactivated
HLA	Human lymphocyte antigen
HSP	Heat shock protein
IC	Immune complex
Id	Idiotype
IDDM	Insulin-dependent-diabetes-mellitus
Ig	Immunoglobulin

IL-2R	IL-2 receptor
JDM	Juvenile dermatomyositis
JRA	Juvenile rheumatoid arthritis
LAK	Lymphokine activated killer
LDA	Limiting dilution analysis
LICC	Lectin-induced-cellular-cytotoxicity
LSP	Liver specific protein
LU	Lytic units
MAb	Monoclonal antibody
MIs	Minor lymphocyte stimulating
NGS	Normal goat serum
NK	Natural killer
O/N	Overnight
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PM	Polymyositis
PPD	Purified protein derivative
PHA	Phytohaemagglutinin
PWM	Pokeweed mitogen
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SE	Suppressor effector
SI	Suppressor inducer
SLE	Systemic lupus erythematosus
SRBC	Sheep red blood cell
SS	Sjogren's syndrome
3-H-T	^{3}H-methyl - thymidine
V	Variable
VCA	Viral capsid antigen

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CHAPTER 1

1.0 INFLAMMATORY MUSCLE DISEASE

Inflammation of skeletal muscle can occur following infection with bacteria, viruses or protozoan parasites. When the infectious organism has been eliminated from host tissues, inflammation, which is generally of short duration, usually resolves (reviewed by Kagan, 1984).

The terms polymyositis (PM) and dermatomysitis (DM) are used to describe chronic conditions, in which inflammation persists (reviewed by Mastaglia and Ojeda, 1985). Since chronic inflammatory muscle diseases are associated (a) with human lymphocyte antigen (HLA) haplotypes common in other diseases of probable autoimmune origin and (b) with the presence of autoantibodies in serum (reviewed by Pachman, 1986), an auto-immune pathology is generally believed to be responsible for the conditions.

The experimental work, described in this thesis, investigated patients with juvenile dermatomyositis (JDM) and in this chapter, the clinical features, proposed aetiology and immuno-pathology of JDM will be presented and considered.

1.1 JUVENILE DERMATOMYOSITIS

1.1.1 Clinical Features

(i) Definition

Dermatomyositis (DM) is defined as a multisystem disease, in which a characteristic skin rash is accompanied by muscle weakness (reviewed by Pachman and Maryjowski, 1984). The term juvenile dermatomyositis

(JDM) is used to describe the disease when it occurs in children below the age of 16 years.

(ii) **Historical Background**

Wagner (1887) described a case of what he called "Polymyositis". The patient had generalised muscle weakness and a pronounced skin rash. Unverricht (1891) was first to use the term "Dermatomyositis" because of the skin involvement, concurrent with muscle weakness, in the condition. Karelitz and Welt (1932) were the first to comment on the prominence of children among patients with DM.

(iii) **Symptoms and Diagnosis**

At presentation, children are generally miserable and show signs of proximal muscle weakness and/or a skin rash.

The criteria of Bohan and Peter (1975) are used by most clinicians to establish the diagnosis of dermatomyositis in both adults and children (Table 1.1). A muscle biopsy is usually performed, and evidence of damage to fibres, together with a mononuclear cell infiltrate in the muscle are diagnostic features (reviewed by Glynn, 1984). Although elevation in the serum of muscle derived enzymes is a criterion for diagnosis, creatine phosphokinase (CPK) estimations are usually unhelpful in monitoring disease activity (Miller, Heckmatt and Dubowitz, 1983). Electromyography is used as an aid to diagnosis and to distinguish myositis from neurological diseases.

TABLE 1

Diagnostic Criteria for Dermatomyositis

1)	Symmetrical weakness of limb-girdle muscles.
2)	Muscle biopsy evidence of necrosis of muscle fibres; perifascicular muscle atrophy; variation in fibre size; an inflammatory exudate, often perivascular.
3)	Elevation in serum of skeletal muscle enzymes, particularly creatine phosphokinase.
4)	Electromyographic abnormalities.
5)	Dermatologic features including lilac discolouration of the eyelids; a scaly erythematous dermatitis over the dorsum of the hands and involvement of the face, neck, upper torso, knees and elbows.

The major pathological abnormalities are localised in muscle although pathology of the small blood vessels in the skin, gastrointestinal tract, fat and small nerves have also been observed where material has been available (Banker and Victor, 1966, reviewed by Pachman, 1986). Gastrointestinal vasculitis, with mucosal ulceration and perforation, is more common in the childhood form of DM than in adults and this may be a cause of death in some patients (Spencer et al,

1984). Patients with JDM may also have a restrictive decrease in ventilatory capacity due to involvement of respiratory muscles and death may occur in some patients due to fibrosing alveolitis (Pachman and Maryjowski, 1984). Cardiac involvement in JDM has been less well substantiated (Askari, 1984), although post-mortem results from 4 out of 8 JDM patients included evidence of subendocardial and myocardial inflammatory cell infiltrates (Banker and Victor, 1966). Soft tissue calcification (calcinosis) can be the most debilitating consequence of JDM (Pachman, 1986). It is often unresponsive to therapy and calcium deposits may localise in tendons, restricting mobility.

(iv) **Classification**

There is controversy over the classification of JDM patients within the spectrum of inflammatory muscle disease. Bohan and Peter (1975) defined primary "idiopathic" DM as a separate classification but did not distinguish juvenile from adult disease. Banker and Victor (1966) and Banker (1975) stressed that the histological and clinical manifestations were unique to children, calling JDM a "systemic angiopathy of childhood". This suggested that myositis might be only one symptom of a primary vasculitis affecting many different tissues. This concept has been endorsed by Carpenter et al (1976) but has been disputed by Hudgson and Peter (1984) who, while accepting that some cases of JDM showed evidence of multi-system disease,

believed that the majority conformed to a disease with muscle as the primary target organ.

Spencer et al (1984) defined three courses of treated JDM.

(A) Patients who responded well to corticosteroid therapy and have only a brief disease.

(B) Patients who relapsed after medication was discontinued.

(C) Patients who relapsed before medication could be withdrawn.

Patients in groups B and C had a recurrent, relapsing pattern of disease with extended duration of rash and muscle weakness. Patients in group C had more severe calcinosis and skin atrophy. Crowe et al (1982) also distinguished a group of patients suffering from the severe visceral form of JDM in which cutaneous and enteric ulceration resulted from vascular occlusion.

(v) **Vascular Lesions and Muscle Pathology**

There have been, during the present decade, two detailed, retrospective studies of the histopathology of JDM patients. Crowe et al (1982) studied the muscle biopsies of 29 patients while Bowyer et al (1986) examined skin biopsies from 14 patients.

Patients who displayed a prolonged unremitting course of disease often presented with severe cutaneous vasculitis early in their illness (Bowyer et al, 1986).

Earliest abnormalities seen on skin biopsy consisted of endothelial injury in dermal capillaries and venules. Patients with extensive cutaneous vasculopathy, manifested by an ulcerating rash, also showed most severe muscle involvement, and responded poorly to treatment.

Within the affected muscle, a spectrum of endothelial cell changes progressing from swelling to necrosis and obliteration has been reported in the small blood vessels (Crowe et al, 1982). Capillaries, venules and small arteries show evidence of damage, with loss of muscle capillary network (Pachman and Maryjowski, 1984), whereas it was of variable occurrence in adult - onset DM (Pachman, 1986). Crowe et al (1982) have suggested that poor perfusion due to vasculopathy was an important element in muscle weakness in patients with both limited and chronic JDM, suggesting a similar pathogenesis for the two conditions.

The extent of inflammatory cell infiltration in vascular and muscle lesions is controversial. Bowyer et al (1986) reported that vascular lesions could occur in the presence or absence of a prominent inflammatory component. Crowe et al (1982) suggested that inflammation in muscle as indicated by mononuclear cell infiltration was usually a minor morphological indicator of disease. Glynn (1984), however, while accepting that the intensity of inflitrations may be extremely variable and that there may be no obvious

correlation between damaged fibres and the degree of inflammatory cell infiltration, stated that the histological diagnosis depended on the unequivocal presence of inflammation and fibre damage.

Although Crowe et al (1982) contended that lymphocytic vasculitis was not generally observed, except in severe chronic cases of JDM, other workers have emphasised the importance of the perivascular inflammatory infiltrate in the disease (Arahata and Engel, 1984). It is possible that discrepancies between different groups regarding the presence of the inflammatory infiltrate were due to (a) variation in progression of disease when patients presented (b) sampling errors (Glynn, 1984).

Damage to fibres, within affected muscle, may be variable (Crowe et al, 1982). Incompletely damaged fibres showed attempts at regeneration but other studies have shown that where regeneration is unsuccessful, collagenous scar tissue and fat replaced destroyed fibres (Glynn, 1984; Pachman, 1986). In contrast to adult PM, where immunohistological evidence of cytotoxic T cells invading basal lamina, destroying and replacing muscle fibres, exists (Arahata and Engel, 1986), there is no evidence of such a pathology in JDM.

(vi) Incidence and Genetics

The incidence of JDM is 10-20 times more frequent than PM in the juvenile age group and female preponderance has been reported with a ratio of 2:1 (Pachman and

Maryjowski, 1984). The association with malignancy in adult PM patients with myositis has not been found in JDM (Pachman, 1986).

These are very few reports of a familial incidence of JDM, although in a recent publication (Horati, Nikayan and Bergman, 1986), identical twins developed JDM, 2 weeks apart, following upper respiratory tract infection.

Tissue typing studies have provided evidence of similar genetic background in patients suffering many diseases of presumed autoimmune origin (Todd et al, 1988).

Typing for HLA antigens A and B showed a significantly increased frequency of HLA - B8 in 16 white JDM patients (Friedman et al, 1983a). In a further study, an association between HLA-DR3 and JDM (estimated relative risk 3.8) was observed (90% of patients positive for DR3) independent of the ethnic origin of the total of 51 patients tested, suggesting that DR3 or a gene in linkage disequilibrium with DR3 may predispose to the development of JDM (Friedman et al, 1983b).

(vii) **Treatment**

Before the use of corticosteroids in therapy, death occurred in a third or more of children with JDM and severe restriction of movement in another third. It is generally believed to be the advent of corticosteroids which has reduced the number of deaths in these

patients (Pachman and Maryjowski, 1984). In active severe cases high doses of prednisolone, up to 2mg/Kg/24h are given. The complications of long term steroid therapy can be bone necrosis, diabetes, hypertension and steroid myopathy.

Since the pathogenesis of the disease is uncertain, the method of drug selection has been empirical. The presence of an inflammatory infiltrate in sites of muscle damage in patients with myositis suggested aspects of autoimmune pathology (Cambridge, 1984b; Arahata and Engel, 1984). Therefore, immunosuppressive drugs have been used in the treatment of JDM patients who do not respond to steroid therapy or who suffer from steroid toxicity (Pachman, 1986).

Azathioprine, methotrexate and cyclophosphamide have all been prescribed but there has been no controlled trial of the use of immunosuppressive drugs in association with corticosteroids. Opportunistic infections, particularly septicaemia and pneumonia may be a potentially serious side effect of giving an immunosuppressive agent, particularly in combination with corticosteroids (Bunch, 1981). A recent trial of cyclosporin A (CsA) in 14 JDM patients refractory to or suffering complications from corticosteroids or cyclophosphamide has demonstrated the beneficial effects of the drug. Mobility increased in 13/14 patients, improvements in the mood of all patients treated were noted, and no incidents of CsA induced

nephrotoxicity occurred (Heckmatt et al, in press). The beneficial effects of immunosuppressive drugs in JDM implies a role for the immune system in the pathogenesis of the disease.

Plasmapheresis has been used in therapy (Dau and Bennington, 1981). Most cases showed a transient relief of symptoms with relapse usual after cessation of treatment (Cambridge, 1984a). Intravenous serum globulin therapy successfully alleviated symptoms in the one reported case (Roifman et al, 1987).

1.1.2 Aetiology

The aetiology of JDM is unknown. Most available evidence seems to implicate an initial insult to skeletal muscle by an infectious agent, and the response to this insult in genetically susceptible individuals, resulting in the disease.

(i) Association of Viral Infection with Myositis

A number of viruses can cause a benign, acute myositis in man including Coxsackievirus A and B, influenza A and B, parainfluenza and adenovirus 2 (Mastaglia and Ojeda, 1985). In the epidemic form of post-influenzal myositis, usually only children are affected. During the active phase, serum CPK levels are usually elevated, electromyographs may show myopathic changes and evidence of necrotising inflammatory myopathy can be found on muscle biopsy (Ruff and Secrist, 1982).

It is presumed that myositis results from infection of muscle by virus but attempts at virus isolation from

patients with post-influenzal myositis have been unsuccessful (Mastaglia and Ojeda, 1985). Influenza virus has, however, been isolated from muscle in a case of severe necrotising myopathy associated with myoglobinuria and in some cases of Reye's syndrome in which myositis may occur (Mastaglia and Ojeda, 1985).

A definite association of a picornavirus, echovirus, with myositis has been observed in a group of patients with hypogammaglobulinaemia, who failed to make an antibody response to virus (Webster, 1984). The picornavirus, Coxsackievirus B, has been isolated from patients with Bornholm disease, a benign acute myositis affecting the chest muscles (Dubovitz, 1985).

During the 1970's there were many reports of ultra-structural changes, resembling picorna virus particles, detected in muscle biopsies from patients with myositis (reviewed by Schiraldi and Iandolo, 1978). More recent interpretations of these electron microscope studies have suggested that the virus-like crystalline arrays were actually either glycogen aggregates (Denman, 1984) or due to the disease process itself (Kagan, 1984). Denman (1984) has stated that there were no consistent ultra-structural changes compatible with virus infection in any adequately controlled study of patients with PM and DM.

(ii) The Association of Coxsackievirus Infection with JDM

Coxsackieviruses A and B, members of the picornavirus family are the most myotropic of human viruses and are

estimated to cause the majority of acute myositis and myocarditis in the Western world (Grist and Bell, 1974). Coxsackieviruses are also known to infect endothelial cells (Estri and Huber, 1987). Coxsackievirus B (CVB) was isolated from a JDM patient 35 years ago (Zweymuller, 1953). Since then there have been only two descriptions of Coxsackievirus isolation from juvenile myositis patients (Tang et al, 1975; Schiraldi and Iandolo, 1978). The isolate obtained by Tang et al (1975) was of Coxsackie A 9 subtype and grown in human amnion cells. Virus crystalline structures were also detected in the patient's muscle biopsy. Schiraldi and Iandolo (1978) isolated Coxsackie B2 from a stool sample. There are several problems associated with the isolation of virus from muscle biopsy including:

(a) lack of sufficient tissue for the isolation of potential organisms

(b) lack of suitable susceptible cell lines.

Serological studies have revealed an association between Coxsackievirus infection and JDM (Denman, 1984). The presence of complement fixing (CF) antibodies to these viruses can be detected for only a few months after infection and in a detailed study, Christensen et al (1986) showed 83% of serum samples taken within 4 months of disease onset in 12 JDM patients had CF antibody to 1 or more CVB antigens. CF antibodies to B1, B2 and B4 Coxsackie subtypes were all significantly raised. Only 25% of serum samples from

children hospitalised because of viral syndromes and 25% of juvenile rheumatoid arthritis (JRA) patients's serum contained CF antibodies to CVB. In serial studies of the 12 JDM patients, remission correlated with loss of CF antibody and persistence of disease with elevation of CF antibody, suggesting persistent antigenic stimulation. There was no difference between JDM and JRA patients in CF antibody levels to 13 other viruses tested.

Travers et al (1977) measured neutralising antibody to Coxsackievirus in two adult DM patients within two months of disease onset. Antibody titres to Coxsackie B4 increased fourfold over a 2-4 week period while antibody titres to 12 other viruses remained unchanged.

The development of gene probing methods now allows the detection of viral genome in human tissues. Using a picorna virus anti-sense DNA probe, Bowles et al (1987) detected viral RNA in 4/8 JDM but not from dystrophic muscle biopsies. However, the cellular localisation of the viral genetic material could not be determined. This observation requires to be confirmed, in a larger panel of patients and controls. It may also be possible to isolate sufficient messenger RNA from tissues for sequence studies which could identify the particular CVB subtype(s) present in muscle from JDM patients. CVB is ubiquitous and yet JDM is a very rare disease. Therefore, it is possible that JDM is associated with a particular subtype of CVB, or that

other genetic and immunological factors, controlling the host response to virus, predispose to disease.

(iii) **Other Possible Aetiological Agents of JDM**

Although influenza virus types A and B have been associated with acute transient leg muscle myositis in children, Koch et al (1975) did not find significantly increased serum antibody levels to influenza viruses in JDM patients.

Parasites such as Toxoplasma can cause DM (Mastaglia and Ojeda, 1985) but in one study only 1/4 JDM patients had complement fixing antibodies to Toxoplasma Gondii (Kagen, Kimball and Christian, 1974).

A DM-like syndrome was observed in a child with chronic graft-versus-host (GVH) disease after bone marrow transplantation (Anderson et al, 1982). Graft-versus-host disease is associated with increased susceptibility to viral infection, altered immune function and autoimmunity (Ch 2.7) although no evidence of viral infection was observed in the above case.

Dermatomyositis has been described in several cases following BCG vaccination (Kass et al, 1979). BCG has been shown to induce IFN-γ production by T cells (Engleman et al, 1981) and these authors also suggested that IFN-γ may potentiate autoimmune disease, arising as a result of antecedent infection. IFN-γ has been detected in the serum of patients with vasculitis (Hooks et al, 1982) and in the basement membrane of

muscle fibres in biopsies from adult PM patients (Isenberg et al, 1986).

(iv) **Conclusion**

Infectious organisms as diverse as viruses and protozoan parasites may cause a DM-like illness in children. In most cases, this is acute, the symptoms resolving. In some patients, chronic disease results. Although CVB may be the principal aetiological agent of the disease, the evidence is limited. The observations of infection of muscle with CVB and the presence of CF antibodies to CVB in JDM patients may be due to opportunist infections (Woodruff, 1980).

1.1.3 Immunopathology of JDM

In this section, the immunohistopathology, cellular immunology and serology of JDM and animal models of the disease will be reviewed.

(i) **Muscle Biopsies**

There have been few immunohistochemical investigations of inflammatory cells in muscle lesions and none of skin lesions. The results of such investigations have, however, aided attempts at unravelling the immunopathology of chronic inflammatory muscle diseases and differentiated these diseases at the cellular level.

Arahata and Engel (1984) examined muscle biopsies from 6 JDM patients and quantitated mononuclear cell infiltrates for 4 of the patients. At both perivascular and endomysial sites, B cells were far more abundant than in adult PM and scleroderma biopsies. B cells

were most abundant at perivascular sites, representing 40% of the infiltrate compared to 36% T cells of which 60% were CD4+. At endomysial sites, CD8+ cells were the predominant T cell type but there were significantly fewer CD8+ cells in JDM compared to PM biopsies. Only 16-20 % of T cells were estimated to express class II antigens but double labelling of sections was not done in order to validate this figure.

Behan et al (1987) only included 1 JDM patient in their immunohistochemical study but found that B cell infiltration was significantly increased in muscle biopsies from 6 adult DM patients with acute (recently diagnosed) disease compared to 4 PM patients also with acute disease. In contrast to B cells, macrophage infiltration was significantly reduced in DM compared to PM biopsies.

Entirely different results were obtained by Miller, Smith and Michael (1987) in their series of 8 JDM muscle biopsies. They found no significant differences in numbers of infiltrating CD3, CD4 or CD8 cells and B cells in JDM compared with adult PM biopsies. They did, however, claim to find significantly increased numbers of CD2 expressing cells in PM as opposed to JDM biopsies. The authors did not comment on the discrepancy between the number of cells staining with CD2 compared to CD3. This was unlikely to be due to infiltrating NK cells, which although they may be present in inflammatory muscle infiltrates, may

represent only 1-2 % of the infiltrate (Behan and Behan, 1987). Miller, Smith and Michael (1987) stated that when 2 samples from a single biopsy were evaluated, different results were obtained and therefore sampling error had to be tken into account. Furthermore 1-2 fold variability was found for each antibody tested.

It is extremely difficult to reconcile these discrepancies in results between different groups. If the inflammatory infiltrate is variable in affected tissue (Glynn, 1984), sampling error is clearly a considerable problem.

The presence of macrophages and activated T cells at perivascular sites suggested that cytokines secreted by these cells may contribute to vasculopathy and muscle fibre damage (Clowes et al, 1983; Talmadge et al, 1987). The presence of B cells in cell clusters with macrophages and T cells (Arhata and Engel, 1984), at perivascular sites, indicated possible secretion of immunoglobulins (Ig).

Whitaker and Engel (1972) showed antibody and complement deposition, usually limited to the walls of small blood vessels, in muscle tissue from 2 JDM patients. Crowe et al (1982) found that vessels exhibiting non-inflammatory vasculopathy were often reactive with anti-IgM and anti-C3 reagents. Kissel, Mendell and Ramohan (1986), using an antiserum to the C5b-9 membrane attack complex (MAC) detected intense

staining for MAC of blood vessels in biopsies from 10/12 JDM patients. Deposition of MAC was predominantly in the endomysial arterioles and capillaries, although some larger perimysial arterioles also showed deposition of MAC. Vessels positive for MAC also had IgM deposition. Although these authors claimed that MAC was not detected in microvasculuture of patients with non-inflammatory neuromuscular diseases, Sewry et al (1987) have detected C8 and C9 deposits in vessels from the biopsies of some Duchenne muscular dystrophy patients.

(ii) **Cell Mediated Immunity**

(a) **Responses to Tissue Specific Antigens**

Immunohistological results, presented in the previous section, could be interpreted as evidence for auto-antigenic stimulation of infiltrating lymphocytes in muscle lesions, resulting in clonal expansion and sequestration of memory T cells within the lesion. There is indeed some evidence of such a pathology in the synovia of patients with rheumatoid arthritis (RA) (Brennan et al, 1988). The well known overlap syndrome between patients with chronic myositis and other auto-immune connective tissue diseases (Mastaglia and Ojeda, 1985) also suggests a similar immuno-pathology in JDM. However, the putative autoantigen(s) remains elusive and uncharacterised in inflammatory connective tissue diseases.

Early reports (Currie et al, 1971; Essiri, MacLennan and Hazleman, 1973) claimed to measure significant proliferation by peripheral blood mononuclear cells (PBMC) from myositis patients in response to human skeletal muscle homogenates. Two JDM patients were included in each of these studies, but not distinguished in the results. Currie et al (1971) claimed that proliferation was specific in that "very little" response to liver and kidney homogenates and collagen was found. These responses were not confined to cells from myositis patients as cells from polymyalgia rheumatica patients gave a similar degree of proliferation. In both these reports the muscle cell antigens responsible for proliferation were not defined and, indeed, even the protein concentrations of the homogenates not given. Experiments in this laboratory have not shown consistent, significant differences in the proliferative responses of 9 myositis patients's (5 adult PM and 4 JDM) PBMC to purified, human, skeletal muscle membrane preparations compared to normal control cells (A Faith and G Cambridge, unpublished data).

Furthermore, there is no convincing or consistent evidence, in the literature, of the presence of cytotoxic T cells with specificity for tissue specific antigens, in the peripheral blood of JDM

patients (Iannacone et al, 1982; Cambridge, 1984b; Pachman, 1986).

Lymphotoxin production by PBMC, in response to autologous skeletal muscle from 4 JDM patients has been reported (Johnson, Fink and Ziff, 1972). The lymphotoxin containing supernatants significantly inhibited uptake of labelled amino acids by cultured human fetal muscle cells and caused the cells to round up or disappear during culture. These experiments have not been confirmed.

There has only been 1 paper published on in vitro culture of lymphocytes from a muscle biopsy of a DM patient, in this case a 70 year old female (Rosenschein et al, 1987). A line comprising 80% CD4 cells was established which (a) gave genetically (DR) restricted proliferation to human skeletal muscle homogenate in the presence of matched antigen presenting cells (b) was cytotoxic for cultured rat skeletal, but not rat heart, muscle cells. Proliferation was, however, not shown to be tissue specific and the effect of anti-DR antibodies on proliferation not tested. The line was expanded with mitogen plus IL-2 and therefore it is possible that genetically unrestricted cytotoxicity was due to T cells activated by the mitogen (Lanier and Phillips, 1986). An alternative explanation may be that NK cells, possibly present in the biopsy (Arahata and

Engel, 1984) may have been activated in the culture conditions to express lymphokine activated killer (LAK) activity. In fact, Cambridge (1984a) demonstrated that the majority of killing of human, fetal, skeletal muscle cells by peripheral blood lymphocytes (PBL) from JDM patients was due to Fc_{γ} receptor positive cells, which are principally NK cells (Lanier et al, 1985). Therefore, on the evidence of this single patient study, it cannot be concluded that sequestration of cytotoxic T cells with specificity for skeletal muscle antigens contributes to the immunopathology of JDM.

(b) **Lymphocyte function *in vitro***

The proliferative responses of PBMC from 5 untreated, active JDM patients to stimulation by mitogens and undefined muscle antigens were reported to be similar to age matched normal children (Pachman and Maryjowsky, 1984).

Cytotoxic activity by NK cells, but not antibody dependent cellular cytotoxicity (ADCC) was significantly reduced for 5 JDM patients compared to normals (Miller, Lantner and Pachman, 1983). In a recent study of NK activity in cells from myositis patients (Gonzalez-Amaro, Alcocer-Varela and Alarcon-Segovia, 1987), 2 untreated JDM patients's cells had greatly reduced cytotoxic activity in the presence and absence of IL-2. The

author suggested, on the basis of studying 4 unclassified myositis patients, that NK cells were reduced in number.

(iii) **Peripheral Blood Lymphocyte Subsets**

Analysis of peripheral blood lymphocyte subsets may provide initial evidence of immuno-regulatory defects in autoimmune diseases. There have been three studies which included JDM patients in subset analysis.

In a study of 3 JDM patients, Strelkauskas, Schauf and Dray (1976) reported decreased T and increased B and null cells compared to normals. Lisak et al (1984), in a study of 15 PM and DM patients, whose ages were not given, showed significantly reduced CD8+ but not CD3+ or CD4+ PBL. In contrast, Iyer, Lawton and Fenichel (1983) found no differences in T cell subsets in a study of 6 PM and 1 JDM patients. Clearly, insufficient JDM patients have been tested to draw any conclusions.

(iv) **Serological Studies**

The identification of immune complexes (IC) and complement components in the involved small blood vessel wall at the site of muscle damage in JDM patients suggested the possibility that patients might develop organ specific antibodies (Whitaker and Engel, 1972). However, there were no differences between patient and control sera in their antibody binding patterns to skeletal or smooth muscle, thyroid or gastric tissue antigens (Pachman and Friedman, 1985).

Furthermore, antibodies to muscle specific proteins, such as myoglobin have been found in a number of myogenic and neurogenic diseases and are not confined to myositis patients (Pachman, 1986). Anti-human sarcolemma antibodies have not been detected in the sera of JDM patients (reviewed by Cambridge, 1984b). Antibodies to normal, human endothelial cells have also not been detected in JDM sera (Behan and Behan, 1985), suggesting that IC deposition in the microvasculature in affected muscle could be due to uptake of Ig by Fc receptors present on endothelial cells.

Attention has recently been focused on serum auto-antibodies to subcellular particles. Anti-nuclear anti-bodies (ANA) have been detected, by immuno-fluorescence, in the serum of some JDM patients (Table 1.2). Sullivan, Cassidy and Petty (1977) found ANA in 8/41 (20%) of JDM sera while Pachman et al (1985) detected IgG anti-ANA in serum from 21/90 (23%) of JDM patients. Pachman and Friedman (1985) also noted that of those JDM sera tested within four months of disease onset and containing anti-CVB antibodies, 60% also contained an ANA which had a coarsely speckled pattern by immunofluorescence. This antibody did not have specificity for the cytoplasmic t-RNA histidine synthetase enzyme (Jo-1). Reichlin (1984) also found that anti-Jo-1 antibodies occurred very infrequently in JDM compared to PM sera, although antibodies to two nuclear proteins, Mi-1 and Mi-2, were found in some JDM sera.

Arad-Dann et al (1987) have used purified 200S ribonucleoprotein (RNP) extracts as antigens in Western blotting and detected IgG antibodies to a 56kD component in sera from 12/16 adult DM, PM and myositis/SLE overlap patients compared to 2/20 SLE patients without muscle involvement. Sera from 15 JDM patients have now been tested in this Western blotting assay and all contained the anti-56kD RNP antibody (G Cambridge, personal communication), implying that a serological marker of myositis may now have been identified.

Significantly increased levels of circulating immune complexes (IC) have been detected in JDM sera (Spencer, Jordan and Hanson, 1980; Pachman and Friedman, 1985) and a correlation found between the presence of ANA and of IC (Pachman and Friedman, 1985). However, no correlation was found between IC levels in JDM sera and disease severity (Pachman et al, 1985), suggesting either that IC detected in sera bore little relation to IC in affected muscle or that IC were not contributing significantly to disease activity (Crowe et al, 1982).

TABLE 1.2

Characteristics of Anti-nuclear Antibodies (ANA) Detected in JDM Sera

Antigen	ANA Pattern	Prevalence in Myositis	Reference
Mi-1	Particulate	3% of JDM, not detected in PM	Reichlin (1984)
Mi-2	Homogeneous	20% of JDM, rare in PM	Reichlin (1984)
Undefined	Speckled	23% of JDM	Pachman and Friedman (1985)
56kD Nucleo-protein	-	75% of Adult PM and DM	Arad-Dann et al (1987)
PM-1	-	7% of JDM	Pachman and Friedman (1985)

(v) **Animal Models of Inflammatory Muscle Disease**

Although several animal models of chronic myositis have been developed, there is no established model of DM. Vasculitis is a minor and transient feature of models of chronic myositis. However, these models have provided strong evidence of autoimmune pathology, which has been lacking in the human disease.

(a) Experimental Allergic Myositis

Repeated immunisations, with homologous muscle tissue in complete Freund's adjuvant, of rats and guinea pigs resulted in the development of experimental allergic myositis (EAM), with muscle lesions consisting of focal segmental necrosis and an accompanying inflammatory infiltration around the affected fibres (reviewed by Whitaker, 1982). The disease could be passively transferred to the same inbred strain of rats by lymphocytes but not by serum.

(b) Coxsackie Virus Induced Inflammatory Muscle Disease

Inoculation of mice with some strains of Coxsackievirus B1 (CVB1) has been shown to induce myositis as well as myocarditis. Genetic factors predisposed to myositis since only some mouse strains, particularly the outbred Swiss COH, were susceptible (Ray, Minnich and Johnson, 1978). Strongwater et al (1984) showed that myositis could be induced in Swiss CD1 mice after challenge with CVB1. By day 8 after challenge, there was

widespread necrosis of muscle cells, inflammatory cell infiltration into affected muscle and, importantly, proximal muscle weakness. Weakness persisted for more than 10 weeks, long after detectable virus had disappeared from the animals, suggesting a possible autoimmune response induced by virus.

Further evidence for the role of the immune system has been provided by experiments showing that T cell deficient, athymic Swiss CD1 mice are not susceptible to chronic myositis induced by CVB1 (Ytterberg, Mahowald and Messner, 1987). When reconstituted with spleen cells from normal Swiss CD1 animals, the athymic mice developed myositis on challenge with CVB1. Myositis was not simply due to acute virally induced muscle damage since both athymic and normal mice had equivalent viral titres in muscle after challenge.

There is strong evidence that myocarditis, following antecedent infection with CVB proceeds by an autoimmune T cell dependent mechanism (Estri, Smith and Huber, 1986; Estri and Huber, 1987). These workers have shown that myocarditis may be induced by recognition of cardiocyte autoantigens by cytotoxic T cells. However, IgG anti-cardiac cell autoantibodies may be the principal mediators of CVB induced myocarditis in some strains of mice (Estri and Huber, 1987). The

cell component against which these autoantibodies are directed has been suggested to be cardiac myosin (Rose et al, 1988).

In CVB induced myositis, the putative autoantigen(s) inducing T cell mediated disease has not been identified. Also, genetic factors such as the role of the MHC and that of genes controlling the ability of CVB to bind to and penetrate skeletal muscle has not been investigated.

(c) Familial Canine Dermatomyositis

A spontaneously appearing DM has been described in Collie dogs (Hargis et al, 1986). The disease first appeared in 7-11 week old dogs and was variably severe. Clinical, electromyogram and histologic evidence of myositis was present in dogs severely or moderately affected by dermatitis. Vascular lesions and necrosis of vessel walls was found in affected sites within muscle and skin. No viruses were isolated from muscle and none of the dogs had a positive ANA titre.

High levels of IC and serum IgG were found in severely affected dogs and elevated levels of IC appeared before or concurrent with dermatitis, myositis developing later. However, immunofluoresence tests for IgG in the skin and IgG and C3 in vessels in muscle were consistently

negative. The authors believed that IC's might be important in the pathogenesis of the disease but evidence was lacking. This may, however, be a very useful model of DM although further advances probably await the application of monoclonal markers for dog lymphocytes and genetic inbreeding studies.

(vi) **Conclusion**

The presence of autoantibodies and IC in patients's sera and perivascular lymphocytic infiltration suggests a role for the immune system in JDM. Further evidence for this role is implied by the HLA associations of the disease, the beneficial effects of immunosuppressive therapy and animal models of related diseases. However, there is no evidence that myocytotoxic T cells contribute to the pathology of JDM.

CHAPTER 2

2.0 AUTOIMMUNITY

This chapter will describe areas of research into autoimmunity of relevance to JDM. These areas will be discussed in the following order:

(a) recognition of self

(b) viruses and autoimmunity

(c) HLA class II associations of self-recognition

(d) regulation of tolerance induction in the thymus and the periphery

(e) immunoregulatory T cells

(f) graft -versus - host disease - a model of autoimmunity.

2.1 Recognition of Self

The immune system must tolerate self-antigens, yet mount a vigorous response to clear and eliminate foreign antigens including pathogenic organisms. However, cross-recognition, by T cells of an epitope present on both a pathogen and a self-molecule can occur. There are 2 reports of such molecular mimicry of mycobacterial antigens. In the first, an arthritogenic T cell clone (Cohen, 1986) responded to an epitope, expressed by both the 65kD mycobacterial antigen and cartilage peptidoglycan (Van Eden et al, 1988). The second report showed that cloned T cells, derived from a normal BCG vaccinated individual, responded to the conserved portion of a 22 amino-acid sequence, expressed by both mycobacteria and

human cells (Young, Mehlert and Smith, 1988). These 2 reports indicate, that although autoimmune disease may result by a breakdown in self-tolerance due to molecular mimicry, this is not an inevitable consequence. Further evidence that recognition of self and autoimmune disease can be distinguished has come from observations that autoantibodies can be detected, in low concentrations, in the serum of otherwise healthy individuals (Hooper et al, 1972).

Autoimmune disease may be the final stage in a graded process which could include the beneficial effects of recognition of self (Cohen and Cooke, 1986). These authors have suggested that natural autoantibodies, present in normal individuals, may serve to blind the immune system to recognition of self-determinants. Coutinho and his associates (Martinez et al, 1988) have suggested that natural autoantibodies may share variable (V) regions with T cell receptor (TCR) V regions, present on a sub-population of activated T cells responsive to self-antigens. This linkage of receptors for self-antigens on antibodies and T cells may provide a mechanism for control of autoreactivity.

However, in some individuals, putative control of autoimmunity may break down and autoimmune disease may result. This could occur in sites of infection, where an inflammatory response may cause normally sequestered host molecules to be exposed to the immune system (Cohen, 1986). Inflammatory mediators such as protease enzymes might also expose neoantigens, 'altered' self, to which the immune system had not been rendered tolerant. Alternatively, the presence of adjuvants such as

bacterial lipopolysaccharides, lymphokines such as IL-2 and IFN-γ, and enhanced MHC antigen expression on epithelial and endothelial cells, in sites of inflammation, may overwhelm counterbalancing self-tolerance mechanisms (Cohen and Cooke, 1986). The animal models of myositis (Ch. 1.1.3v) suggest that potentially autoreactive T cells are present in the periphery of normal animals and an initiating event, such as CVB infection, precipitates the abrogation of self-tolerance and the development of autoimmune disease.

2.2 Viruses and Autoimmunity

It has long been recognised that infection may be one of the factors contributing to the development of autoimmune disease (Williams, 1977). Most infectious organisms possess tropisms for specific tissues and induce local acute inflammatory responses. Acute inflammation of the joints has been associated with infection by mycoplasma, bacteria such as salmonella, and viruses such as rubella and parvovirus. Often, children are particularly susceptible during epidemic outbreaks.

In a limited number of patients, inflammation may persist and become chronic. Several theories have been proposed as to why chronic inflammation should result from infection, but all emphasise the centrality of the host immune response in the process (Denman, 1984).

There are many ways in which viruses may interact with the immune system after infection of host tissue. A virus may induce cytopathic effects in infected cells, self-components may be exposed and autoimmune reactions may occur in a

genetically susceptible host. This process has recently been suggested as occurring in a model of myocarditis induced by Coxsackievirus B3 infection (Rose et al, 1988), where the self-component was identified as cardiac myosin.

Viral infection of cells may cause the appearance of virally encoded neoantigens on the cell membrane and the presentation of these antigens to the immune system. Antibodies to viral components may cross-react with self antigens. Such cross-reactivities have been postulated as explaining the presence of antibodies to the autoantigens, histidyl t-RNA and alanyl t-RNA synthetase in some adult PM patients (Walker and Jeffrey, 1986).

Molecular mimicry has also been postulated as a cause of auto-immunity in patients with RA (reviewed by Fox et al, 1985). These patients were found to have high titres of antibodies to a 62kD nuclear protein expressing unusual glycine-alanine repeat sequences, also expressed by the Epstein-Barr virus (EBV) encoded nuclear antigen, EBNA-1. Anti-EBNA-1 antibodies were shown to react with determinants present in the synovial lining cells of RA but not normals or osteoarthritic patients. However, since the vast majority of normal adults have been infected with EBV and yet do not apparently express the 62kD protein in synovial tissue, the concept of molecular mimicry as postulated by Vaughan and his colleagues (Fox et al, 1987), appears to have other implications. Their results imply 'altered' self, biochemical differences between the synovial lining cells of RA patients compared to normals. Such alterations in self could result from infection. However, EBV

genomic DNA has not been isolated from RA synovium. It is of course possible that the original antigenic insult in synovium was induced by EBV, which was then cleared from the site by the immune system.

There is more substantial evidence of an association between EBV and another autoimmune connective tissue disease, Sjogren's syndrome. Genomic DNA from EBV has been isolated from the salivary glands of patients with Sjogren's syndrome (SS) (Fox et al, 1987). EBV replication occurs in salivary glands during both primary infection and reactivation. Induction of MHC class II expression has been observed with IFN-γ containing supernatants, secreted by T cells derived from salivary glands of patients with SS (Fox et al, 1986). The epitopes recognised by these T cells are unknown, but, anti-bodies to the cytoplasmic antigen, SS-B, detected in the serum of SS patients, precipitate a protein capable of complexing with EBV encoded RNA. Intracellular proteins such as SS-B are believed to regulate post-transcriptional processing of messenger RNA (Denman, 1984), and by binding to viral RNA, may be recognised as altered self and presented to host T cells. However, it is also possible that, rather than inducing inflammation in salivary glands, reactivation of EBV infection may be a consequence of inflammation of the glands.

2.3 HLA Class II Associations of Autoimmune Diseases

Sjogren's syndrome, in common with several other autoimmune diseases, including JDM, is associated with the HLA class II antigen, DR3. Genetic restrictions are imposed upon recognition of epitopes by T cells, by amino acid sequences

encoded by the polymorphic MHC class I and class II genes (Rothbard et al, 1988; Lamb et al, 1988). Skewing of the antigenic repertoire of T cells, due to specificities imposed by the antigen binding site of class II molecules, may be particularly associated with autoimmune disease (Todd et al, 1988). These authors have shown that a single amino acid, at position 57, of the DQ_B chain, correlated strongly with susceptibility and resistance to insulin-dependent-diabetes-mellitus. Residue 57 is situated in the antigen binding cleft of the proposed MHC class II binding site (Rothbard et al, 1988). However, it is not understood if and how MHC class II restrictions skew the T cell repertoire towards recognition of autoantigens.

A simple explanation would be that certain class II haplotypes can present autoantigens to T cells. However, it is difficult to explain how a single, polymorphic class II determinant, such as DR3, could present so many different epitopes derived from a diversity of tissues within sites of inflammation. One possibility is that each polymorphic class II determinant can indeed bind to and present many different epitopes. Peptide binding and competition studies have indicated that this may be the case (reviewed by Bersofsky et al, 1989). Alternatively, it is possible that homologous T cell epitopes are expressed by a variety of autoantigens within different tissues. Heat shock proteins (HSP) are intracellular molecules synthesised by cells of many lineages in response to temperature increase, oxidative injury or viral infection (reviewed by Polla, 1988). Antibodies to the 70kD HSP have

been detected in the serum of patients with both systemic lupus erythematosus (SLE) and RA (Young, Mehlert and Smith, 1988). Although no HLA class II restriction has yet been observed for the 70kD HSP, it has been shown that the 19kD HSP is restricted by DR1 (Lamb et al, 1988). Therefore the ubiquitous HSP, possibly restricted to certain class II haplotypes and synthesised in sites of infection and inflammation, could provide an antigenic source for autoimmune attack.

2.4 Tolerance and Clonal Deletion

In the previous sections, mechanisms by which autoimmune disease could be precipitated, have been discussed. However, recent experimental evidence has demonstrated that the normal immune system has evolved ways to prevent self-recognition.

Burnet (1957) proposed that clones of lymphocytes which had receptors for self-antigens were deleted during development in order to prevent auto-immune reactivity. Several recent studies have indicated that clonal deletion takes place in the thymus, the lymphoid organ in which the mature T cell repertoire is selected (reviewed by Sprent et al, 1988). Evidence of clonal deletion of T cells recognising murine self antigens such as the MHC class II molecule, I-E (Kappler et al, 1988), the male antigen H-Y (Kisielow et al, 1988) and an allele of the minor lymphocyte stimulating locus, Mls_a (Kappler et al, 1988), has been presented. The results indicated:

(a) the process of deletion is associated with the disappearance of T cells expressing certain gene products

of the variable (V_B) region of the T cell receptor (TCR). The implication is that antigen specificity of the TCR resides, at least partially, in that particular V_B region amino acid sequence;

(b) if a self antigen cannot be presented in the context of a particular MHC haplotype, due to failure of the antigen to bind to the MHC molecule, then T cells expressing the antigen binding V_B region need not be deleted;

(c) both class I and class II MHC restricted T cells may be deleted.

The theory of clonal deletion has been unable to explain how self-epitopes, not expressed intrathymically, can be presented to developing T cells. However, it now appears that thymic antigen presenting cells (APC) may constitutively express self-epitopes, believed until recently, to be restricted to the periphery (Lorenz and Allen, 1988). Furthermore, APC isolated from the peripheral organs (spleen and liver) of normal animals also constitutively expressed self-peptides, derived from the haemoglobin molecule, and could present these peptides to cloned T cells. It is therefore possible that if autoreactive T cells escaped clonal deletion and seeded the periphery, they could then respond to constitutively expressed self-peptides, unless subjected to tolerance induction.

2.5 Role of IL-2 in Tolerance Induction and Abrogation

It is well established that peripheral T cells can be tolerised to foreign antigens (reviewed by Jenkins et al, 1987). Tolerance induction was associated with greatly

reduced transcription of message for IL-2, the most important soluble factor required for T cell growth. It is possible that a similar mechanism exists to tolerise autoreactive T cells. There is indeed some evidence to support this hypothesis, based on observations that administration of IL-2 can abrogate tolerance to autoantigens (reviewed by Kromer, Schaustein and Wick, 1986; Malkovsky and Medawar, 1984; Riemann and Diamanstein, 1981).

Further evidence for this hypothesis is the ability of exogeneously administered IL-2 to exacerbate autoimmune disease. The BB rat is characterised by the spontaneous development of immune-mediated insulin-dependent diabetes (Kolb et al, 1986). The administration of IL-2 to BB rats accelerated the development of diabetes.

If IL-2 were capable of inducing autoimmunity, high levels of IL-2 might be expected in sites of autoimmune disease, and, in fact, high levels of IL-2 have been detected in the synovial fluid of RA patients (Wilkins et al, 1983). Another important question is whether IL-2 dependent auto-reactive T cells can be isolated from affected tissues. Such T cells have been isolated from thyroids of patients with autoimmune thyroiditis (Londei, Bottazzo and Feldmann, 1985). When cloned, many of these T cells responded to class II expressing autologous thyrocytes.

2.6 Immunoregulation and Autoimmune Disease

Tolerance induction and abrogation may be partially regulated by secretion of, and response to, IL-2 by T cells, via an autocrine mechanism, forming one part of a wider system of

immunoregulation. This system may be maintained by antigen-receptor interactions, in which a homeostatic balance is maintained by positive and negative stimuli. These stimuli are conveyed by regulatory T cell subsets, providing help or suppression for the immune response. The following section will consider features of regulatory T cells delivering signals resulting in suppression.

2.6.1 Human T Cell Subsets

T cell populations are divided, on the basis of membrane antigen expression, into CD4+ and CD8+ subsets. The CD4+ subset can now be further divided (Table 2.1) into cells which provide help to B cells and those which appear to be essential intermediary cells in the induction of suppression (Kansas and Engleman, 1987; Takeuchi et al, 1987).

Evidence for discrimination between CD4+ T cell subsets has been provided by Schlossman and associates (Morimoto et al, 1986) and confirmed independently by Beverley's group (Beverley, 1987). Two essentially reciprocal subsets were identified by monoclonal antibodies (MAb's) recognising determinants now designated as CD45R and UCHL1. These 2 antigens are expressed by reciprocal subsets of CD4+ cells due to differential splicing of messenger RNA coding for the leucocyte common antigen, T200 complex (Beverley, 1987). The function of suppressor-inducer (SI) cells was found to be a property of the CD4+ CD45R+ subsets, while CD4+ UCHL1+ cells were required as helpers for antibody production. A marker, identified by the MAb, Leu8, has also been shown to differentiate SI (Leu8+) and helper (Leu8-) CD4+ subset (Damle

et al, 1984). CD45R has recently been reported to be a differentiation antigen (Akbar et al, 1988) and it is therefore possible that Leu8, a lineage marker (Beverley, 1987), may more precisely define the SI subset.

Reciprocal subsets of CD8+ T cells have also been described (Rich, El Masry and Fox, 1986) (Table 2.1). CD8+ CD28+ T cells were found to be precursor and effector cytotoxic cells while suppression of antibody synthesis was restricted to the CD8+ CD11+ subset.

Table 2.1

Phenotypes and functions of human T cell subsets

Subset	Proposed Function	Reference	
CD4+ UCHL1+	Helper	Beverley	(1987)
CD4+ CDw29 (4B4)	Helper	Morimoto et al	(1986)
CD4+ Leu 8+	Suppressor-inducer	Kansas and Engleman	(1987)
CD4+ CD45R (2H4)	Suppressor-inducer	Takeuchi et al	(1987)
CD8+ CD28+	Cytotoxic	Rich, El Masry and Fox	(1986)
CD8+ CD11+	Suppressor-effector	Rich, El Masry and Fox	(1986)

2.6.2 Functional Evidence for Suppressor-inducer Subset of CD4+ T Cells

Engleman and colleagues have identified CD4+ Leu 8+ cells as inducing antigen specific suppression in CD8+ T cells (Damle et al, 1984). In these experiments CD4+ Leu 8+ T cells were primed with the mycobacterial antigen, purified protein derivative (PPD) in the presence of APC. The PPD activated CD4+ Leu 8+ cells were then cocultured with purified autologous CD8+ T cells. Addition of cocultured but not control CD8+ cells resulted in dose-dependent suppression of the proliferative response of fresh CD4+ T cells to PPD. Suppression was antigen-specific as the responses of CD4+ cells to tetanus toxoid were not suppressed.

These experiments have been confirmed by Brines and Lehner (1988), who induced CD8+ T suppressor cells specific for a soluble bacterial antigen. Depletion of suppressor-inducer (SI) CD4+ Leu 8+ cells completely inhibited induction of suppressor-effector (SE) CD8+ cells.

2.6.3 Genetic Restrictions of Suppressor T Cells

Studies in humans have not associated SI or SE cells with expression of unique HLA-encoded or associated determinants (Rich, El Masry and Fox, 1986), although one report (Navarrete et al, 1985) suggested that DQ determinants of the HLA class II region might restrict suppressor T cell induction in the mixed lymphocyte reaction.

2.6.4 Recognition Elements of SI and SE Cells

That part of the TCR variable region, forming the antigen binding site and defining the clonality of the T cell, is known as the idiotype (reviewed by Geha, 1986).

Jerne (1974) proposed a model of the immune system in which idiotype-antiidiotype recognition may provide a regulatory mechanism for the immune response. One consequence of this theory is that T cell idiotypes may be regulated by an anti-idiotypic mechanism. The first experimental evidence of this was provided by Lamb and Feldmann (1982). They showed that recognition by a cloned T suppressor cell of an influenza antigen specific autologous cloned T helper cell was probably due to recognition of the TCR idiotype. The activation of antigen-specific CD8+ SE cells by primed CD4+ SI cells in the apparent absence of the initial antigenic stimulus (Damle et al, 1984; Brines and Lehner, 1988) also indicated recognition of the CD4+ TCR idiotype by CD8+ cells. SE cells are CD8+ and therefore would be expected to recognise antigen in the context of class I MHC products (Bjorkman et al, 1987). The idiotope may therefore be associated with class I products on the SI cell membrane.

If SE cells recognise TCR idiotypes, their targets for suppression may be T helper cells expressing idiotopes for priming antigenic epitopes. This is indeed suggested, at the clonal level, by Lamb and Feldmann's results (Lamb and Feldmann, 1982) and by Ottenhoff et al (1986). These authors isolated and cloned suppressor T cells from skin lesions of leprosy patients. The clones suppressed M. leprae specific responses by CD4+ cells. Since the clones did not themselves recognise and respond to M. leprae antigens, it is possible that they recognised the idiotope expressed by M. leprae specific CD4+ cells. Since T cells can also recognise and

respond to epitopes expressed by the Ig variable region, (Geha, 1986), the antibody idiotype, it is possible that SE cells can recognise the idiotope expressed by the B cell and directly suppress B cell function.

The most detailed investigations of idiotypic regulation in autoimmune conditions are by Cohen (1986). He and his collaborators have prepared murine T cell clones recognising autoantigens such as myelin basic protein (MBP). Adoptive transfer experiments have demonstrated the ability of these clones to transfer experimental allergic encephalomyelitis (EAE), the disease caused by immunisation with MBP, to syngeneic animals. However, when these T cell clones were treated by membrane modifiers, such as hydrostatic pressure, which may have increased the immunogenicity of the cell membrane components, and then used to immunise animals, protection was afforded against the disease. Similar results were obtained with arthritogenic T cell clones (Van Eden et al, 1988). Cohen has suggested that the immunizing clones may act as SI cells which can stimulate SE cells capable of recognising the T cell receptor idiotype of the SI cells. However, other explanations are possible. Membrane alterations may have allowed putative "immunising" T cells to tolerise pathogenic T cells. Alternately, the recirculation patterns of pathogenic T cells may have been altered due to the presence of "immunising" T cells in inflammatory sites.

Cohen's experiments undoubtedly open the possibility of vaccinating patients against autoimmune disease, using antigen-specific T cells. However, the identification of

autoreactive T cells has still to be made in most autoimmune connective tissue diseases, including JDM.

2.6.5 Regulation of the Immune Response to Autoantigens

The Middlesex group have extensively studied T cell suppression exerted on autoantigen induced immune responses. Mice primed with rat red blood cells may develop Coombs positive haemolytic anaemia. Transfer of T cells from primed animals into normal, syngeneic recipients specifically suppressed subsequent induction of the Coombs autoantibody response (Cooke, Hutchings and Playfair, 1978). Induction of tolerance to thyroglobulin was also shown to depend on the presence of active suppressor cells specific for thyroglobulin (Parish et al, 1988).

The active control of responses to self-antigens by suppressor T cells has also been demonstrated in man. Patients with chronic active hepatitis (CAH) may give in vitro T cell responses to a hepatocellular membrane component of liver specific protein (LSP) (Vento and Eddleston, 1987). This response could be suppressed by T cells from healthy normals but not by T cells from 50% of first degree relatives of the patients, suggesting genetic factors in the ability to mount a suppressor T cell response. Although CAH is associated with the HLA antigens B8 and DR3, the inability of relatives to induce suppressor cells was not restricted to individuals with this haplotype.

Suppressor mechanisms specific for self antigens were also implied from the results of Mach et al (1984), showing that

DNA could stimulate specific anti-DNA antibody synthesis in PBMC from SLE patients but not from control cells. Purified B cells from some normal individuals could however be induced to secrete anti-DNA antibodies.

Therefore, lack of induction of autoantigen specific suppressor T cells may be a predisposing factor in the development of autoimmune disease.

2.6.6 Conclusion

The existence of suppression as a component of the immune response has been verified in many experimental systems, but the role of suppressor T cells remains in doubt (Mitchison, 1988). This is due to the complexity of systems in which suppression is observed and the lack of data defining the molecular interactions between epitopes and TCR of suppressor cells.

The work of Engleman and his associates (reviewed by Fox, Elmasry and Rich, 1986) suggested that SI cells represent a separate lineage. However, until the epitopes expressed by SI cells and recognised by suppressor-effector cells have been defined in molecular terms and their MHC restrictions analysed and confirmed, the possibility remains that the phenomenon of suppression-induction may be an *in vitro* artefact.

2.7 Graft -versus- Host Reaction: A Model of Autoimmune Disease

Several theories of autoimmunity, including molecular mimicry, altered self, abrogation of tolerance and defects in immunoregulation by suppressor T cells have been discussed in previous sections. Is it therefore possible to construct a

model of autoimmune disease, of relevance to JDM, and encompassing at least some of these concepts?

Patients given bone marrow transplants may develop DM (Gale, 1985) as a consequence of graft -versus- host disease (GVHD) due to MHC incompatabilities between donor and host. Animal models have shown that autoimmune features of GVHD arise due to recognition by donor T cells of host MHC class II antigens (Gleichmann, 1984). Recognition of allo class II may be compared to responses against altered-self antigens, and class II antigens, expressed by tissue macrophages and endothelial cells (Pober et al, 1983), are widely distributed in many tissues of the host.

It is well established that organ-specific autoantibodies, such as anti-thymocyte and anti-smooth muscle antibodies, as well as anti-DNA and anti-nuclear antibodies are produced in GVHD. Although these results could be interpreted as due to non-specific polyclonal reactivity (Gleichmann, 1984), they could also result from recognition by the immune system of self-epitopes exposed by the disease process. This latter interpretation might have more relevance to other autoimmune connective tissue diseases, including JDM, in which the range of autoantibodies observed is more restricted than in GVHD (Pisetsky, 1987).

Anti-lymphocyte antibodies have also been detected in animals with autoimmune GVHD and patients with connective tissue diseases (reviewed by Winfield, 1985). Antibodies in the serum of patients with juvenile rheumatoid arthritis, with

specificity for an antigen expressed by suppressor-inducer T cells, may contribute to the reduction of this subset in patient peripheral blood (Borel, 1984). The determinants recognised by anti-lymphocyte antibodies are the subject of continuing controversy (Winfield, 1985). Their relevance to disease processes are unknown, but complement fixation by anti-lymphocyte antibodies could result in cell lysis and disregulation of the host immune response. Anti-lymphocyte antibodies have also been shown to contribute to immunosuppression (Pruzanski, 1986) and, by interfering with the function of regulatory subsets, could promote autoimmune disease.

Via and Shearer (1987) have suggested that the host may attempt to control disregulation of the immune system during GVHD by altering the balance of the immune response towards suppression. Reduced CD4+ T cell dependent responses have been shown to be mediated by suppressor cells generated during GVHD. Anergic lymphocyte responses are also a feature of RA and SLE, and impaired T lymphocyte function _in vitro_, including diminished proliferative responses to mitogens and antigens have been described (reviewed by Alcocer-Varela, Jouanen and Alarcon-Segovia, 1985; Emery et al, 1984; Warrington, 1987).

Opportunist viral infection could also contribute to immunosuppression of the host immune response, during autoimmune disease (Williams, 1977). There are an increasing number of observations that viruses may infect cells, including lymphocytes and macrophages, "silently" (reviewed by McChesney

and Oldstone, 1987), in which case cell lysis and cytopathic effects may not be observed, even when viral replication takes place. Furthermore, "silent" infection of immunocompetent cells has been shown to suppress immune function.

It is paradoxical that anergic lymphocyte responses may coexist with autoimmune disease in the same individual. There is no satisfactory explanation for this observation as yet. It is possible that immunoregulatory cells may suppress primary immune responses more efficiently than memory T cells responding to putative autoantigens. Also, the balance of immunoregulation within the inflammatory site may be tilted towards autoimmunity. The local environment, such as the precise proportions of different T cell subsets, their state of activation, physical associations with each other, the presence of cytokines and their inhibitors, would determine whether the equilibrium between tolerance and autoimmunity was abrogated.

CHAPTER 3

3.0 NATURAL KILLER CELLS IN INFECTION AND AUTOIMMUNITY

During the early 1970's Herberman and associates reported that lymphocytes isolated from unimmunised, normal hosts could lyse certain tumour cell targets (Herberman and Gaylord, 1973). Killing was observed not to be restricted by the MHC (Bolhuis et al, 1978). This is known as natural killer (NK) activity. NK cells may play an important innate role in combating a variety of viral infections (reviewed by Herberman and Ortaldo, 1981), and may also have important immuno-regulatory functions, concerned with both antibody production (Kuwano et al, 1986) and the activation of cytotoxic and suppressor T cells (Stephens et al, 1985). Reduced NK activity has been reported in JDM patients and in patients with other autoimmune connective tissue diseases (Miller, Lantner and Pachman, 1983; reviewed by Sibbitt and Bankhurst, 1985).

3.1 Immunoregulatory Role of NK Cells

Studies in the early 1980's suggested phenotypic similarities between NK cells and cells with suppressor function, in that most cells of both lymphocyte populations shared expression of the CD8 and Leu7 markers (Abo, Cooper and Balch, 1982a). This led to examination of the ability of NK cells to mediate suppressor function.

NK cells were found to suppress T and B cell function in a variety of *in vitro* assay systems, by a mechanism distinct from cytotoxic activity (Kuwano et al, 1986). NK suppressor cells differed from T suppressor cells in being relatively

radiation resistant (Tilden, Abo and Balch, 1983). As well as acting as effectors of suppression, NK cells may also be capable of inducing suppressor activity in T cells (Stephens et al, 1985). These authors reported that lymphokine containing supernatants secreted by activated NK cells induced non-specific suppressor activity in T cells. One of the lymphokines mediating this activity was identified as IFN-γ. The activation and proliferation of NK cells have also been observed in the mixed lymphocyte reaction (Salmon et al, 1985) and the autologous mixed lymphocyte reaction (Goto and Zwaifler, 1983), *in vitro* assay systems in which the generation of immunoregulatory cells has been well established (Klein, 1982; Fitzharris and Knight, 1981).

Therefore, NK cells may form part of a homeostatic mechanism with the capacity to regulate the immune response, as well as possessing a putative role in anti-tumour immunity. The decline of NK activity with old age is typically accompanied by increased concentrations of circulating autoantibodies (Abruzzo and Rowley, 1983). Low or absent NK activity is particularly associated with strains of mice expressing the beige gene mutation. These mice are prone to development of glomerulonephritis and other SLE-like disorders (Roder et al, 1982).

3.2 NK Activity and Autoimmune Disease

Reduced levels of NK activity have been observed in PBMC from patients with autoimmune connective tissue diseases, including JDM. There is some evidence, based on phenotyping studies, of decreased numbers of NK cells in patients with SLE (Egan et

al, 1983), although it has also been claimed that the number of effector-target conjugates, formed by PBL from SLE patients, were not significantly reduced compared to controls (Sibbit, Mathews and Bankhurst, 1984). This discrepancy, however, was probably due to binding of a T cell subpopulation to targets (Phillips and Lanier, 1985).

Analysis of function of NK cells from SLE patients has demonstrated significant decreases in levels of cytotoxic factors secreted in response to target cells compared to controls (Sibbitt and Bankhurst, 1985). It is possible that functional defects of NK cells from patients with autoimmune diseases are partly due to binding of immune complexes to the low affinity Fc receptor (CD16), expressed on 80-90% of human NK cells (Perussia et al, 1984).

3.3 Properties of NK Cells

The evidence of recent experimental results indicates that NK cells form a separate lineage, distinct from T and B cells. Scid mice exhibit a severed combined immunodeficiency due to defects in early T and B cell differentiation. Nevertheless, bone marrow transplant experiments showed that NK progenitor cells from scid mice could differentiate into mature NK cells with normal lytic function (Hackett et al, 1986). This suggests that NK cells represent a more primitive lymphocyte than T or B cells. This concept is suported by the evidence that NK cells do not rearrange or transcribe TCR genes (Lanier et al, 1986) and utilise a clonally invariant target cell receptor (reviewed by Hersey and Bolhuis, 1987).

The nature of the antigen receptor for NK cells is still unclear and controversial (Lanier et al, 1986; Kaplan, 1986; Hersey and Bolhuis, 1987). Cold competition experiments have shown the ability of different target cell lines to compete for killing by NK cells, inferring the existence of cross-reacting target antigens and a complementary receptor on the effector cell. The CD2 complex has been shown to be an accessory, clonally invariant receptor expressed by T cells (O'Flynn et al, 1985). Also, the CD2 receptor is expressed by most NK cells (Perussia et al, 1984), and recent experiments have demonstrated that binding of anti-CD2 antibodies to NK cells augmented killing, suggesting that signals required for lysis were transduced by the CD2 complex (Schmidt et al, 1987). Therefore, CD2 may be the antigen receptor, or associated with the antigen receptor, on NK cells. Comparison of the phenotypes of NK cells and other leucocytes is presented in Table 3.1.

TABLE 3.1

Phenotypic Relationship Between NK Cells and Other Leucocytes

Phenotypes[a]	T LYMPHOCYTES	NK CELLS	MONOCYTES	GRANULOCYTES
CD2	+	+	-	-
CD3	+	-	-	-
CD4	+	-	+	-
CD7	+	+	-	-
CD8	+	+	-	-
CD11a	+	+	+	+
CD16	-	+	-	+
CD18	+	+	+	+
Leu7	+	+	-	-
Leu19	+	+	-	-

[a]Taken from Phillips and Lanier (1986).

3.4 IFN Receptors and NK Cells

The interferons are a family of proteins defined as possessing antiviral activities (Trinchieri et al, 1978). Receptors for each IFN type have been detected on NK cells (Faltynek, Princler and Ortaldo, 1986) and strong enhancement of NK activity can be induced by treatment of effectors with IFN and IFN inducing agents (Trinchieri and Perussia, 1982).

In many viral infections, such as Coxsackievirus B3 induced myocarditis, in which IFN's are secreted by infected cardiocytes, NK cells may be important mediators of early resistance against infection (Godeny and Gauntt, 1987). It is probable that the latter stages of resistance against viral infections are mediated by antigen-specific cytotoxic T cells, but it is possible that IFN's secreted by NK cells, in response to virally infected cells, may enhance killing by cytotoxic T cells (McIntyre and Welsh, 1986; Christmas, Meager and Moore, 1987). The central role of IFN's in promoting resistance to some viral infections is suggested by experiments in which the administration of anti-IFN antibodies reduced resistance of animals infected with cytomegalovirus and hepatotropic viruses (Shellam, Grundy and Allan, 1982).

3.5 IL-2 Receptors and NK Cells

The high affinity IL-2 receptor is a heterodimer composed of 2 proteins, each capable separately of binding to IL-2 (reviewed by Smith, 1988). The 2 components of the heterodimer have been designated IL-2Rα and IL-2Rβ, the latter expressing the epitope (CD25) recognised by the anti-Tac antibody. IL-2Rα and β appear to be combined during the process of lymphocyte

activation.

Freshly isolated NK cells were found to be highly enriched in IL-2Rα compared to resting T cells (Siegel et al, 1987; Dukovich, 1987). Since IL-2Rα has been proposed as the biologically active component of the IL-2R (Smith, 1988), it is possible that NK cells are more responsive to the effects of IL-2 than resting T cells. Metaphase inhibition experiments have produced evidence supporting this view. Most NK cells, but only a minimal number of T cells, were induced into S phase in the presence of IL-2 and colchicine (London, Perussia and Trinchieri, 1986). Immunoprecipitation experiments have demonstrated the induction of Tac antigen on purified NK cells during culture in IL-2. Therefore, it appears that the initial stimulatory signals mediated by IL-2 are transduced by IL-2Rα, present constitutively on NK cells. These activation signals then induce the expression of high affinity IL-2 receptors on NK cells.

The significance of these observations lies in the fact that lymphocytes cultured in IL-2 may acquire non-specific lytic activity against a variety of target cells, including autologous and allogeneic tumours, resistant to NK cells (reviewed by Hersey and Bolhuis, 1987). Cells acquiring these functions on culture in IL-2 have been termed lymphokine activated killer (LAK) cells. The major feature of this lytic activity is that is MHC unrestricted.

Much publicity has attended the exploitation of LAK cells _in vivo_, in the treatment of patients with cancer. However, in sites of viral infection, secretion of IL-2 by antigen

specific T cells may induce activation of LAK cells which could contribute to resistance to infection. A reduced frequency of such cells or their precursors could restrict the ability of the host to combat infection.

3.6 Conclusion

NK cells form a distinct lymphocyte subset, with the potential to exercise immunoregulatory functions. Lack of regulation by NK cells of reactivity against self may be a predisposing factor in the development of autoimmune disease. Activation of NK cells by lymphokines secreted in disease sites may provide the host with an important defence against viral infection. Defective generation of such activated NK cells could contribute to an inappropriate response to virus.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Patients

4.1.1 **Children.** Parental approval was obtained when blood was taken from children in these studies. All the children were attending the Muscle Clinic at Hammersmith Hospital under the care of Prof V Dubowitz, in the Department of Paediatrics and Neonatal Medicine.

4.1.2 **Adults.** Adult patients studied were attending the Rheumatology Clinic at the Middlesex Hospital under the care of Dr's M Snaith and D Isenberg.

Clinical details of patients are given in Chapter 5.

4.2 Monoclonal Antibodies and Phenotype Analysis

The monoclonal antibodies (MAb's) used in these studies, are given in Table 4.1. For determination of lymphocyte phenotype, pre-determined saturating concentrations of MAb's (50 μl) were added to 2×10^5 mononuclear cells in suspension in LP3 tubes and incubated on ice for 20 minutes. Cells were washed twice in 0.1% bovine serum albumen in phosphate buffered saline containing 0.05% sodium azide (PBS/BSA) and 50 ul $F(ab')_2$ rabbit anti-mouse Ig - FITC (1/10 dilution, DAKO) then added to cells for 20 min. at 4°C. All dilutions were made in PBS/BSA. Cells were again washed twice and resuspended in 50% PBS - glycerol before examination at 280 nm using a fluorescence microscope (Optiphot, Nikon, Japan). At least 100 cells were counted for estimation of percentage of

TABLE 4.1. MONOCLONAL ANTIBODIES (MAb)

MAb	Class	CD Designation	Source	Reference
OKT3	IgG_{2a}	CD3	Ortho Diagnostics	Van Wauwe, Goosens & Beverley (1983)
MT-310	IgG_{2a}	CD4	Dr P Beverley[g]	Beverley (1982)
UCHT4	IgG_{2a}	CD8	Dr P Beverley	Beverley (1982)
OKB7	IgG_{2a}	CD21	Ortho Diagnostics	Golay & Webster (1987)
Leu 11	IgM	CD16	Becton & Dickinson	Lanier et al (1983)
Leu 19[a]	IgG_1	--	Becton & Dickinson	Lanier et al (1986b)
Anti-IL-2 Receptor	IgG_1	CD25	Becton & Dickinson	Becton & Dickinson MAb Source Book (1986)
DA2[c]	IgG_1	--	Dr P Beverley	Brooy et al (1979)
Leu 10[b]	IgG_1	--	Dr K Welsh[h]	Becton & Dickinson MAb Source Book (1986)
L243[c]	IgG_1	--	Dr K Welsh	Lampson & Levy (1980)
B7/21[d]	IgG_1	--	Dr K Welsh	Watson et al (1983)
2A1[e]	IgG_1	--	Dr P Beverley	Beverley (1980)
2D1[f]	IgG_1	--	Dr P Beverley	Beverley (1980)
Leu 18	IgG_1	CD45R	Becton & Dickinson	Morimoto et al (1985)
UCHL1	IgG_{2a}		Dr P Beverley	Beverley (1987)

a Leu 19 recognises an antigen present on 90% of CD16 cells.

b Leu 10 recognises a common polymorphic determinant present on HLA-DQ encoded antigens.

c DA2 and L243 recognises a non-polymorphic determinant present on HLA-DR encoded antigens.

d B7/21 recognises a common polymorphic determinant present on HLA-DP encoded antigens.

e 2A1 recognises a non-polymorphic determinant present on HLA-class I encoded antigens.

f 2D1 recognises an antigen common to all leucocytes.

g These MAb's were kindly donated by Dr P Beverley.

h These MAb's were kindly donated by Dr K Welsh (Guy's Hospital).

cells stained. Viability of cell populations was always greater than 90%.

In double staining experiments, the above procedure was followed. However, after washing to remove excess FITC labelled conjugate, cells were incubated with 50 µl of 2% normal mouse serum on ice for 20 min. The cells were washed twice in PBS/BSA and then 50 ul of phycoerythrin conjugated anti-IL-2 receptor antibody (1/15 dilution) was added to cells for 20 min. at 4°C. Cells were washed and resuspended in PBS/glycerol. The phycoerythrin conjugate stained cells were examined at 340 nm.

Flow cytometry analysis was kindly performed by Mr S Patel and Dr P Lydyard, Middlesex Hospital on an EPIC V flow cytometer (Coulter Instruments).

4.3 Treatment of Sera Used in Experiments

It was possible that JDM and other pathological sera contained immune complexes. Therefore, all sera were centrifuged at 5,000 x g for 30 min on a microfuge (MSE, London) and supernatants retained. Immune complex levels before and after centrifugation were determined by PEG precipitation. This method is in routine use in the Middlesex Hospital Immunology Dept. Briefly, immune complexes were precipitated from sera in 12% polyethylene glycol (BDH, Poole), washed, and class specific complexes measured by single radial immunodiffusion. This method demonstrated that all immune complexes were cleared from sera by centrifugation.

All human sera and serum fractions were sterilised using 0.22 µm membrane filters (Gelman, Nottingham) before addition to *in vitro* assays.

4.4 Biological Response Modifiers

Recombinant IL-2 (Specific activity 3.6 x 10^6 U/mg) was the kind gift of Biogen SA, Geneva).

Recombinant IFN-α (Specific activity 1 x 10^5 U/ml) was the kind gift of Dr P Beverley (ICRF).

4.5 Fractionation of Whole Blood into Lymphocyte Sub-populations

4.5.1 Separation of PBMC from Whole Blood

Blood was taken by venepuncture from healthy controls and patients and anticoagulated with 20 U/ml of preservative free heparin (mucous heparin sodium, Wedel Pharmaceuticals, London) Blood was layered onto a Ficoll-Hypaque solution of density 1.077 g/ml (Lymphopaque, Nyegaard, Oslo). The gradient was centrifuged at 850 x g for 20 min. and PBMC recovered from the interface, washed twice in calcium and magnesium free Hanks balanced salt solution (HBSS, Gibco, Paisley) and the majority of platelets removed by centrifugation at 200 x g. PBMC were resuspended in RPM1 1640 (Gibco), supplemented with 20 mM Hepes, 2 mM L-glutamine and 1 µg/ml erythromycin (CM). Viability was determined using Acridine orange/Ethidium bromide (Becton and Dickinson Source Book).

4.5.2 Preparation of Sheep Red Blood Cells (SRBC)

SRBC (Burroughs Welcome, Beckenham, England) were washed 3 times in HBSS prior to treatment with S-2 aminoethyl isothiourium bromide hydrobromide (AER, Aldrich, Wis. USA). 1

vol of packed SRBC was treated with 4 vol of 143 mM AET, pH 9.8, for 30 min. at 37°C. The SRBC were then washed 4 times before resuspension and storage in CM. AET treated SRBC could be used for up to 14 days and were then discarded.

4.5.3 Separation of E+ and E- Fractions

One vol PBM (5×10^6/ml) was mixed with 1 vol of a 4% suspension of AET-treated SRBC plus ¼ vol of foetal calf serum (FCS) or autologous serum in 15 ml Falcon conical tubes (Becton and Dickinson, Grenoble, France). The cell mixture was centrifuged at 20 x g for 5 min, incubated for 40 min on ice, then gently resuspended by axial rotation.

The suspension (1 vol) was layered over Ficoll/ Hypaque (2 vol) in 15 ml Falcon conical tubes and centrifuged at 850 x g for 15 min. The E- fraction was recovered from the interface and washed twice. The SRBC rosettes were treated with 1 ml of sterile distilled water to lyse red cells for 5-10 sec, then rapidly diluted with 10 vol of CM. The recovered E+ cells were then washed twice in CM. The E+ populations contained greater than 90% CD3 cells from normal adult, normal child and JDM donors. E- populations varied between 42 and 60% surface Ig positive cells for both normal adults and normal children and 44 - 71% surface Ig positive cells for JDM patients.

4.5.4 Plastic Adherence

Isolated PBMC E+ and E- fractions (2×10^6/ml) were incubated in 50mm plastic petri dishes (Nunc, Gibco Biocult, Paisley) in 10ml CM containing 10% heat inactivated (HI) foetal calf serum (CM-FCS) for 30 min at 37°C. The non-adherent cells were

recovered by gently aspirating the dish with CM. Contamination of peripheral blood lymphocytes (PBL) with monocytes was determined by examining E+ and E- populations for the presence of non-specific esterase staining cells (Yam et al, 1971). A single cycle of depletion usually reduced monocyte contamination to 1 - 2% of non-adherent cells. Cell preparations with higher monocyte concentrations were given a second cycle of plastic adherence.

4.5.5 Mitomycin C and Cycloheximide Treatment

Cells (1×10^7/ml) were added to mitomycin C (50 μg/ml; Sigma) or cycloheximide (1 μg/ml; Sigma) in 1 ml CM in 15 ml Falcon tubes. Cells were incubated at 37°C for 45 min and then washed 3 times.

4.5.6 Complement Dependent Lysis

The appropriate concentration of MAb was determined in initial titration experiments. OKT3, OKB7 and Leu 11b were used at 0.1μg, 0.2μg and 0.1μg/10^6 cells respectively. 2 - 5 x 10^6 lymphocytes were incubated with 0.5 ml of the appropriate MAb or combination of MAb's, diluted in CM, for 30 min at 4°C. Cells were then washed once and 1 ml of rabbit complement (PEL-Freeze, London), diluted 1/5 in CM supplemented with 1% FCS added to the cells. The cells were then incubated for 45 min at 37°C, washed twice and resuspended in CM. Viable cells were then obtained by centrifugation over Lymphoprep, washed twice and an aliquot retained for phenotypic analysis. Two cycles of lysis were usually employed for depletion.

4.5.7 **Panning**

This method was used to isolate CD3+ and CD16+ cells. E+ and E- lymphocyte subpopulations were prepared by 2 cycles of SRBC rosetting. 0.5 ml of OKT3 (0.2μg/10^6 cells) and 0.5 ml of Leu 11b (1μg/10^6 cells) were added to 5 x 10^6 E+ (for CD3+) and E- cells (for CD16+) respectively, for 30 min at 4°C. E- cells were then washed twice and added to 60 x 15 mm Petri dishes (Falcon) coated O/N at 4°C with 2 ml goat anti-mouse IgM (1 mg/ml; Birmingham University Research Institute). E+ cells were washed and added to plastic Petri dishes coated with 2 ml goat anti-mouse Ig (1 mg/ml; DAKO, Denmark). Cells were left to adhere for 45 min at 4°C. Non-adherent cells were removed by aspiration and washing. Adherent cells were obtained by careful dislodging with a 2 ml rubber tipped syringe barrel. Cells were washed twice and an aliquot retained for phenotyping.

4.6 In Vitro Assays of Lymphocyte Function

4.6.1 Proliferative Responses to Mitogens

Proliferative responses of normal control and patient cells to varying concentrations of each mitogen tested were obtained in preliminary experiments. Dose-response curves indicated optimal and sub-optimal concentrations for each mitogen. These experiments also showed that maximal proliferation occurred at the same time (48 - 72h) for both control and patient cells. Optimal and sub-optimal concentrations of phytohaemagglutinin (PHA) and concanavalin A (ConA) (Sigma Chemical Co, USA), pokeweed mitogen (PWM) (Gibco Biocult, UK) and OKT3 (Orthodiagnostics Ltd) were added to triplicate cultures of patient and control PBM in microtitre plates.

Cells were cultured in CM supplemented with 10% heat inactivated (HI) FCS (CM - FCS) ('Myoclone' - Gibco Biocult). The batch of FCS used had previously been screened and was not mitogenic for human cells. Each well contained 2×10^5 PBMC in a volume of 100µl with the appropriate concentrations of mitogens added in a further 100ul of culture medium. Proliferation was measured by the uptake of (methyl - ^3H) thymidine (^3H - T, 37 KBq/well Amersham International) added in 50ul to cultures for the final 16h incubation. Cells were harvested on a cell harvester (Ilacon Ltd, UK) and incorporated ^3H - T measured on a liquid scintillation counter (LKB, England). All proliferation assays were set up in flat-bottomed 96 well microtitre plates (Nunc, Gibco, UK).

4.6.2 Mixed Lymphocyte Reaction

Mitomycin C treated E- cells (0.5×10^5) were added to 1.5×10^5 allogeneic E+ cells in a total volume of 200ul in microtitre plates and cultured in triplicate in CM supplemented with 10% HI normal human serum. Cells were cultured for 5 days and ^3H - T added to each well for the final 16h incubation period. Cells were harvested and ^3H - T uptake measured.

4.6.3 Autologous Mixed Lymphocyte Reaction (AMLR)

Mitomycin - C treated E- cells (0.5×10^5) were added to autologous E+ cells (1.5×10^5) in microtitre plates, in triplicate, in a total volume of 200ul CM supplemented with 10% HI human serum. In other experiments, 1×10^6 mitomycin C treated E- cells were cultured with 3×10^6 autologous E+ cells in Multidish 24- well cluster plates (Costar, USA), in

duplicate, in a total volume of 2ml CM supplemented with 10% human serum. E+ cells were also cultured alone. As an estimate of proliferation, 2 aliquots of 100µl each were transferred from cluster plate wells to microtitre plate cells when required, to be pulsed for 16h with 37 KBq/well ^{3}H - T added in 100ul CM. Cell proliferation in cultures initially established in 96 well plates was measured by addition of 37 KBq ^{3}H-T/well in 50ul CM. Cells were then harvested and ^{3}H-T incorporation measured. In other experiments, after 3 and 5 days culture, microtitre plates were centrifuged (150 x g) and 100µl supernatant harvested and stored at -70°C for IL-2 production measurement.

4.6.4 Generation of Suppressor Cells in the AMLR

Mitomycin C treated E- cells (1×10^{6}) were added to autologous E+ cells (3×10^{6}) in 24 well cluster plates, in duplicate, and cultured for 7 days as 4.6.3 above. Viable T cells were then harvested by SRBC rosetting, before use in suppressor cell assays.

4.6.5 Suppression of IgM Production by T Cells Generated in the AMLR

Fractionated E- cells ($0.5 - 1 \times 10^{6}$) were incubated with 1ml of a 1:10 dilution of supernatant from Epstein-Barr virus (EBV) secreting B95-8 cell line, for 1h at 37°C. Cells were then washed and resuspended in CM - FCS and cultured for 7 days. Increasing concentrations of T cells (5×10^{4} - 3×10^{5}) were added to 5×10^{4} autologous, EBV treated E- cells in 200 ul CM- FCS final volume in microtitre plates. T cells were derived from control E+ cells cultured alone, and AMLR cultures. Supernatants from EBV-treated B cells cultured

alone and from cocultures of EBV-treated B and T cells were harvested after 7 days and IgM production measured. All cultures were performed in triplicate.

Preliminary experiments using cells from normal adults showed that:

(a) optimal suppression was achieved with a T:B cell ratio of 3:1;

(b) treatment of T cells with mitomycin C decreased levels of suppression of IgM production obtained by 42 - 60%;

(c) adding fresh medium after 4 days coculture of B and T cells did not significantly affect the levels of suppression achieved.

4.6.6 IL-2 Assay

In experiments where IL-2 production was to be measured supernatants were tested for their ability to restimulate mitogen activated PBMC. PBMC (2×10^6/ml) were incubated in CM-FCS supplemented with PHA (1 μg/ml) for 5 days. Blasts from these cultures were isolated over FCS gradients (1 x g) for 1h, washed 5 times and added (1×10^5/well) to microtitre plate wells, in triplicate in 100μl CM supplemented with 1% FCS (CM - 1%). Serial dilutions of supernatants (1/2 - 1/16 in CM - 1%) were added in 100ul aliquots to wells and incubation continued for 48h. ^{3}H - T (37 KBq)/well) was added for the final 16h of culture. Cells were then harvested and proliferation measured. Lymphoblasts were also stimulated with serial dilutions of recombinant human IL-2 and this was used to construct a standard curve. IL-2 concentrations were obtained by interpolation on the standard curve by regression

analysis.

4.6.7 Antigen Specific Proliferation

100ul PBMC (2×10^6/ml) were added to microtitre plate wells, in triplicate, in CM supplemented with 10% normal adult serum. Cells were cultured with or without purified protein derivative (PPD) (50µl) at the required concentrations. PPD was obtained from the Veterinary Research Institute, Weybridge. The effect on the antigen specific proliferative response of culturing cells in purified IgG from patient and control sera was assessed by adding purified IgG (50 µl) to wells to give a final concentration of 1mg/ml. Cells were cultured for 7d and ^{3}H - T (37 KBq/well) was added during the final 16h of culture. Cells were then harvested and ^{3}H - T incorporation measured.

4.7 Cytotoxicity Assays

4.7.1 Target Cell Labelling

100µl sodium (^{51}Cr) chromate (3.7 MBq; Radiochemical Centre, Amersham) was added to a cell suspension of 1 - 3 x 10^6 target cells in 100µl CM. After incubation at 37°C for 1h, cells were washed three times and resuspended at 5×10^4/ml in CM.

4.7.2 Cytotoxic Assay

100µl of effector cells, at appropriate dilutions in CM, in triplicate, were added to 100µl of ^{51}Cr labelled target cells in CM supplemented with 20% serum. Spontaneous release was measured by incubating target cells in medium alone and maximal release by incubating target cells in 6% sodium dodecyl sulphate. The length of incubation for assays measuring cytotoxicity against K-562 and RD cells was 4h,

while cytotoxicity against lymphoblastoid cell lines was measured in an 8h assay. 100ul of supernatant from each well was transferred to individual LP3 tubes (Luckham, Sussex) for counting on a γ - counter (LKB, England). The percentage cytotoxicity was calculated according to the formula -

$$\% \text{ specific lysis} = \frac{\text{Mean } ^{51}\text{Cr release in test wells - mean spontaneous release}}{\text{Mean maximal release - mean spontaneous release}}$$

The standard deviation of the mean of triplicate ^{51}Cr release values never exceeded 10%.

For calculation of cytotoxicity by clones derived in limiting dilution experiments a cytotoxicity index was calculated based on the following formula -

$$\text{Cytotoxicity index} = - \ln \left(1 - \frac{\% \text{ specific lysis}}{100}\right) \times 100$$

The cytotoxicity index was preferred to % specific lysis as only the former has been shown to be linearly proportional to clone size (Rozans et al, 1986).

Calculation of Lytic Units (LU)

Graphs were plotted of % specific lysis against effector: target cell ratio. A value of % specific lysis, lying on the linear portion of the curves (15 - 30% lysis) was then chosen. Effector : target ratios were then read for each curve at the chosen % specific lysis value and lytic units/10^7 cells calculated according to the formula -

$$\text{LU}/10^7 \text{ cells} = \frac{10^7}{\text{E:T ratio} \times \text{target cell number } (5 \times 10^3)}$$

4.7.3 **Lectin Induced Cellular Cytotoxicity (LICC)**

PHA (2 µg/ml) was added to cells at various E:T ratios and cytotoxicity against RD targets measured after 4h culture.

4.7.4 **α Interferon Induced Cellular Cytotoxicity**

Donor PBL (5×10^6/ml) were incubated with α IFN (1000 U/ml) (Wellcome, Beckenham, Kent) for 4h. Cells were washed 3 times and cytotoxicity against K-562 and RD cells measured after 4h culture.

4.8 Continuous Cell Lines

4.8.1 K-562

K-562 cells grow in suspension and were derived from a patient with chronic myeloid leukaemia in terminal blast crisis (Lozzio and Lozzio, 1976). K-562 cells do not express HLA antigens.

4.8.2 RD

RD cells grow as an adherent monolayer and were derived from a human embryonal rhabdomyosarcoma (McAllister et al, 1969). RD cells express class I but not class II MHC antigens (Fig 4.1). Trypsinisation did not affect the expression of class I antigens. RD cells were grown in 25 cm^2 tissue culture flasks (Nunc) until confluent and then subcultured following harvesting using a mixture of 0.025% trypsin (Flow Laboratories) and 0.5 mM disodium ethylene diamine tetra-acetic acid (EDTA) in PBS.

4.8.3 B95-8

B95-8 is an Epstein-Barr (EBV) virus secreting marmoset lymphoblastoid cell line obtained from Dr P Lydyard (Middlesex Hospital). B95-8 cells were cultured, in 25 cm^2 tissue culture flasks, at a concentration of 1 x 10^6/ml. Cell supernatants were obtained by centrifugation and stored at -70°C.

4.8.4 Lymphoblastoid B Cell Lines

PBL (2 x 10^6/ml) were incubated for 1h in 1ml of EBV containing supernatant in 15ml Falcon tubes. 5ml of CM-FCS, containing 4ug/ml cyclosporin A (kindly donated by Dr D Crawford, University College Hospital) was added to each culture and cells were centrifuged at 400 x g for 5 min. Cells were cultured for 1 week before washing and resuspension in 24 well cluster plates at a concentration of 1 x 10^6/ml. All continuous cell lines were cultured in CM-FCS.

(A)

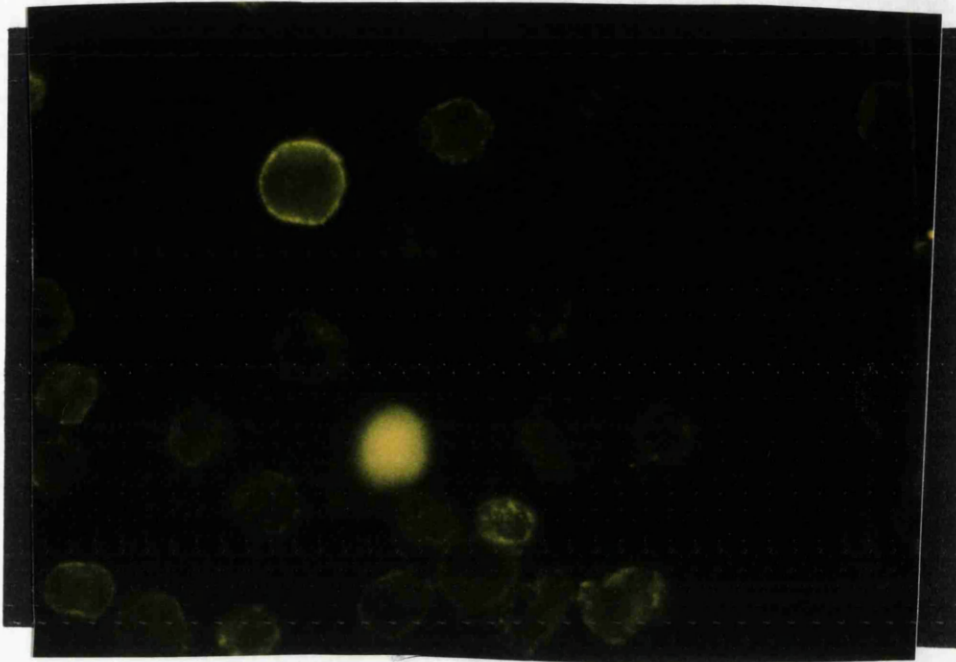
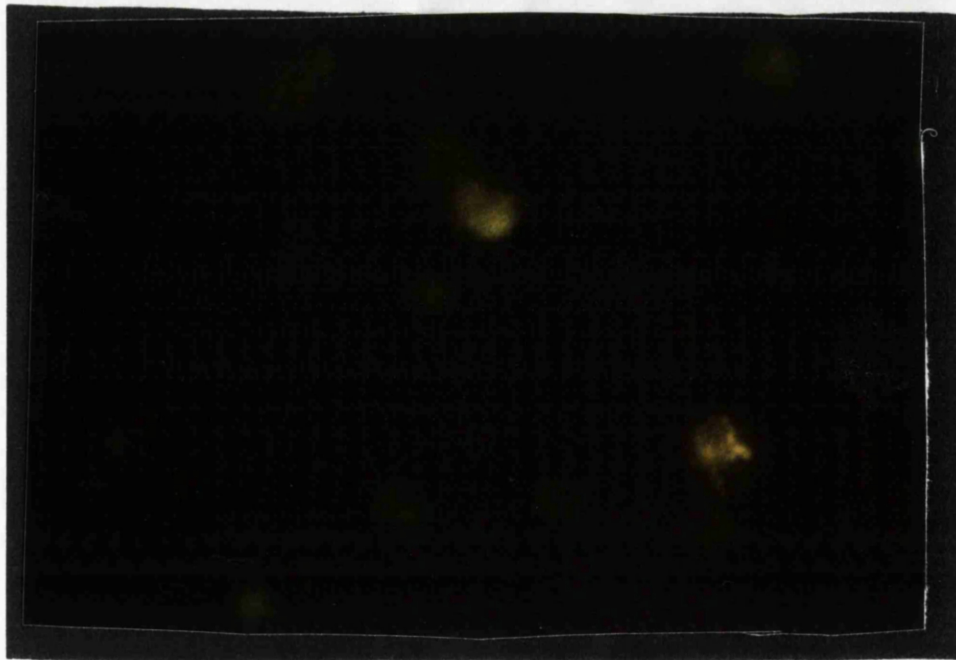


Fig 4.1. Indirect immunofluorescence staining of RD cells in suspension with (A) anti-class I MAb (B) conjugate alone.

(B)



4.9 Generation of Effector Cells in IL-2 Containing Medium

4.9.1 Bulk Cultures

PBL and lymphocyte subpopulations were cultured (2 x 10^6/ml) in 24 well cluster plates in CM- FCS and IL-2 (50 U/ml). Cells were cultured in 2ml volumes and half the medium in each well was replenished with fresh medium, including IL-2, twice a week. Cells were harvested, washed and cultured O/N in medium without IL-2 before analysing phenotypes or measuring cytotoxicity or proliferation.

4.9.2 Coculture Experiments

The ability of normal and patient E- and CD16+ cells cultured in IL-2, to induce cytotoxic activity in fresh, normal, allogeneic T cells was assessed by cocultivation experiments. Donor PBL (2 x 10^6/ml) were cultured in 24 well cluster plates for 6 days in CM -FCS plus IL-2 (50 U/ml). Cells were then washed, cultured O/N in medium without IL-2, harvested, then fractionated by SRBC rosetting, and E- cells, designated "activated", retained. Increasing concentrations of activated E- cells were then added to fresh, normal, allogeneic E+ cells (8 x 10^4) in 200µl CM -FCS in round-bottomed 96 well culture plates (Nunc), in triplicate. In some experiments:

(a) PBL were cultured for 7 days in medium without IL-2 and then fractionated to give E- cells which were cocultured with fresh E+ cells;

(b) activated E- cells were depleted of CD16+ lymphocytes before coculturing with fresh E+ cells;

(c) CD3+ cells were purified from fresh E+ cells and mixed with activated E- cells.

All cell combinations were cocultured O/N, 100µl of supernatant then aspirated from each well and cells (in remaining 100 ul of medium) harvested and added to ^{51}Cr labelled RD cells (5×10^3) in 100µl CM-FCS in round-bottomed 96 well Linbro plates (Flow Laboratories). After 4h incubation, plates were centrifuged at 150 x g for 5 min and 100µl supernatant removed from each well for estimation of ^{51}Cr release.

4.9.3 Limiting Dilution Analysis (LDA) of Lymphokine Activated Killer (LAK) Cells

Limiting dilutions of normal ($200-3.2\times10^3$) and JDM ($800-2\times10^4$) PBL were cultured in CM-FCS, supplemented with IL-2 (50 U/ml), in flat-bottomed 96 well microtitre trays. Feeder cells, which were pooled, allogeneic, normal PBMC (1×10^5) were irradiated (4000 Rads) and added to cells cultured at limiting dilutions or cultured alone. Twenty-four wells were used for each limiting dilution and for feeder cells alone. Cells were cultured in a final volume of 200ul and medium was changed by replacing half the volume of each well by fresh medium, containing IL-2, at days 4 and 7 of culture. On day 9, 150µl of medium was aspirated from each well and replaced by an equivalent volume of CM-FCS. On day 10, cells were harvested from each well and 2 aliquots of 100µl each added to 5×10^3 ^{51}Cr labelled RD or K-562 cells in CM-FCS (100µl) in 96 well round-bottomed Linbro plates. After 4h incubation plates were centrifuged and 100µl supernatant was removed from each well and counted. A computer program (Dr P Beverley, ICRF, Middlesex Hospital) based on the methods of Tasswell (1981) was used to estimate precursor frequencies. This program also

determined the 95% confidence limits for each frequency estimate, and fitted the experimental values to the zero order Poisson equation by the chi-square minimisation method. The probability of the fitness of experimental values to a straight line equation (p) was determined by the chi-square method. Tests in which the probability was greater than 0.05 were rejected.

Initial experiments, using cells from normal adults, showed (a) that 1×10^5 feeder cells/well were optimal (b) cytotoxic activity was maximal after culture for 10 days (c) feeder cells consisting of 50% autologous EBV transformed cells and 50% pooled allogeneic PBM did not give significantly different results from PBM alone.

4.10 Purified IgG Preparations

4.10.1 Preparation

3ml of Sepharose 4B - Protein A (Pharmacia, Sweden) was added to a K9 column (10 x 1 cm; Pharmacia) and equilibrated in 20ml PBS. 1ml of human serum was added to the top of the gel and allowed to equilibrate with the gel for 1h at 4°C. The effluent was removed with PBS at a flow rate of 40 ml/h. Acetic acid (0.5M, pH 4.0) was then added to the column and the eluate obtained by descending chromatography at a flow rate of 40 ml/h. The eluate was dialysed against 3 changes of PBS at 4°C. The concentration of the eluate was obtained by absorbance at 280 nm in a double-beam spectrophotometer (Perkin-Elmer, Surrey). The purity of the IgG preparation was determined by immunodiffusion in 1% agar using polyclonal antisera against normal human serum and normal human IgG

(Dako).

4.10.2 Addition of Purified IgG to AMLR Assays

Cells were cultured in 10% normal human serum to which the appropriate volume of purified IgG was added to give the required final concentration. When cells were cultured in the non-IgG containing serum fraction, the volume of this fraction was adjusted to give a final concentration of 10% of the original serum.

4.11 In Vitro IgM Production

4.11.1 PWM Stimulation of PBMC

Initial experiments showed (a) that a 1/100 dilution of PWM mitogen induced optimal levels of IgM production in both normal and patient cell cultures (b) spontaneous and PWM induced IgM production by normal and patient cells were maximal after culture for 8 days. PBMC (2×10^5/well) were added to flat-bottomed 96 well microtitre plates in CM-FCS and cultured in triplicate, either alone or with PWM in a total volume of 200µl for 8 days. Plates were then centrifuged (150 x g) and supernatants (100µl) removed from each well and stored at -20°C, before determination of IgM concentration.

4.11.2 Autologous and Allogeneic Combinations

E+ cells (1×10^5) were added to autologous or allogeneic E- cells (1×10^5) in CM-FCS in a final volume of 200µl with or without the addition of PWM (1/100 dilution). Supernatants were harvested after culture for 8 days for estimation of IgM production.

4.11.3 **Effect of IL-2 and MLR Supernatant on Spontaneous and PWM Induced IgM Production**

Equal numbers (5×10^6) of PBMC from 3 or 4 HLA mismatched normal donors were cocultured in CM-FCS in 25 cm^2 tissue culture flasks, for 5 days, and supernatants pooled and stored at -70°C. MLR supernatants and IL-2 were added to PBM with or without an optimal concentration of PWM. Supernatants were harvested after culture for 8 days and IgM production measured.

4.11.4 **IgM ELISA Assay**

100μl of affinity purified goat anti-human IgM (1 μg/ml; Sigma) was added to each well of ELISA plates (Immuno Plate I, Nunc) and incubated for 1h at room temperature (RT). The plates were washed with 0.1M carbonate buffer, pH 8.4, and 100μl of 4% normal goat serum (NGS) in carbonate buffer added to each well for 1h at RT. The plates were washed 3 times with 0.1% Tween 20 in PBS (PBS/T) and 100μl of serial dilutions of test supernatants (1/2 - 1/16 in NGS/PBS-T) were added to each well. Dilutions of a standard serum (Dako, Holland) were added to the wells in order to construct a standard curve. Plates were incubated for 1½h at RT. Plates were then washed 3 times with NGS-PBS/T and 100μl alkaline phosphatase conjugated anti-human IgM (1/1000 dilution in NGS/PBS-T; Sigma) added to each well for 1h at RT. The plates were then washed 3 times with PBS-T and once with carbonate buffer and then 100 ul of phosphatase substrate (1 mg/ml; Sigma) diluted in carbonate buffer supplemented with 0.01M magnesium chloride, pH 9.2, was added to each well and plates were incubated at 37°C for 15 - 30 min. The reaction was

stopped by addition of 100µl of 3N NaOH to each well and the reaction read at 405 nm on an automated ELISA reader (Dynatech). IgM concentrations were obtained by interpolation on the standard curve by regression analysis.

4.12 Absorption of JDM Sera

Absorption assays were conducted to characterise the inhibitory factor present in JDM sera. Serum, diluted 1/3 in PBS, was added to 1 x 10^6 EBV transformed B lymphoblastoid cells or 1 x 10^6 K-562 cells, in LP3 tubes. Serum samples were absorbed for 1h on a rocker at 4°C. Supernatants were obtained by centrifugation of LP3 tubes at 600 x g. Supernatants were then absorbed again and stored at -70°C.

4.13 Analysis of JDM Sera for the Presence of Anti-DR Antibodies by an Inhibition Assay

PBMC (1 x 10^7) from several normal donors were cultured in CM-FCS supplemented with 1 µg/ml PHA in 25 cm^2 tissue culture flasks. Fresh medium, containing IL-2 (50 U/ml) was added to flasks after 6 and 10d culture. Cells were harvested over FCS after 14 days. These cell populations were greater than 98% CD3+ and from 45-65% of T cells coexpressed DR antigens. Lymphoblasts (2 x 10^5) were incubated with 200µl of human serum, diluted 1/10 in PBS/BSA containing 0.1% BSA and 0.05% sodium azide for 30 min at 4°C, washed 3 times in PBS/BSA and then incubated with 50µl of either DA2 (neat) or RFDR2 (neat) MAb's, for 30 min at 4°C. Cells were then washed and incubated in $F(ab)_2$ rabbit anti-mouse IgG (DAKO), diluted 1/10 in PBS/BSA, for 30 min at 4°C. Cells were washed and resuspended in 0.1% formaldehyde in PBS containing 1% FCS.

Cells could then be stored for up to 7 days at 4°C before analysis by flow cytometry.

4.14 Statistical Analysis

The majority of data presented in this thesis were not normally distributed. Therefore, data was analysed by non-parametric statistical tests. Standard computer software (programs included Statworks and Statview 512) was used. The advice of Brian Newman, University College London, particularly on limiting dilution analysis, is gratefully acknowledged.

CHAPTER 5

5.1 INTRODUCTION TO STUDY OF PATIENTS WITH JDM

This chapter includes the clinical details of JDM and adult PM patients studied. Immunological parameters of JDM patients, including PBL subset phenotypes, HLA haplotypes, complement allotypes, complement consumption (CH50) levels, serum Ig and immune complex levels are also presented.

There are many inherent difficulties in studying patients with a rare disease such as JDM. The difficulties are compounded as the patients are children. The major referral centre in the South East of England is Professor Dubowitz's Paediatric Clinic at the Hammersmith Hospital. A group of 20 JDM patients are seen there at regular intervals. Blood taken from patients was despatched to UCH/Middlesex hospitals where the studies reported here were carried out. Most of the patients had already presented with symptoms before the study began and were being treated with corticosteroids and other drugs. Sequential studies were performed on some patients, but many attended clinics at irregular intervals and therefore, not all assays could be performed on the same group. The drug treatment, which patients were receiving when blood was taken for experiments is given in the appropriate chapters. Clinical assessment of disease activity is given for sequential studies of NK function. Clinical details of patients are given in Table 5.1.

TABLE 5.1. Summary of Clinical Details of JDM Patients

INITIALS OF PATIENT	AGE/SEX		DISEASE DURATION (YEARS)	FAMILY HISTORY OF AUTOIMMUNITY
KA	3	F	1	-
AB	5	M	1	-
NC	13	M	1	-
SC	4	M	1	-
TC	11	F	1	-
GH	18	M	7	JRA
NH	11	F	2	RA
EK	13	M	7	RA
JK	16	F	3	THY
MK	7	M	1	IDDM
PL	6	M	1	Unknown
HM	4	F	1	THY
LM	16	F	5	THY
PO	10	F	4	-
LR	3	M	1	-
SR	9	F	1	-
HV	9	F	2	Unknown
SW	6	M	1	-

JRA = Juvenile rheumatoid arthritis

THY = Thyroid disease

IDDM = Insulin-dependent diabetes mellitus

RA = Rheumatoid arthritis

5.2 Control Groups

Ideally, control groups should have consisted of:

(a) children with non-inflammatory muscle diseases, not receiving steroid therapy;

(b) children with inflammatory disease, but without muscle involvement, and receiving steroid therapy;

(c) normal, control children.

Ethical, and logistic problems meant that only sera from patients in group (a) could be readily obtained. Heparinised blood from group (a) patients could only be obtained very irregularly and then not on the same day as JDM patients. Group (a) patients are therefore considered only in terms of serological studies. Material from group (b) patients could not be obtained although attempts were made to obtain blood from asthmatic children at the Brompton Hospital. Blood from normal, healthy children could be obtained, and was used at least for initial studies in each of the assay systems tested. Blood was obtained from 3 children of colleagues, 5 children attending paediatric clinics with fractures and 3 siblings of JDM patients. Blood was also obtained from 5 children attending the Metabolic Clinic at UCH, none of whom were known to have an immunological component to their disease. The mean age of these control children was 7 years, range 5-14 years. Blood was also obtained from normal adult controls, medical, laboratory and clerical staff at UCH/Middlesex Hospitals.

5.3 Adult Polymyositis Patients

Eight adult PM patients were included in part of this study. These patients were attending the Rheumatology Clinic at the Middlesex Hospital under the care of Dr D Isenberg. Clinical details of the patients are given in Table 5.2.

5.4 PBL Subset Analysis of JDM Patients

An analysis of PBL subsets of JDM patients was carried out by Dr's G Cambridge and P Lydyard (Middlesex Hospital).

Of the 15 JDM patients analysed, 3 were untreated, 6 were receiving prednisolone and azathioprine and 2 were receiving azathioprine alone.

A significant decrease in CD8+ cells from JDM patients compared to both adult and childhood controls was observed ($p < 0.01$) (Fig 5.1). The 3 untreated patients (KA, HV, SW) all had reduced numbers of CD8+ cells, 12, 6 and 8% respectively. Decreased numbers of CD8+ cells have been reported in the peripheral blood of SLE patients and it is possible that this may correlate with depressed suppressor T cell function (Alarcon-Segovia, Alcocer-Varela and Diaz-Jouanen, 1985). No information is available on the functional characteristics of suppressor T cells in JDM patients.

5.5 HLA Haplotypes of JDM Patients

The HLA haplotypes and complement allotypes of 16/18 of the JDM patients in this study are given in Table 5.3. Typing was performed in Professor Batchelor's Department of Immunology, Hammersmith Hospital. The information on which the table is based has been taken from Robb et al (1988).

Table 5.2. Summary of Clinical Details of Adult PM Patients

PATIENT	DRUGS (DURATION YEARS)	DISEASE (DURATION YEARS)	CPK (U/ml)
TAN	Pred (1)	2	16,585
THO	None	6	1,256
JAQ	Pred, Aza	6	-
ZEF[a]	None	1	-
GOO	Pred, Aza (5)	6	60
OSM	CsA (1)	1	208
FOU[b]	Aza (2)	3	199
VER	Pred, Aza (4)	5	1,057

Pred = Prednisolone

Aza = Azathioprine

CsA = Cyclosporin A

[a] Concurrent scleroderma

[b] Concurrent psoriatic arthropathy

The results showed that the raised frequency of B8 and DR3 in JDM were due almost completely to an association with the common haplotype B8, DR3, C4AQ0, C4B1, C2C, BfS, which 13/20 patients carried. Eighteen patients carried a null allele for C4, 16 at C4A and 2 at C4B. The difference in frequencies of C4 null alleles between the patient group (0.9) and the control panel (0.46) was highly significant ($p < 0.001$). The presence of null alleles in 18/20 patients, including 14/16 patients presented in this thesis suggests that they may be one of the disease susceptibility genes or in very close linkage disequilibrium with the disease susceptibility genes.

5.6 Serum Ig and Immune Complexes

The concentrations of Ig and immune complexes (IC) in sera from 10 JDM patients and 9 childhood controls were determined (data not shown). The concentrations of IgG, IgM, IgA, IgG IC and IgMIC in JDM sera were not significantly different from controls. The concentrations of IgA IC were significantly elevated in the sera of 8/10 JDM patients compared to controls (Drs J Ludlow and F Hay, Middlesex Hospital).

5.7 Complement Consumption

Complement consumption (CH50) levels were measured sequentially in 15 JDM patients (Immunology Dept, Hammersmith Hospital). Patient PO had reduced CH50 levels (31, 36, 56 - normal CH50 range 65 - 135) on 3 separate occasions and her vasculitis was moderate to severe at each time of testing. When this patient's CH50 levels were within the normal range, her vasculitis was either mild or in remission. Patients JK

Table 5.3. HLA Haplotypes and Complement Allotypes of 20 JDM Patients

PATIENT	HLA-A	HLA-Cw	HLA-B	C2	Bf	C4A	C4B	HLA-DR	DELETION OF C4 GENE	DELETION OF 21- OH GENE
EK	2	-	40	C	S	QO	2	4	-	-
	1	-	8	C	S	QO	1	3	A	A
LM	1	7	8	C	S	QO	1	3	A	A
	30/31	2	27	C	S	3	1	4	-	-
HM	28	2	27	C	S	QO	1	3	A	A
	1	4	35	C	S	3	1	1	-	-
NC	2	-	8	C	S	QO	1	3	A	A
	1	-	8	C	S	QO	1	3	A	A
JK	1	-	8	C	S	QO	1	3	A	A
	28	3	13	C	S	3	1	3	-	-
SW	2	-	40	C	S	QO	1	2	ND	ND
	24	-	13	C	S	4	2	5/-	ND	ND
SC	2	5	44	C	S	3	QO	7	-	-
	24	3	55	C	S	3	QO	7	B	A
TC	3	-	14	C	F	3	1	7	-	-
	1	-	8	C	S	QO	1	3	A	A
GH	3	2	27	C	S	QO	1	7	A	A
	1	-	8	C	F	3	1	3	-	-
SR	-	-	7	C	S	3	1	2	ND	ND
	3	-	13	C	S	3	1	5	ND	ND
NH	1	-	44	C	F	3	1	5	-	-
	3	-	8	C	S	QO	1	3	A	A
1	3	-	7	C	S	3	1	2	-	-
	3	2	27	C	S	3	1	2/-	-	-
2	32	2	16	C	F	QO	1	3	-	-
	29	3	44	C	F	3	1	7	-	-
3	1/-	2	27	C	S	3	1	7	-	-
	1	-	8	C	S	QO	1	3	A	A
PO	24	-	49	C	S	3	1	2	-	-
	1	-	8	C	S	QO	1	3	A	A
KA	2	2	51	C	S	3	1	2	-	-
	1	-	8	C	S	QO	1	3	A	A
MK	1	-	8	C	S	QO	1	3	A	A
	2	-	40	C	S	2	2	2	-	-
4	3	5	7	C	F	3,2	QO	4	-	-
	29	5	44	C	F	3	1	7	-	-
AB	30	-	8	C	S	QO	1	3	A	A
	2	2	27	C	S	3	1	2	-	-
SR	1	-	8	C	S	QO	1	3	A	A
	1	2	27	C	S	3	1	5	-	-

ND = Not done

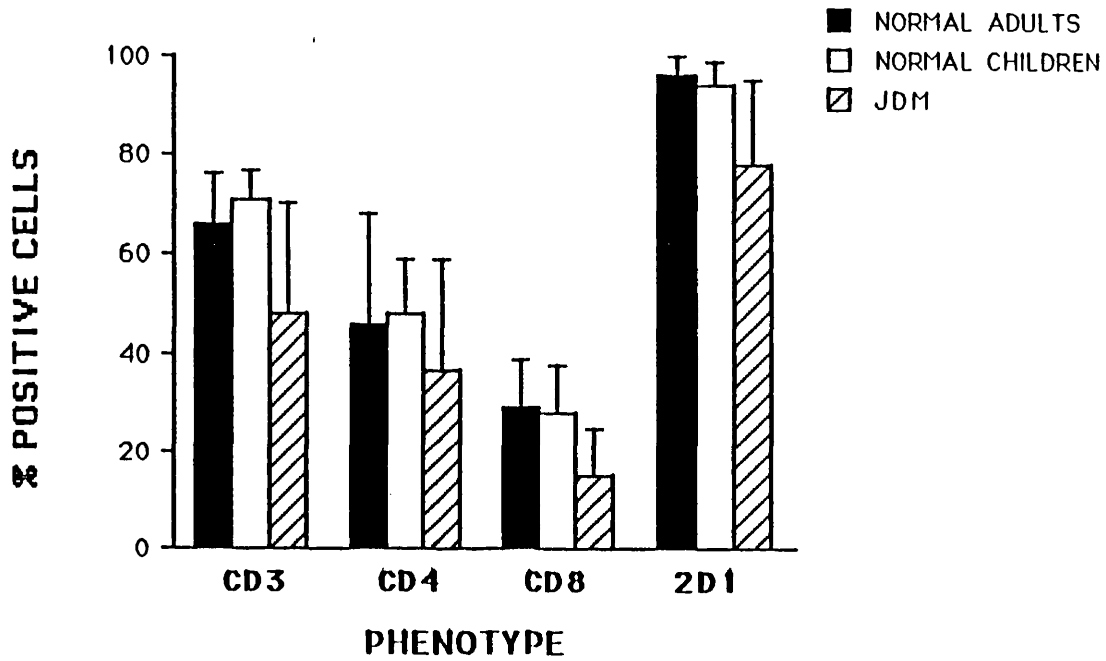


FIGURE 5.1.

Analysis of lymphocyte subsets from normal adults (12), normal children (8) and JDM patients (15). PBMC were tested for expression of the designated markers.

and SC both had reduced CH50 levels on 2 occasions when their vasculitis was of moderate severity and CH50 levels returned within the normal range when their vasculitis remitted. No other patients, however, had reduced CH50 levels when tested, although patients NC and EK had moderate to severe vasculitis on these occasions. Serum C3 and C4 levels, followed sequentially, were always found to lie within the normal range for all 15 JDM patients.

Immune complex mediated vasculitis has been reported in 14/16 patients with homozygous C4 deficiency (Schifferli and Peters, 1983). Although only 2 JDM (EK, NC) patients had homozygous C4A deficiency they were in fact 2 of the most severely ill patients, with vasculitis as a prominent component of their disease, and also subject to frequent bacterial infections which could be due partly to defective clearance of organisms due to complement deficiencies. This was suggested by the observation that these patients's CH50 levels were not reduced during intercurrent infections.

5.8 Conclusion

These studies have demonstrated 2 findings which may contribute to JDM (a) reduced numbers of CD8+ cells (b) an extended MHC haplotype, common to the majority of patients. This information became available after most of the work described in this thesis was completed.

5.9 Aims Of The Project

This project was initially designed to investigate the responses of peripheral blood lymphocytes from patients with myositis to purified normal human skeletal muscle membrane

antigens. However, no significant differences in the proliferative responses of either adult or childhood patients compared to controls were observed (data not shown). More recent studies have also shown no significant differences in proliferative responses of cells from myositis patients to light chain skeletal muscle rabbit myosin. Unfortunately it was not possible to obtain muscle biopsies from an adequate number of patients, particularly children, in order to extend these studies.

Initial experiments by the author, using immunofluorescence techniques, did not demonstrate the presence of tissue specific antibodies in sera from JDM patients to either normal human fetal muscle or human placental endothelial cell anigens. Similar results have been obtained by Dr Cambridge, employing Western blotting.

No information has been published on immunoregulatory circuits in patients with JDM. Yet, abnormalities in the control of the immune response are well established in, and may contribute to, putative autoimmune connective tissue diseases. Therefore the project became an investigation of immunoregulation in patients with JDM. A second complementary approach to this work was the exploration of possible anergic responses by lymphocyte populations from JDM patients, as this phenomenon is also associated with autoimmune connective tissue disease. Initial mitogen stimulation experiments allowed both aspects of the project to be investigated. These experiments identified defects of T-B cell cooperation and the role of regulatory T cell subsets was further studied in

responses to autologous antigens. The presence of an anti-lymphocyte antibody was identified in patient serum and its immunoregulatory characteristics and possible contribution to anergy analysed. Finally, the regulatory interactions between T and NK cells were investigated, original data obtained and related to the responses of patient cells at both the population and clonal levels.

CHAPTER 6

6.0 RESPONSES OF CELLS FROM JDM AND ADULT PM PATIENTS TO MITOGENIC STIMULATION

6.1 INTRODUCTION

Antigen stimulated activation of T and B cells and subsequent proliferation and differentiation into antigen specific effector T and Ig secreting cells involves a complex series of receptor mediated stimuli. Antigen binding to the CD3 - Ti complex on T cells and to surface Ig on B cells are the initial events in the generation of a conventional antigen specific response. Antibodies directed against these receptors (anti-CD3; anti-IgM) can mimic antigen induced activation pathways, potentially resulting in polyclonal expansion of appropriate receptor bearing cells.

Certain carbohydrate binding proteins, or lectins, also have the capacity to activate different subpopulations of lymphocytes by cross-linking sugar residues present on lymphocyte receptor molecules. Many lectins are known to possess mitogenic activity, stimulating lymphocytes to grow and divide. Some, such as phytohaemagglutinin (PHA) and concanavalin A (ConA) stimulate only T cells, while pokeweed mitogen (PWM) is a T cell dependent B cell mitogen.

6.1.1 T Cell Mitogens

The CD2 molecule, a receptor complex present on all T cells, has been shown to be the ligand binding site for PHA (O'Flynn et al, 1985). Binding of PHA to CD2 induces a conformational change in the receptor which induces an increase in intra-cellular free calcium followed by protein kinase C activation

and phosphorylation of membrane proteins including CD3. Phosphorylation may be a signal for down-regulation of the CD3-Ti antigen receptor, thus inhibiting further membrane activation (Cantrell et al, 1987). A second message is required to drive resting T cells in G_0 to G_1 phase and this may be provided by IL-1 secreted by accessory cells. Within hours of initial triggering, activated T cells release IL-2, which by binding to its receptor, transduces the signals required to switch T cells from G_1 phase to mitosis (reviewed by Smith, 1984).

6.1.2 B-Cell Mitogens

A recent review (Jelinek and Lipsky, 1987) has suggested that all polyclonal human B cell activators, with the exception of EBV, require T cells or T cell derived factors for the generation of Ig secreting cells, although some will induce proliferation in a T cell independent manner. Both proliferation and differentiation of B cells induced by PWM are T cell dependent although T cell proliferation is not required for maximal responsiveness. The role of PWM is probably to facilitate T-B interactions, perhaps by increased adherence of the 2 cell populations. There is no requirement for MHC compatibility between cooperating T and B cells for PWM stimulation but recognition of non-polymorphic class II determinants may be involved in the interaction (Suzuki et al, 1986).

In contrast to PHA and ConA responsive T cell precursors, the precursor frequency of the PWM responsive B cell population is small, being approximately 1/1000 - 1/2000 of B cells

(Martinez-Maza and Britton, 1983). The principal responder population has been shown to be surface IgD-. This appears to comprise resting as well as activated B cells. The resting population may comprise a subset of post-switch memory B cells capable of IgG and IgA as well as IgM secretion (Jelinek and Lipsky, 1987).

6.1.3 Responses of Lymphocytes from Patients with Connective Tissue Diseases to Mitogens

Impaired proliferative responses to mitogens by T cells from patients with connective tissue diseases, including adult PM, has been reviewed by Alarcon-Segovia, Alcocer-Varela and Diaz-Jouanen (1985). Depressed proliferative responses to PHA by lymphocytes from adult PM patients has also been reported by Behan and Behan (1985), but a study of 5 untreated, active JDM patients showed no significant differences from controls for proliferative responses to ConA and PHA (Pachman, Christensen and Scott, 1980).

Impaired secretion of Ig by lymphocytes from both SLE and RA patients in response to PWM has been reported (Manny, Datta and Scwartz, 1979; Patel, Panayi and Unger, 1983). In contrast to defective PWM responses, lymphocytes from some RA and SLE patients spontaneously secrete elevated levels of Ig (Patel, Panayi and Unger, 1983; Blaese, Grayson and Steinberg, 1980).

No studies of in vitro Ig production by cells from myositis patients have been published. However, autoantibodies, have been detected in the serum of both adult and childhood myositis patients (Bernstein, 1987; Pachman and Friedman,

1985). Spontaneous Ig production, a proportion of which represents autoantibody secretion, appears to be associated with inflammatory connective tissue diseases (Alarcon-Segovia, Alcocer-Varela and Diaz-Jouanen, 1985).

6.2 EXPERIMENTAL DESIGN

Mitogen induced proliferation and IgM production from PBMC obtained from JDM and adult PM patients, adult and childhood controls were measured. It was not possible to detect quantitative ANA production _in vitro_ by JDM cells due to lack of access to purified nucleoprotein antigens (Ch 1.1.3) and therefore total IgM production by cells from myositis patients and controls was recorded. The mean age of control children was 7 years. The mean age of the 14 JDM patients tested was 10 years. All except 2 of the children studied were receiving either alternate day or daily prednisolone at doses of less than 1mg/Kg and 4 were also receiving azathioprine. The mean age of the adult PM patients was 40 years and their clinical details are given in Table 6.2. Adult controls were normal, healthy volunteers (age range 20 - 49 years).

6.3 RESULTS

6.3.1 Proliferative Responses to Mitogens

The mean level of proliferation to the mitogens PHA, ConA and PWM by PBMC from 13 JDM patients did not differ significantly from that of cells from the normal control group at either optimal or sub-optimal concentrations (Table 6.1). The mean proliferative responses of PBMC from 8 adult PM patients to the three T cell mitogens was significantly lower than controls at both optimal and sub-optimal concentrations. The

PM patients could be divided into 2 groups of 4 (VER, FOU, THO, TAN) and (OSM, ZEF, JAQ, GOO), the former group having significantly reduced mitogen proliferative responses compared to the latter ($p < 0.05$). Three out of 4 patients with reduced responses had considerably elevated serum CPK levels. There were no differences between the two groups in immuno-suppressive drug therapy (Table 5.2)

6.3.2 Spontaneous and PWM Induced IgM Production

Spontaneous and PWM induced IgM production by PBMC from 9 normal adults, 8 PM patients, 8 normal children and 12 JDM patients are shown in Fig 6.1. PBMC from the adult patients did not differ in either their spontaneous (65 $\pm$ 16 ng/ml) or PWM induced (2022 $\pm$ 418ng/ml) IgM production from that shown by cells from adult control individuals (50 $\pm$ 10 and 2183 $\pm$ 492 ng/ml respectively). The mean level of spontaneous IgM production by PBMC from the juvenile patients, 232 $\pm$ 148 ng/ml was significantly higher than childhood controls (131 $\pm$ 207 ng/ml; $p < 0.01$). Further, high spontaneous IgM production was associated with an inability to produce significantly increased amounts of IgM in response to PWM stimulation. The mean level of IgM production by PBMC from JDM patients, in response to PWM, (712 $\pm$ 560 ng/ml) was significantly less than that by child controls (1913 $\pm$ 418ng/ml; $p < 0.01$).

TABLE 6.1. Proliferative responses of PBMC from normal controls and from patients with PM and JDM to mitogens added at optimal and sub-optimal concentrations.

MITOGEN		CONTROL ADULTS (n= 11)	CONTROL CHILDREN (n = 6)	JDM PATIENTS (n = 13)	ADULT PM PATIENTS (n = 8)
		^{3}H-THYMIDINE INCORPORATION (cpm ± SD)			
PHA	(10ug/ml)	51,776 ± 42,776	75,209 ± 18,901	67,499 ± 57,611	26,664 ± 21,651[a]
	(1ug/ml)	23,498 ± 18,312	34,028 ± 14,811	28,191 ± 20,019	8,038 ± 5,974[a]
Con A	(10ug/ml)	69,079 ± 25,072	43,890 ± 40,290	51,138 ± 16,499	24,235 ± 30,836[a]
	(1ug/ml)	26,432 ± 9,103	25,034 ± 10,027	23,341 ± 12,005	9,086 ± 11,664[a]
OKT 3	(25ng/ml)	33,609 ± 17,113	ND[b]	26,761 ± 17,184	10,682 ± 12,351[a]
	(2.5ng/ml)	5,111 ± 2,317	ND	4,219 ± 2,308	2,437 ± 1,423[a]
PWM	(1/100)	37,945 ± 8,312	29,076 ± 13,241	43,100 ± 20,369	ND
	(1/1000)	13,769 ± 8,312	11,698 ± 5,989	14,969 ± 6,326	ND
Medium Alone		1,000 ± 567	2,000 ± 1,949	2,096 ± 3,886	876 ± 415

[a] $p < 0.05$. Significance determined by comparison with values given by normal control populations at the same mitogen concentration.

[b] ND = Not determined

6.3.3 Contribution of T-Cells to Spontaneous IgM Production by JDM Lymphocytes

Although JDM immunoglobulin secreting cells produced low levels of IgM spontaneously in the relative absence of T cells (Table 6.2b), co-culturing equal numbers of autologuous T and non-T cells resulted in a five fold increase in IgM production.

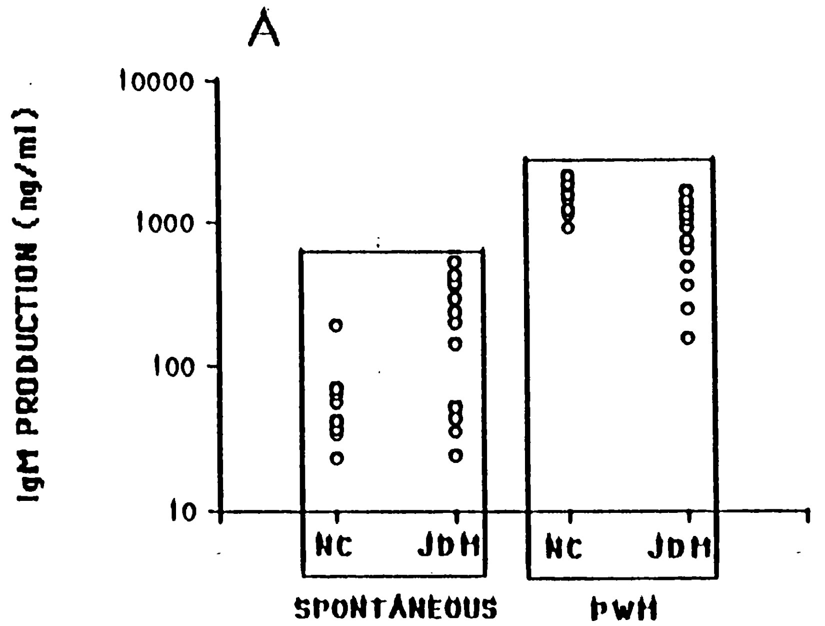
6.3.4 Requirement for Protein and DNA Synthesis by JDM non-T Cells for Spontaneous IgM Production

E- cells from 4 JDM patients were treated with cycloheximide or mitomycin C before coculturing with autologous E+ cells. Spontaneous IgM production was then measured (Figure 6.2). The effect of cycloheximide and mitomycin C was to suppress IgM production by 90% and 85% respectively.

6.3.5 Effect of Cytokines on Spontaneous and PWM Induced IgM Production

Addition of MLR supernatants to PBMC from 3 normal children and 4 JDM patients resulted in a dose-dependent increase in spontaneous IgM production (Fig 6.3) which was significant ($p < 0.005$) for both MLR concentrations tested. The addition of MLR supernatant to the cultures had no effect on PWM stimulated IgM production, while the addition of IL-2 had no effect on spontaneous or PWM induced IgM production.

There were no significant differences between spontaneous or PWM induced IgM production by normal adult and childhood controls. Therefore, in view of the considerable problems involved in obtaining material from normal children, it was felt reasonable, if not ideal, to conduct the following



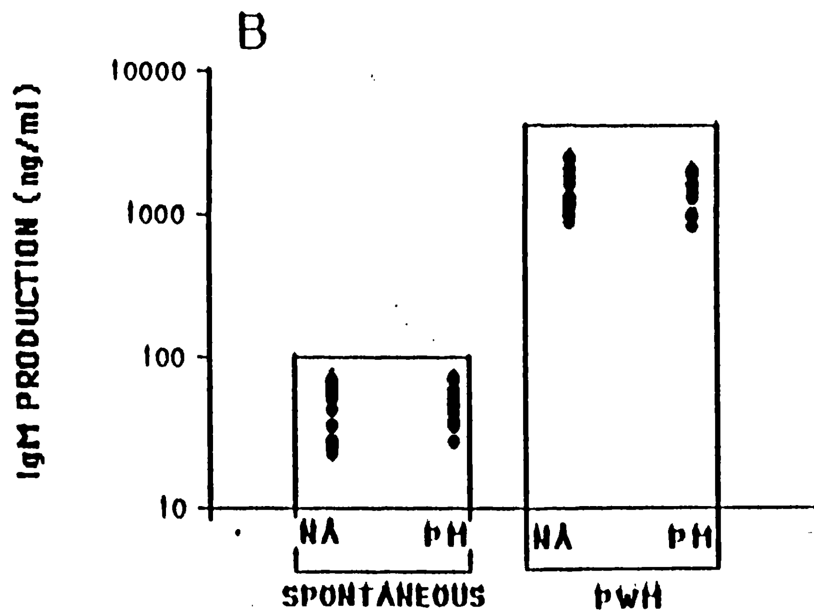
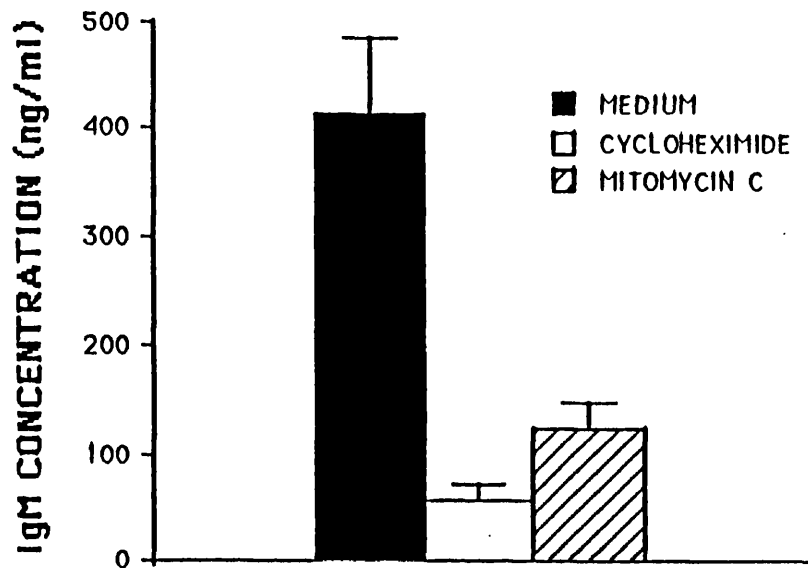


Figure 6.1.

Spontaneous and PWM induced IgM production by PBMC from (A) normal children (NC) and JDM patients and (B) normal adults (NA) and polymyositis (PM) patients. Cells ($2x10^5$) were cultured for 8 days and supernatants then harvested for IgM measurement.



<u>Figure 6.2</u>.

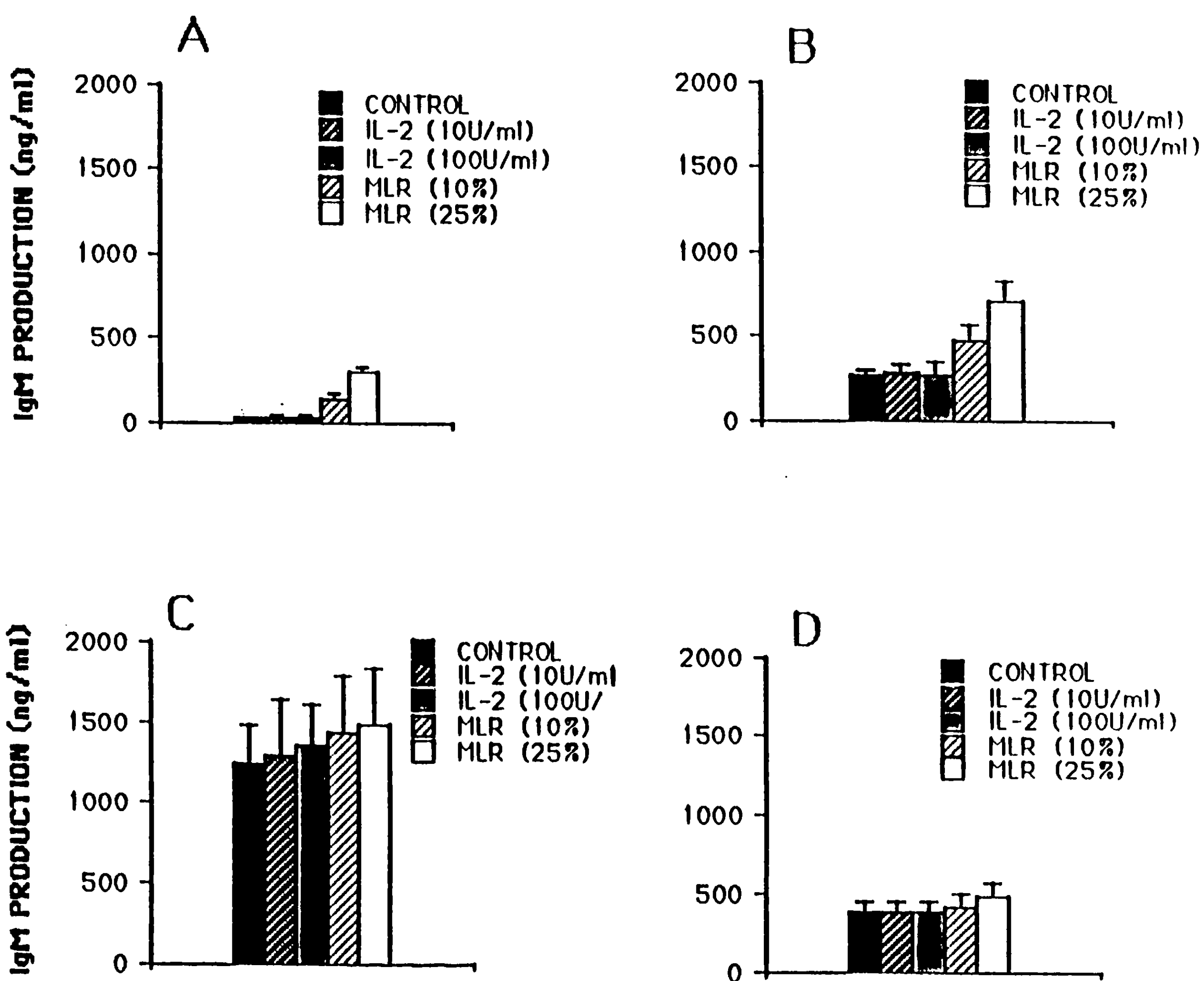
Effect of pre-treatment of E- cells with cycloheximide or mitomycin C on spontaneous IgM production by cocultures of autologous E- (1×10^5) and E+ (1×10^5) cells from 4 JDM patients.

experiments, examining IgM production by allogeneic combinations, using normal adult cells as controls.

6.3.6 Effect of Allogeneic Combinations of T and non-T Cells on Spontaneous and PWM Induced IgM Production

Autologous and allogeneic combinations of T and non-T cells from 3 normal adults and 3 JDM patients were cocultured, and spontaneous and PWM induced IgM production measured after 8 days. Individual combination results are given in Table 6.2a and grouped data in Table 6.2b. Grouped data results showed that allogeneic combinations of normal T and non-T cells did not differ significantly from autologous combinations for PWM induced or spontaneous IgM production. Individual results showed that of 5 allogeneic combinations of normal cells, 2 gave significantly reduced ($p < 0.05$) spontaneous IgM production compared to autologous combinations. Individual results for PWM stimulated IgM production showed no significant differences between allogeneic and autologous cocultures of normal cells.

When JDM T cells were cocultured with normal non-T cells, spontaneous IgM production was significantly enhanced (grouped data, $p < 0.01$) and IgM production in response to PWM was significantly reduced (grouped data, $p < 0.01$) compared with autologous combinations of normal cells. When normal T cells were cocultured with JDM non-T cells, spontaneous IgM production was significantly reduced (grouped data, $p < 0.01$) and PWM induced IgM production significantly enhanced (grouped data, $p < 0.01$) compared with autologous combinations of JDM cells. Similar results were obtained from every individual combination tested within each group. These results strongly



<u>Figure 6.3.</u>

Effect of the addition of IL-2 and MLR supernatants on (A, B) spontaneous and (C, D) PWM induced IgM production by PBMC from (A, C) normal children and (B, D) JDM patients. PBMC (2×10^5) were cultured in the presence or absence of cytokines and IgM production measured after 8 days. The concentration of IgM in the neat MLR supernatant was 158 ng/ml.

TABLE 6.2a

Spontaneous and PWM induced IgM production by individual autologous and allogeneic combinations of T and non-T cells from normal adults (N) and JDM patients.

(A) Autologous Combinations[a]

E+	E-	IgM PRODUCTION (ng/ml) SPONTANEOUS	PWM
N1	N1	48 + 10[b]	2,050 + 208
N2	N2	38 + 6	1,644 + 224
N3	N3	44 + 10	1,387 + 128
JDM 1	JDM1	380 + 45	344 + 38
JDM2	JDM2	261 + 28	363 + 28
JDM3	JDM3	230 + 28	184 + 22

(B) Allogeneic Combinations[a]

E+	E-	SPONTANEOUS	PWM
N1	N2	26 + 4[c]	2,111 + 47
N2	N1	18 + 6[c]	1,870 + 251
N1	N3	43 + 12	1,575 + 261
N3	N2	36 + 10	1,435 + 205
N3	N1	75 + 15	1,858 + 244
N1	JDM1	144 + 20	1,257 + 84
N1	JDM2	117 + 34	1,761 + 116
N3	JDM1	124 + 40	1,121 + 174
JDM1	N1	128 + 16	208 + 33
JDM1	N3	184 + 18	474 + 138
JDM 2	N2	108 + 25	220 + 24
JDM3	N1	154 + 44	164 + 20

a 1×10^5 E+ cells were cocultured with 1×10^5 E- cells in the presence or absence of PWM. Culture supernatants were harvested after 8 days and IgM production measured.

b The results are given as the mean + SD IgM production of triplicate wells in each experiment.

c Allogeneic combinations of normal cells giving significantly reduced spontaneous IgM production compared to autologous combinations.

TABLE 6.2b

Spontaneous and PWM induced IgM production by grouped autologous and allogeneic combinations of T and non-T cells from normal adults (N) and JDM patients.

(A) Autologous Combinations

E+	E-	n[a]	IgM PRODUCTION (ng/ml) SPONTANEOUS	PWM
N	N	3	43 + 4[c]	1,694 + 273
JDM	JDM	3	290 + 65	297 + 80
-	N[b]	3	10	ND
-	JDM[b]	3	54 + 15	ND

(B) Allogeneic Combinations

E+	E-	n	SPONTANEOUS	PWM
N	N	5	40 + 20	1,770 + 238
N	JDM	3	128 + 11	1,380 + 275
JDM	N	4	144 + 29	267 + 122

a Number of combinations of 1×10^5 E+ and E- lymphocytes from patients or controls.

b 1×10^5 E- cells cultured alone.

c The results are given as the mean + SD IgM production of triplicate wells cultured in the presence or absence of PWM.

suggested that B cells from JDM patients were responsive to regulatory signals from T cells.

6.4 DISCUSSION

The proliferative responses of PBMC from a group of adult PM patients to 3 T cell mitogens were significantly reduced compared to normal adult controls. Lack of access to patients unfortunately did not allow follow up of these observations. Examination of (a) CD4/CD8 ratios (b) production of and response to IL-2 by PM T cells (c) precursor frequencies of mitogen reactive T cells (d) effect of coculture of normal PBMC and PM T cells on mitogen responsiveness (e) role of monocytes and monocyte derived factors, may give clues to the reasons for defective proliferation.

PM patients could be divided into patients who gave proliferative responses within the normal range and those whose responses were significantly reduced. The differences between the 2 groups could not be ascribed to drug treatment, but CPK levels were considerably elevated in patients with poor responses, perhaps reflecting disease exacerbation.

In contrast to adult patients, mitogen induced proliferation of cells from JDM patients was not significantly different from normal children. One study, conducted on children with various diseases including JDM, indicated that a single, oral dose of prednisolone (0.5mg/Kg or greater) may significantly reduce the PHA induced proliferative responses of lymphocytes taken 4 hours after drug administration. The effect of prednisolone appeared to depend on the plasma level achieved and this may vary considerably because of slow and erratic

absorption found in many JDM patients (Green et al, 1978). In the present study, proliferative responses of lymphocytes to mitogens appeared to be unrelated to therapy.

Marked differences in spontaneous and PWM induced IgM production between adult myositis and JDM patients were also observed (Fig 6.1). PBMC from most JDM, but not adult, patients produced elevated levels of IgM spontaneously *in vitro*. There were no apparent differences in drug treatment or length of history of disease between those patients whose cells produced IgM spontaneously and those who did not. Cells from the 2 untreated patients (GH and SW) secreted the highest levels of IgM spontaneously.

Although JDM E- populations were shown to produce IgM spontaneously in the relative absence of T cells, coculturing equal numbers of autologous JDM T and non-T cells resulted in augmented IgM production, suggesting that *in vivo* activated JDM T cells were co-operating with B cells to induce Ig secretion. Both proliferation and protein synthesis by JDM non-T cells were required to maintain spontaneous IgM production, indicating that carry over of IgM or release of IgM by damaged cells were unlikely to contribute to these results.

Spontaneous Ig production has been described in autoimmune disorders and animal models of autoimmune disease (Budman et al, 1977; Patel, Panayi and Unger, 1983; Manny, Datta and Scwartz, 1979). The reasons for this have been ascribed to hyperreactive B cells, activated T helper cells or diminished

numbers of T suppressor cells (Beale, Nash and Bertavich, 1982; Morimoto, Abe and Homma, 1979). Examination of spontaneous IgM secretion by allogeneic combinations of normal and JDM T and B cells produced evidence suggesting there was no intrinsic defect of JDM B cells leading to spontaneous IgM production. The results implicated JDM T cells as being primarily responsible for the response.

No enhancement of IgM production by allogeneic compared to autologous combinations of normal T and non-T cells was observed, suggesting that augmented helper function by JDM T cells for spontaneous IgM production by normal non-T cells, was not the result of an allogeneic effect. Suppression of Ig production due to allogeneic interactions has been described (Brenner and North, 1983; Rumke et al, 1982) and was in fact found for some normal, allogeneic combinations described here. This suppressive effect has been reported to be partially overcome by the addition of supernatants from activated T cells (Brenner and North, 1983), providing a possible mechanism for spontaneous IgM production by E- cells in response to JDM T cells.

Therefore, experiments investigating the role of lymphokines in inducing spontaneous Ig production were conducted (Fig 6.3). MLR supernatants significantly enhanced spontaneous IgM production by both normal and JDM cells. The effects of MLR supernatants may have been directed to activated B cells within normal and JDM lymphocyte populations (Jelinek and Lipsky, 1987).

Although highly purified tonsillar B cells have been shown to secrete Ig in response to IL-2 alone, peripheral blood B cells had poor responsiveness, possibly reflecting different levels of B cell activation in different anatomical sites (Romagnani et al, 1986). Normal and JDM cells were found not to give enhanced spontaneous secretion of IgM in response to IL-2. It was also possible that IgM production by PBMC in response to IL-2 was inhibited by the presence of T cells in the cultures (Bich-Thuy and Fauci, 1985). Experiments examining whether purified JDM B cells could be induced to secrete Ig in response to IL-2 could give information on the activation state of the cells.

Activated B cells may express IL-2 receptors and IL-2 has been claimed to be an essential factor for normal B cell differentiation in response to PWM (Nakagawa et al, 1986). The inability of JDM T cells to provide help to B cells in the presence of PWM was unlikely to be due to defective IL-2 production, as the proliferative responses of JDM cells to PWM were not significantly different from that of normal cells. Furthermore, the addition of exogenous IL-2 had no effect on defective IgM production by JDM cells in response to PWM. The results obtained suggested there was a failure of cooperation between JDM T and B cell populations in response to PWM. Although allosuppression or suppression induced by PWM (Rumke et al, 1982) could have contributed to this effect, none of the normal, allogeneic combinations gave significantly reduced responses compared to autologous combinations in the presence of PWM. Similarly, Callard (1984) could not detect an allosuppressor effect in PWM induced Ig production.

Significantly increased IgM production in response to PWM by combinations of JDM E- and normal T cells suggested that the PWM responsive B cell population was probably not defective in JDM patients. PWM induced differentiation of B cells appears to require the presence of T cells as well as T cell factors for optimal responsiveness (Jelinek and Lipsky, 1987). Therefore defective PWM facilitated T-B interactions by JDM cells, for which T cell factors were unable to substitute, may have contributed to the impaired response. It is possible that a T helper population(s), required for the PWM response, is defective in JDM patients.

The T cell population recognised by the MAb UCHL1 has been reported to be the subset responsible for cooperating with B cells in Ig production in response to PWM (Beverley, 1987).

The CD4+ UCHL1+ T cell population has recently been shown to be significantly reduced in the peripheral blood from JDM patients compared to childhood controls (G Cambridge and P Lydyard, unpublished results), although functional analysis of the subset in the PWM response of cells from JDM patients has still to be performed.

An alternative explanation was that suppressor T cells within the JDM lymphocyte population were inhibiting PWM induced Ig production. This could not be ruled out, but preliminary experiments, adding JDM T cells to PWM stimulated normal PBMC showed no evidence of suppression (data not shown).

6.5 CONCLUSION

No evidence of anergy was observed for responses of cells from JDM, in contrast to adult PM patients. The results indicated defective cooperation between the PWM responsive JDM helper-inducer T cell subset and B cells in Ig production. However, B cell differentiation factors secreted by JDM T cells could have been responsible for enhanced spontaneous Ig production.

It was possible that inability of T cell subpopulations to co-operate normally in response to certain activation signals could result in failure to down-regulate an immune response resulting in spontaneous Ig production. A sensitive assay system was therefore required to investigate T cell interactions and regulatory cell function. The following chapter presents results using the response to autologous antigens to investigate these functions.

CHAPTER 7

7.0 THE AUTOLOGOUS MIXED LYMPHOCYTE REACTION

7.1 INTRODUCTION

7.1.1 Responder T Cell Populations

Normal human T cells proliferate *in vitro* when stimulated with MHC class II expressing non-T cells from the same donor in the autologous mixed lymphocyte reaction (AMLR). T cells are believed to recognise and respond to autologous D region antigens, present in peripheral blood, on monocytes and B cells (Winchester and Kunkel, 1979). The AMLR has been shown to be dependent on the production of and response to IL-2 (Palacios and Moller, 1981). Following initial recognition of HLA-D region antigens by resting T cells, both CD4+ and CD8+ lymphocytes are induced to express IL-2 receptors but only CD4 lymphocytes can secrete IL-2 (Takada et al, 1985). The first 2-3 days of the AMLR are believed to result in the activation of at least 2 T cell populations (i) T cells which can cooperate with B cells in antibody responses (Volk and Diamanstein, 1986), and which may belong to the CD4+ UCHL1+ subpopulation (Beverley, 1987) (ii) T cells which can give help for the activation of suppressor cells, which are the main functional cell type detected during the stage of maximal proliferation in the AMLR (Fitzharris and Knight, 1981). The human suppressor-inducer T cell subset has been characterised (Morimoto et al, 1985) and evidence for its role has been presented in Chapter 2.6.2.

7.1.2 Clonal Analysis

Greater understanding of the AMLR has derived from the generation of Class II MHC reactive murine and human T cell clones. Human clones have been obtained and grown in serum free medium and shown to proliferate in response to purified, autologous DR antigen incorporated into liposomes (Coeshott et al, 1986). This evidence strongly suggests that autologous reactivity is not solely due to artefacts such as xenogeneic serum or sheep red blood cells (Bretscher, 1986). Human class II reactive clones have also been obtained after (a) influenza virus immunisation (b) allogeneic stimulation _in vitro_ (Tilkin et al, 1987). The clones generated in these experiments proliferated to epitopes of DR molecules and could be inhibited by monoclonal anti-DR antibodies. The DR epitopes recognised by autologous reactive clones also functioned as restriction elements for influenza virus specific T cell clones. Autoreactive T cells could therefore belong to the antigen specific repertoire (Faherty, Johnson and Zunderer, 1985) with a high affinity for self MHC molecules, making them independent of nominal antigen.

7.1.3 Autoimmunity

The AMLR represents a useful assay in autoimmune disease since it (a) is a measure of cellular co-operation (b) allows the testing of functional cells generated during the reaction (c) allows the measurement of IL-2 and IL-2 receptor expression by T cells activated in the immunoregulatory circuit which comprises the AMLR.

Significantly reduced or absent AMLR proliferative responses of peripheral blood T cells have been reported in autoimmune diseases such as SLE, RA and autoimmune thyroid disease (Volk and Diamanstein, 1986; Pope et al, 1984; Canonica et al, 1984). There is one report (Ransohoff and Dustoor, 1983) describing a defective AMLR proliferative response in a group of 8 adult PM/DM patients, but PBL from JDM patients have not been tested for their AMLR responses. Sera from patients with SLE have been tested for its effect on the AMLR of normal cells (Stephens et al, 1982). Most SLE sera totally suppressed the proliferative response. The effect of SLE sera has been ascribed to anti-DR antibodies present in the sera (Okudaira et al, 1982). The effect of JDM sera on the AMLR of normal cells has not been examined.

7.2 Experimental Design

This Chapter will describe experiments examining various characteristics of the AMLR including:

(a) the role of cell surface determinants on stimulator and responding cell populations;

(b) the role of B cells as stimulators;

(c) the production of and response to IL-2, believed to be central to the AMLR by most workers (Palacios, 1982; Takada et al, 1985);

(d) the induction of functionally active suppressor cells.

The responses of cells from normal controls and JDM patients will be compared according to the above criteria.

7.3 Effect of Corticosteroid Treatment

Ideally, blood should have been taken 24h after drug administration in order to minimise the effects of steroid treatment on the AMLR. Since patients were being treated at a different centre this was not possible. There is no information available as to the possible effects of long term corticosteroid therapy on the AMLR in patients with rheumatic diseases. Studies in mice have shown that the responder cells in the AMLR reside in the cortisone resistant T-cell populations (Hahn et al, 1980). In one study, when prednisolone (10-200 ng/dl) was added to AMLR cultures of cells from normal adult individuals, proliferation of responder T cells was significantly reduced if monocyte derived cells were used as stimulators. However, when B and null cells were used as the stimulator population, as in the experiments described in this thesis, the AMLR was not affected (MacDermott and Stacey, 1981).

7.4 RESULTS

7.4.1 Time Course of the AMLR

The kinetics of the proliferative response of cells from a normal child and a JDM patient, cultured in serum from either the patient or the control, were determined over 7 days (Fig 7.1). When patient and normal cells were cultured in normal serum, patient lymphocyte proliferation was significantly lower ($p < 0.01$) at all time points tested. When serum from this patient was substituted for normal human serum in the experiment, the proliferation kinetics followed the same time course, but reduced the maximum response by normal cells at

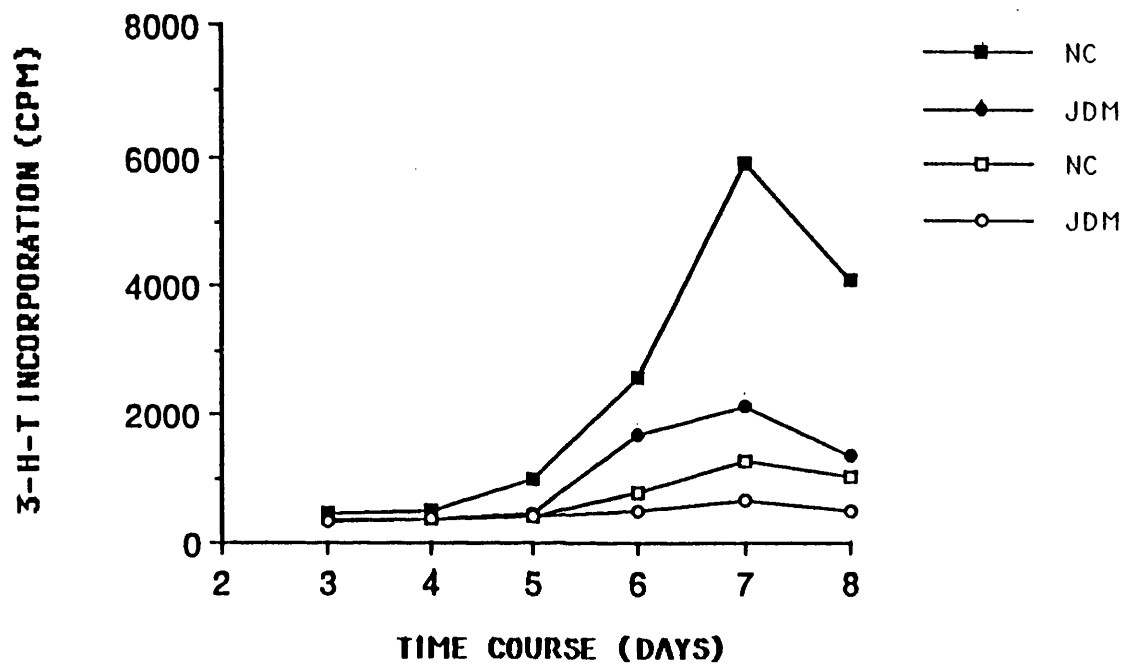


Fig 7.1

Time course of the AMLR proliferative response of cells from a normal child (NC) and a JDM patient. Cells were cultured in autologous normal serum (closed symbols) or autologous JDM serum (open symbols).

day 7 by 70%. The response of autologous patient lymphocytes was ablated. In a series of further experiments, similar results were obtained (data not shown). In no case was an early or biphasic proliferative response of JDM cells observed. The proliferation kinetics of cells from normal adult donors also followed the same time course, with maximum proliferation on day 7 (data not shown).

7.4.2 Effect of Antibodies to Lymphocyte Markets on the AMLR of Normal Cells

The effect of adding MAb's, recognising T and B cell antigens, on the response of normal adult cells in the AMLR was examined (Table 7.1). The addition of anti-DR or anti-IL-2 receptor antibodies ablated the proliferative response in each experiment. Inhibition of the responses (mean of 3 experiments) when anti-CD3 was added was 95% and 89% for the 2 concentrations of antibody used. The mean inhibition of the responses when anti-CD4, anti-CD8 and anti-class I were added were 76%, 58% and 60% respectively for the higher concentrations of antibodies. These levels of inhibition were significant ($p < 0.01$).

7.4.3 Response of T Cells to E- and EBV Transformed Stimulator Cells

The proliferative responses of T cells from 12 JDM patients, 6 healthy children and 8 healthy adults was measured in the AMLR using either fresh peripheral blood E- cells or EBV transformed B cell lines as the stimulator cell population (Table 7.2). The mean value of $^3H - T$ incorporation for patient T cells in response to autologous E- cells was significantly less than that given by responder cells from either control group ($p < 0.01$). When EBV transformed B cells

Table 7.1.

Effect on the AMLR proliferative response of culturing normal adult cells in the presence of MAb's to lymphocyte markers.

		^{3}H - T INCORPORATION (CPM)		
		EXP 1	EXP 2	EXP 3
Medium Alone		5,516 ± 530[a]	9,721 ± 1,768	8,314 ± 874
+ DA2	(1/100)[b]	157 ± 15	117 ± 25	151 ± 15
	(1/1000)	194 ± 26	135 ± 18	121 ± 20
+ 2A1	(1/100)	2,217 ± 312	4,318 ± 543	3,874 ± 407
	(1/1000)	4,138 ± 427	7,384 ± 1,124	6,124 ± 324
+ Anti-Tac	(1/10)	224 ± 31	193 ± 20	254 ± 41
	(1/100)	305 ± 65	204 ± 24	387 ± 34
+ UCHT1	(1/10)	816 ± 134	2,312 ± 526	403 ± 198
	(1/50)	1,740 ± 190	5,718 ± 631	903 ± 694
+ UCHT4	(1/10)	1,851 ± 222	6,464 ± 298	1,587 ± 685
	(1/50)	4,184 ± 637	7,932 ± 1,080	2,723 ± 821
+ MT310	(1/10)	1,295 ± 111	3,980 ± 475	1,487 ± 907
	(1/50)	3,704 ± 399	7,113 ± 621	3,104 ± 655
+ 2D1	(1/10)	5,108 ± 738	9,914 ± 837	8,181 ± 907
	(1/50)	4,813 ± 574	10,734 ± 2,132	8,528 ± 1,223

[a] Results are expressed as the mean ± 1SD.

[b] Monoclonal antibodies, at the concentrations indicated, were added at the initiation of the culture period.

were used as the stimulator population, responder cells from adult controls, child controls and JDM patients, showed significantly increased proliferative responses ($p < 0.05$), although JDM T cells still showed a significantly lower mean value for ^3H - T incorporation than either control group ($p < 0.05$). Flow cytometry did not reveal any difference between the numbers of DR antigen expressing E- cells and EBV lines of patients and childhood controls (data not shown).

7.4.4 AMLR Proliferative Responses of JDM Lymphocytes Cultured in Autologous and Normal Sera

The proliferative responses of T cells, stimulated by autologous non-T cells, from 8 JDM patients are given in Fig. 7.2. Three patients were tested on 2 separate occasions.

Two patients's cells gave no proliferative response in the AMLR. These patients (Fig 7.2A) (SW, NC), tested at the time of diagnosis, were untreated. Patient TC's cells (Fig 7.2B) made no proliferative response in the first AMLR assay (TC1) and a poor response (SI 2.8) on retesting 3 months later (TC2). The patient was not receiving drugs at the time of the first assay but was receiving prednisolone (7 mg/day) when retested. Patient JK's cells (Fig 7.2C) made a moderate proliferative response at the first test (JK1) (SI 5.0) but no response on retesting 3 months later (JK2). When first tested this patient had begun treatment with azathioprine but when retested she was untreated. Patient PO (Fig. 7.2D) was tested on 2 occasions, 2 months apart and was receiving prednisolone (20 mg on alternate days) at the time of both assays (PO1, PO2). Her T cells made a moderate proliferative response (SI

6.4) at both assays. Patients HM, KA and LM's cells (Fig 7.2E) gave proliferative responses with SI 15.4, 7.1 and 4.8 respectively. HM was being treated with 15 mg prednisolone daily, KA with 2 mg prednisolone daily and LM with 10 mg prednisolone on alternate days at the time the assays were performed. Two patients's cells (HM and PO2) gave significantly reduced proliferative responses ($p < 0.01$) when cultured in autologous compared to normal adult serum. The data on untreated patients and patients tested on separate occasions indicated that the responses of patient cells could not be explained as a result of corticosteroid treatment.

7.4.5 Effects of Cytophillic Antibodies

The possibility that cytophillic antibodies might have bound to JDM lymphocytes and inhibited the AMLR was examined. Incubation of JDM cells for 1h or O/N at 37°C, in 10% normal serum, did not significantly affect the proliferative responses of JDM cells (data not shown), suggesting that membrane bound antibodies did not contribute to the defective AMLR.

7.4.6 Response of Normal T Cells to Allogeneic Normal or JDM Non-T Cells

A series of mixed lymphocyte reactions provided information on the ability of normal adult T cells to respond to allogeneic normal and JDM non-T cells from 3 normal adults and 4 patients (Table 7.3). There was no significant difference between mean levels of response to normal compared to JDM non-T cells.

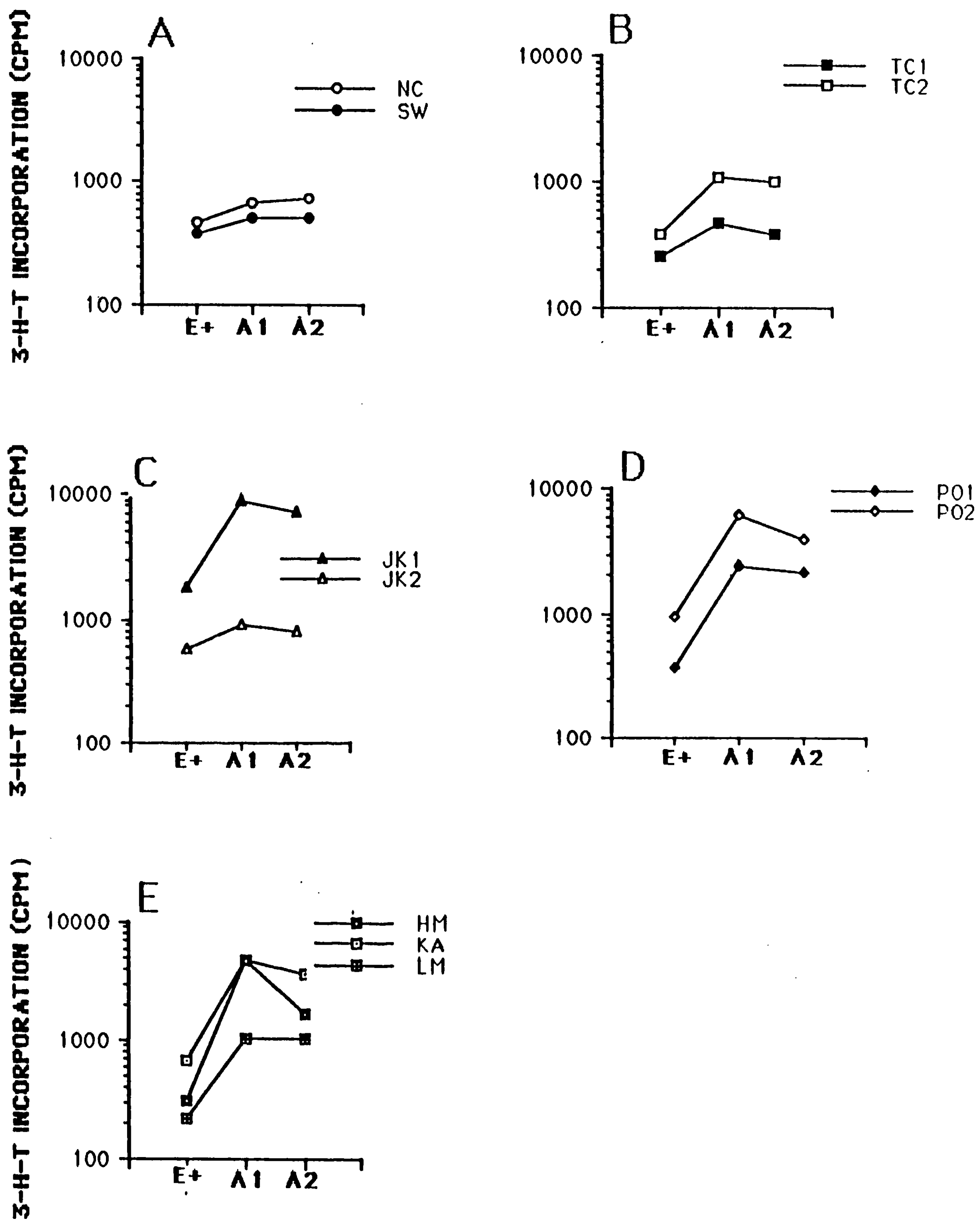


Fig 7.2 Proliferative responses in the AMLR of T cells from JDM patients, 3 of whom (B, C, D) were tested on 2 separate occasions. Cells were cultured in serum from a normal child (A1) or autologous serum (A2). The responses of T cells cultured alone in normal serum (E+) are also shown.

7.4.7 **Production of IL-2 by JDM Cells in the AMLR**

Secretion of IL-2, in the AMLR, was measured for cells from 3 normal adults and 3 JDM patients.

IL-2 production by JDM cells, was greatly reduced compared with normal adult cells (Table 7.4). In 2/3 experiments, the levels of IL-2 generated in the AMLR by JDM cells was the same as that of E+ cells alone. The normal adult cells in experiment 2 had a very high background ^{3}H-T incorporation and IL-2 production. This person was subsequently found to have been suffering from uveitis at the time blood was taken. IL-2 activity in supernatants reflected the proliferative response of cultures of normal and JDM cells, suggesting that failure to detect IL-2 activity in JDM cultures did not simply reflect absorption of free lymphokine to activated cells. Patients LM and JK were receiving prednisolone (< 1 mg/Kg/day), while patient HM was untreated.

7.4.8 **Phenotypes of Cells Expressing IL-2 Receptors in the AMLR**

The phenotypes and proportion of T cells expressing IL-2 receptors (CD 25) after 3 and 7 days of culture in the AMLR were determined (Table 7.5). Experiment 1 showed that 30% of normal child CD3+ cells expressed CD 25 after culture for 3 days in the AMLR. Experiments 2 and 3 showed that 30% and 22% respectively of normal child CD4+ cells expressed CD 25 after culture in the AMLR for 3 days. The proportion of normal child T cells expressing CD45R increased after 3 days culture in the AMLR and CD 25 expression on these cells after 3 days varied from 35 to 50%. Adult control T cells showed similar phenotypic changes to childhood control T cells in the AMLR.

TABLE 7.2.

Proliferative responses of T-cells from control individuals and JDM patients to stimulation with autologous E-cells and EBV transformed B-cell lines (EBV-tf).

		STIMULATOR CELLS	
	N	E-	EBV-tf
ADULTS	8	7,626 ± 9,555[a]	12,822 ± 11,329
CHILDREN	6	11,986 ± 10,483	19,806 ± 12,107
JDM PATIENTS	12	1,833 ± 2,573	5,114 ± 6,570

a Results are expressed as mean cpm ± SD of ^{3}H-Thymidine incorporation.

TABLE 7.3.

Proliferative responses of T cells from normal adult donors to allogeneic non-T cells from normal adults and JDM patients[a]

E-	DR[b]	E+	DR	^{3}H - T INCORPORATION (CPM)
N	4,5	N	1,2	70,034 ± 6,819
JDM	3,4	N	1,2	65,381 ± 10,327
N	4,5	N	-	51,775 ± 7,824
JDM	3	N	-	41,919 ± 4,380
N	3,4	N	1,2	75,921 ± 6,842
JDM	3,4	N	1,2	89,755 ± 9,304
JDM	2,3	N	1,2	57,138 ± 6,131
N	1,2	N	4,5	50,865 ± 8,312
JDM	2,3	N	4,5	61,903 ± 8,194
JDM	3,6	N	4,5	41,384 ± 3,119

a 1×10^{5} normal (N) or JDM E- cells cocultured with 1×10^{5} normal E+ cells. Proliferative responses were measured after 5 days.

b DR types of normals and patients are given where known.

Table 7.4.

Production of IL-2 by JDM Cells in the AMLR

			IL-2 PRODUCTION[a] (U/ml)		^{3}H-T INCORPORATION[b] (CPM)
			3 DAYS	5 DAYS	7 DAYS
EXP. 1	NORMAL	E+	4	5	1,363 ± 178
		AMLR	80	88	21,816 ± 1,017
	LM	E+	6	5	2,005 ± 313
		AMLR	2	6	3,142 ± 287
EXP. 2	NORMAL	E+	40	44	12,347 ± 849
		AMLR	84	96	40,251 ± 1,725
	HM	E+	<2	<2	238 ± 43
		AMLR	<2	<2	471 ± 99
EXP. 3	NORMAL	E+	<2	<2	474 ± 50
		AMLR	8	10	3,129 ± 315
	JK	E+	<2	<2	425 ± 42
		AMLR	4	4	1,223 ± 187

a Supernatant aliquots from control and AMLR cultures were harvested at days 3 and 5 and IL-2 concentration measured by stimulation of PHA blasts and interpolation on a standard curve.

b T cell proliferation of control and AMLR cultures were measured at day 7.

The results showed some increase in number of CD4+ and CD45R+ JDM T cells expressing CD 25 after 3 days culture in the AMLR, but this increase was far less than for normal cells. There was a marked increase in normal CD8+ cells by day 7 of the AMLR and 16 to 24% of these cells expressed CD 25. In contrast, the 5 patients tested showed only minimal increases in the proportion of CD8+ cells and only in experiment 3 was there any increase in CD 25 expression by CD8 cells in the AMLR culture compared to control T cells. JDM patients in experiments 1 and 2 were untreated. In experiments 3-5 patients were receiving prednisolone (<0.5mg/Kg/day).

7.4.9 Effect of Adding IL-2 and Indomethacin to the AMLR of Normal Adult and JDM Cells

The addition of indomethacin at a concentration (1ug/ml) previously shown to inhibit PGE_2 production in vitro, (Staite et al, 1982) had no significant effect on the response of cells from 6 normal adults (Fig 7.3A), 4 normal children (Fig 7.3B) and 6 JDM patients (Fig 7.3C). The addition of IL-2 gave dose-dependent increases in the responses of cells from controls and patients (Fig 7.3), although the mean responses of normal cells in the presence of IL-2 was still significantly greater than that of JDM cells in IL-2 ($p < 0.01$). Responses of E+ cells cultured alone in the presence or absence of IL-2 were subtracted from the results.

Table 7.5. IL-2 receptor expression on lymphocyte subsets during the AMLR by cells from 3 normal children, 2 normal adults and 5 JDM patients.

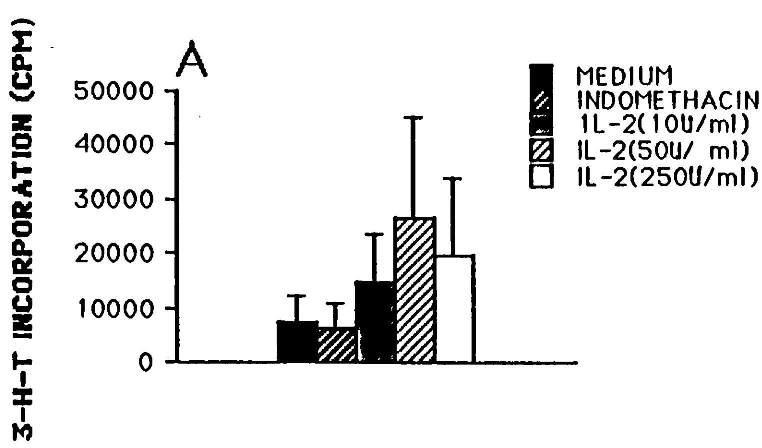
			3 DAYS			7 DAYS
			CD3	**CD4**	**CD45R**	**CD8**
EXPT. 1	NORMAL CHILD	- E+	95[a](1)[b]	63 (1)	25(1)	40(1)
		- AMLR	95 (30)	65(46)	45(35)	65(20)
	JDM	- E+	92 (1)	74 (1)	26(1)	20 (5)
		- AMLR	94 (8)	76 (3)	39 (6)	33 (6)
EXPT. 2	NORMAL CHILD	E+	ND	75 (7)	40 (5)	27 (2)
		AMLR	ND	60(50)	52(50)	56(24)
	JDM	E+	ND	68 (3)	35 (1)	25(1)
		AMLR	ND	65 (5)	38 (3)	35(1)
EXPT. 3	NORMAL CHILD	E+	ND	64 (3)	28 (5)	32 (5)
		AMLR	ND	64(35)	38(36)	58(16)
	JDM	E+	ND	70(1)	30(1)	28 (5)
		AMLR	ND	64 (8)	34(10)	33(10)
EXPT. 4	NORMAL ADULT	E+	95 (1)	61 (1)	28(1)	34 (1)
		AMLR	95(33)	64(45)	37(65)	59(24)
	JDM	E+	96 (1)	69 (1)	31 (1)	22 (1)
		AMLR	93 (4)	69 (1)	35 (1)	24 (1)
EXPT. 5	NORMAL ADULT	E+	ND	60 (2)	34 (1)	36 (2)
		AMLR	ND	64(50)	42(44)	58(20)
	JDM	E+	ND	69 (3)	27 (3)	27(1)
		AMLR	ND	73 (6)	28 (4)	29(1)

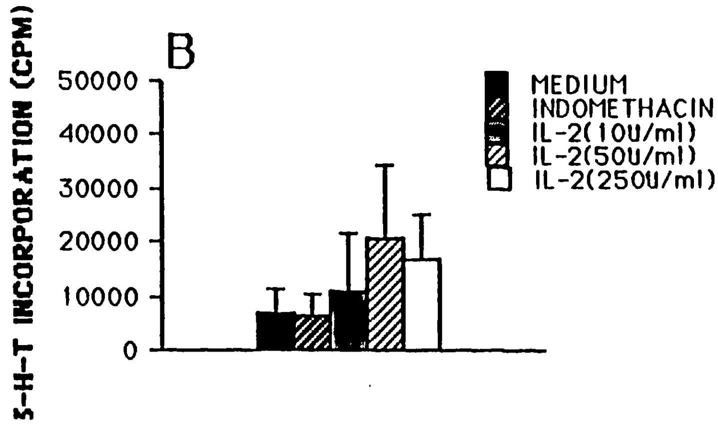
a Figures represent percentage of cells expressing the designated marker. Cells were harvested after 3 days or 7days culture. Expression of CD3, CD4 and CD45R were determined after 3 days culture, CD8 expression after 7 days.

b Figures in parenthesis represent the percentage of cells expressing the designated marker which coexpressed CD25.

7.4.10 Generation of Suppressor Cells in the AMLR

Experiments were undertaken to investigate the generation of functionally active suppressor cells during the AMLR (Fig 7.4). T cells were harvested at day 7 from cultures of normal childhood and JDM cells. These cells were then added to autologous B cells which had been cultured for 7 days in the presence of EBV. IgM production was then measured after a further 7 days culture. T cells generated during the AMLR of cells from 3 normal children, 1 tested on 2 separate occasions, inhibited IgM production by a mean of 42% (range 35-48%). The addition of IL-2 (50U/ml) to the AMLR increased the suppressive effect of normal childhood T cells on IgM production to 63% (range 55-70%). IgM production was significantly reduced ($p < 0.01$) when normal T cells, generated in the AMLR in the presence or absence of IL-2, were cocultured with B cells compared with B cells cultured alone or with B cells cultured with control T cells. Functionally active suppressor cells were not generated during the AMLR of cells from 4 JDM patients (Fig 7.4), one of whom (GH) was untreated at the time of testing. The addition of IL-2 to the AMLR, although increasing proliferation of T cells from JDM patients (Fig. 7.3), had no effect on suppressor cell generation. Two out of 4 JDM patients tested had serum antibodies to EBV viral capsid antigens (VCA) (Dr D Crawford, UCH).





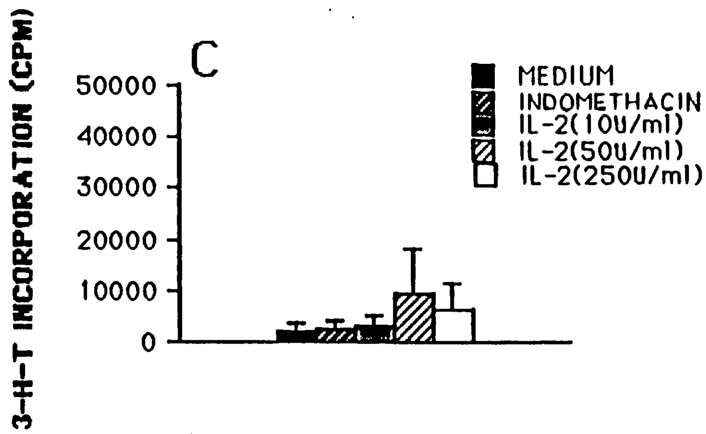
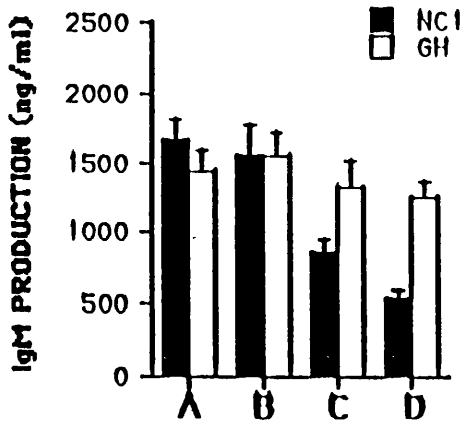
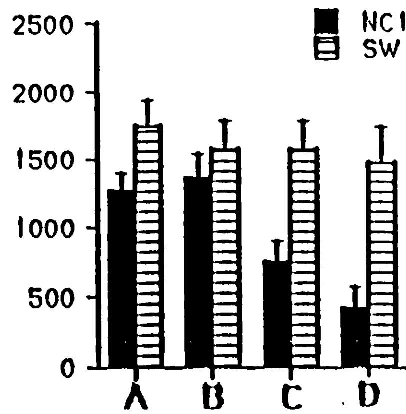
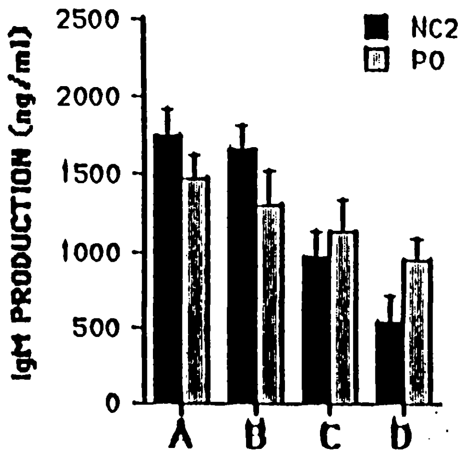


Figure 7.3. Effect of the addition of indomethacin and IL-2 on mean levels of proliferation in the AMLR of T cells from (A) normal adults (B) normal children and (C) JDM patients.







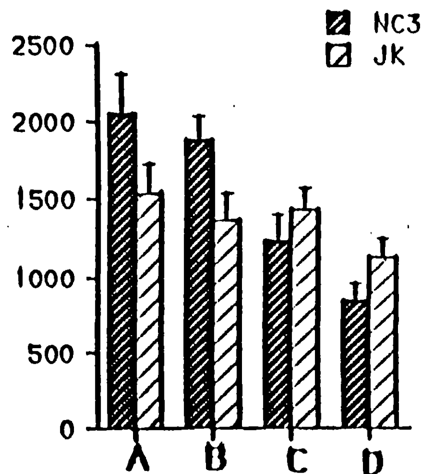


Figure 7.4.
Effect on IgM production by EBV infected B cells (5×10^4) of coculture with control or AMLR generated autologous T cells (1.5×10^5) from 3 normal children (NC1-3) and 4 JDM patients. T cells were harvested from control and AMLR cultures after 7 days and then cocultured with EBV infected B cells. IgM production was measured after 7 days. (A) B cells alone (B) B cells + T cells from control cultures to which IL-2 (50 U/ml) had been added (C) B cells + T cells from AMLR cultures (D) B cells + T cells from AMLR cultures to which IL-2 (50 U/ml) had been added.

7.5 Discussion

Time course experiments showed that the proliferative response kinetics in the AMLR were the same for normal and JDM cells. An early or biphasic response has been described for cells from scleroderma patients in the AMLR (Alarcon-Segovia, Alcocer-Varela and Diaz-Jouanen, 1986). One interpretation of these results could be that scleroderma patients possess a population of in vivo activated AMLR reactive T cells, not present in the peripheral blood of JDM patients.

Anti-CD3, CD4, CD8, DR and anti-class I antibodies all significantly inhibited the proliferative responses of normal cells in the AMLR (Table 7.1). The effects of these antibodies suggested that the recognition structures present on and necessary for activation and proliferation of autologous reactive T cells are the same as those for antigen-specific T cells. Inhibition of the AMLR by anti-CD8 antibodies was also noted by Romain et al (1984), who showed that CD8+ as well as CD4+ lymphocytes could proliferate in response to autologous stimulation. Inhibition of the responses by anti-class I antibodies indicated that CD8+ cells were responding to epitopes presented in the context of class I antigens (Ch 2.6.4). Anti-CD25 significantly inhibited the proliferative response of normal cells in the AMLR. This result and the detection of IL-2 secreted by AMLR reactive normal cells (Table 7.4), support the IL-2 dependency of the AMLR.

T cells from JDM patients as well as normal controls gave significantly enhanced responses to EBV transformed cells compared to fresh E- cells. EBV transformed cells have been shown to induce equivalent proliferative responses of normal, autologous T cells from VCA positive and negative donors (Konttinen, Bluestein and Zvaifler, 1985), indicating that responses were probably not due to recognition of virally encoded antigens.

Expression of DR antigens was increased on EBV transformed compared to autologous E- cells. Proliferative response in the AMLR has been shown to correlate with DR antigen density on stimulator cells (Palacios, 1982). Therefore it was possible that increased expression of class II determinants after EBV transformation enhanced the proliferative response. However, some authors have been unable to demonstrate a correlation between class II expression on stimulator cells and proliferative response in the AMLR (Crow and Kunkel, 1985; Duke-Cohan et al, 1987). Duke-Cohan et al (1987) have suggested that soluble factors secreted by stimulator cells may induce proliferation in the AMLR. It is therefore possible that lymphokines secreted by EBV transformed cells may contribute to enhanced T cell responses.

T cells from JDM patients gave significantly lower proliferation than T cells from normal adults or children in response to (a) fresh autologous E- cells (b) EBV transformed autologous B cells.

Abnormalities within the stimulator or responder cell populations could have contributed to the defective proliferative response by JDM T cells in the AMLR. Two pieces of evidence suggested that JDM stimulator cells were not defective:

(a) there was no difference in expression of DR antigens on JDM compared to normal E- cells or EBV transformed cells;

(b) the proliferative responses of normal T cells to allogeneic JDM non-T cells and to allogeneic normal non-T cells were not significantly different.

Furthermore, the addition of indomethacin to AMLR cultures had no effect on the response of JDM T cells indicating that prostaglandins secreted by contaminating monocytes were not inhibiting proliferation.

Therefore, since T cells appeared to be implicated in the defective AMLR, the phenotypes of normal and JDM T cells generated during the AMLR were examined. A high percentage of normal CD4+ and CD45R+ T cells, up to 50%, expressed CD25 after autologous stimulation. A similar proportion of CD4+ normal T cells, expressing CD25 during the AMLR, has been reported (Takada et al, 1985). An expansion of CD8+ cells was also observed. One group (Goto and Zvaifler, 1983) has reported finding no change in CD4/CD8 ratios during the AMLR. However, the conditions used by these authors differed from those in this thesis, including using a 1:1 ratio of E+ to E- cells and culturing in 20% autologous plasma. Furthermore, since CD8+ T suppressor cells have been shown to be generated

in the AMLR (Gatenby and Engleman, 1986), suppression of proliferation of CD4+ T cells is not unexpected. Rich, ElMasry and Fox (1986) have also observed feedback suppression of CD4+ T cells by autologous, activated CD8+ T suppressor cells.

The results indicated the relative failure of T cells from JDM patients express CD25 and of CD8+ T cells to proliferate. Defective IL-2 receptor expression was associated with significantly reduced IL-2 production by JDM cells during the AMLR. CD4+ T cells from SLE patients have also been reported to show significantly reduced IL-2 receptor expression and IL-2 production during the AMLR (Takada et al, 1985). These abnormalities were implicated as causes of the defective proliferative response of CD8+ T cells, which could be corrected by the addition of exogenous IL-2 to cultures. In contrast, the addition of IL-2 to JDM cells only partially reversed the defective proliferative response, since the response was still significantly less than that of normal cells in the presence of IL-2.

In the AMLR of PBL from normal children, T cells were generated which could directly suppress Ig production by EBV infected autologous B cells. This was unlikely to be due to EBV specific cytotoxic T cells as such cells are not detected in the first 7 days of coculture with EBV infected autologous B cells (Konttinen, Bluestein and Zvaifler, 1985).

A trivial explanation, that T cells were competing with EBV infected B cells for nutrients was investigated.

Replenishment of medium during the assay had no effect on levels of suppression attained (data not shown). It was also unlikely that suppression was due to competition for IL-2 (Palacios and Moller, 1981) as EBV infected B cells as did not express CD25 (data not shown) and therefore could not utilise IL-2 as a growth factor. The addition of IL-2 to the AMLR increased the potency of suppressor cells induced in the response. This result was in agreement with that of Volk and Diamanstein (1986) and indicated that generation of T suppressor cells as well as T cell proliferation in the AMLR was IL-2 dependent.

Cells from JDM patients generated during the AMLR failed to induce suppression of EBV induced antibody production, suggesting defects of suppressor T cell generation. Another possibility was that JDM B cells were defective in their response to suppressor T cells. This was unlikely as patient B cells were capable of responding to normal T cells (Ch. 6.3.6). Tsokos, Magrath and Balow (1983) showed that Ig production by EBV transformed normal and SLE B cells could be suppressed by normal T cells and suggested that SLE B cells were therefore capable of receiving regulatory signals.

The addition of IL-2 to JDM AMLR cultures did not allow the generation of suppressor cells in the AMLR. Therefore, although IL-2 could partially restore the proliferative response of JDM cells, it had no effect on suppressor cell generation. This contrasts with the reported restoration of suppressor cell activation by addition of IL-2 to the AMLR of SLE cells (Volk and Diamanstein, 1986). It was possible that

growth factors other than IL-2, were required for the generation of activated T suppressor cells, (Rich, ElMasry and Fox, 1987). Lack of such factor production by JDM CD4+ T cells might explain the failure to generate functional suppressor cells even in the presence of IL-2.

It could not be excluded that suppressor cells present in JDM T cell populations were inhibiting the AMLR. Fractionation of PBL into CD4+ and CD8+ cells would allow examination of the 2 cell populations's ability to respond to autologous antigens in the presence and absence of IL-2.

7.6 Conclusion

These experiments demonstrated the inability of T cell sub-populations from JDM patients to cooperate in the activation of regulatory T cells. Although CD4+ T cells failed to respond to autologous stimulation by the secretion of IL-2 and the induction of IL-2 receptors, lack of suppressor cell activation could not be ascribed solely to insufficiency of IL-2.

The inhibitory effects of sera from JDM patients suggested the possibility of a host response to autoimmunity, an attempt to down-regulate the immune response. The characteristics of serum inhibitory factor(s) are presented in the following 2 chapters.

CHAPTER 8

8.0 THE IMMUNOSUPPRESSIVE EFFECTS OF SERA FROM PATIENTS WITH JDM

8.1 Introduction and Experimental Design

Initial experiments showed that JDM sera reduced the proliferative responses of normal childhood lymphocytes in the AMLR (Ch 7.4.1). The experiments described in this chapter were conducted to determine whether the effect of sera (a) was reproducible (b) was due to allogeneic suppression (c) altered production of and responses to IL-2 (d) induced phenotypic changes in cells generated during the AMLR.

8.2 RESULTS

8.2.1 Effect of JDM Sera on Proliferation in the AMLR

When cells from 5 normal adult donors were cultured in sera from 16 JDM patients, proliferative responses in the AMLR were significantly reduced ($p < 0.05$) compared to culture in sera from 6 normal children (data not shown). Five representative sera were chosen and the effect on the AMLR of culturing cells from 4 different normal adult donors examined (Table 8.1).

The mean level of inhibition induced by each of these 5 sera was significant at the 1% level. Comparison of DR types of JDM serum donors and normal lymphocyte donors showed that the degree of inhibition of the proliferative response was not related to possession of any particular haplotype by serum and lymphocyte donors (data not shown). The possible presence of prednisolone in sera at the time of testing could not be excluded as contributing to inhibition although it was significant that 3 patients were not receiving drug therapy at

the time serum was obtained. Therefore, the immunosuppressive effect of JDM sera on the AMLR were reproducible.

TABLE 8.1.

Effect on the AMLR of culturing cells from 4 normal adult donors in JDM sera

Serum Donors	Prednisolone Therapy	% Inhibition of AMLR Proliferative Response
PD	5 mg daily	71 (55 - 96)[a]
JK	18 mg daily	47 (38 - 69)
GH	Untreated	53 (41 - 75)
HM	Untreated	65 (46 - 81)
NC	Untreated	68 (44 - 94)

[a] Mean inhibitory effect and range for each JDM serum tested compared to culture in normal serum.

8.2.2 Effect of Culturing Normal Adult Cells in Allogeneic Normal and Childhood Disease Control Sera

Preliminary experiments showed that proliferative responses of normal cells varied when allogeneic normal sera were substituted for autologous serum in the AMLR. It was therefore necessary to determine the range of variation in the AMLR of cells from normal individuals tested in the presence of normal childhood sera and sera from children with diseases not known to have immunological abnormalities as a major component of their disease. Sera were obtained from 9 children, 7 of whom had idiopathic scoliosis and 2, Duchenne muscular dystrophy. Table 8.2 represents the mean proliferative responses of normal cells in these sera as percentage suppression (+ 1 SD) of the response of each lymphocyte donor's cells in autologous serum.

TABLE 8.2.

The Effect of Sera from Normal Children and Childhood Disease Controls on the AMLR of Cells from 2 Normal Adults.

SERA	n	% SUPPRESSION OF AMLR Exp 1	Exp 2
NORMAL CHILDREN	5	19.7 + 8.9[a]	-14.3 + 19.0
CHILDHOOD DISEASE CONTROL	9	9.0 + 25.8	-19.4 + 22.4

[a] The mean percentage inhibition +1 SD, by two groups of sera on the AMLR compared to cells in autologous serum was calculated from the mean +1 SD CPM for each group of sera.

There was some variation in the effect of both normal and disease control sera on the AMLR, apparently depending on the donor. A mean enhancement of the AMLR by normal and disease control sera was evident in the case of the donor in experiment 2. However, there was no significant difference between the effect of normal and disease control sera on the AMLR in either experiment. The cumulative 95% confidence limits of variation in the 2 experiments lay between 34.8 and -41.8% suppression. It was of note that the level of suppression, by sera from JDM patients, was consistently greater than 40% and no enhancement of the proliferative response was ever observed.

8.2.3 Effect of JDM Sera on IL-2 Production in the AMLR

Donor lymphocytes were obtained from 4 normal adults and cultured in serum from a normal child or 1 of 2 JDM sera (Table 8.3). The sera chosen for study were those from patients HM and NC, who were not receiving drug treatment at the time blood was taken. Supernatants were obtained after 3

and 5 days of culture and IL-2 production measured and compared with T cell proliferation, measured at 7 days. Concentrations of IL-2 in sera from JDM patients were below the limits of detection in the assay (1 unit of IL-2/ml). Secretion of IL-2, by donor cells cultured in normal serum, was inhibited by 56-82% when cells were cultured in sera from the JDM patients. Inhibition of IL-2 production by JDM sera was also reflected in suppression of T cell proliferation.

8.2.4 Effect of the Addition of IL-2 to the AMLR of Normal Cells Cultured in Normal or JDM Sera

Since JDM sera suppressed T cell proliferation in the AMLR, apparently by limiting IL-2, the effect on proliferation of adding exogenous IL-2 to cultures was examined.

Cells from 3 normal donors were cultured in sera from 3 normal children or sera from 3 JDM patients (HM, NC, GH) not receiving drug therapy, with or without the addition of exogenous IL-2. Results are shown for cells from 1 normal adult donor (Fig 8.1). The addition of IL-2 gave dose-dependent enhancement of the proliferative response of T cells cultured in normal or patient sera. However, suppression of the response obtained by culturing cells in JDM sera was not reversed by the addition of IL-2, since the response was still significantly less ($p < 0.01$) than that obtained by the addition of IL-2 to cells cultured in normal serum. Similar results were obtained for cells from 2 other normal adults (data not shown). This suggested that suppression of proliferation in the AMLR was not due to an IL-2 inhibitor present in JDM sera.

TABLE 8.3.

Effect of JDM Sera on IL-2 Production in the AMLR

		IL-2 PRODUCTION (U/ml) 3 DAYS	IL-2 PRODUCTION (U/ml) 5 DAYS	^{3}H - T INCORPORATION (CPM) 7 DAYS
DONOR 1	E^{+}(N)[a]	6	8	1,363 ± 179
	AMLR(N)	188	200	21,816 ± 1,017
	AMLR(HM)	36	40	8,433 ± 1,459
	AMLR(NC)	40	26	6,328 ± 2,208
DONOR 2	E^{+}(N)	8	8	216 ± 30
	AMLR(N)	200	200	12,419 ± 1,921
	AMLR(HM)	36	44	3,219 ± 481
	AMLR(NC)	15	18	5,384 ± 874
DONOR 3	E^{+}(N)	14	12	469 ± 87
	AMLR(N)	128	152	6,102 ± 884
	AMLR(HM)	56	38	2,651 ± 383
	AMLR(NC)	32	56	2,103 ± 284
DONOR 4	E^{+}(N)	10	10	461 ± 124
	AMLR(N)	108	124	8,274 ± 620
	AMLR(HM)	36	40	4,138 ± 525
	AMLR(NC)	22	28	3,765 ± 491

a E^{+} control cells from 4 normal adult donors were cultured in serum from a normal child (N). AMLR cells were cultured in normal serum or serum from JDM patients HM and NC.

8.2.5 Effect of JDM Sera on the Phenotype of Responder Lymphocytes in the AMLR

The effect of sera from patients with autoimmune connective tissue diseases on phenotypes of responding T cells in the AMLR has not previously been reported.

Normal adult cells from 3 donors were cultured in serum from a normal child or 2 JDM sera (Table 8.4). When cells were cultured in normal serum for 7 days, the CD4/CD8 ratio was 1.8 to 1.9 for E+ control cells and 0.6 - 0.8 for AMLR cells, indicating the proliferation of CD8+ cells. When cells were cultured in JDM sera the CD4/CD8 ratio for AMLR cells was 1.6 to 2.0, similar to E+ controls. The percentage of class II positive cells increased by an average of 8-fold during the AMLR when cells were cultured in normal serum, but only an average of 3-fold in JDM sera. Therefore, the principal observed effect on phenotype of culture in sera from JDM patients was lack of expansion of the CD8+ population.

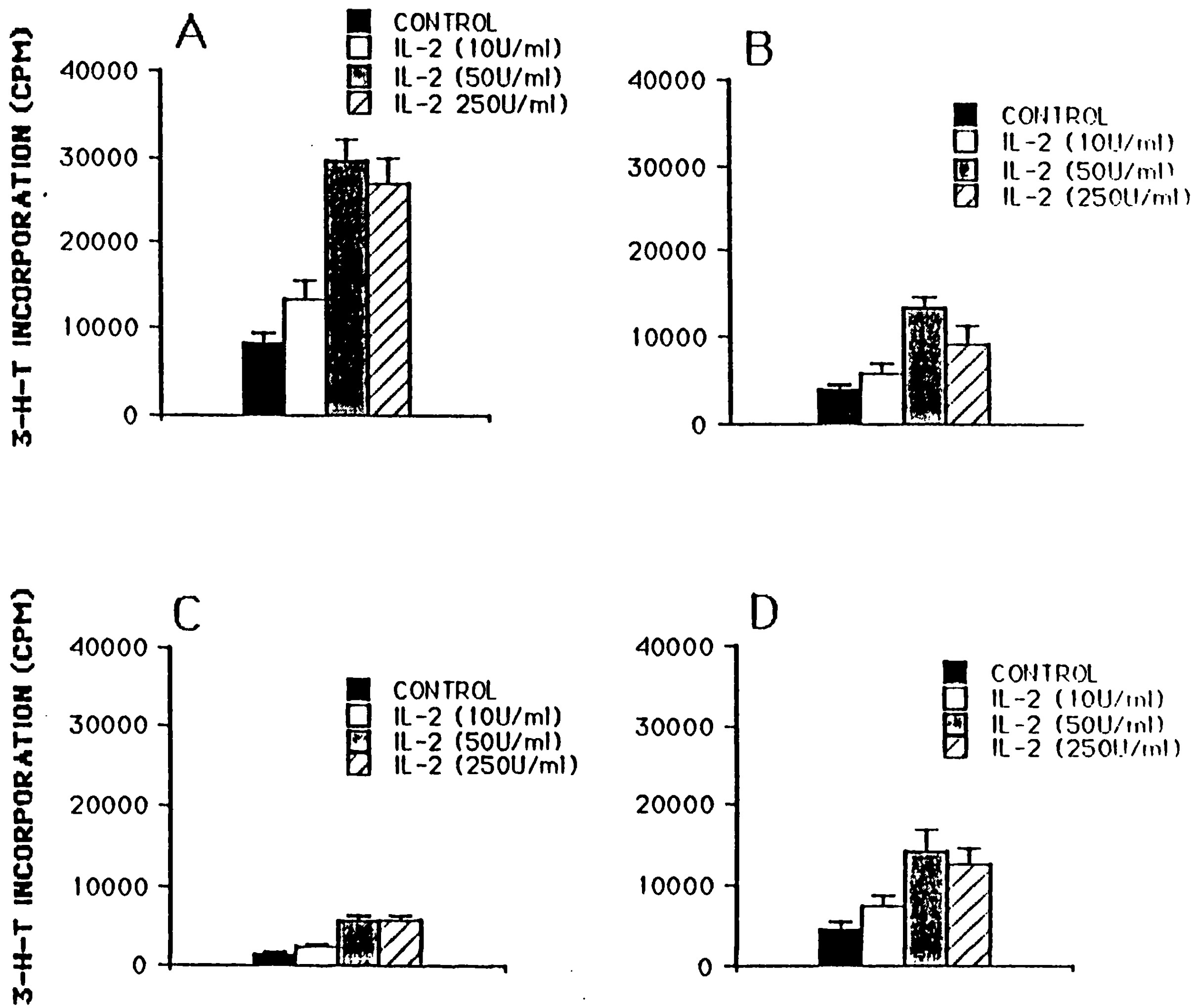


Figure 8.1.

Effect of the addition of IL-2 on proliferation in the AMLR. Cells from a normal adult donor were cultured (A) in serum from a normal child or (B-D) sera from 3 JDM patients - HM, GH and NC respectively.

TABLE 8.4.

Effect of JDM Sera on the Phenotype of Cells Generated During the AMLR

		CD3	CD4	CD8	DR
	E+(N)[a]	83[b]	56	30	5
DONOR 1	AMLR(N)	85	35	56	28
	AMLR(HM)	84	59	29	18
	AMLR(NC)	92	59	30	4
	E+(N)	88	59	33	3
DONOR 2	AMLR(N)	84	41	50	25
	AMLR(HM)	80	54	28	7
	E+(N)	77	50	28	2
DONOR 3	AMLR(N)	78	32	46	24
	AMLR(NC)	74	47	29	15

a Control E+ cells from 3 normal adult donors were cultured in serum from a normal child (N). AMLR cells were cultured either in normal serum or serum from JDM patients HM and NC. Cells were harvested after 7 days and phenotypes determined.

b Figures represent percentage of cells expressing the designated marker.

8.3 Discussion

In initial experiments it was observed that the mean proliferative response of adult cells cultured in JDM sera was significantly less than that of normal cells cultured in autologous serum. This effect was reproducible and not dependent on donor lymphocyte population.

It was possible that the suppression induced by JDM sera was due to an allogeneic effect, perhaps associated with the presence of elevated levels of non-specific suppressor factors such as α_2- globulins or cholesterol in some sera (Maisch et al, 1979). When donor lymphocytes were cultured in sera from allogeneic healthy children and disease control children, neither group of sera induced significant suppression of the proliferative response.

Suppressor factors, present in JDM sera, might have bound to cell membrane determinants responsible for transducing activation signals. The AMLR is IL-2 dependent and therefore, one mechanism for the suppressive effect of JDM sera could have been inhibition of IL-2 production and cell membrane expression of the IL-2 receptor. Low levels of proliferation were indeed associated with defective IL-2 production, when cells were cultured in JDM sera. Another possible explanation of these results could have been that an IL-2 inhibitor was present in JDM sera. Such an inhibitor has been reported in sera from patients with acquired immunodeficiency syndrome (Spiegel et al, 1985).

In an attempt to determine whether suppression was due to an IL-2 inhibitor, exogenous IL-2 was added to normal cells cultured in patient serum. The results showed that IL-2 could only partially reverse suppression of the AMLR by sera from JDM patients. This was unlikely to be due to alterations in IL-2 dose-responses for donor cells cultured in patient serum, since higher concentrations of exogenous IL-2 clearly induced maximal proliferation of responding cells i.e. proliferation was on the plateau region of the dose-response curve for culture in both normal and patient sera (Fig 8.1). Since an IL-2 inhibitor, if present, should be saturable, this strongly suggested that the inhibitor was not exerting its effects by competing with the IL-2 receptor for IL-2. An alternative was that the serum inhibitor was competing with IL-2 for its receptor. Such an inhibitor would probably have to be (a) in molar excess of IL-2 added or (b) possess a greater affinity for the receptor than IL-2 itself. The first possibility seemed unlikely since the dose-response curve, for cells cultured in patient serum, would be expected to shift to the right, which was not observed. The affinity of IL-2 for its receptor varies with the nature of the receptor (Smith, 1988), but no other soluble factors have been shown to compete with IL-2 for binding to the high affinity receptor on activated T cells. Therefore, suppression of the AMLR by sera from JDM patients was unlikely to be due either to an IL-2 inhibitor or to a factor inhibiting T cell activation by competing with IL-2 for binding to the IL-2 receptor.

8.4 Conclusion

Sera from JDM patients had an inhibitory effect on the AMLR resulting in reduced T cell proliferation, and in the production of and response to IL-2. These effects were not mediated by inhibitors of IL-2. It seemed more plausible that inhibition was directed at lymphocyte surface antigens which were important (a) in stimulating an AMLR response, or (b) conveying activation signals to potential responder cells. Attempts to characterise JDM serum inhibitory factors will be described in the following chapter.

CHAPTER 9

9.0 CHARACTERISATION OF THE SUPPRESSOR FACTORS PRESENT IN SERA FROM JDM PATIENTS

9.1 Introduction and Experimental Design

This chapter describes attempts to characterise factors in sera from JDM patients, capable of suppressing the proliferation of responder cells in the AMLR. Experiments were conducted to determine whether (a) suppression was directed at stimulator or responder cells (b) whether an MHC class II bearing cell line could absorb the suppressor factors from sera (c) the suppressor factors were contained within the IgG or non-IgG serum fractions. Since results indicated that the factor responsible for suppression in JDM sera was an IgG antibody, further experiments investigated (a) the effect of the antibody on antigen-specific immune responses and (b) the possible antigenic determinants recognised by the antibody.

9.2 RESULTS

9.2.1 Effect of Incubating Stimulator non-T and Responder T Cells in JDM Sera on the AMLR

Donor lymphocytes, obtained from 3 normal adults, were separated into T and non-T cells and preincubated O/N in serum from either a normal child or from 3 JDM patients. The cells were then washed and the non-T cells were treated with mitomycin C. Both T and non-T fractions were resuspended in 10% autologous serum, recombined and cultured for 7 days, when proliferation was measured. When both T and non-T cells were preincubated in each of 3 JDM sera, proliferative responses were significantly inhibited ($p < 0.01$), compared to T and non-T cells preincubated O/N in normal serum (Fig 9.1).

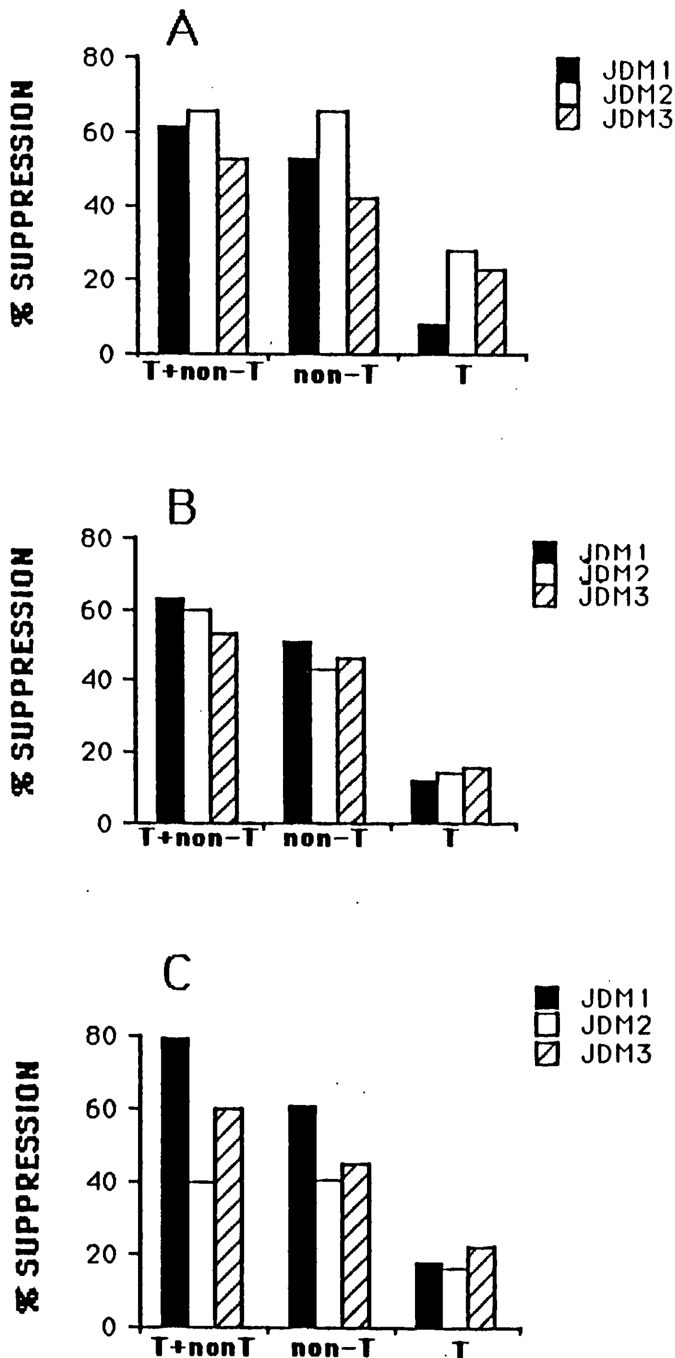


Fig 9.1. Effect on the AMLR of preculturing T or non-T cells in sera from JDM patients. Cells from 3 normal adult donors (A-C) were fractionated into T and non-T cells and then precultured separately O/N in serum from a normal child or serum from 1 of 3 JDM patients. The cells were then washed, the E- cells mitomycin C treated, recombined and culured for 7 days in autologous serum and proliferation then measured. T+ non-T = both populations precultured in JDM serum. T = T cells precultured in JDM serum and non-T cells precultured in normal childhood serum. Non-T = non-T cells precultured in JDM serum and T cells precultured in normal childhood serum. The results are expressed as % suppression of the proliferative response when T and non-T cells were precultured in normal childhood serum. These responses were (A) 3803 + 386 (B) 4,812 + 590 (C) 6,109 + 383.

The effect of preculturing non-T cells in each of the 3 JDM sera and T cells in normal serum was again to significantly inhibit ($p < 0.01$) the proliferative response. Conversely, preincubating non-T cells in normal serum and T cells in each of the JDM sera, resulted in proliferative responses not significantly different from responses obtained when T and non-T cells were precultured in normal serum.

Suppression directed against the stimulator non-T cell population could imply the presence of serum factors binding to and perhaps masking class II antigens present on stimulator cells.

9.2.2 Effect of Absorbing Normal and JDM Sera with K-562 or EBV-Transformed Cells on the AMLR

The effect of absorbing normal and JDM sera on a class II bearing B cell line was examined. Sera were untreated or absorbed with EBV transformed or K-562 cells. Lymphocytes from 2 normal adult donors were then cultured in absorbed or control sera from 2 allogeneic normal child donors and 3 JDM patients. Absorption of normal sera with either cell line had no effect on the AMLR proliferative response (Table 9.1). Absorption of JDM sera with K-562 cells had no effect on the observed inhibition, but absorption with EBV transformed cells significantly ($p < 0.01$) reduced the inhibitory effect of patient sera on the AMLR.

TABLE 9.1

Effect of absorbing normal and JDM sera with EBV- transformed cells on the serum suppressor factor.

		ABSORPTION[a]	^{3}H-T INCORPORATION (CPM)	% INHIBITION
Expt. 1		-	2632 ± 367[b]	
	NC	EBV - tr	3167 ± 322	-20
		K-562	2711 ± 411	- 3
		-	465 ± 44	82
	JDM 1	EBV - tr	1846 ± 192	20
		K-562	456 ± 175	83
		-	252 ± 29	90
	JDM 2	EBV - tr	864 ± 159	67
		K-562	212 ± 36	92
		-	839 ± 108	68
	JDM 3	EBV - tr	2075 ± 384	21
		K-562	1120 ± 190	57
Expt. 2		-	4875 ± 392	
	NC	EBV - tr	4684 ± 489	4
		K-562	4738 ± 687	3
		-	2104 ± 236	57
	JDM 1	EBv - tr	3841 ± 409	21
		K-562	2117 ± 299	57
		-	1120 ± 358	77
	JDM 2	EBV - tr	2784 ± 322	57
		K-562	898 ± 102	82
		-	1864 ± 295	62
	JDM 3	EBV - tr	5202 ± 484	-7
		K-562	2348 ± 414	52

a Normal child (NC) or JDM sera were absorbed twice with 1 X 10^6 EBV transformed (EBV-tr) or K-562 cells.

b Mean ± SD.

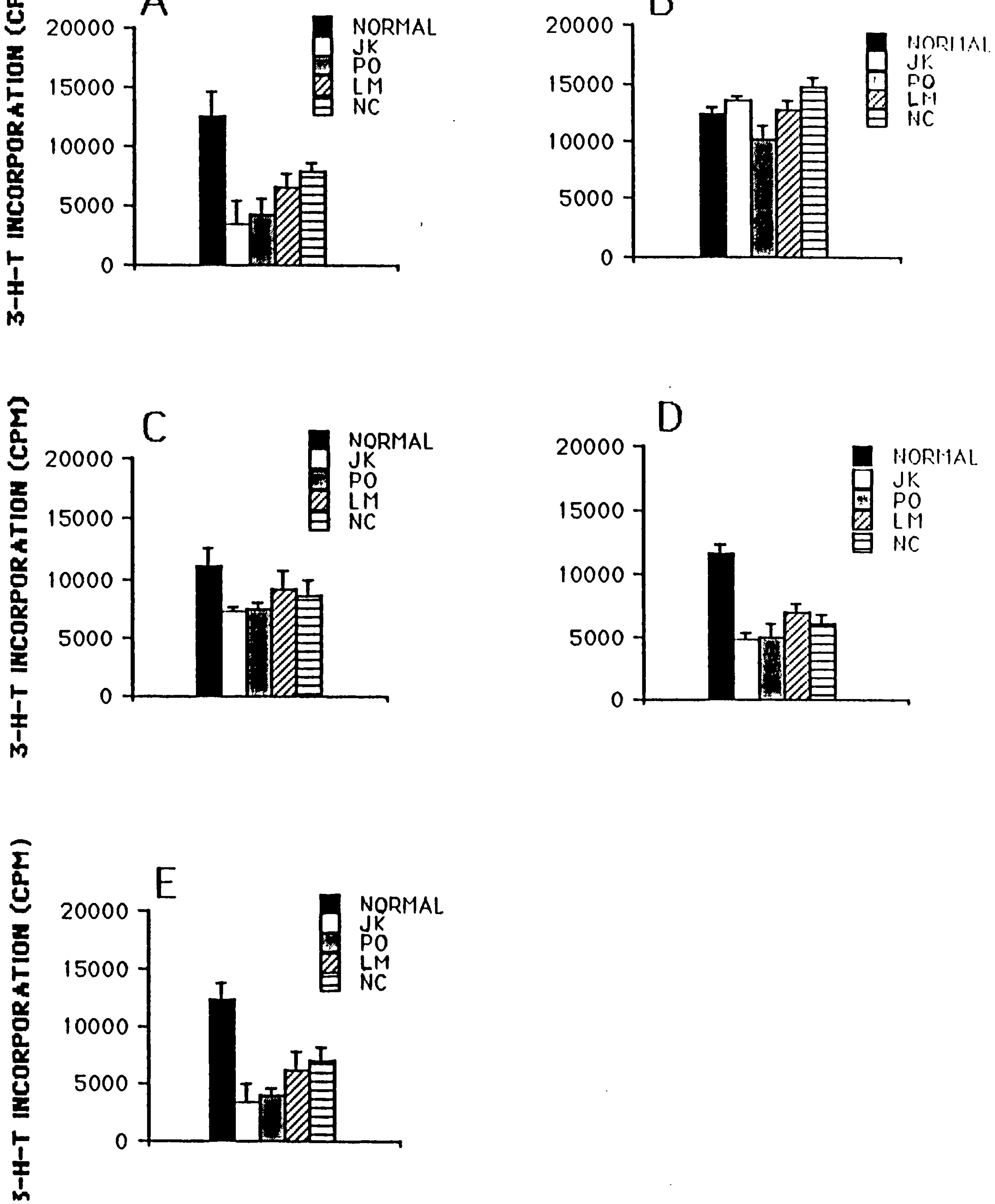


Figure 9.2. Effect on the AMLR of culturing cells from a normal adult donor in serum fractions from a normal child and 4 JDM patients. Cells were cultured (A) in whole serum (B) non-IgG containing fraction of serum (10% of original serum concentration) (C) 0.25 mg/ml of purified serum IgG (D) 0.5 mg/ml of serum IgG (E) 1 mg/ml of serum IgG.

9.2.3 Effect of Purified IgG from Normal and JDM Sera on the AMLR

Purified IgG was prepared from 2 normal children and 5 JDM patients. Lymphocytes from normal adult donors were then cultured in (a) the unfractionated sera (b) the non-IgG containing fractions of the sera (c) different concentrations of purified IgG from the sera. The AMLR proliferative responses were then measured. The results of a representative experiment are shown in Figure 9.2. Significant inhibition ($p < 0.005$) of the proliferative response was obtained by culturing cells in 0.5 or 1mg/ml purified IgG from the JDM sera but not by (a) IgG from the normal childhood serum (b) the non-IgG containing fractions of patient and normal sera. The inhibition induced by IgG from JDM sera was dose-dependent.

Further, purified IgG from JDM sera tested gave dose-dependent inhibition of the AMLR responses of 3 normal adult donors. The degree of suppression of the AMLR obtained with JDM IgG varied depending on the source of donor lymphocytes, but was still reproducible. The non-IgG containing fraction of these patients's serum did not significantly affect the AMLR response. Table 10.2 gives the mean inhibition and ranges for the 4 serum IgG (1mg/ml) preparations tested.

Serum fractions from normal children did not significantly inhibit the AMLR of lymphocytes from the 3 donors tested (data not shown). Two patients (GH and NC) were not receiving drugs when serum was obtained.

TABLE 9.2. Suppression of T cell proliferation in the AMLR by purified IgG from JDM Sera

SERUM	% INHIBITION OF AMLR
PO	77 (63 - 86)
GH	76 (69 - 86)
LM	56 (44 - 70)
NC	70 (55 - 80)

9.2.4 Time Course for Inhibition of the AMLR by Purified IgG from JDM Sera

Purified IgG (1mg/ml) from 2 JDM sera (NC and GH) was added on succeeding days to the AMLR of cells from 2 normal adult donors (Table 9.3). When IgG was added on days 1-3, significant inhibition of the proliferative response ($p < 0.01$) was obtained. When added on days 4 or 5 no significant change in the proliferative response was observed. Addition of purified IgG from an allogeneic, normal child serum had no effect on the AMLR.

9.2.5 Inhibition by Purified IgG from JDM Sera of Antigen Specific Normal T cell Proliferative Responses

The effect of purified IgG from normal and JDM sera on the proliferative responses of T cells from 2 normal adult donors were examined. These experiments were performed by Dr V Gant, Immunology Dept, St Thomas's Hospital (Fig 9.3). Purified IgG (1mg/ml) from the 2 JDM sera significantly inhibited ($p < 0.01$) proliferative responses to PPD at the 3 dose points tested.

Culture of cells in the non-IgG containing fractions of patient sera had no effect on the proliferative responses of donor cells.

TABLE 9.3. Time Course for Inhibition of the AMLR by Purified IgG From JDM Sera.

IgG	Day Added	^{3}H - T INCORPORATION (CPM) Donor 1[a]	Donor 2
	-	3830 ± 464[b]	5085 ± 691
JDM (NC)	1	1387 ± 125 (74)[c]	3181 ± 413 (38)
	2	1643 ± 182 (58)	3019 ± 485 (42)
	3	2320 ± 261 (40)	3816 ± 324 (25)
	4	3658 ± 584 (5)	4881 ± 624 (4)
	5	3418 ± 516 (10)	5206 ± 1581 (-2)
JDM (GH)	1	798 ± 240 (80)	2408 ± 284 (53)
	2	1184 ± 261 (70)	2310 ± 305 (55)
	3	1762 ± 214 (54)	3407 ± 281 (33)
	4	2991 ± 491 (22)	4910 ± 640 (3)
	5	3308 ± 475 (14)	5184 ± 468 (0)
NORMAL CHILD	1	3310 ± 561 (14)	5491 ± 570 (-8)
	2	3364 ± 459 (12)	5476 ± 438 (-8)
	3	3876 ± 323 (0)	5163 ± 384 (0)
	4	3491 ± 385 (1)	4784 ± 1240 (2)
	5	3784 ± 424 (0)	4916 ± 306 (0)

a Lymphocytes from 2 adult donors were cultured in autologous sera with or without purified IgG (1mg/ml) from an allogeneic normal or 2 JDM sera. IgG was added on succeeding days, starting at the initiation of culture.

b Mean ± SD.

c Figures in parenthesis represent % suppression of control response.

9.2.6 Attempts to Characterise Structures Recognised by Anti-Lymphocyte Antibodies in JDM Sera

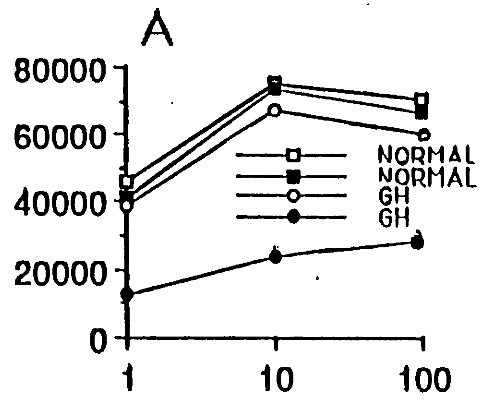
Several techniques have been employed to try to characterise anti-lymphocyte antibodies in JDM sera -

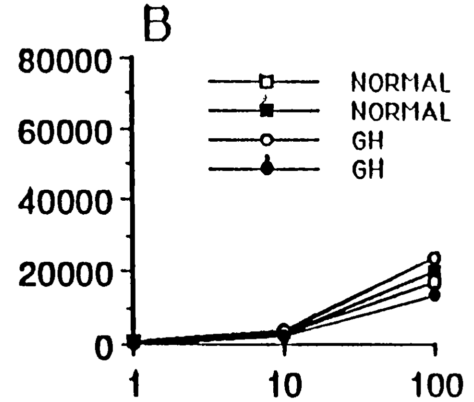
(a) Direct binding assay to EBV transformed lines and PHA blasts derived from normal donors.

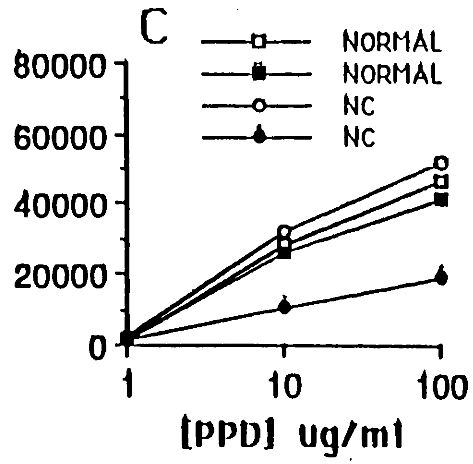
(b) Inhibition of binding of anti- DR, anti- DP and anti- DQ MAb's to PHA blasts derived from normal donors.

(c) immunoprecipitation of radio labelled EBV transformed lines by autologous sera.

Methods (a) and (b) did not show significant differences between JDM and control sera. Method (c) (experiments carried out by Dr J Lanchbury, Guy's Hospital) has proved inconclusive as yet (data not shown). However, more patients's sera need to be tested, including those (PO and JK) whose purified IgG gave the greatest inhibition of the AMLR. Immunoprecipitation of normal, allogeneic EBV transformed B cells and activated T cells by JDM sera are also experiments in progress.







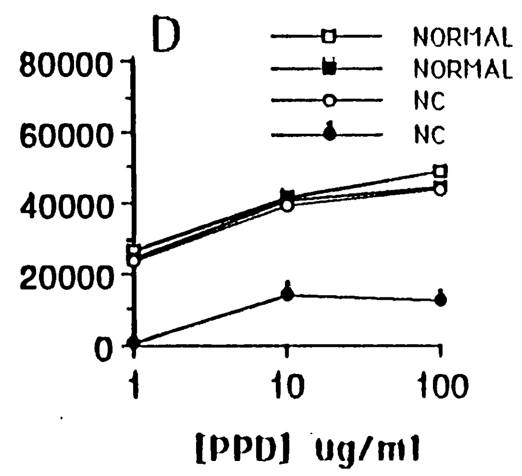


Fig 9.3. Effect on PPD induced proliferation of addition of purified IgG from JDM sera. PBMC ($2x10^6$/ml) from 4 normal adult donors (A, B, C, D), were cultured in the presence of purified IgG (closed symbols) or the non-IgG containing serum fraction (open symbols) from 2 JDM patients (GH, NC) and 1 normal child. Cells were cultured in 10% normal adult serum plus serum fraction (1mg/ml). Responses of cells in the absence of PPD were subtracted from the results.

9.3 DISCUSSION

Antigens expressed by stimulator and/or responder cells in the AMLR were possible targets for inhibitory factors in JDM sera. Sera were found to preferentially inhibit the stimulator population in the AMLR. This indicated possible masking of class II determinants, which was investigated by attempting to absorb out the inhibitory factors from sera. Absorption was indeed observed with a class II bearing cell line (Table 9.1). K-562 cells, a myeloid cell line which may express high levels of Fc recepors, were unable to absorb the inhibitory factor, indicating that non-specific binding to EBV transformed cells could not explain these results.

Two pieces of evidence suggested that the inhibitory factor might be an IgG antibody (a) previous reports of IgG anti-DR antibodies in sera from patients with other connective tissue diseases (Okudaira et al, 1984) (b) the inhibitory factor was warm-reacting suggesting an effect mediated by an IgG rather than an IgM antibody (Yamada and Winfield, 1984).

The factor present in JDM sera, responsible for inhibiting the AMLR, bound to Protein A. This factor was unlikely to be IgG or IgM immune complexes, as these could not be detected in JDM sera (Ch 5.6). Futhermore, such complexes, if present in serum, would be expected to inhibit NK activity, which was not observed (Ch. 10.3.5). The inhibitory factor was therefore probably IgG. Class II restricted responses to PPD were inhibited by purified IgG from JDM sera (Fig 9.3), supporting the evidence for anti-class II antibodies in patient sera.

Unfortunately, experiments designed to detect anti-class II antibodies in JDM sera have not so far been successful. A major problem encountered in attempting to discriminate between binding of sera from JDM patients and normal sera to human cells was the high background binding of normal sera

(data not shown). Although blocking sera reduced background binding, this still did not allow discrimination between human sera. Indeed, xenogeneic blocking sera may contain heterophile antibodies to human cell membrane components, possibly inhibiting binding of antibodies present in human sera. The failure to detect anti-class II antibodies in JDM sera may reflect the low concentrations and/or affinity of these antibodies. If the affinities of MAb's to class II antigens, used in competition experiments, were greater than that of serum antibodies, the former may well displace the latter from membrane binding sites.

Evidence was also obtained that antibodies in JDM sera might be directed at determinants other than class II antigens. Time course experiments indicated that serum IgG could inhibit the AMLR up to 72 hours after initiation of the response (Fig 9.3). Therefore, activation antigens, expressed by responding T cells, might be recognised by antibodies in JDM sera. One explanation could be that antibodies to several lymphocyte surface antigens may be present in JDM sera.

9.4 Conclusion

Evidence was obtained for the presence of at least 2 anti-lymphocyte antibodies in sera from JDM patients. One antibody was directed at determinants expressed by stimulator cells in the AMLR and the second may have had specificity for activation antigens expressed by T cells.

CHAPTER 10

10.0 ACTIVITY OF NK CELLS FROM JDM PATIENTS

10.1 INTRODUCTION

Reduced levels of NK activity have been detected in peripheral blood cells from 5 untreated JDM patients (Miller, Lantner and Pachman, 1983). The reported role of NK cells in maintaining host defence against virally infected targets (Shellam, Grundry and Allan, 1982) and in exercising an immunoregulatory role over T and B cell responses (Tilden, Abo and Balch, 1983; Kuwano et al, 1986) suggested that abnormal NK activity in JDM patients might contribute (a) to the proposed viral aetiology of JDM (Ch 1.1.2) and (b) to disregulation of the immune response, such as high levels of spontaneous IgM synthesis (Ch 6.3.2).

10.2 Experimental Design

The normal ranges of NK activity were first determined for PBL from normal adults and children. The range of NK activity for PBL from JDM patients was then measured. The relationship between corticosteroid therapy and NK activity of JDM patients's cells was examined by regression analysis. Sequential measurements of NK activity were related to skin vasculitis, as a measurement of disease activity. Finally the enhancing effects of IFN added *in vitro* on NK function was measured against K-562 cells and an NK resistant tumour cell line, RD.

10.3 RESULTS

10.3.1 NK Activity of PBL from Normal Adults, Normal Children and JDM Patients

Cytotoxic activity of PBL from 12 normal adults, 8 normal children and 12 JDM patients was measured against K-562 targets, at an E:T ratio of 50:1 (Fig 10.1). Nine JDM patients were receiving prednisolone alone and 3 were not receiving drugs at the time of testing. The mean level of cytotoxicity of cells from the normal adults (42.1 $\pm$ 17.0) and the normal children (31.9 $\pm$ 7.3) against K-562 targets were not significantly different. The mean level of cytotoxicity of cells from JDM patients against K-562 (14.8 $\pm$ 17.2) was significantly less ($p < 0.01$) than that of the 2 control groups. Cells from the 3 untreated patients (GH, NC and SW) had among the lowest levels of cytotoxicity, 11, 8 and 6%, respectively.

10.3.2 Effect of Prednisolone Dose on NK Activity of PBL from 2 JDM Patients

The possibility that reduced NK function by JDM PBL could be due to prednisolone therapy was examined. Fig 10.2 shows the cytotoxic activity against K-562 cells plotted against prednisolone therapy at time of testing for 2 JDM patients (JK and PO). Regression analysis showed no correlation between NK activity and prednisolone dose, and the lowest NK values were obtained when patients were receiving the lowest dosage of steroids.

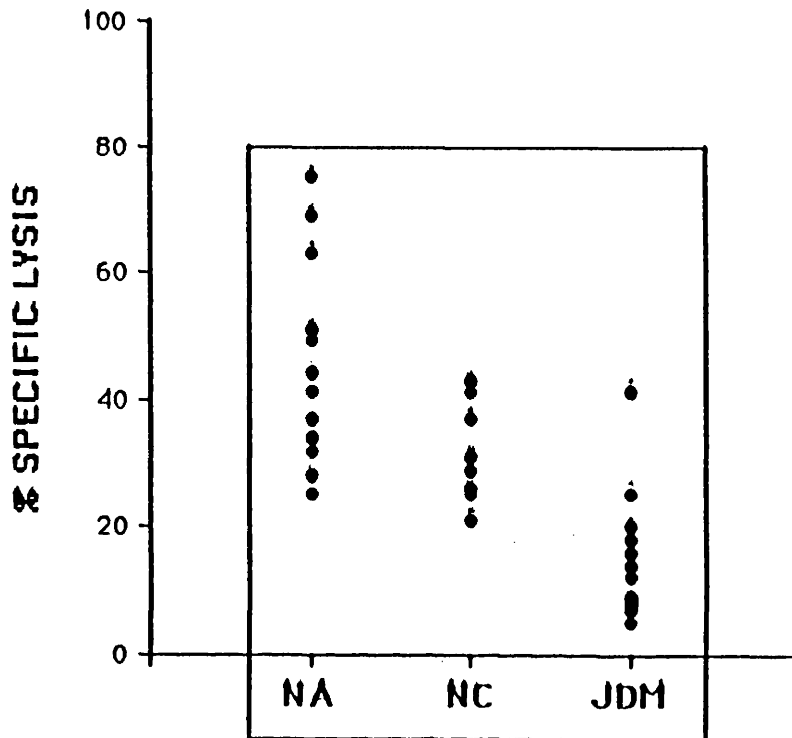
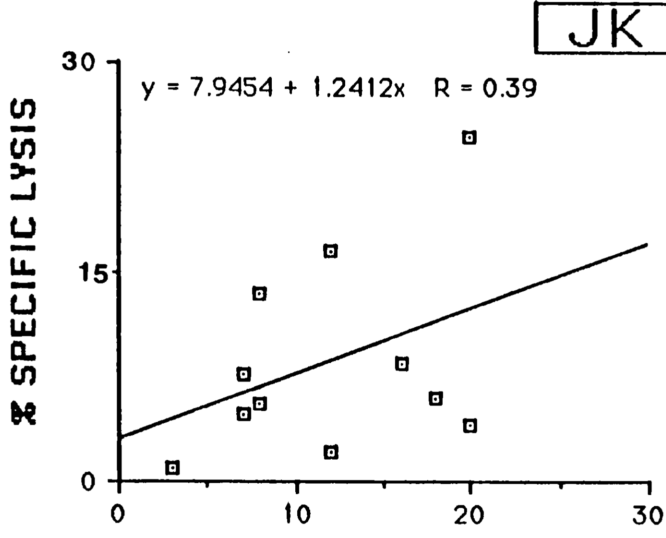


Figure 10.1.

Cytotoxic activity against K-562 cells by PBL from normal adults (NA), normal children (NC) and JDM patients. E:T ratio 50:1.



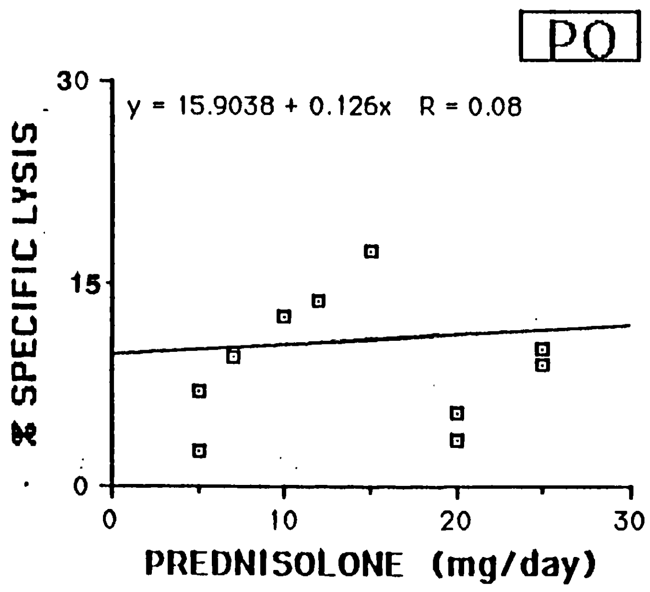


Figure 10.2.

Relationship of NK activity to prednisolone therapy for 2 JDM patients (JK and PO). The regression line and correlation coefficient (R) are shown for each set of data. E:T ration was 50:1.

10.3.3 Relationship of NK Activity to Degree of Vasculitis

The possibility that low NK activity was associated with severe skin vasculitis had been suggested by single-point determinations of NK activity for a number of patients (G Cambridge, unpublished observations). Examination of sequential measurements of NK activity for 2 JDM patients (PO and JK) indicated (Fig 10.3) that there was no convincing relationship between NK activity and vasculitis, for these 2 patients.

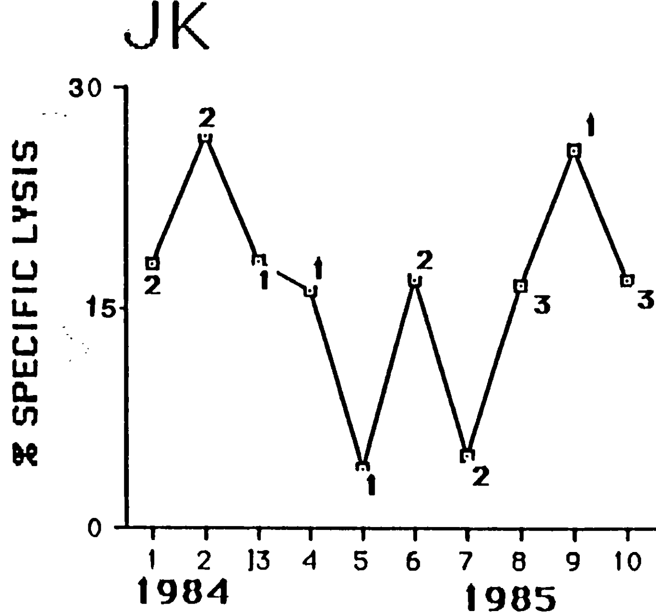
10.3.4 Effect of Preincubation with IFN- on the NK Activity of PBL from Normals and JDM Patients

The ability of NK cells to respond to IFN's may determine their capacity to respond to and kill target cells (Ch 3.4).

Cytotoxic activity of PBL from 3 normal children and 3 JDM patients was assessed against K-562 and RD cells. Donor PBL had previously been cultured for 4h in the presence of IFN- (1000 U/ml) (Fig 10.4). Mean levels of cytotoxicity against both targets were significantly less for JDM than normal PBL at each E:T ratio tested ($p < 0.005$). When a further 4 JDM patients's cells were tested, similar results were obtained (data not shown).

10.3.5 Effect on NK activity of Incubating PBL in JDM Sera

Anti-lymphocyte antibodies, recognising determinants present on NK cells, have been detected in sera from SLE patients (Sibbitt and Bankhurst, 1985). Therefore, cytotoxic activity of normal and patient PBL was always tested during culture in both normal and JDM sera. There was no significant difference in levels of cytotoxicity between the two groups of sera used (data not shown).



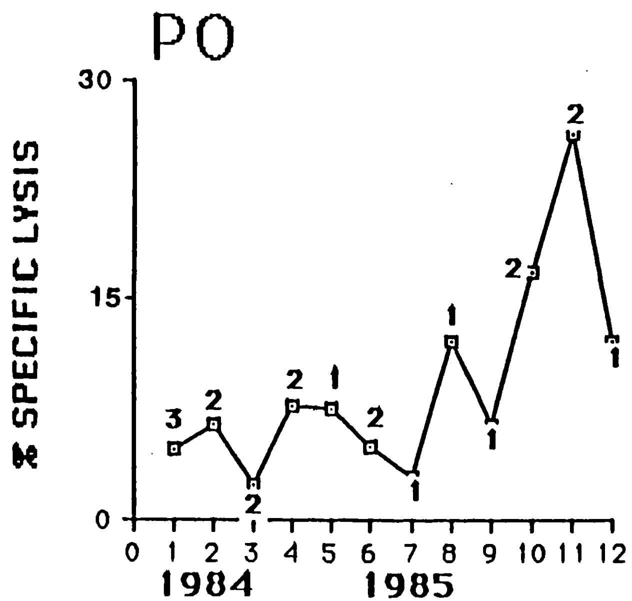
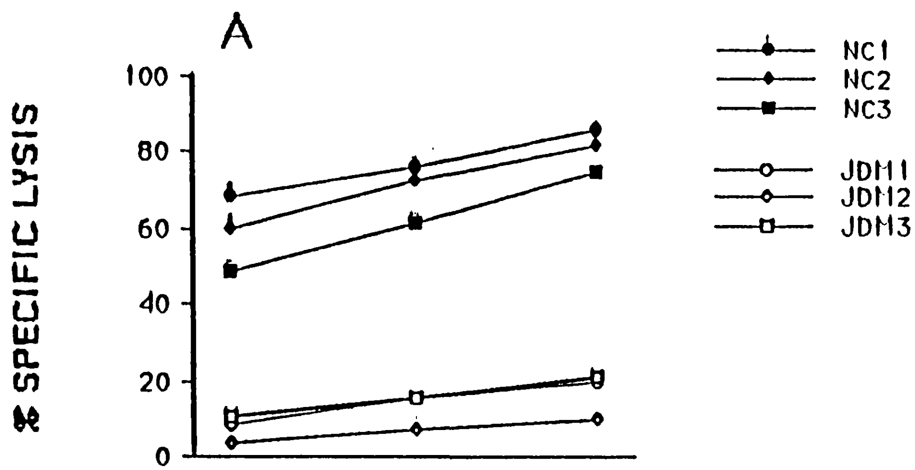


Figure 10.3.

Relationship of NK activity (E:T 50:1) to degree of skin vasculitis at time of testing for 2 JDM patients. Assays were conducted over a 10 month time period. Vasculitis was assessed on a score of 1 to 3. 1 = mild 2 = moderate 3 = severe.



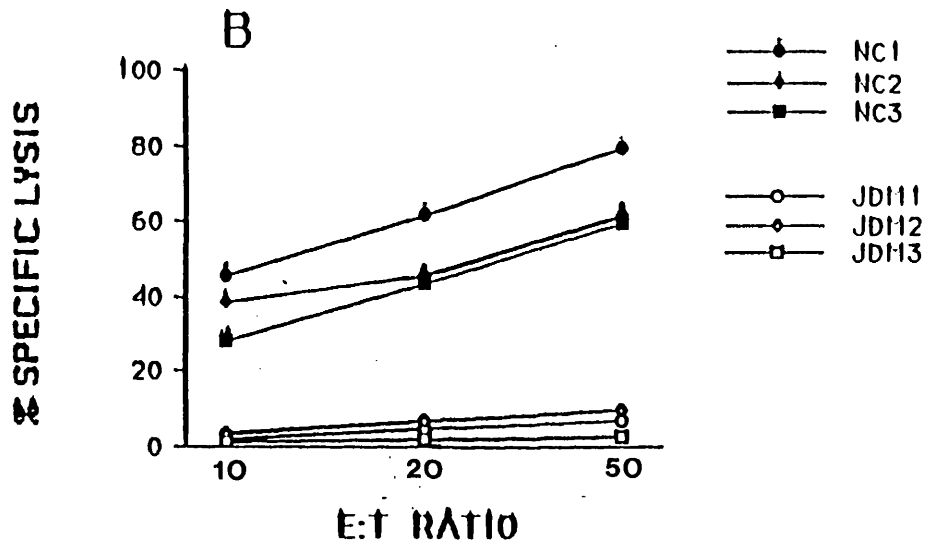


Fig 10.4.

Effect of IFN-α (1000U/ml) on NK activity of PBL from 3 normal children (NC) and 3 JDM patients. PBL were incubated for 4h in IFN-α before cytotoxicity against A) K-562 and B) RD targets was measured.

10.4 Discussion

There was no significant difference in NK activity between adult and childhood controls, although there was a trend for the children to be within the lower range of adult cytotoxicity values. A recent report (Tilden et al, 1986) examined CD16+ lymphocytes, during ontogeny. These were low in number during the first decade of life and reached maximum levels during the second decade. NK functional activity increased moderately to reach maximum levels during the second decade. Males and females exhibited comparable cytotoxic activity.

The majority of JDM patients (9/12) had significantly reduced NK function compared to childhood controls, suggesting a defect unrelated to age. These results were taken at single time points and when NK activity of normal adults was followed sequentially, it was clear that NK activity varied even when blood was drawn at the same time of day (data not shown). This suggested that studies of NK function taken at a single time point may have less validity than serial measurements. Serial measurements showed that NK activity by cells from 2 patients remained low during a period of 10 months (Fig 10.3).

Regression analysis showed that there was no relation between NK activity and corticosteroid dose for the 2 patients followed sequentially. A previous study also demonstrated that at the levels of prednisolone achieved therapeutically in children, NK function *in vitro* was unaffected (Miller, Lantner and Pachman, 1983). There is controversy over the effects of corticosteroids on NK activity *in vivo* and *in vitro*. One

group (Katz, Zaytoun and Lee, 1984) reported that a single dose of 400 mg hydrocortisone significantly increased NK activity 4h after administration. This augmentation was not due to changes in NK effector function but was secondary to a relative increase in NK cells as a result of depletion from the circulation of CD4+ T cells. The authors did not assess whether there were any changes in the absolute number of NK cells. Therefore the increased NK activity might also be due to NK cells entering the peripheral blood. Parillo and Fauci (1979) administered 12mg dexamethasone to normal adults and showed significantly depressed NK function lasting from 24 to 48h after treatment. Different corticosteroids may therefore have contrary effects on NK activity _in vivo_. Hoffman (1980) reported that pharmacologic (10^{-5}M) doses of hydrocortisone added _in vitro_ inhibited NK activity while Katz, Zaytown and Lee (1984) found the drug to have no effect at pharmacologic or suprapharmacologic (10^{-4}M) concentrations _in vitro._ These results appear to remain unreconciled.

There is no information on the long term administration of steroids on NK activity of normals. When patients with vasculitis were treated with prednisone daily for 3 weeks, the lymphopaenia induced by the drug was of much shorter duration than at the beginning of therapy, suggesting reduced responsiveness to steroids (Cooper et al, 1977). No association was found in the present study between total lymphocyte count and NK activity for JDM patients (data not shown). An interpretation of this result is extremely difficult since NK cells may possess different recirculation patterns than T and B cells (Fox et al, 1984). It is possible

that low NK activity in JDM patients could result from sequestration of NK cells. It is also possible that bone marrow derived precursors of NK cells may be reduced in JDM patients.

There appeared to be little association between NK activity and severity of skin vasculitis in JDM patients. There was also no association between NK activity and other measures of disease activity such as serum CPK level, muscle weakness and mood of the patient (data not shown).

The release of IFN by effector cells after interaction with targets may augment the recycling capacity of NK cells (Herberman and Ortaldo, 1981). It was therefore possible that patient NK cells (a) failed to make sufficient IFN to attain normal levels of cytotoxicity (b) did not respond normally to IFN. Pretreatment of JDM cells with IFN-α did not significantly increase cytotoxicity against target cells. The defective response was probably not due to the possibly inhibitory effects of IFN in vivo (Sibbitt and Bankhurst, 1985) as JDM patients were found to have no detectable IFN in their serum (G. Cambridge, unpublished results) when tested by radioimmunoassay. The production of IFN by NK cells from patients in response to targets has not been examined in the present study.

10.5 Conclusion

Reduced levels of NK activity by PBL from JDM patients were not associated with age group of patients, corticosteroid therapy, lymphopaenia or disease activity. Lack of enhanced cytotoxicity to IFN suggested an inability of NK cells from

JDM patients to respond normally to stimulation mediated by lymphokines. Therefore, further experiments described in the following chapter, examined the response of NK cells from normals and patients to IL-2.

CHAPTER 11

11.0 LYMPHOKINE ACTIVATED KILLER CELLS

11.1 INTRODUCTION

This chapter will describe experiments investigating the properties of LAK cells derived from normal controls and JDM patients. There has been very little investigation of cooperation between NK and T cells in the induction of LAK activity, although there is some evidence of a role for T cells as both precursor and effector populations.

11.1.1 LAK Precursor Cells

It is important to recognise that different groups use varying procedures to measure LAK activity such as (a) different tumour target cells (b) varying IL-2 concentrations which may not be optimal for induction of cytotoxic activity in effector cells (c) different incubation times for induction of LAK activity. Therefore it is difficult to relate the results of one group to another. The results from bulk culture experiments have suggested that it may be possible for T cells to respond to culture in IL-2 by acquiring the ability to kill NK resistant targets (Hersey and Bolhuis, 1987). However, although the evidence suggests that there is no single peripheral blood LAK precursor cell (Itoh et al, 1985), the majority of LAK cytotoxic activity appears to be due to activation of NK cells (Phillips and Lanier, 1986).

Limiting dilution analysis has confirmed this interpretation. Precursor frequencies for LAK cells were 10-50 times higher in the CD16+ Leu19+ population compared to the CD3+ population (Ortaldo, Mason and Overton, 1986). Ferrini et al (1987), using cloning procedures, also showed that the majority of LAK clones were derived from CD2+ CD3- precursors.

11.1.2 Phenotype and Specificity of LAK Cells

The main factors which determine the phenotype and specificity of LAK cells have been described as (a) the method of stimulation (b) the type of lymphoid cell stimulated (Hersey and Bolhuis, 1987). When PBL were stimulated with IL-2 alone, predominantly CD3- CD16+ effector cells were generated (Ortaldo, Mason and Overton, 1986).

The paterns of specificity of cloned LAK cells vary considerably (Allavena and Ortaldo, 1984; Van de Griend et al, 1984; Christmas, Meager and Moore; 1987). Some clones were found to possess very broad paterns of specificity and others had far more restricted reactivity. There did not appear to be any antigenic pattern which could distinguish the varying specificities of LAK clones. CD16+ CD3- clones were found to make significantly less IFNγ - and TNFα than CD3+ CD16- clones in response to PHA (Christmas, Meager and Moore, 1987). Nevertheless, CD16+ CD3- clones were the most potent cytotoxic cells.

11.2 Experimental Design

These experiments were designed to examine the ability of IL-2 to induce LAK activity in normal and JDM PBL populations. Defective generation of LAK activity was found for JDM cell populations and this was further analysed at the precursor level.

Furher experiments were conducted to investigate whether cooperation between lymphocyte subpopulations was required to induce optimal LAK activity and to identify these sub-populations. Evidence of such cooperation was observed between normal but not JDM lymphocyte subpopulations.

11.3 RESULTS

11.3.1 Time Course for the Generation of LAK Cells from Normal PBL

PBL were obtained from 3 normal adults and 3 normal children. One aliquot of PBL was fractionated into E+ and E- cells and cytotoxicity activity of each fraction measured. The remainder of the PBL were cultured in IL-2 containing medium for 24h or 13 days, then washed and cultured O/N in medium without IL-2. Cultured cells were then fractionated into E+ and E- populations and cytotoxicity of each fraction obtained at 4 E:T ratios. Table 11.1 shows the results expressed as % ^{51}Cr release at 2 E:T ratios and lytic units determined for the E- effectors at 30% and 15% ^{51}Cr release for K-562 and RD targets respectively. The results are not expressed in lytic units for E+ effectors since augmentation of cytotoxicity by IL-2 was greater than for E- effectors resulting in cytotoxicity values at different time periods which could not be connected by a single ^{51}Cr release point.

(i) **E- Effectors.** Culture of PBL in IL-2 augmented the cytotoxic activity of E- cells and the degree of enhancement varied considerably for both adults and children. Increased killing for K-562 targets varied between 31 and 511% at 48 hours. Two of the 3 childrens's cells had enhanced cytotoxicity at 48 hours with no further increase at 14 days. Cytotoxic activity by the other subjects's cells increased between 48 hours and 14 days, the range being 34-109%. Two adults's and 2 childrens's cells responded to IL-2 by giving considerably enhanced killing against RD targets, augmentation ranging from 165 to 333% at 48 hours and 114 to 298% between 48 hours and 14 days. One adult's and 1 child's E- cells responded poorly to IL-2 and gave a very limited increase in killing against either target.

(ii) **E+ Effectors** - augmentation of cytotoxicity by E+ effectors from both adults and children again varied considerably. For K-562 targets, at E:T ratio 50:1, enhanced killing ranged from 83 to 302% at 48 hours and 97 to 236% between 48 hours and 14 days. Enhanced cytotoxicity against RD targets at 50:1, reflecting the almost complete lack of killing by freshly isolated E+ effectors from PBL, ranged from 570 to almost 2000% at 48 hours and 107 to 2983% between 48 hours and 14 days. All adults and children showed enhanced cytotoxicity against both K-562 and RD targets.

TABLE 11.1

Time course for induction of LAK activity in PBL from normal controls.

(A) K-562 TARGETS

(i) E^- EFFECTORS

	CYTOTOXICITY								
	0[a]			48h[b]			14d		
NORMAL ADULTS E:T	20:1	50:1		20:1	50:1		20:1	50:1	
1	27.1[c]	42.8	(80.0)[d]	33.8	60.8	(105.2)	43.0	75.5	(142.8)
2	22.2	30.1	(40.0)	24.3	39.5	(64.5)	42.7	60.5	(133.3)
3	41.6	65.2	(120.5)	62.3	86.9	(235.3)	80.4	81.5	(404.0)
NORMAL CHILDREN									
1	21.9	34.8	(55.6)	27.3	46.5	(80.0)	28.8	52.2	(87.0)
2	13.8	30.7	(34.5)	18.8	51.3	(90.9)	28.7	66.4	(95.2)
3	27.1	44.3	(41.7)	49.7	65.5	(266.7)	64.1	76.8	(357.1)

(ii) E^+ EFFECTORS

	0		48h		14d	
	20:1	50:1	20:1	50:1	20:1	50:1
NORMAL ADULTS						
1	11.1	17.9	18.5	36.0	58.4	71.2
2	8.1	10.8	14.9	31.4	38.7	66.5
3	6.3	8.7	16.4	26.5	41.3	67.4
NORMAL CHILDREN						
1	8.4	12.1	10.1	21.2	27.4	58.3
2	6.5	7.4	15.8	32.6	38.9	76.4
3	5.3	5.3	11.8	21.4	43.7	71.9

TABLE 11.1 continued

(B) RD TARGETS

(i) E^- EFFECTORS	CYTOTOXICITY					
NORMAL ADULTS	0^a		48h		14d	
1	6.7	11.7 (18.2)	7.1	11.8 (18.5)	11.0	11.8 (25.0)
2	6.8	8.6 (20.6)	9.5	15.4 (60.6)	19.5	32.8 (153.8)
3	6.5	8.8 (22.4)	11.8	16.2 (59.5)	21.3	37.4 (181.8)
NORMAL CHILDREN						
1	7.2	7.3 (13.3)	6.4	7.5 (16.7)	8.4	12.6 (29.4)
2	6.1	8.3 (15.6)	7.8	22.6 (62.5)	28.7	45.8 (248.0)
3	5.9	9.7 (12.3)	9.5	16.5 (53.5)	17.4	31.2 (114.9)
(ii) E^+ EFFECTORS						
NORMAL ADULTS						
1	0.1	0.8	7.4	15.1	37.2	57.8
2	1.3	1.3	12.6	25.8	81.3	84.7
3	2.0	2.4	12.3	24.3	24.3	50.6
NORMAL CHILDREN						
1	1.7	1.1	7.1	8.4	12.6	18.3
2	2.8	3.1	12.3	20.8	27.4	52.8
3	2.1	2.4	15.1	26.5	39.3	60.8

a) NK activity of E+ and E- effectors after fractionation of PBL

b) PBL were cultured for 24h or 13d in IL-2 (50U/ml), then washed and cultured O/N in medium without IL-2. Cells were then separated into E+ and E- fractions, and cytotoxicity assayed

c) Figures represent % ^{51}Cr release

d) Figures in parenthesis represent lytic units /10^7 cells, calculated for E- effectors at 4 E:T ratios K-562 (30%) RD (15%).

Levels of cytotoxicity by both effector populations did not increase after 2 weeks culture and cell viability began to decrease between 3 and 4 weeks, most cultured cells dying by 6 weeks.

The ability of PBL from 4 normal adults cultured in IL-2 for 2 weeks, to kill autologous and allogeneic EBV transformed lymphoblastoid cells was examined. None of the donor effector cells tested showed significant cytotoxicity against these targets (data not shown).

11.3.2 Induction of LAK Activity in PBL from JDM Patients

(i) Time Course

The ability of JDM patients's PBL to develop LAK activity in response to IL-2 was assessed by measuring cytotoxicity against RD and K-562 targets. (Fig 11.1). Results for whole lymphocyte populations are given as there were insufficient cells to fractionate into E+ and E- subpopulations at the start of culture. Lymphocytes were obtained from 6 JDM patients, 2 of whom were tested on 2 separate occasions. Cells from all patients tested responded to IL-2 by giving enhanced levels of cytotoxicity after 7 or 14 days in culture. The levels of enhancement varied from 20-100% for K-562 targets and 20-150% for RD targets. Cytotoxicity by cultured cells was found to be maximal after 1 - 2 weeks and viability decreased after 2 - 3 weeks culture. Most cells had died after 4 - 5 weeks.

All patients were receiving corticosteroids. Patient LM was receiving 12 mg and 4 mg prednisolone daily at

the time of the first and second tests, respectively. Patient PO was receiving 20 mg and 5 mg prednisolone daily at the time of the first and second tests, respectively.

(ii) **Comparison of LAK Activity in Cells From Controls and JDM Patients**

PBL obtained from 8 normal adults, 6 normal children and 12 JDM patients were cultured in IL-2 (50 U/ml) containing medium. Cells were separated into E+ and E- fractions after culture for 14 days and cytotoxic activity of each fraction measured. Cytotoxicity values for E:T ratio of 20:1 are shown in Fig 11.2.

There were no significant differences in levels of cytotoxicity induced in lymphocytes from normal adults and normal children. The mean levels of cytotoxicity by normal childrens's cells against K-562 and RD targets respectively were:- E- effectors 62, 33%; E+ effectors 31, 34%. The mean levels of cytotoxicity by normal adults's cells against K-562 and RD targets respectively were: E- effectors 74, 40%; E+ effectors 29, 26%. Cytotoxicity by E- effectors against K-562 but not RD targets was significantly greater than E+ effectors ($p < 0.01$).

The mean level of cytotoxicity by JDM patients's E- cells against K-562 and RD targets were 28 and 12% respectively. These means were significantly lower ($p < 0.01$) than the corresponding mean levels of cytotoxicity of normal E- cells. The mean levels of

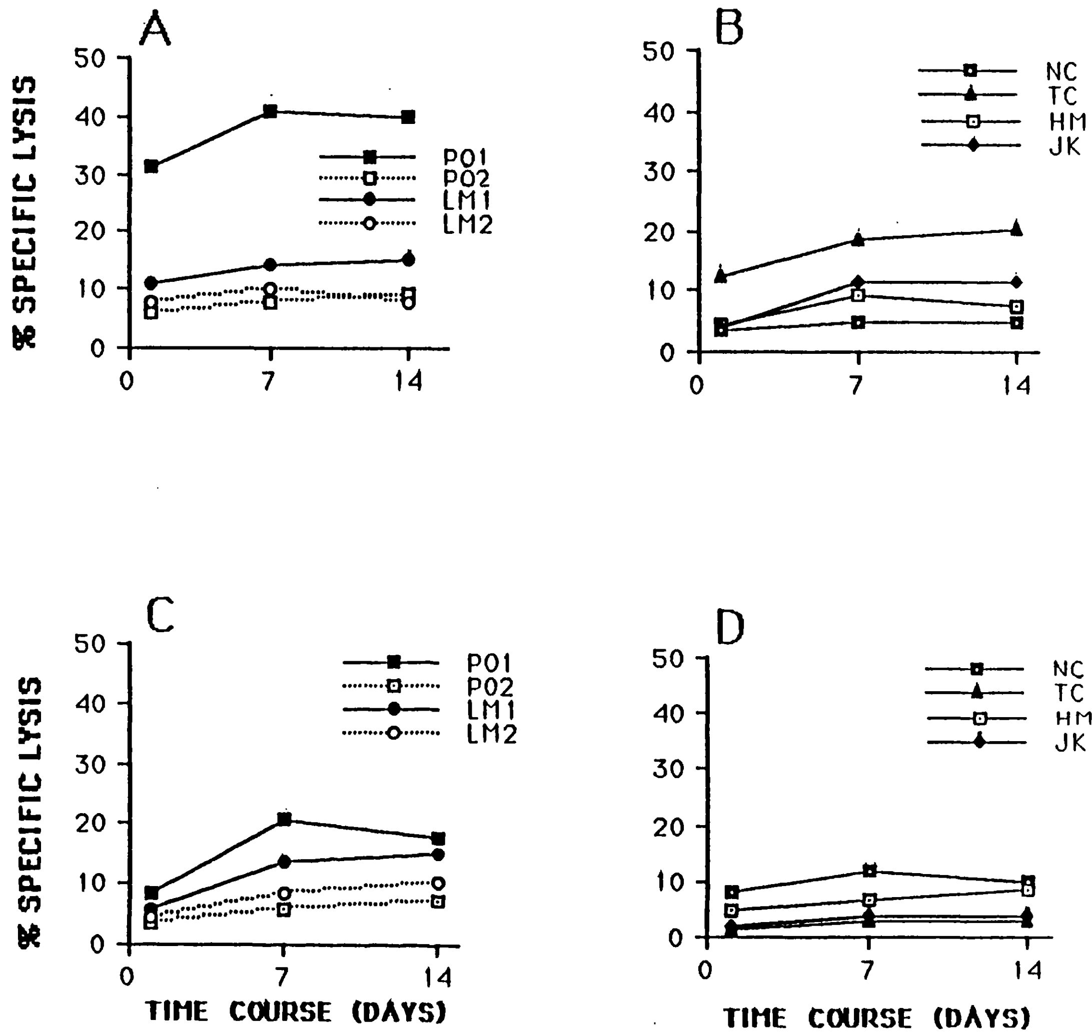
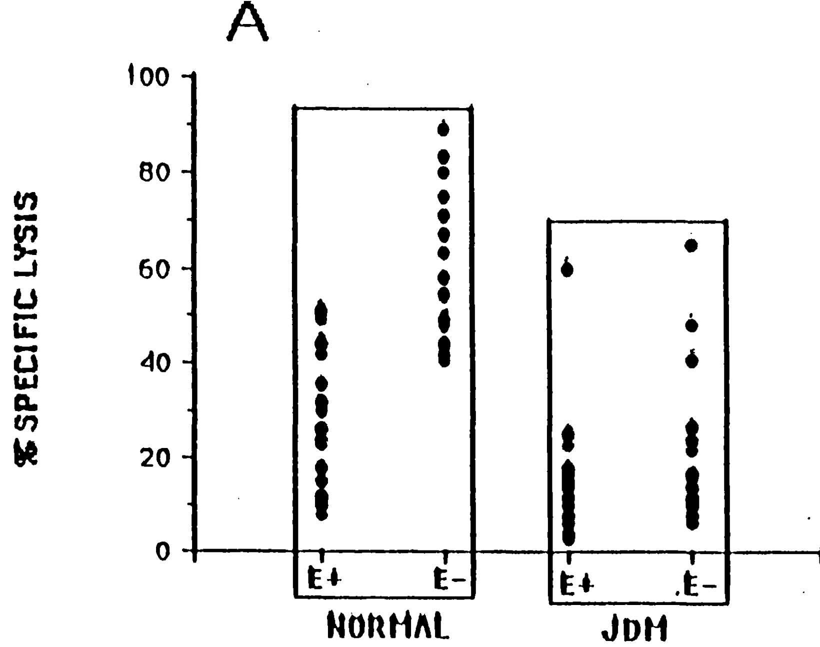


Figure 11.1 Time course for induction of LAK activity in cells from JDM patients. PBL were cultured in IL-2 (50 U/ml) and cytotoxicity against (A, B) K-562 targets and (C, D) RD targets, measured at days 1, 7 and 14. Cells were obtained from patients PO and LM on 2 separate occasions. The E:T ratio was 20:1.



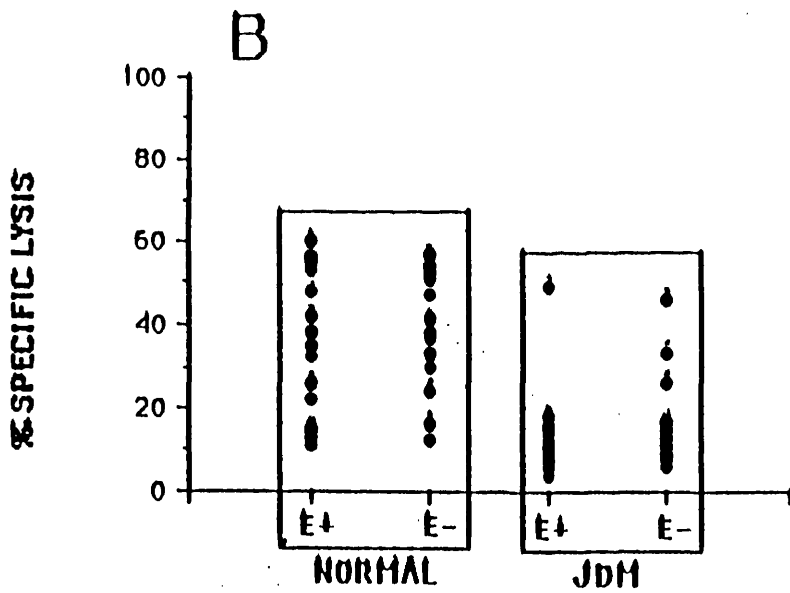


Figure 11.2. LAK activity by cells from normal controls and JDM patients. PBL were cultured in IL-2 (50 U/ml) for 14 days, then fractionated into E+ and E- populations and cytotoxicity measured against (A) K-562 targets and (B) RD targets. The E:T ratio was 20:1.

cytotoxicity by JDM patients's E+ cells against K-562 and RD targets were 14 and 11% respectively. These means were significantly lower ($p < 0.01$) than the corresponding mean levels of cytotoxicity of normal E+ cells.

Normal and patient PBL (3 normal adults, 3 JDM patients) were also cultured for 6 days in a higher concentration of IL-2 (500U/ml). The levels of cytotoxicity induced in patient lymphocyte populations were again significantly reduced ($p < 0.01$) compared to normal adults (data not shown). Therefore:

(1) Development of LAK activity by JDM patients's cells was impaired compared to controls.

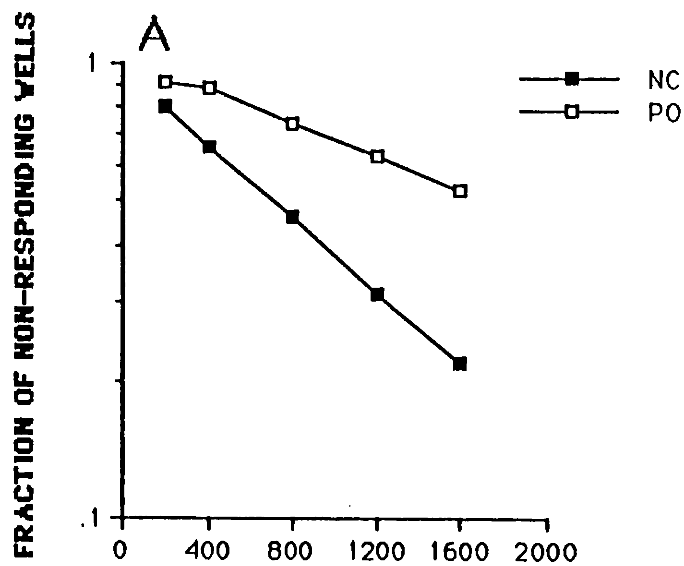
(2) Impaired development of LAK activity by cells from JDM patients was not due to a higher threshhold for IL-2 responsiveness than control cells.

The complexity of interactions which must be taken into account with polyclonal cell populations can be simplified and clarified by experiments on clonal populations. Therefore limiting dilution analysis was applied to determine LAK cell precursor frequencies and provide information on the functional activity of LAK cells at the clonal level.

11.3.3 Precursor Frequencies of LAK Cells From Normal Controls and JDM Patients

Precursor frequencies were determined for LAK cells from 4 normal adults, 4 normal children and 6 JDM patients. Two patients (GH and NC) were untreated when tested. The rest of the patients were receiving prednisolone alone, except JK who was also receiving azathioprine. Fig 11.3 shows a representative experiment in which LAK cell precursor frequencies were determined for a normal child and a patient at 5 different responder cell concentrations. The precursor frequencies are shown in Table 11.2. The data obtained suggested that a single cell was limiting as the regression lines fitted the Poisson distribution ($p < 0.05$) in all cases. The frequencies of LAK cells from normal adults and children did not differ significantly and frequencies ranged from 1/685 - 1/2080 and 1/550 - 1/1765 for cells from controls against K-562 and RD cells respectively. Comparison of frequencies of LAK cells for the 2 target cells showed no significant differences. Frequencies of LAK cells from JDM patients ranged from 1/2830 - 1/15830 and 1/2180 - 1/13860 for K-562 and RD cells respectively. The frequencies of LAK cells from patients differed significantly from controls ($p < 0.01$) for both target cells.

The 2 untreated patients had the lowest numbers of precursors. Two normal adults and 2 JDM patients (JK and PO) were tested on 2 separate occasions. No differences in LAK precursor frequencies between the 2 occasions were observed (data not shown). Patient JK had severe vasculitis when first tested and mild vasculitis when tested subsequently.



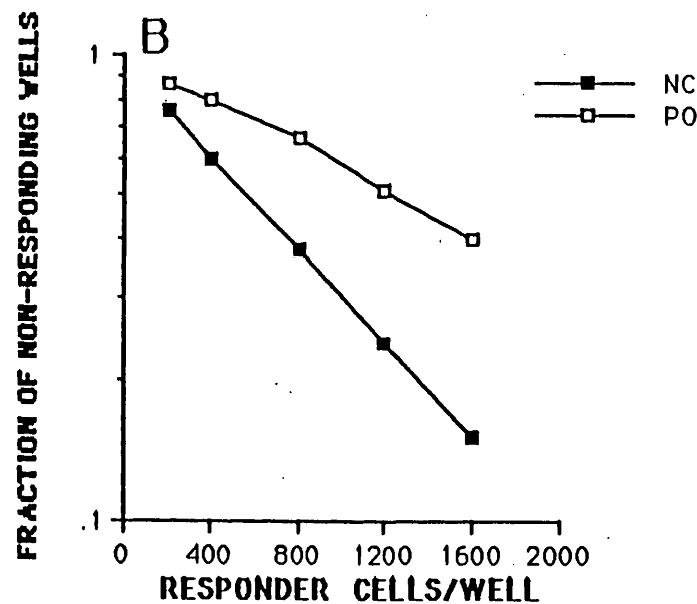


Figure 11.3.

Representative precursor frequency determination of LAK cells from a normal child (NC) and a JDM patient (PO). Limiting dilutions of PBL were cultured with 1×10^5 pooled allogeneic normal PBM and IL-2 (50 U/ml). LAK activity against (A) K-562 and (B) RD targets was determined after 10 days. The Poisson equation states that at the point where the proportion of non-responder cultures equals 0.37, the frequency of LAK cells in the responder cell cultures is 1.

TABLE 11.2.

Precursor Frequencies of LAK Cells from Normal Controls and JDM Patients

Donors	Frequency[a]	
Controls	K-562	RD
NA[c]	1/1460 (1295 - 1690)[b]	1/1185 (1010 - 1375)
NA	1/685 (600 - 825)	1/550 (480 - 675)
NA	1/1280 (1150 - 1470)	1/960 (840 - 1225)
NA	1/1660 (1405 - 2150)	1/1235 (1055 - 1580)
NC	1/1125 (975 - 1260)	1/840 (715 - 1060)
NC	1/780 (710 - 870)	1/665 (595 - 775)
NC	1/965 (840 - 1150)	1/910 (805 - 1090)
NC	1/2080 (1735 - 2135)	1/1765 (1525 - 2180)
JDM Patients		
PO	1/2830 (2540 - 3260)	1/2180 (1840 - 2560)
JK	1/5180 (4455 - 5965)	1/4735 (4250 - 5490)
LM	1/12860 (10995 - 14435)	1/11760 (10425 - 13280)
HM	1/4585 (4260 - 5250)	1/4320 (3865 - 4985)
NC	1/15830 (13360 - 19345)	1/13860 (12130 - 16315)
GH	1/10190 (8580 - 11775)	1/9125 (7680 - 11345)

[a] Precursor frequencies were determined for varying numbers of PBL cultured with 1 x 10^5 pooled, allogeneic, normal PBM and IL-2 (50 U/ml)

[b] Data in parenthesis are the 95% confidence intervals

[c] NA = Normal Adult NC = Normal Child

In order to compare functional activity as well as precursor frequencies, cytotoxicity was also determined in limiting dilution cultures at a cell concentration when there was less than 1 precursor cell present/well, and these results are shown in Fig 11.4. Normal donors had mean levels of cytotoxicity of 52 and 58 against K-562 and RD targets respectively, whereas the values for JDM patients were 18 and 25 against K-562 and RD respectively. The mean levels of cytotoxicity by clonal LAK cells from patients were significantly less ($p < 0.05$) than that from normal controls for both target cells. The number of LAK cells obtained from clonal wells was approximately 2×10^4 and the E:T ratio was therefore 4:1.

Individual wells containing LAK cells cultured at a precursor frequency of less than 1 cell/well were examined for target specificity. Greater than 80% of positive wells from both normal controls and patients contained LAK cells which killed both K-562 and RD targets.

These experiments indicated that a single precursor LAK cell was limiting. However, cooperation between lymphocyte sub-populations in the induction of LAK activity had been observed by other workers (Stephens et al, 1985). Evidence of such cooperation was therefore sought in bulk culure experiments.

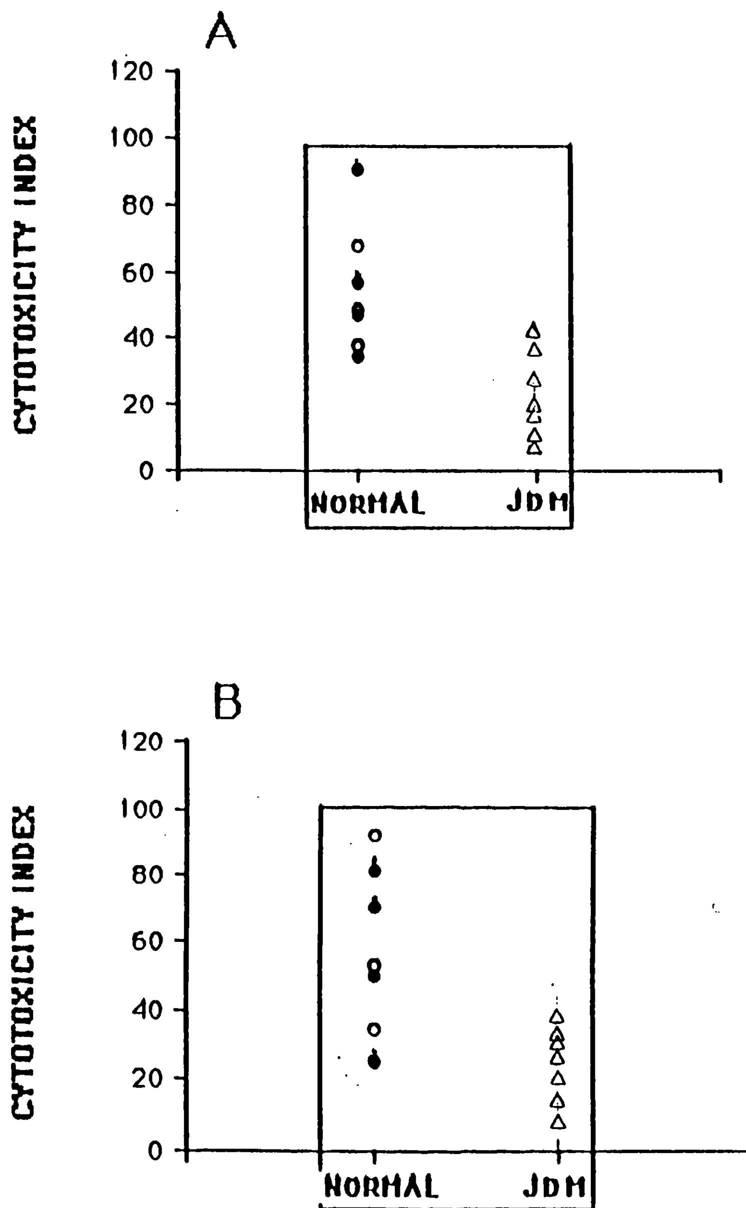


Fig 11.4.

Cytotoxic activity of LAK precursor cells from normal controls, including normal adults (open circle), normal children (closed circles) and JDM patients. Each point represents the mean cytotoxicity (cytotoxicity index) of multiple clonal wells from a single individual. The responder cell concentration chosen contained less than 1 LAK precursor cell/well according to the Poisson distribution.

11.3.4 Generation of LAK Activity in Purified CD3+ and CD16+ Cells

Purified CD3+ and CD16+ cells were obtained from 3 normal adults by E rosetting, followed by deletion of residual contaminating cells by complement mediated lysis.

(i) **CD3 Cells.** Purified T cells were 97-98% CD3+. CD16+ or CD21+ cells comprised less than 1% of the population. The cells were cultured in IL-2 (100U/ml) and phenotypes, cytotoxic function and proliferation measured at weekly intervals (Table 11.3). T cells grew well in IL-2 alone and multiplied 8-15 fold by 14 days. High levels of proliferation were obtained at 7 and 14 days. Proliferation decreased after 14 days and cultures died after 4-5 weeks. Phenotypic analysis showed that T cells were not induced to express CD16 in culture, although the percentage of CD25 expressing T cells increased to between 27-36% after 14 days. Cytotoxicity against RD targets was not enhanced by culture in IL-2 and was also not enhanced against K-562 cells (data not shown). Similar levels of lectin-induced-cellular-cytotoxicity (LICC), 584 $\pm$ 43 and 585 $\pm$ 52 LU, were observed for uncultured and cultured cells respectively.

(ii) **CD16 Cells.** Purity of CD16+ cells was 73-81% after fractionation and depletion. A minor population of CD16- Leu7+ NK cells has been reported (Lanier et al, 1983) which may comprise most of the CD16- CD3- population. The purified population contained 1-2% monocytes and less than 1% CD3+ and CD21+ cells. The

TABLE 11.3

Phenotypic and functional characteristics of purified normal CD3 and CD16 cells cultured in IL-2.

	Phenotype			^{3}H - T Uptake[b]	LU/10^7 Cells[c]	
	CD3	CD16	CD25	(CPM)		PHA
I. CD3						
0	98[a]	<1	2	395 ± 84	48	625
7 days	98	<1	18	43,266 ± 3,729	66	714
14 days	98	<1	27	38,215 ± 2,431	57	526
CD16						
0	<1	81	1	318 ± 27	118	133
14 days	<1	79	36	7,484 ± 1,050	1,120	852
II. CD3						
0	98	<1	1	474 ± 59	125	588
14 days	98	<1	33	53,237 ± 5,318	180	610
CD16						
0	<1	73		383 ± 37	160	180
14 days	<1	77	28	18,432 ± 3,413	1,340	750
III. CD3						
0	97	<1	1	532 ± 210	78	540
14 days	98	<1	27	42,141 ± 6,184	83	620

a Figures represent % positive cells.

b ^{3}H - T was added to triplicate cultures of 2 x 10^5 cells, at the time points indicated, and ^{3}H - T incorporation measured.

c Cytotoxicity against RD targets measured at 3 E:T rations in the presence or absence of PHA (2 ug/ml). Lytic units (LU) measured at 15% cytotoxicity.

purified NK cells grew very poorly in IL-2 (50U/ml) and had died by 14 days. Increasing the concentration of IL-2 to 500 U/ml had no effect on maintaining viability, probably due to lack of cofactors, as has previously been reported (Christmas, Meager and Moore, 1987). CD16+ cells were therefore grown in the presence of irradiated, autologous PBMC and PHA (Dr S Christmas, personal communication) as well as IL-2. Under these conditions, 2 out of 3 cultures grew well for 14 days, multiplying 8 fold, although there were insufficient cells to assay at 7 days. Proliferation decreased from 2-3 weeks and cells had died by 4 weeks. The 3rd culture did not respond and died within 14 days.

Proliferation of NK cells in the 2 surviving cultures at 14 days, was 2.5 to 6 fold less than for the same number of initial T cells. The phenotype at 14 days showed that the percentage of CD16+ cells had varied by less than 5%. There was no increase in CD3+ cells but CD25 expression increased to 28-36% (Fig 11.5). Cytotoxic activity against RD targets had increased 9-10 fold at 14 days, but addition of PHA to the cytotoxic assay inhibited killing by 25-44%. Similar results were obtained for K-562 targets (data not shown).

Therefore, cooperation between both CD16+ and CD3+ subsets and other mononuclear cells appeared to be required to achieve optimum LAK activity by both

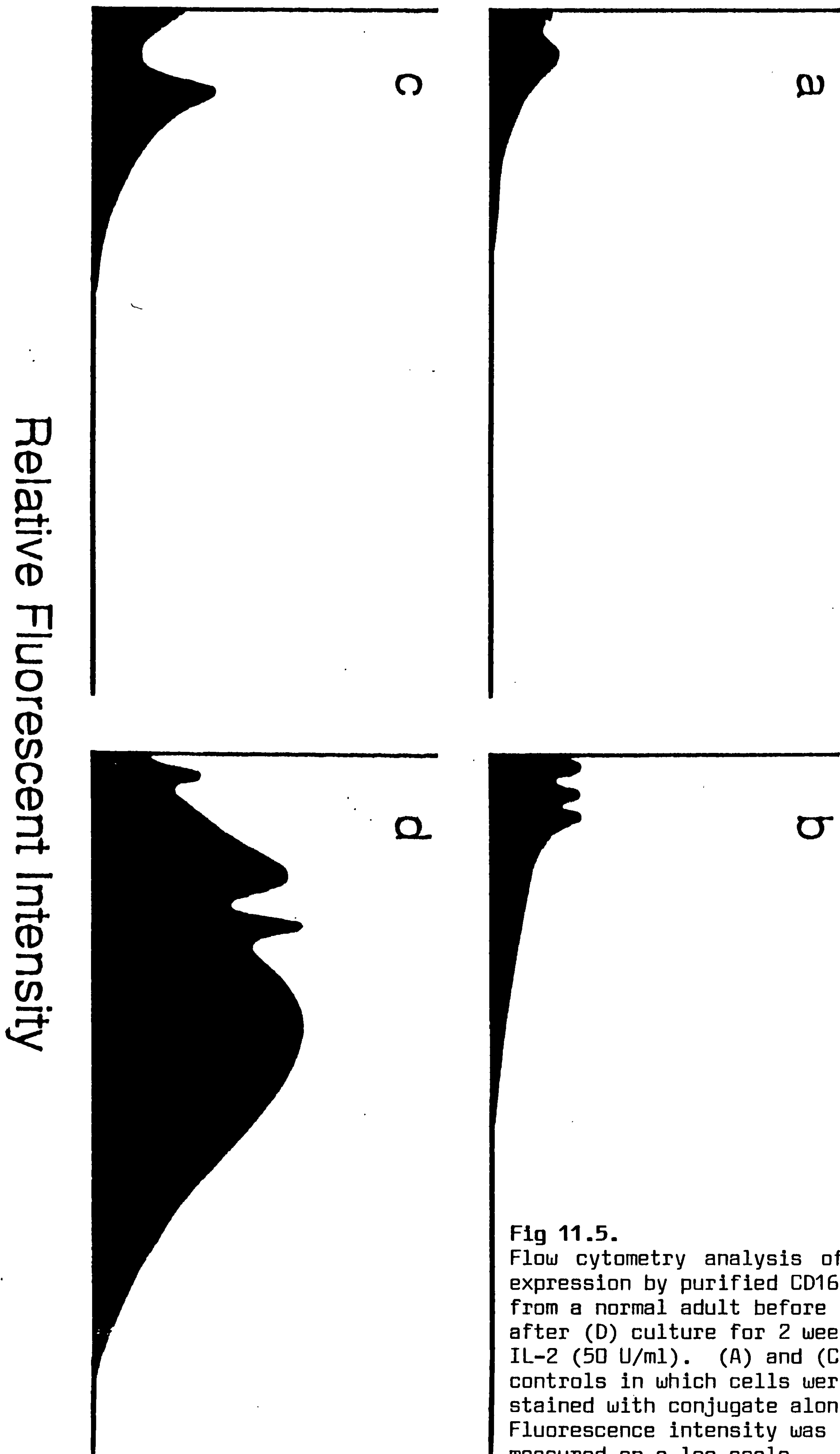


Fig 11.5.
Flow cytometry analysis of CD25 expression by purified CD16+ cells from a normal adult before (B) and after (D) culture for 2 weeks in IL-2 (50 U/ml). (A) and (C) are controls in which cells were stained with conjugate alone. Fluorescence intensity was measured on a log scale.

lymphocyte populations. Although purified CD3+ cells could not express LAK activity when cultured alone, it was possible that cocultures of CD3+ with other mononuclear cells could generate LAK activity in CD3+ cells.

11.3.5 Effect of Depletion of CD3+ and CD16+ Cells on Cytotoxicity of Cultured PBL

This experiment was designed to examine whether CD3+ cells could express LAK activity in cooperation with other cells in a normal PBL population. A further intention was to explore the presence of cytotoxic CD16 cells within the E+ population. PBL from 2 normal children were cultured for 2 weeks in IL-2 containing medium (50 U/ml). The cells were then harvested and separated into E+ and E- fractions. The E+ fraction was then divided into 3 aliquots. One aliquot was depleted of CD3+ cells and the second of CD16+ cells by complement lysis. The depletion of CD3+ cells varied from 78-83%, while that of CD16+ cells varied from 87-95%. The 3rd aliquot was treated with complement alone. The depleted and control aliquots were then tested for cytotoxicity (Fig 11.6). Comparison of lytic units at 20% cytotoxicity showed that depletion of CD3+ cells had no significant effect on K-562 killing. However, the results for RD cells showed significant reductions in cytotoxicity ($p < 0.05$) by the CD3 depleted aliquot compared with control. The mean cytotoxicity values for RD targets before and after depletion of CD3+ cells were 273 and 184 LU/10^7 cells respectively.

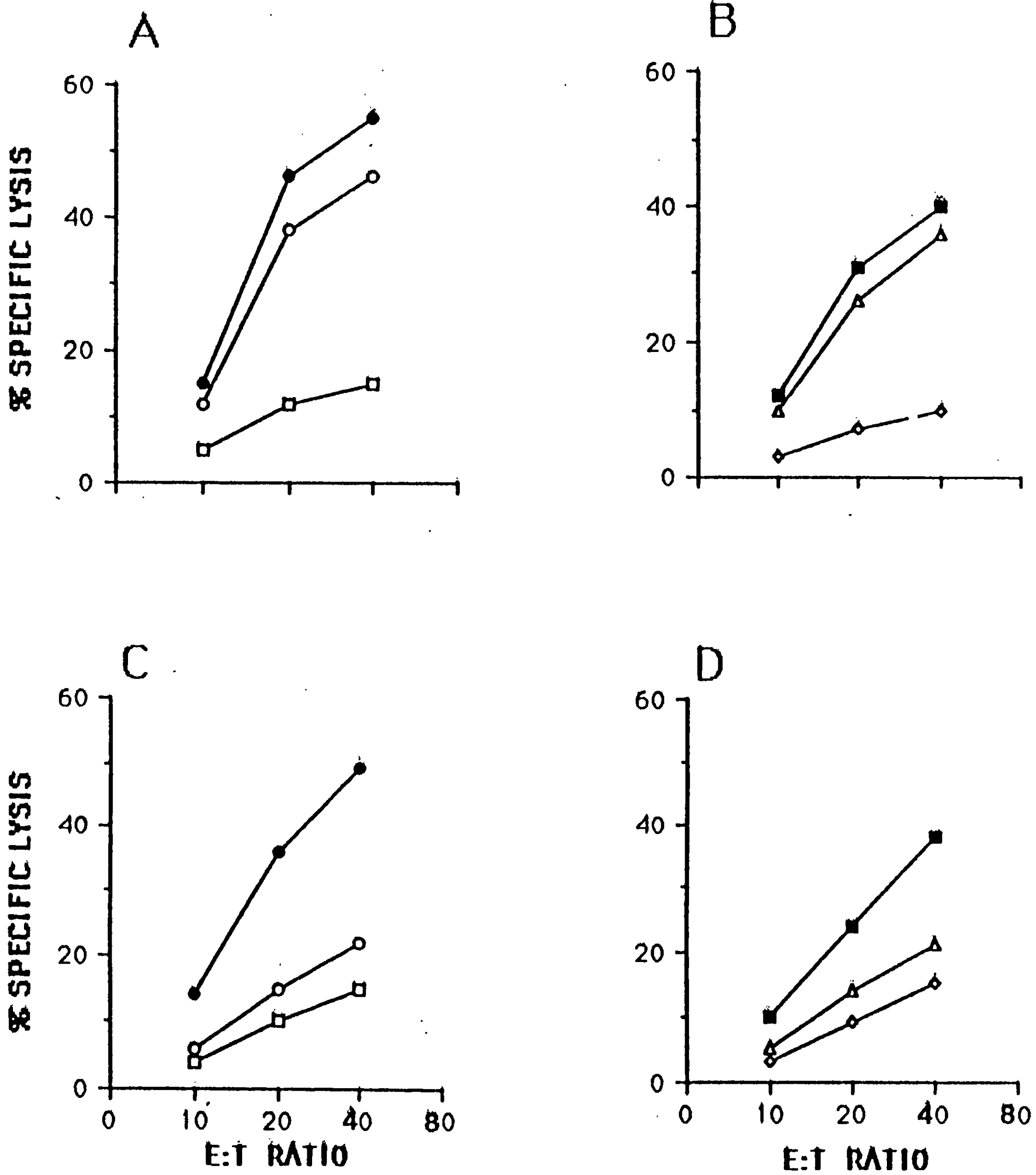


Fig 11.6. Effect of depletion of CD16+ and CD3+ cells on cytotoxic activity of E+ LAK cells. PBL from 2 normal childhood donors were cultured for 2 weeks in IL-2 (50 U/ml). Cells were then fractionated and the E+ population depleted of CD16+ or CD3+ cells. Closed symbols represent cytotoxicity by undepleted control E+ populations from (A, C) donor 1 and (B, D) donor 2 against (A, B) K-562 and (C, D) RD targets. Open symbols represent cytotoxicity by CD3+ depleted cells (circles and triangles) and CD16+ depleted cells (squares and diamonds), respectively.

Depletion of E+ effectors of CD16+ cells also significantly reduced cytotoxicity against RD targets ($p < 0.01$).

Treatment of effector cells with complement alone had no significant effect on cytotoxic activity.

Therefore, CD3+ cells could develop LAK activity when cultured with other PBL. However, a minor population of CD16+ cells within the E+ population also contributed to LAK activity.

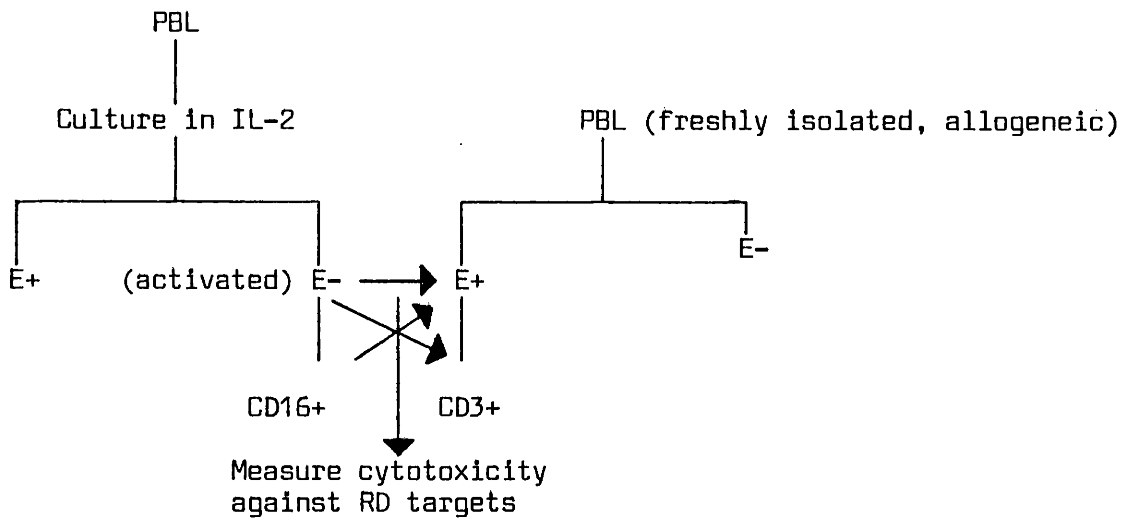
These experiments indicated that CD16+ cells were the principal LAK effectors. Since the majority of precursor LAK cells were shown to kill both target cell lines (Ch 12.3.3), most precursor LAK cells were probably also CD16+.

11.3.6 Induction of Cytotoxic Activity in Normal T Cells Through a CD16+ Cell Intermediary

(i) The Effect of Addition of LAK E- Cells on Cytotoxic Activity of Fresh E+ and CD3+, Allogeneic Cells

The following 2 experiments were designed to conclusively show that CD3+ T cells could develop LAK activity through the cooperation of an intermediary cell and to identify this intermediary.

PBL from normal childhod donors were cultured for 6 days in IL-2 (50 U/ml) containing medium. The cells were then separated into E+ and E- fractions, referred to as the 'activated' fractions. The experimental design is outlined schematically as follows:



→ Coculture O/N of lymphocyte subpopulations.

Increasing numbers of activated E- cells from 2 normal, childhood donors were added to a constant number (8×10^4) of fresh, normal allogeneic E+ or purified CD3+ cells from 2 normal, adult donors and cocultured O/N. An aliquot of each cell fraction was also cultured separately O/N. Cytotoxic activity of the various cell fractions was then determined against RD targets (Figs 11.7 and 11.8). Cytotoxicity by the cocultured cells showed a dose-response effect i.e. increasing cytotoxicity with increasing numbers of added activated E- cells.

The results showed that enhanced cytotoxicity was not due to a simply additive effect and furthermore, that E- cells cultured in the presence but not the absence of IL-2 could induce enhanced cytotoxicity in fresh, normal, allogeneic E+ or CD3+ cells (data not shown).

(ii) Induction of Cytotoxicity in Normal E+ Cells Through A CD16+ Intermediary Cells

The role of CD16+ LAK E- cells in inducing cytotoxicity in normal E+ cells was investigated by depleting CD16+ cells from LAK E- populations derived from culturing PBL from 2 normal children for 6 days in IL-2 (50 U/ml). The CD16 depleted, and control, LAK E- cells, treated with complement alone, from each donor were cocultured O/N with fresh, normal, allogeneic E+ cells and cytotoxicity of the cocultured cells against RD targets then measured (Fig 11.9). Depletion of CD16+ cells ablated the ability of LAK E- cells to induce cytotoxicity in E+ cells.

Therefore, CD16+ LAK cells act as intermediaries in conveying the signals required by T cells to develop non-MHC restricted cytotoxicity. Since LAK cells from normals could perform regulatory functions in relation to T cells, the ability of LAK cells from JDM patients to exercise these functions was investigated.

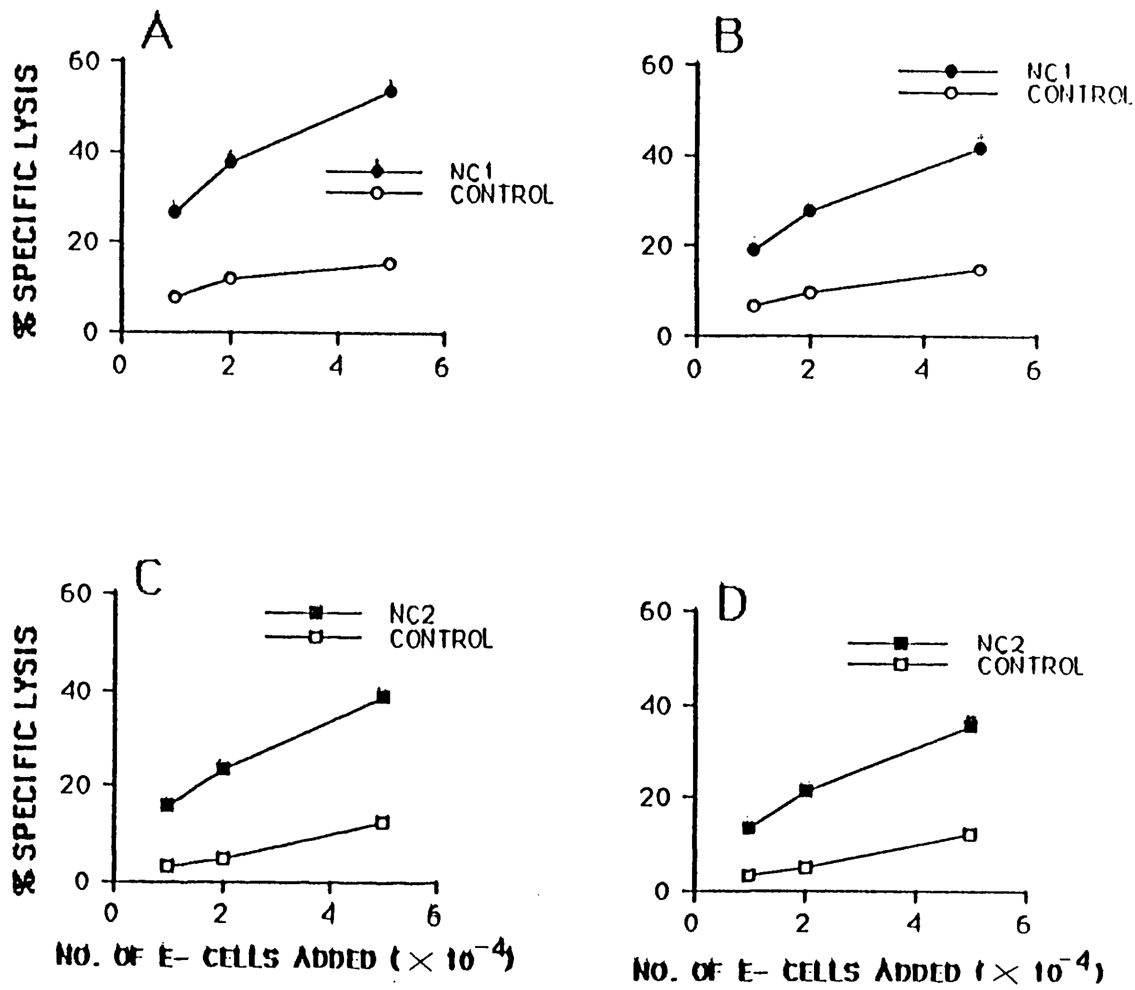


Fig 11.7. Ability of E- LAK cells to induce cytotoxic activity against RD targets in E+ cells. PBL from 2 normal children (NC1 and NC2) were cultured for 6 days in IL-2 (50 U/ml), E- cells then fractionated and added at 3 separate concentrations to a constant number ($8x10^4$) of fresh, allogeneic E+ cells from (A, C), normal adult donor 1, and (B, D), normal adult donor 2. Cells were cocultured O/N and cytotoxicity against RD targets ($5x10^4$) then measured. Controls represent cytotoxicity by E- LAK cells alone. Cytotoxicity by E+ cells from the normal adult donors was 2.1 and 1.1% respectively.

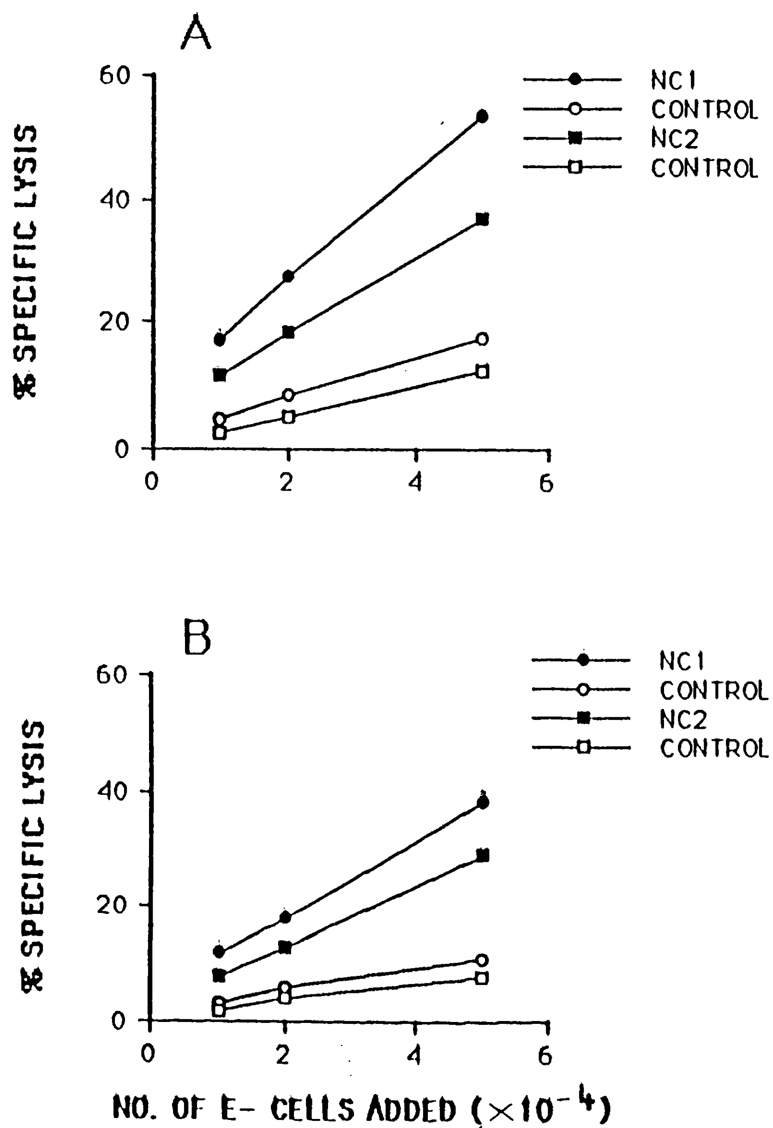
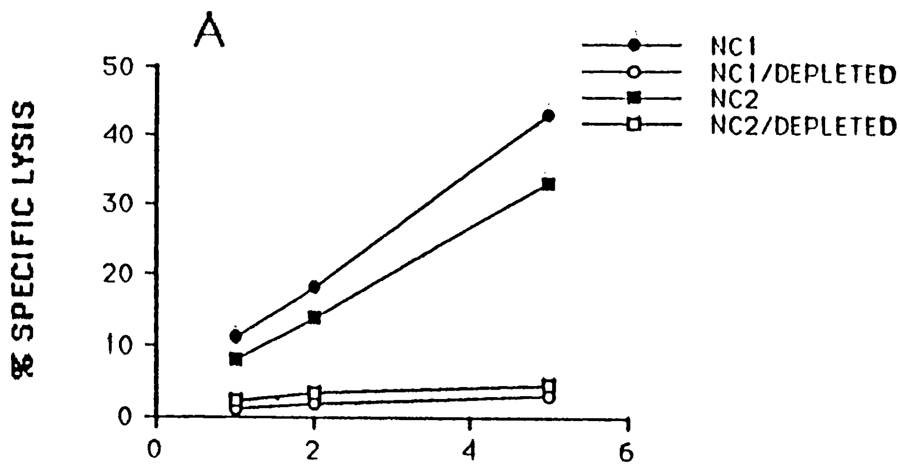


Fig 11.8 Ability of E- LAK cells to induce cytotoxic activity against RD targets in CD3+ cells. PBL from 2 normal children (NC1 and NC2) were cultured for 6 days in IL-2 (50 U/ml), E- cells then fractionated and added at 3 separate concentrations to a constant number (8×10^4) of fresh, allogeneic CD3+ cells from (A) normal adult donor 1 and (B) normal adult donor 2. Cells were cocultured O/N and cytotoxicity against RD targets (5×10^4) then measured. Controls represent cytotoxicity by E- LAK cells alone. Cytotoxicity by CD3+ cells from the normal adult donors was 1.8 and 1.4% respectively.



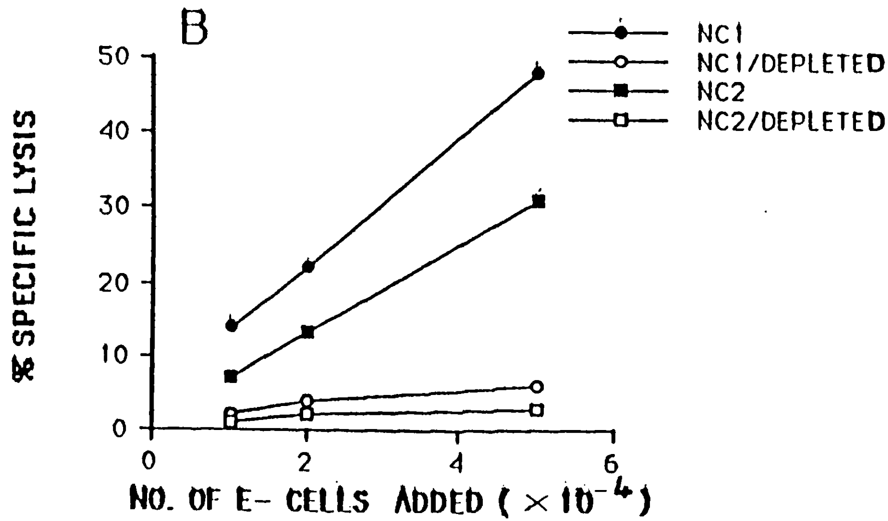


Fig 11.9. Effect of depleting the LAK E- population of CD16+ cells on its ability to induce cytotoxic activity against RD targets in E+ cells. PBL from 2 normal children (NC1 and NC2) were cultured in IL-2 (50 U/ml) for 6 days, E- cells fractionated and one aliquot depleted of CD16+ cells. Control undepleted and CD16+ depleted E- cells were then added at 3 separate concentrations to fresh, allogeneic E+ cells (8×10^4) from (A) normal adult donor 1 and (B) normal adult donor 2. Cells were cocultured O/N and cytotoxicity against RD targets then measured.

11.3.7 **The Effect of Addition of LAK E- Cells from JDM Patients on Cytotoxic Function of Fresh Normal, Allogeneic E+ Cells**

Donor PBL from 6 JDM patients were cultured for 6 days in IL-2 (50 U/ml). The cells were then fractionated and the E- fractions, referred to as activated, retained. Increasing numbers of activated JDM E- cells were added to a constant number (8×10^4) of fresh, E+ cells from 3 normal adult donors and cocultured O/N. Cytotoxic activity of the cocultured cells were then determined against RD targets. Results are shown for 3 JDM patients's cells cocultured with 1 normal adult donor's cells (Fig 11.10). Cells from JDM patients were unable to induce cytotoxicity in normal E+ cells. Similar results were obtained using cells from 2 other normal adult donors (data not shown).

These results therefore implied that inability to generate normal levels of LAK activity in cells from JDM patients could be due to defective cooperation between lymphocyte populations, as well as or as a consequence of low precursor frequencies of LAK cells.

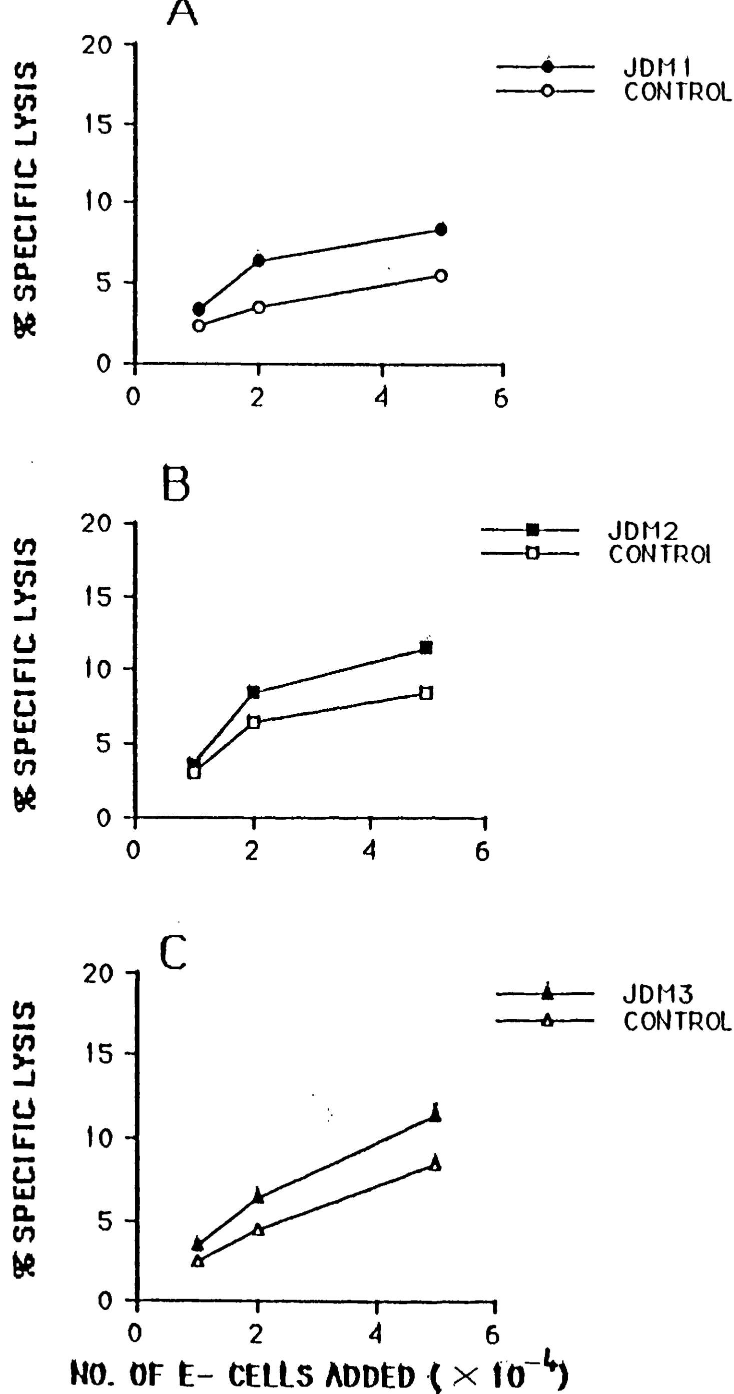


Fig 11.10. Lack of ability of E- LAK cells derived from JDM patients to induce cytotoxic activity against RD targets in E+ cells. PBL from 3 JDM patients (JDM 1, 2, 3) were cultured for 6 days in IL-2 (50 U/ml), E- cells then fractionated and added at 3 concentrations to a constant number ($8x10^4$) of fresh, allogeneic E+ cells from a normal adult donor. Cells were cocultured O/N and cytotoxicity against RD targets ($5x10^4$) then measured. Controls represent cytotoxicity by E-LAK cells alone. Cytotoxicity by E+ cells from the normal adult donor was 2.4%.

11.4 DISCUSSION

11.4.1 Induction of LAK Activity in Normal PBL Populations

The levels of LAK cytotoxic activity induced in lymphocyte subpopulations from normal adults and children were not significantly different. Similar results were reported by Tilden et al (1986).

The kinetics of induction of LAK activity has been shown, in other studies, to be dependent on the IL-2 concentration used. Concentrations of IL-2 in excess of 500U/ml induced maximal cytotoxicity after culture for 18 hours (Phillips and Lanier, 1986). It seemed possible that the use of lower concentrations of IL-2 would require longer periods of time for generation of maximal cytotoxicity. This was found to be the case in these experiments, which showed that maximal cytotoxicity by normal PBL and purified CD16+ cells was achieved after culture for 7 days in 50U/ml IL-2.

Initial experiments demonstrated that purified CD16+ cells did not proliferate in response to IL-2 alone and died in culture (Ch 11.3.4). The slow growth rate of CD16+ cells in IL-2 compared to CD3+ cells has been observed previously (Allavena and Ortaldo, 1984). CD16+ cells have been shown to require higher concentrations of IL-2 to maintain growth than CD3+ cells (Van de Griend et al, 1984), and therefore, purified CD16+ cells were cultured in a 10 fold increase in IL-2 concentrations. CD16+ cells were still not viable in this concentration of IL-2. Support for these results comes from the studies of Lange (A Lange, personal communication), who found that CD16+ cells do not survive longer than 1 week

in IL-2 alone and suggested that an autocrine negative feedback pathway, induced by factors such as TNFα, might be inhibiting cell growth.

The addition of irradiated PBMC plus PHA supported the growth of purified CD16+ cells from some but not all normal individuals in the presence of IL-2. It is therefore possible that factors, other than IL-2, released by PBMC in response to PHA may synergise with IL-2 to promote growth of CD16+ cells (Christmas, Meager and Moore, 1987). Several cytokines, including IL-1 (Oppenheim et al, 1986), IL-2 and IL-4 (O'Garra et al, 1988) have been shown to promote the growth of cytotoxic T cells. Alternatively, an IL-2 independent pathway of CD16+ cell proliferation, such as has been reported for stimulation by K-562 cells (Phillips and Lanier, 1985) could have occurred. This seemed less likely, though, since the percentage of CD16+ cells coexpressing CD25 was greatly enhanced in culture.

Several pieces of evidence indicated that PHA had not stimulated proliferation of contaminating T cells within the CD16+ population:

(a) there was no increase in cells expressing CD3 phenotype during culture in IL-2;

(b) LICC could only be induced in cultures of purified CD3+ not CD16+ cells;

(c) LAK activity could only be induced in cultures of CD16+ but not CD3+ cells.

Although, apparent cooperation between lymphocyte subsets in the induction of LAK activity was therefore observed in bulk culture, this was not observed at limiting dilution (Ch 11.3.3) where a single cell was limiting. It is possible that the negative feedback pathway, suggested by Lange, is not observed under limiting dilution conditions.

The observation that CD16+ cells were not induced to express CD3 during culture in IL-2 is in accordance with a previous report (London, Perussia and Trinchieri, 1986). However, cloned CD16+ cells have been reported to express T cell markers including CD3 (Christmas, Meager and Moore, 1987). It seems possible that the latter result was due to clonal expansion of CD16+ cells coexpressing CD3 (Lanier et al, 1985) rather than induction *de novo* of CD3 during culture.

Purified CD3+ cells appeared to proliferate more strongly in response to IL-2 than CD16+ cells. Proliferation may, however, be due to a minor population of low density T cells (London, Perussia and Trinchieri, 1986) which then rapidly divide over the period in culture. However, these T cells did not express LAK activity. The precursor population of these IL-2 reactive T cells may be a minor population of activated T cells, present in the peripheral blood of normal individuals (London, Perussia and Trinchieri, 1986).

11.4.2 Are T Cells Capable of Expressing LAK Activity?

Since E+ cells derived from cultures of normal PBL could express LAK activity, yet cultures of purified CD3+ cells could not, the question arose as to whether the E+ population expressing LAK activity were in fact T cells. CD3+ cell

depletion from cultured PBL showed that T cells were capable of LAK activity against RD but not K-562 targets. Bolhuis (1978) has also demonstrated that lysis of K-562 cells is mediated solely by cells expressing antibody-dependent-cellular-cytotoxicity (ADCC) activity, a function of NK cells. An interaction between an E- population and T cells in the presence of IL-2 may have been required in order to induce LAK activity in T cells.

Such an interaction was demonstrated by coculture experiments. When PBL were activated by culture in IL-2 and then separated into E+ and E- fractions, the activated E- fraction was able to induce LAK activity in fresh, normal, allogeneic T cells. The E- fraction capable of inducing this activity were shown to be CD16+ cells. Induction of MHC non-restricted cytotoxicity in T cells by E- lymphocytes, recovered from cocultures of tumour cells and lymphocytes, has also been described by Stephens et al (1985). It is not clear whether cell contact is required for induction of cytotoxicity in T cells or whether lymphokines such as TNF and IFN-γ, secreted by CD16+ LAK cells (Christmas, Meager and Moore, 1987) can synergise to induce cytotoxicity.

However, depletion experiments had also indicated that E+ LAK activity was also mediated by CD16+ cells. Stephens and Knight (Stephens, 1983) have also reported non-MHC restricted cytotoxic activity mediated by an E+ CD3- subset.

The cytotoxicity induced by E- subsets in T cells was unlikely to be due to allo-reactivity since:

(a) viable E- cells obtained from PBL cultured in the absence of IL-2 were unable to induce LAK cytotoxicity in fresh, allogeneic cells;

(b) LAK E- cells depleted of CD16+ lymphocytes were unable to induce cytotoxicity in allogeneic E+ cells.

11.4.3 Generation of LAK Activity by JDM Patients's Lymphocytes

Patients's E+ and E- cells were found to possess significantly lower LAK activity than control cell populations. This did not appear to be related to steroid dose at time of testing (Ch 11.3.2). The addition of exogenous IL-2 has been shown to ablate the suppressive effects of corticosteroids on T lymphocyte function in vitro (Walker, Potter and House, 1987). The mechanism for this effect is still unknown but was not due to steroid-IL-2 binding. Conceivably, exogenous IL-2 can induce the re-expression of high affinity receptors on corticosteroid sensitive cells. The effect of steroids in vivo on the generation of LAK activity of normal human cells has yet to be established.

The defective generation of LAK activity by JDM patients's cells was probably related to defective NK activity since the experiments on normal PBL had shown that the majority of LAK activity was generated from the CD16+ population (Ch 11.3.4). Precursor frequencies of LAK cells were 3-10 fold lower for JDM compared to normal cells. Furthermore levels of cytotoxicity induced in cloned JDM LAK cells were significantly lower than those obtained for control clones.

These results strongly indicated that impaired generation of LAK activity in JDM patients's cells was principally due to defects at the precursor level, although failure of cooperation between lymphocyte subpopulations (Ch 11.3.7) may also have contributed.

The LAK activity of PBL from adult PM patients was reduced compared to controls, but the cells appeared to function normally when recycling capacity was measured (Gonzalez-Amaro, Alcocer-Varela and Alarcon-Segovia, 1987). The results for JDM patients suggested a functional abnormality of LAK cells as well as reduced precursor frequencies, and measurement of (a) the recycling capacity (b) secreion of cytotoxic factors from LAK cells from JDM patients may provide further evidence of functional defects.

11.5 Conclusion

These experiments provided new information on the development of LAK activity in PBL. CD16+ cells were shown to be he principal effectors of LAK activity, but differences in the induction of CD16+ LAK cells were observed at limiting dilution and in bulk culture. Induction of optimal LAK activity in bulk culture required cooperation with other cell populations.

The acquisition of LAK activity by T cells was shown to be dependent on a CD16+ cell intermediary and not a direct effect of IL-2. CD16+ cells from JDM patients were unable to provide this cooperation with T cells, which was probably principally due to the low frequency of CD16+ LAK precursors within patient PBL.

CHAPTER 12

GENERAL DISCUSSION

Aetiology And Pathogenesis of JDM

In this chapter the presently held concepts of the development of JDM will be related to findings presented in this thesis.

12.1 Coxsackievirus B Infection and JDM

Coxsackievirus B has been shown to infect endothelial cells *in vitro* (Huber, Job and Woodruff, 1984). The cell receptors for a number of picornaviruses have recently been identified as adhesion molecules such as intercellular-adhesion-molecule-1 (ICAM-1) (Harrison, 1989). It is significant that a related adhesion molecule, endothelial-leucocyte-adhesion-molecule-1 (E-LAM1) has now been shown to be expressed in 50% of the vessels in both involved and uninvolved skin biopsies from JDM patients. ELAM-1 is expressed by very few vessels in normal skin (G Cambridge and D Haskard, unpublished results). It is possible that CVB could infect endothelial cells, perhaps through attachment to ICAM-1, and induce increased expression of other vascular adhesion molecules such as ELAM-1.

Alimentary tract infection, allowing local replication of an enterovirus such as CVB provokes a host immune response, with secretory IgA antibody detectable within 2-4 weeks (Moore and Morens, 1984). Since the half-life of IgA is short, its presence indicates continuous production in response to antigenic stimulation. The presence of elevated levels of IgA immune complexes (IC) in sera from JDM patients (Ch 5.6) may therefore indicate continued antigenic stimulation. If JDM

patients were found to make secretory IgA antibodies to CVB, this could indicate that the virus may reside in epithelial cells for prolonged periods, providing a source for infection of other tissues. The molecular analysis of IgA immune complexes from JDM sera may allow identification of CVB antigens as components of the complexes.

Juvenile patients with allergic vasculitis, Henoch-Schonlein purpura, were also reported to have elevated levels of IgA immune complexes in serum (Fink, 1986). Allergic vasculitis can coincide with bacterial and viral infections (Clemmensen et al, 1986) and in hepatitus B virus induced vasculitis, hepatitus B surface antigen, Ig and complement components have been found at sites of inflamed blood vessels (Gupta and Kohler, 1984). Reports of examination of JDM muscle or skin biopsy material for the presence of CVB antigens at sites of vascular lesions have not been published. Since CVB complementary DNA probes are now available (Bowles et al, 1987), in situ hybridisation may provide evidence of vascular localisation of virus.

12.2 Anti-Lymphocyte Antibodies - Autoantibodies Induced by Viral Infection

Viral infection is well known to often result in the production of anti-lymphocyte antibodies (Williams, 1977) and anti-lymphocyte antibodies were detected in the serum of patients with JDM (Ch's 8 and 9). Viral receptors such as ICAM-1 are expressed on lymphocytes as well as endothelial cells. Plotz (1983) has proposed that autoantibodies may be anti-idiotypic antibodies recognising anti-viral antigen idiotypes and binding to viral receptors. Therefore, the

inhibitory effects of anti-lymphocyte antibodies, present in JDM sera, might be ascribed to cross-reactions, with idiotypic regions expressed by receptors such as ICAM-1. Since ICAM-1 is an adhesion molecule which may promote lymphocyte interactions (Rothlein et al, 1986), binding of antibodies to epitopes expressed by ICAM-1 could result in suppression of lymphocyte responses, such as the AMLR and PPD stimulation.

Anti-lymphocyte antibodies might also cross react with endothelial cell antigens. The MAb Leu 13 has been shown to react with an antigen present on both lymphocytes and endothelial cells (Pumarola-Sune et al, 1986) and IgG anti-endothelial cell antibodies, cross-reacting with lymphocyte antigens, have been detected in SLE sera (Cines, 1984). Although, in initial experiments, antibodies to placental cord endothelial cells could not be detected in sera from JDM patients (data not shown), infection of endothelial cells with CVB could result in the induction of antigens which may be recognised by antibodies in JDM sera.

12.3 Anti-56Kd RNP Antibodies - A Marker of Myositis

Although anti-endothelial cell antibodies have yet to be detected in sera from JDM patients, the recent finding (R. Sperling et al, unpublished results) that sera from 15 JDM patients, 6 studied serially, all contained ANA to a 56kD RNP suggests that this antibody may be a specific marker of disease.

It is possible that non-specific immuno-stimulation, may result in secretion of ANA (Pisetsky, 1987). However, the autoantibody profile in JDM sera is highly restricted

(Ch 1.1.4). Furthermore, although the relative proportion of ANA to total Ig has not been determined in sera from JDM patients compared to controls, hypergammaglobulinaemia was not found to be a feature of sera from patients presented in this study (Ch 5.6). Therefore, anti-56kD RNP autoantibodies probably arise as a specific response to the 56kD RNP or to a cross-reacting determinant.

The observation that anti-56kD antibodies have been detected in sera from most adult PM patients (Arad-Dann et al, 1987) indicates that the cross-reacting determinant could be a muscle cell component. Cloning of the 56kD antigen would allow its amino-acid sequence to be determined. The sequence could then be compared with known sequences of muscle antigens such as myosin and actin. However, since anti-56kD RNP, in contrast to anti-myosin and anti-actin antibodies, have not been detected in sera of patients with non-inflammatory muscle disease, responses to normal muscle components do not adequately explain the presence of this antibody.

An alternative explanation is that the 56kD RNP can bind to and is involved in the processing of viral messenger RNA. Binding to viral RNA may expose an epitope recognised by T cells, with concomitant stimulation of antibody producing cells.

12.4 Autoantibodies - Possible Relationship to Pathogenesis

It is possible that anti-lymphocyte antibody production may be a host response to perturbation of the immune system as a consequence of autoimmunity. The accumulation of T cells

expressing activation antigens within the inflammatory site may provide the necessary conditions for abrogation of tolerance to activation antigens. Anti-lymphocyte antibodies do not appear to suppress the autoimmune response giving rise to JDM. It is, however, possible that in the absence of anti-lymphocyte antibodies, the disease would be exacerbated.

Although anti-lymphocyte antibodies might be induced as a host response to autoimmunity, the response might still have pathogenetic potential. The selective depletion of CD8+ cells in the peripheral blood of JDM patients may be related to the presence of lymphocytotoxic antibodies, as has been observed for some patients with SLE (Alpert et al, 1987). The lack of generation of suppressor cells in the AMLR may be related to reduced numbers of CD8+ lymphocytes.

If antibodies bound to endothelial cell antigens, the normal function of the cells might be affected. One function of apparently specialised endothelial cells (Pals et al, 1989) in inflammatory sites is the extravasation of lymphocytes. Binding of antibodies to vascular adhesion molecules could alter the ability of endothelial cells to adhere to lymphocytes. This could be beneficial, if lymphocytes, responding to autoantigens and enhancing the inflammatory response, were excluded from the inflammatory site, but detrimental, if passage of regulatory lymphocytes suppressing inflammation were impeded. Complement fixation by antibody bound receptors could exacerbate vascular damage. If MAC were deposited on uninvolved vessels in inflammatory muscle and skin of JDM patients, this could be interpreted as involvement

of MAC in pathogenesis, rather than uptake by diseased vessels.

Elevated levels of IgG and IgM IC were not detected in sera from JDM patients (Ch 5.6), suggesting a lack of association of JDM with systemic IC mediated vasculitis, although production of autoantibodies within affected muscle may contribute to local IC deposition. However, there is no evidence of defective solubilisation or defective clearance of IC from inflammatory sites. Furthermore, although Robb et al (1987) proposed that C4A null alleles were the disease susceptibility genes in JDM, there is no evidence that patients heterozygous for the C4A null allele expressed complement deficiencies.

There is extremely little evidence in the literature to suggest that ANA contribute to pathogenesis. However, this may be due to technical difficulties in performing experiments in the inflammatory site in animal models of autoimmune disease. A high concentration of ANA produced locally in the inflammatory site may have important pathological consequences. Immune complexes, comprising ANA may bind to Fc receptors expressed in high density by endothelial cells in inflammatory sites. If endothelial cells were already compromised by infection with virus, the deposition of immune complexes could aggravate vascular damage.

12.5 Endothelial Cell Autoantigens in JDM

The autoantigens inducing disease in JDM patients are unknown. Although there is some evidence for the presence of myocytotoxic lymphocytes in adult PM patients (Cambridge,

1984; Arahata and Engel, 1986), there is none in JDM. No evidence of proliferative responses by PBMC from JDM patients to purified, normal, human skeletal membrane preparations was obtained by the author.

An alternative hypothesis is that myositis is consequent on vascular damage perpetuated by autoimmune reactivity. I would postulate that the original antigenic insult in JDM is due to CVB infection of endothelial cells. An autoantigenic or altered self epitope(s) is exposed to the immune system and immunopathogenesis is dependent on the presence, within inflammatory sites, of T cells having specificity for these epitopes.

The limited evidence available (Arahata and Engel, 1984; Pachman, 1986) implicates perivascular clusters of macrophages and lymphocytes as promoting the inflammatory response in JDM. One possibility is that T cell responses are directed against endothelial cell determinants expressed in different tissues including muscle, skin and gut. Certain organs, such as kidney, may be spared because of variation in antigenic composition of endothelial cells in different tissues. This is suggested by observations of distinct vascular adhesion molecules expressed by inflammed synovial membrane (Pals et al, 1989).

If epitopes common to muscle and skin derived endothelial cells were identified, their significnce could be determined by precursor frequency analysis, comparing peripheral blood and inflammatory site derived polyclonal T cells with specificity for these epitopes. Sequestration of T cells with

these specificities would be strong evidence for their role in the disease.

12.6 Lymphokine Production by Activated T Cells

Animal models of autoimmune disease have shown that auto-reactive T cells probably recirculate from the inflammatory site to the secondary lymphoid organs via blood and lymph (Cohen, 1986). Therefore, the detection of activated, lymphokine secreting T cells in peripheral blood of JDM patients may represent events within the inflammatory site. Secretion of B cell differentiation factors by these T cells (Ch 6.3.3) suggests that autoantibody production may take place within the muscle lesion. No evidence was found for excessive spontaneous production of IL-2 by T cells from JDM patients (Ch 7.4.7). It is possible that T cells recirculating from inflammatory sites may represent a subset of post-activated cells, down-regulating IL-2 genes, yet still capable of secreting other lymphokines, such as B cell differentiation factors.

12.7 Immunoregulation in JDM

(i) Immunoregulation and Autoimmune Disease

Immunoregulatory studies of the peripheral blood in autoimmune diseases may be criticised for observing systemic rather than organ-specific features of disease. However, a strong body of evidence (Ch 1.1.1) suggests that JDM has many systemic features, which have been characterised as a systemic angiopathy. Furthermore, since lymphocytes recirculate between inflammatory sites and the secondary lymphoid organs,

the peripheral blood will contain lymphocytes mediating and responding to inflammation within the disease site.

Cohen and his collaborators (Cohen, 1986) have isolated arthritogenic T cells from the spleen as well as the regional draining lymph nodes of animals with adjuvant arthritis. Systemic treatments of autoimmune diseases have proved beneficial. Administration of anti-CD4 antibodies has been shown to prevent induction of collagen induced arthritic lesions (Wooley et al, 1987) and total lymphoid irradiation of patients with RA has given remission of disease (Alpert et al, 1987). Therefore, analysis of immunoregulation within the peripheral blood may also reflect events within the inflammatory site.

(ii) **Immunoregulation in JDM and Adult PM**

Anergic lymphocyte responses have been proposed to be the result of host attempts to combat autoimmunity, by down-regulating CD4+ T cell dependent responses through suppressor mechanisms (Via and Shearer, 1987). Although anergic mitogen responses were observed for cells from adult PM patients, this was not the case for cells from JDM patients (Ch 6.3.1). These results could imply down-regulation of immune responses in PM patients. This was supported by the observation that cells from adult PM, in contrast to JDM patients, did not spontaneously secrete IgM (Ch 6.3.2). Adult PM patients have been reported to develop ConA induced suppressor function within the normal range (Ransohoff

and Dustoor, 1983), whereas results presented in this thesis indicated defects of suppressor cell generation by cells from JDM patients (Ch 7.4.10). The immune system in patients with JDM may thus be unable to combat autoaggression by deploying suppressor mechanisms.

The theory, proposed by Wick and colleagues (Kromer, Schaustein and Wick, 1986), implicating IL-2 hyper-reactivity in autoimmune disease could not be substantiated by results presented in this thesis. No evidence of hyperproliferation of cells in response to mitogens, spontaneous IL-2 production or the presence of IL-2 in serum was obtained. However, it is possible that IL-2 secreting T cells may be sequestered in inflammatory sites. Activated cells within these sites, including B cells, macrophages and endothelial cells as well as T cells, may express IL-2 receptors and utilise IL-2 within the lesion, preventing detection in the periphery.

(iii) Idiotypic Recognition - A Possible Role for the AMLR

Suppression of B cell response by T cells generated in the AMLR (Ch 7.4.10) might be explained as T cell recognition of the idiotypes expressed by B cells.

If idiotopes are recognised by CD8+ T cells in the contest of class I, it is possible that AMLR reactive CD4+ T cells may have specificity for idiotope in the context of class II. This could explain the failure of

some workers to observe a correlation between increased class II expression on stimulator B cells and T cell responses in the AMLR (Crowe and Kunkel, 1985; Duke-Cohan et al, 1987). Increased class II expression by antigen presenting cells may not correlate with increased idiotope expression.

Certain idiotypes are shared by T cells with specificity for a variety of exogenous antigens (Coutinho et al, 1987). These idiotypes are degenerate and may be recognised by a high frequency of AMLR reactive T cells. Such recognition would normally be under the control of regulatory suppressor T cells with specificity for these idiotopes. However, in JDM, the degenerate idiotope might possess sequence homology with an endothelial cell epitope. Sequestration of AMLR reactive T cells in perivascular sites might then occur. If these T cells were not subject to effective control by suppressor cells, they could contribute to or even initiate autoimmune disease.

(iv) **T-B Cell Cooperation - Role in Antibody Responses**

Defects in cooperation between T and B cells from JDM patients were observed in response to PWM (Ch 6.3.6). This was explicable in terms of cognate recognition of B and T cells in antibody production. However, B cell proliferation in response to PWM, also dependent on cognate recognition, was not defective. A division of human CD4+ influenza antigen specific T cell clones into helper and non-helper status has been reported

(Lamb et al, 1983). Recent experiments (G Cambridge, unpublished results) have shown that the CD4+ UCHL1+ subset is significantly reduced in peripheral blood of JDM patients. If the CD4+ UCHL1+ subset providing help for antibody production were selectively reduced, then defective PWM induced antibody responses could result. The precursor frequencies of CD4+ UCHL1+ cells could be determined for PWM induced proliferative and antibody responses in order to answer this question.

There have been no studies investigating antigen specific antibody responses in JDM. Although patients mount anti-CVB antibody responses, it is possible that the titres, specificities and/or affinities of antibodies produced may be abnormal, thus delaying clearance of the virus. Comparison of serum anti-CVB antibody responses by JDM patients and their siblings, at time of presentation, might provide evidence of abnormal responses to CVB.

(v) **Reduced frequency of LAK Cells Precursors - Role in JDM**

New evidence was obtained for cooperation between LAK cells and T cells in the induction of non-specific T cell cytotoxicity (Ch. 11.3.5). This form of cooperation was not evident in cells from patients. Although T cell mediated mechanisms of resistance to CVB infection may be unimportant in certain mouse strains (Estri and Huber, 1987), this may not be the case for humans. The combination of reduced frequency of LAK precursor cells and defective induction of non-

specific cytotoxic T cells may deprive the host of important measures of resistance against CVB, allowing the virus to localise in endothelial cells. Furthermore, LAK cells and non-specific cytotoxic T cells may have an immunoregulatory role, and defective function of these cells could contribute to the autoimmune response.

CHAPTER 13

FUTURE DEVELOPMENTS IN JDM

13.1 Future Research in JDM

(i) Autoreactive T Cells

At present, there is no understanding of the autoimmune process in JDM. This can only be achieved by the isolation and identification of the specificity of autoreactive T cells.

Detailed phenotypic analysis will reveal similarities or differences between infiltrating T cells in muscle and skin. I have postulated a similar immunopathogenesis for both organs and therefore would expect to observe similar proportions of each subset within the muscle and skin lesions. If this were not the case, it might be necessary to postulate distinct disease mechanisms for the 2 tissues.

It is expected that within the next year, MAb's to the known human TCR V gene families will become available. Comparison of TCR families in peripheral blood and inflammatory sites will allow the identification of probably clonal populations of infiltrating T cells. Identification of the specificities of these T cells will depend on progress in T cell cloning technology. Although normal endothelial cell membranes might be considered as sources of antigen for cloned T cells, it is possible (a) that the autoantigenic epitope is a cytoplasmic or nuclear component (b) that the epitope

is exposed or expressed during an initial antigenic insult such as viral infection or inflammation due to infection (c) that the epitope may be derived from connective tissue.

(ii) Lymphokines as Mediators of JDM

I have suggested that the effector stage of autoimmunity in JDM is not mediated by cytotoxic T cells. Organ specific autoantibodies have also not yet been detected in the disease. The possible contributions of individual and combinations of lymphokines to muscle damage can now be analysed using recombinant IL-1, IFN-γ and TNF-α, and other cytokines. It is well established that IL-1 can induce muscle proteolysis (reviewed by Oppenheim, 1986). Muscle damage usually radiates from inflammatory foci, suggesting the involvement of soluble factors. An assessment of the effects of cytokines in skin might be made using cultured fibroblasts and keratinocytes.

Transfection of the IFN-γ gene into pancreatic islet B cells in mice induced inflammation of the islet cells and autoantibodies to islet cell antigens (Pujol-Borel and Bottazzo, 1988).

Introduction of the IFN-γ gene into cultured endothelial cells could provide information of the response of cells to inflammation. Soluble factors secreted by endothelial cells, such as IL-1 and prostaglandins, could be measured and alterations in membrane and cytoplasmic antigens analysed.

(iii) Animal Model of JDM

Further progress in analysis of immunoregulatory defects in JDM may be dependent on the development of an animal model of the disease. Reduced NK cell activity and frequencies of LAK precursors in patients suggest that CVB infection of mice of different MHC backgrounds, expressing the beige mutation, could provide a disease model. If susceptibility to disease partially depended on H-2 haplotype, this would provide the required model to investigate an autoimmune disease.

13.2 Immunotherapeutic Intervention in JDM

Intervention in the aberrant immune system, by the use of immunosuppressive drugs, has proven beneficial in the treatment of JDM patients. New forms of immunotherapy might be designed (a) to deplete autoreactive T cells (b) alter T cell recirculation patterns (c) inhibit T cell effector function.

Depletion of autoreactive T cells could be achieved by thoracic duct drainage or by non-invasive techniques such as total lymphoid irradiation. However, bone marrow derived stem cells could subsequently undergo thymic processing into mature autoreactive T cells.

The identification of T cells receptors, such as LFA-1, binding to endothelial cell ligands such as ICAM-1, could provide a means of altering the circulation pattern of pathological T cells. Adhesion molecules are not normally capped from the cell surface and therefore, treatment with

antibodies to these molecules could provide a relatively long lasting means of preventing passage of T cells into inflammatory sites.

Infusion of patients with antibodies to T cell activation markers, such as IL-2 receptors, has been successfully employed to treat patients with autoimmune diseases (Kromer, Schaustein and Wick, 1986). Other methods of intervening in effector function might target directly on the TCR, thus providing a means of specific intervention.

Assuming the identification of TCR in muscle and skin lesions of JDM patients, treatment with anti-clonotypic antibodies, recognising V gene encoded segments of the TCR would be possible.

Identification of epitopes recognised by autoreactive T cells would allow the synthesis of peptide analogues. An analogue which competed for binding to class II with the native peptide, but which was non-stimulatory for T cells, would provide a useful therapeutic agent. Antibodies to molecules such ICAM-1 could be used to transport and target peptide analogues to sites of lymphocyte infiltration.

Finally, attempts might be made to tolerise patients to auto-antigenic epitopes. Tolerance might be achieved by administering the epitopes bound to autologous APC (Jenkins et al, 1987), perhaps with concomitant administration of anti-CD4 antibodies.

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A comparative study of *in vitro* proliferative responses to mitogens and immunoglobulin production in patients with inflammatory muscle disease

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ABSTRACT. *The classification of inflammatory muscle disorders is at best confusing owing to the wide spectrum of clinical, histological and serological findings within this group of patients. Although it is generally agreed that most cases reflect an autoimmune disorder with skeletal muscle as the primary target tissue, additional features of the more common form of the disease in juvenile patients, dermatomyositis, have suggested that it may differ in aetiology and pathogenesis from other forms of inflammatory muscle disease. Some adults may also be included within this category. In this study, we have divided patients with inflammatory muscle disease into two groups, 8 adult patients with 'pure' polymyositis and 13 children with dermatomyositis, and compared their* in vitro *lymphocyte function. Abnormal proliferative responses to T-cell mitogens were shown by peripheral blood mononuclear cells (PBM) from adult patients. PBM from the children gave values similar to normal control children.* In vitro *immunoglobulin production by cells from adult patients was comparable with appropriate controls both in the presence and absence of pokeweed mitogen. Childhood controls responded similarly to normal adults. PBM from the juvenile patients with dermatomyositis, however, produced significantly elevated levels of immunoglobulin spontaneously, with little or no increase for most patients following mitogen stimulation. This defective response was shown to be a consequence of activated T- and B-cells present in their peripheral blood and was not related to clinical parameters or drug therapy.*

Key words: inflammatory muscle disease, *in vitro* lymphocyte function.

Introduction

Dermatomyositis and polymyositis are defined as inflammatory myopathies of unknown aetiology. Muscle weakness, which is accompanied by a characteristic skin rash in dermatomyositis, is the primary feature (1). The condition in children, usually taking the form of dermatomyositis, has also been described as a 'systemic angiopathy', with a strong vasculitic component unique to the childhood disease (2). Gastrointestinal vasculitis (3) and marked restrictive decreases in the ventilatory capacity of the lungs (4), have provided evidence that the disease in children may involve organs other than muscle and skin. Immunohistochemical studies have shown that the distribution and composition of the inflammatory infiltrate within affected muscle in juvenile dermatomyositis (JDM) is usually perivascular, containing a high proportion of B cells whereas in adult patients with polymyositis, the infiltrate is usually more diffuse and endomysial, with activated T-cells being the most commonly seen cell type (5, 6).

Whilst it is thought that immune responses direct-

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ed against blood vessel and muscle components contribute to the pathogenesis of JDM, supporting evidence is lacking. Serum antibodies specific for these tissues or for the disease have not been described, although immunoglobulin and complement components are often found deposited in blood vessels within sites of muscle damage (7). Membrane attack complex has also been demonstrated on sarcolemmal and endothelial cell membranes (8). Immune complexes and anti-nuclear antibodies are present in sera from a proportion of both adults and children with inflammatory muscle disease, although reports differ as to the frequency with which they are found (9-12).

There have been very few studies of *in vitro* lymphocyte function in children with JDM. Natural killer cell activity has however, been described as defective (13). A small study of 5 untreated patients with JDM showed that the proliferative responses of their peripheral blood lymphocytes to mitogens and muscle antigen did not differ from that of normal individuals (14). A reduced proliferative response to PHA and autologous stimulation has been reported in adult patients with polymyositis (15, 16). The following comparative studies of both treated and untreated patients with PM and JDM were undertaken to determine whether there were any differences of *in vitro* lymphocyte function between the two groups of patients, compared with appropriate childhood and adult controls.

Materials and methods

Patients

All JDM patients were attending the Muscle Clinic at Hammersmith Hospital, under the care of one of us (VD). Adult patients with polymyositis, were attending the Rheumatology Outpatients Department at the Middlesex Hospital. Patients were diagnosed on the basis of Bohan and Peters' 1975 classification (1). The mean age of the 13 JDM patients studied was 11 years (range 3-19 years) and that of the 8 adults, 40 years. Of the adult patients tested, two were untreated, one was receiving azathioprine alone (125 mg/day) and one, cyclosporine A. The other four adult patients were receiving prednisolone (5-20 mgs/day) and three were also taking azathioprine (50 mgs/day). All except two of the children studied were receiving either alternate day or daily prednisolone at doses of less than 1 mg/kg and four were also receiving azathioprine. Control peripheral blood samples were supplied by normal healthy adults (age range 20-49 years). Of the 6 control children included, three had broken limbs and the other 3 were the normal healthy children of colleagues (age range 3-15 years).

Separation and purification of mononuclear cells

Peripheral blood samples were drawn into heparinised syringes and centrifuged over Ficoll/Hypaque (density 1.077 g/l). Mononuclear cells (PBM), were recovered from the interface and washed three times with HEPES buffered RPMI 1640 culture medium (CM). T-cell enriched (E+) and depleted (E—) populations were separated on the basis of rosetting with S-(2-Aminoethyl)isothiouronium bromide hydrobromide (AET) treated sheep red blood cells (AET-SRBC) after the method of Palacios (17). Both E+ and E— populations were washed twice with CM and resuspended in complete culture medium (CCM; CM supplemented with 2 mM 1-glutamine and 1 μg/ml erythromycin). The viability of all cell populations was determined by Acridine Orange uptake. The E+ populations contained greater than 90% CD3 + ve cells and the E-population, < 5% CD3 + ve cells.

Proliferative response to mitogens

Preliminary experiments showed that PBM from patients and controls did not differ in the kinetics of their proliferative response to mitogens. Predetermined optimal and sub-optimal concentrations of phytohaemagglutinin (PHA; Sigma Chemical Co.), pokeweed mitogen (PWM; Gibco Biocult UK), Concanavalin A (Con A; Sigma), and a monoclonal antibody recognising the CD3 antigen on human T-cells (18), (OKT3; Orthodiagnostics Ltd.), were used to stimulate triplicate cultures of patient and control PBM in flat bottomed microtitre plates. Culture was in CCM supplemented with 10% heat inactivated FCS (Myoclone; Gibco Biocult). The batch of FCS used had been previously screened and was not mitogenic for human cells. Briefly, 2×10^5 PBMC per well in a volume of 100 μl were cultured with the appropriate concentrations of mitogens added in a further 100 μl of culture medium. Proliferation was measured by the uptake of [methyl-^{3}H]thymidine (^{3}H-T; 3.7 kBq/well) added to the cultures for the final 18 hours of incubation. Incorporated ^{3}H-T was measured using standard liquid scintillation counting techniques.

In vitro *IgM production*

PBM were cultured at a concentration of 2×10^5/well either in culture medium alone or with a 1/100 dilution of PWM in a total volume of 200 μl for 8 days. Initial experiments showed that these conditions were optimal for IgM production. Spontaneous and PWM induced IgM production was measured in supernatants of patient and control PBM by an ELISA technique (19). Maximal production of IgM by patient and control cells was after 8 days of culture. Results are expressed in ng/ml of culture supernatant as determined by referral to a human IgM standard.

Effect of cytokines on in vitro *IgM production*

Recombinant human IL2 (rIL2) was kindly supplied by Genzyme Ltd and recombinant human gamma interferon (γIFN) by Boehringer Mannheim UK Ltd. MLR supernatants were from 3-day cultures of allogeneic lymphocyte combinations. Briefly, equal numbers (5×10^6) of PBM from two HLA mismatched normal donors and 2×10^6 K562 cells were co-cultured in CCM supplemented with 10% FCS. Supernatants from several experiments were pooled and stored in aliquots at —70°C until use. rIL2, γIFN and MLR supernatants were added at the initiation of appropriate experiments to PBM, at a concentration of 2×10^5 per microtitre well, either cultured alone or with an optimal concentration of PWM.

Autologous and allogenic combinations: effect on spontaneous and PWM induced IgM production

Equal numbers (1×10^5) of E+ and E— cells or 1×10^5 E-cells alone from normal donors or JDM patients were cultured in autologous and allogeneic combinations in CCM supplemented with 10% FCS in the presence or absence of an optimal concentration of PWM. Supernatants from triplicate wells were collected after 8 days incubation and tested for IgM content as described above.

Statistical analysis

Grouped data was compared using the Wilcoxon Rank Sum Test except when comparing results for patients and controls in the mitogen proliferation studies where the Student's T-test for unpaired samples was applied.

Table I. Proliferative response of PBM from normal adult and childhood controls and from patients with PM and IDM to mitogens added at optimal and sub-optimal concentrations.

		^{3}H-THYMIDINE INCORPORATION (cpm ± SD)			
		Control Adults (n = 11)	Control Children (n = 5)	JDM Patients (n = 13)	Adult PM Patients (n = 8)
MITOGEN					
PHA	(10 µg/ml)	51776 ± 42776	75209 ± 18901	67499 ± 57611	26664 ± 21651*
	(1 µg/ml)	ND**	ND**	ND**	8038 ± 5974
Con A	(10 µg/ml)	69079 ± 25072	43890 ± 40290	51138 ± 16499	24235 ± 30836*
	(1 µg/ml)	26432 ± 9103	25034 ± 10027	23341 ± 12005	9086 ± 11664*
OKT 3	(25 µg/ml)	33609 ± 17113	ND	26761 ± 17184	10682 ± 12351*
	(2.5 µg/ml)	5111 ± 2317	ND	4219 ± 2308	2438 ± 1423*
PWM	(1/100)	37945 ± 8312	29076 ± 13241	43100 ± 20369	ND
	(1/1000)	13769 ± 8312	ND	14969 ± 6326	ND
medium alone		100 ± 567	2036 ± 1949	2096 ± 3886	876 ± 415

* $p \leq 0.05$; Student's T-test. Significance determined by comparison with values given by appropriate age-matched normal control populations at the same mitogen concentration.
** ND not determined.

Results

Proliferative responses to mitogens

The mean level of proliferation to the T-cell mitogens PHA, Con A and OKT3 by PBM from 13 JDM patients did not differ significantly from that of cells from the normal adult or childhood control groups (Table I). PBM from 8 adult patients with PM did not show increased background levels of proliferation in the absence of mitogen. However, the mean proliferative response of their PBM to all three T-cell mitogens was lower ($p < 0.05$) than adult controls at both optimal and sub-optimal concentrations. Although not shown here, the mean decrease of proliferative response was due to a subgroup of 4 patients who gave significantly lower responses than the other 4. Although we compared patients in each subgroup in terms of their drug treatment, length of history, ambulatory status and CPK there appeared to be no correlation with reduced mitogen responses. The mean proliferative responses to PWM by PBM from juvenile patients did not differ from those of adult or childhood controls.

Spontaneous and PWM-induced IgM production

Spontaneous and PWM-induced IgM production by PBM from 9 normal adults, 11 patients with JDM, 6 normal children and 8 adults with PM are shown in fig. 1. PBM from the adult patients did not differ in either their spontaneous (mean ± SD; 65 ± 16 ng/ml) or PWM-induced (2022 ± 418 ng/ml) IgM production from that shown by cells from adult control individuals (50 ± 10 and 2183 ± 492 ng/ml respectively). The mean level of spontaneous IgM production by PBM from the juvenile patients (232 ± 148 ng/ml) was significantly higher than childhood controls (128 ± 187 ng/ml), ($p < 0.01$). Although it appeared that spontaneous IgM production by childhood controls was greater than that by cells from normal adults, the difference was attributable to 1 high value within this childhood group. Comparison of the means showed no significant difference. Only 3/11 of the JDM patients produced IgM levels within the normal range. The mean levels of PWM-induced IgM production by JDM patients (712 ± 560 ng/ml) were significantly less ($p < 0.01$) than that of appropriate age-matched controls (1828 ± 709 ng/ml). Further, in 7/11 patients, high

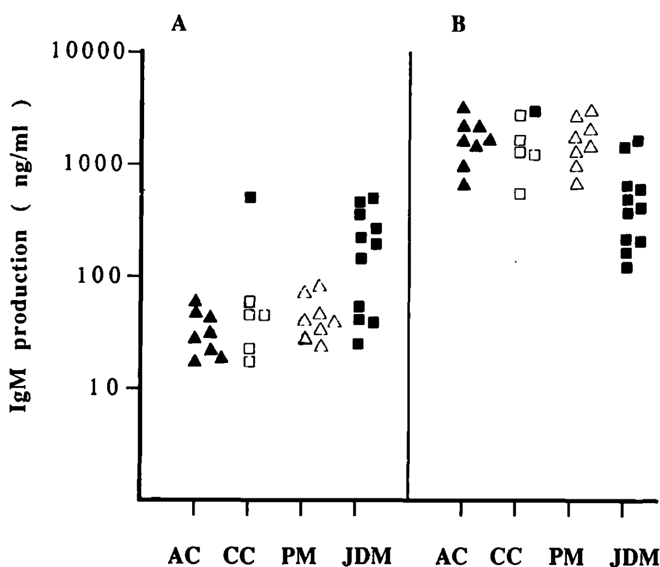


Fig. 1 - Spontaneous (panel A) and PWM-induced (panel B) IgM production by PBM from 8 normal adult controls (AC), 6 control children (CC), 11 patients with JDM and 8 adult PM patients. The closed square in CC panels indicate spontaneous and PWM response of the same normal child.

spontaneous immunoglobulin production was correlated with an inability to produce significantly increased amounts of IgM in response to PWM stimulation.

Effect of recombining autologous and allogeneic T and non-T cells on spontaneous and PWM-induced IgM production

The PWM-stimulated immunoglobulin response is not MHC restricted (20). Combinations of T and non-T cells from different individuals can therefore be used to investigate PWM-induced immunoglobulin production. In the experiments shown in Table II, E+ and non-T populations were obtained by fractionation of PBM from a total of 5 normal adults and 3 JDM patients. Allogeneic combinations of normal T and non-T cells did not differ significantly from their autologous combinations for PWM-induced or spontaneous IgM production. Of the 5 normal allogeneic combinations examined, 2 significantly enhanced and 1 suppressed Ig production (data not shown). However, when these results were combined (Table II), there was no overall difference between allo and autologous combinations for normal cells with respect to mean Ig production. When JDM E+ cells were co-cultured with normal non-T cells a) spontaneous IgM production was significantly enhanced compared with allogeneic combinations of cells from normal donors b) IgM production in response to PWM was significantly reduced compared with the autologous combination of normal cells cultured with PWM. When E+ cells from normal donors were co-cultured with JDM non-T cells spontaneous IgM production was significantly reduced

Table II. Spontaneous and PWM-induced IgM production in supernatants from autologous and allogenic combinations of equal numbers of T and non-T cells from normal adults (N) and JDM patients.

^{3}H-THYMIDINE INCORPORATION (cpm = SD)

a) *Autologous combinations*

E+	E—	n*	Spontaneous [a)]	PWM
N	N	3	43 ± 4	1694 ± 273
JDM	JDM	3	290 ± 65	297 ± 80
—	N**	3	< 10	ND
—	JDM**	3	54 ± 15	ND

b) *Allogeneic combinations*

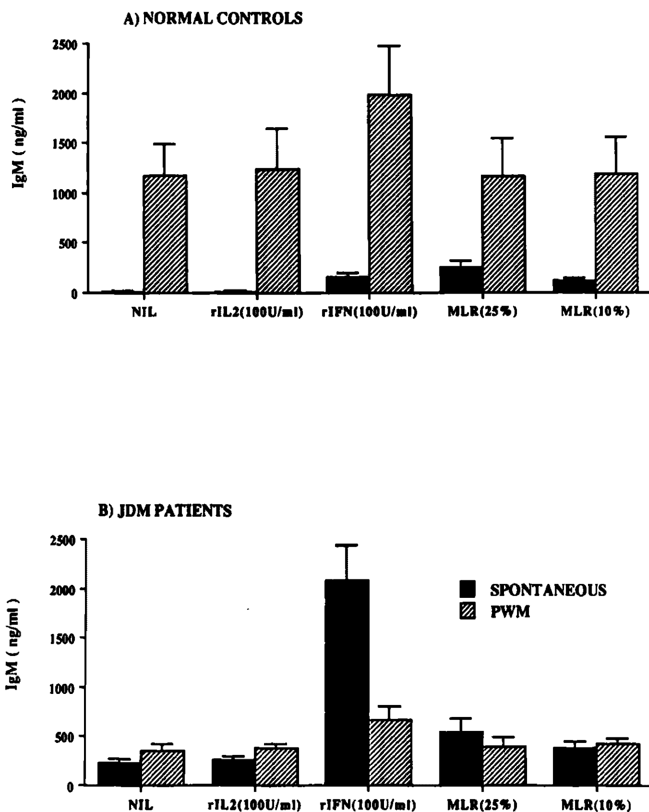
E+	E—	n	Spontaneous	PWM
N	N	5	40 ± 20	1770 ± 238
N	JDM	3	128 ± 11	1380 ± 275
JDM	N	4	144 ± 29	267 ± 122

* Number of combinations of 1×10^5 E+ and E— lymphocytes from patients or controls.
** 1×10^5 E— cells cultured alone.
a) The results are given as the mean ± SD IgM production of triplicate wells cultured in the presence or absence of PWM.
Significance levels for the various combinations of patient and control populations compared with appropriate autologous combinations are given in the Results section.

compared to combinations of autologous JDM cells. This combination also showed that IgM production in response to PWM was significantly enhanced compared with the autologous mixture of JDM cells. Significance levels were at the 1% level.

Effect of cytokines on spontaneous and PWM-induced IgM production

From the results presented in fig. 2A and B, it was apparent that JDM T-cells could not enhance IgM production by normal or patient E-cells in the presence of PWM. The following studies were conducted to determine whether the addition of cytokines to the cultures could correct this defect. Addition of γIFN and MLR supernatants to PBM from normal individuals and JDM patients resulted in a significant increase in spontaneous IgM production ($p < 0.01$). IFNγ also significantly enhanced IgM production from PWM-stimulated cultures of cells from both groups of subjects, although IgM production by JDM lymphocytes in response to γIFN was significantly greater than normal lymphocytes ($p < 0.01$). The addition of γIFN to



Figs. 2 A, B - Effect of the addition of rIL2, rIFNγ and MLR supernatants on *in vitro* spontaneous and PWM-induced IgM production by PBM from (a) 4 normal individuals and (b) from 4 JDM patients.

cultures of patient cells to which PWM had also been added increased IgM production but did not approach values shown by parallel control cultures both in the presence and absence of exogenous γIFN. MLR supernatants did not increase PWM-induced IgM production at the concentrations tested and rIL2 had no effect on either spontaneous or PWM-induced IgM production in either patients or controls.

Discussion

In this study of a series of patients with inflammatory muscle disease, we have described differences of *in vitro* lymphocyte function between adult PM patients and children with dermatomyositis, when compared with appropriate age-matched controls. Further, the nature of the defect differed between each patient group. The significantly lower proliferative response of PBM from adult PM patients to T-cell mitogens confirmed and extended the results of another study, in which the proliferative response to PHA was measured (15). Whether there are decreased frequencies of mitogen responsive cells in PBM from PM patients or inadequate production of, or responsiveness to, IL2 is currently under investigation.

In contrast, there was no defect in the ability of cells from JDM patients to undergo proliferation, presumably via the IL2 autocrine pathway, in response to stimulation with T-cell mitogens. Previous studies of a small group of JDM patients (14) reported similar results. Proliferation of cells from JDM patients following stimulation with PWM, was also not impaired. However, when spontaneous and PWM-induced IgM production was measured, marked differences in responses between the two patient groups were seen. PBM from most (8/11) JDM patients, but not from adult patients, produced elevated levels of immunoglobulin spontaneously *in vitro*. There were no apparent differences in drug treatment or length of history of disease between those patients whose cells produced IgM spontaneously and those who did not. Cells from one of the control children (a male aged 13 years with a fractured radius) also produced high levels of IgM spontaneously. Unlike the children with JDM however, he showed the expected increase in IgM production following PWM stimulation (Fig. 1).

As the mean IgM responses of normal children tested did not differ significantly from those of adult cells, we decided that cells from adult controls were suitable for further studies in which the effect of cytokines on the *in vitro* response and T-B cell interactions were studied. Two pieces of evidence suggested the presence of activated B cells in the peripheral blood of JDM patients. Firstly, patient E— cells spontaneously secreted 5 times more IgM than normal E— cells. Further, combinations of JDM E— and normal E+ cells spontaneously produced 3 times more IgM than normal allogeneic cocultures.

In experiments where control and patient T and non-T cells were mixed and IgM production measured, evidence was also found for the presence of activated T as well as B cells in PBL from JDM patients. The addition of JDM T-cells to autologous or allogeneic non-T cells resulted in significantly enhanced IgM production, suggesting that T-cells from these patients were providing the signals, presumably via the release of cytokines, necessary for spontaneous IgM production (Table II). Although recombining autologous T and non-T cells in a 1:1 ratio may result in enhanced IgM production *in vitro* (21), we found no differences between the amount of immunoglobulin in these supernatants from that of unseparated PBM in either patients or controls (data not shown). Allogeneic combinations of normal T and B cells may induce either augmented or reduced Ig production compared with autologous combinations (22, 23). Although there was considerable variation in the results shown by individual combinations of allogeneic cells, when these results were combined (Table II), there was no overall difference between allo and autologous combinations with respect to mean Ig production. In contrast, combinations of JDM E+ and normal E— cells *always* result-

ed in augmented IgM production. Additionally, in the experiments shown here, the mixing of normal E+ and JDM E— cells *always* resulted in significantly lower IgM production. We have concluded from these experiments that autologous and allogeneic effects made only a minor contribution to the results.

As shown in fig. 1, PWM stimulation of JDM cells did not result in the expected increase of *in vitro* immunoglobulin production, whereas PBM from adult patients gave values for spontaneous and PWM-induced IgM production similar to those of normal and age-matched controls. The addition of allogeneic T-cells could partially restore the response of patient B-cells to this mitogen. The PWM responsive B-cell populations from these patients were thus shown to be able to respond normally to T-cell signals and did not appear to be intrinsically defective. Another explanation could be that PBM from JDM patients contain a suppressor T-cell population, capable of inhibiting immunoglobulin production following PWM stimulation, which has been described in PBM from SLE patients (24). This 'suppressive' effect was unlikely to be due to allogeneic or PWM-induced suppression as all normal allogeneic combinations of E+ and E— cells (Table II) gave similar levels of IgM in response to PWM as the autologous combinations. This is in agreement with previous studies which have shown that allogeneic cells do not suppress the PWM response (25). Alternatively, it could be that the lack of a suitable helper population in JDM PBM is responsible for the defective PWM response. This population of cells is possibly analogous to a subpopulation of CD4+ ve lymphocytes which express the antigen recognised by the UCHL1 monoclonal antibody (26). We are currently investigating the functional activity of cells with this phenotype in JDM patients.

A variety of lymphokines produced by activated T-cells (for example IL4, IL2, BCGFII and IFNγ) could be responsible for causing the activation and differentiation of B-cells into immunoglobulin secreting cells (20). The ability of cytokines to reverse the defective PWM response of patient cells was therefore examined. Activated B-cells express low affinity IL2 receptors and, in the PWM response, IL2 has been claimed to be essential for the differentiation of normal B-cells to plasma cells (27). The inability of JDM T-cells to provide 'help' to B-cells in the presence of PWM was unlikely to be due to a failure of IL2 production, as the proliferative response of JDM cells to PWM was similar to that of normal cells (Table I). In addition, rIL2 added to the cultures failed to result in increased IgM production. The inability of IL2 to induce IgM production by B cells could have been due to excess T cells in the cultures which have been shown to inhibit IgM production by B cells in response to IL2 (28). MLR supernatants had no effect but γIFN doubled IgM secretion by JDM cells in response to PWM. JDM cells, however still produced significantly less IgM than cells from normal donors.

Activated T cells have been shown to produce γIFN (29) and our results showed that addition of γIFN to cultures greatly increased spontaneous IgM production by both normal and patient PBM (Figs. 2a and b). In contrast to PWM stimulated cultures, response was particularly enhanced for JDM cells. Purified normal human B cells do not produce IgM in response to IFN alone (20). Therefore the effects of γIFN are more likely to be mediated through interaction with monocytes or T-cells. γIFN has been shown to induce the release of IL1 from monocytes (30) and IL1 has also been implicated as a cofactor in B-cell differentiation (31). MLR supernatants, which reportedly contain IFNγ, as well as other factors capable of affecting B-cell activation (32), were found to enhance spontaneous IgM production in both groups of subjects ($p < 0.01$). The results suggested that γIFN could be one of the cytokines involved in stimulating IgM production from pre-activated subpopulations of B cells. The proportion of such cells appeared to be increased in JDM patients.

Although the finding of activated T- and B-cells in the peripheral blood of JDM patients is not surprising, it is perhaps of interest that such cells are not found in PBL from adult patients also suffering from what is thought to be an inflammatory muscle disease. Most of the patients included in these studies were receiving therapy and average length of history of disease was similar for both patient groups. Although not yet adequately investigated in adults, the possible involvement of a viral agent in JDM patients (32, 34) suggests that our observations reflect the immune response to a chronic infection of muscle. The understanding of the autoimmune state in inflammatory muscle disease involves a consideration of many factors including those of genetic predisposition, defects in immunoregulatory circuits and possible viral involvement. We have presented evidence of differences of *in vitro* immune function between adult PM and JDM patients, which supports previous smaller studies and provided initial evidence of hitherto undescribed immunoregulatory defects in peripheral blood lymphocyte populations from patients with JDM.

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trol children. We also wish to thank the children with JDM and their parents for their willingness to supply samples used in these studies.

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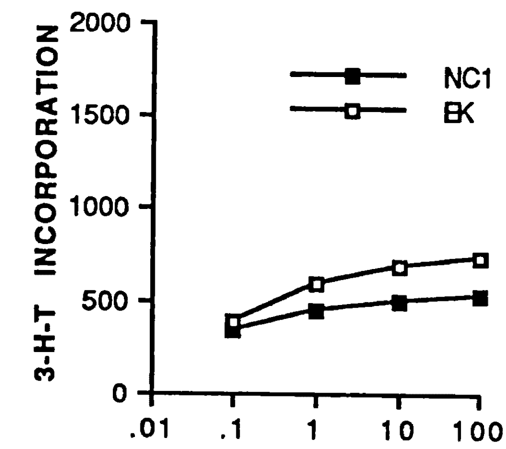
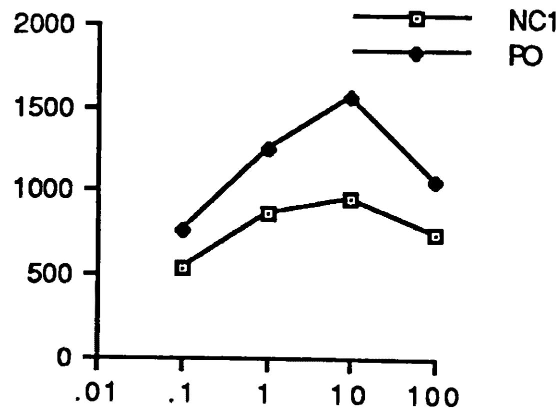
Proliferative responses of PBMC from JDM patients to purified human skeletal muscle membrane

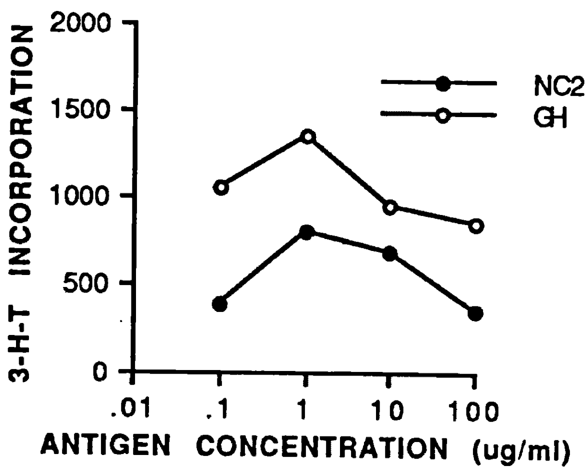
The proliferative responses of PBMC from 4 JDM patients to purified human skeletal muscle membrane were determined (Fig A). There was no significant difference between responses of patients and controls, although responses of patient cells were consistently slightly higher than controls.

Time course of proliferative responses of PBMC from JDM patients to purified human skeletal muscle membrane

Proliferative responses of PBMC from 2 patients and 2 controls to purified human skeletal muscle membrane were determined over a 5 day time course. Proliferation was measured from day 3 to day 7 of culture (Fig B). No significant difference in response of patients and controls was observed.







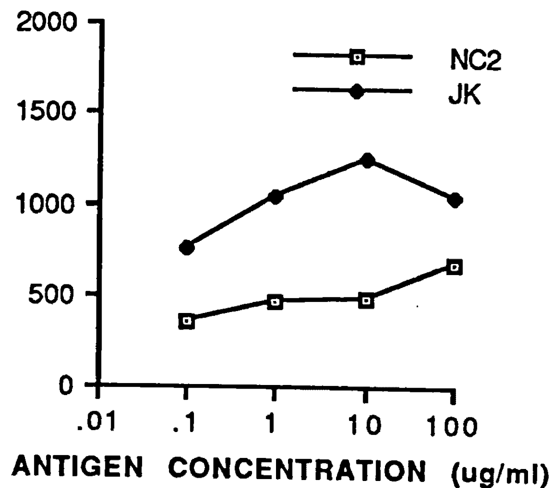


Fig A Proliferative responses of PBMC (2×10^5) from 4 JDM patients and childhood controls to purified normal human skeletal muscle membrane. Proliferative responses in the absence of antigen have been subtracted from the results. METHODS Skeletal muscle, obtained at surgery, was cut into small pieces, dissociated using a Buhler apparatus (3000rev/min) and mechanically homogenised. The muscle (100mg/ml in PBS) was then centrifuged (750g) to remove nuclear material and ultracentrifuged (50000g) to obtain the membrane pellet. The membrane pellet was recovered and homogenised in PBS(1mg/ml) containing Vitamin E as anti-oxidant. Purity of the preparation was assessed by scanning electron microscopy and enzyme analysis. Proliferation was measured after 7days culture.

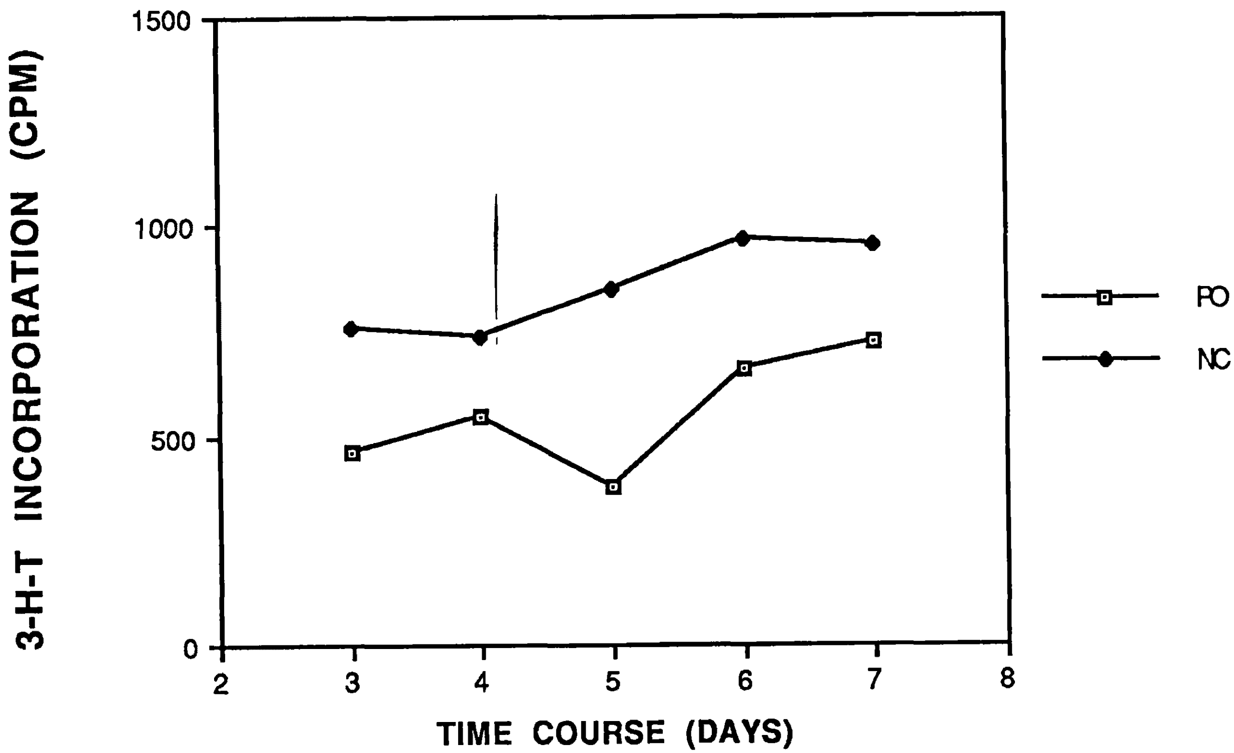


Fig B Time course of proliferative responses of PBMC from 2 JDM patients and 2 childhood controls to purified human skeletal muscle membrane. PBMC (2×10^5) were cultured for 3-7 days and proliferation measured