INTERACTIONS BETWEEN THE NEURAL, ENDOCRINE AND IMMUNE SYSTEMS DURING THE COURSE OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE).

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of London.

By

John Patrick Leonard

Department of Neurochemistry, Institute of Neurology, London.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

Interactions between the neural, endocrine and immune systems have been investigated during the course of experimental allergic encephalomyelitis (EAE), a cell-mediated autoimmune disease, induced by immunization with myelin basic protein (MBP) in complete Freund's adjuvant (CFA).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis and splenic sympathetic noradrenergic pathways was evident during the period of expected lymphoproliferation following immunization with MBP/CFA, or CFA alone, which coincided with an increase in lymphocyte ß-adrenergic receptor density. During the stressful clinical stage of EAE, further activation of neural and endocrine pathways was indicated by increased splenic noradrenaline (NA) and serum corticosterone (CS) levels.

Similar responses were observed when EAE was induced by the transfer of MBP-sensitized splenocytes, despite the absence of the primary immune challenge from the mycobacterial components of the adjuvant. Activation of both neural and endocrine pathways occurred prior to clinical signs. This is most likely a general response to cytokine production from stimulated cells, since comparable changes were seen in control animals following the transfer of cells sensitized to the antigenic component of the adjuvant.

The importance of intact noradrenergic pathways was demonstrated by the altered clinical severity of EAE following either the destruction of central or peripheral sympathetic nerve terminals, or the administration of centrally acting

sympathetic drugs. A significant inverse correlation was found between hypothalamic NA content and circulating CS, which was most evident during peak clinical signs of disease and suggests a role for central sympathetic pathways in the regulation of the HPA-axis during the course of EAE.

Consistent with the changes in circulating CS, increases in pituitary pro-opiomelanocortin mRNA were found during the pre-clinical and clinical stages of the disease. However the reduced levels of hypothalamic corticotropin-releasing factor mRNA at the time of maximum clinical signs, suggests that activation of the HPA-axis results from either vasopressin release or direct activation at the level of the pituitary.

The overriding immunoregulatory influence of CS was demonstrated by the rapid onset of EAE and morbidity in adrenalectomised animals. However, the compensatory increases in splenic NA and lymphocyte ß-adrenergic receptor density seen in these animals and the significant correlation between central NA and circulating CS, indicates that both central and peripheral sympathetic pathways may be activated following immune challenge and play a pivotal role in determining the outcome of disease.

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
Ad	adrenaline
BME	basal medium Eagle
cAMP	cyclic adenosine monophosphate
CFA	complete Freund's adjuvant
CNS	central nervous system
Con A	concanavalin A
cpm	counts per minute
CRF	corticotropin-releasing factor
CS	corticosterone
CYP	cyanopindolol
d.p.i.	days post immunization
d.p.t.	days post transfer
DEX	dexamethasone
DHA	dihydroalprenolol
dpm	disintegrations per minute
EAE	experimental allergic encephalomyelitis
FCS	foetal calf serum
GPSC	guinea pig spinal cord
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IL	interleukin
ir	immunoreactive
ISO	isoproterenol

LPS	lipopolysaccharide
M	molar
MBP	myelin basic protein
mRNA	messenger ribonucleic acid
NA	noradrenaline
nM	nanomolar
6-OHDA	6-hydroxydopamine
PBS	phosphate bufferd saline
pM	picomolar
PPD	purified protein derivative
PVN	paraventricular nucleus
S.I.	stimulation index
SNS	sympathetic nervous system
Thy	thymidine
μg	microgram
μl	microlitre
μM	micromolar

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Louise Cuzner, for her advice and encouragement throughout the course of this work. I am also grateful to; Dr Fiona MacKenzie, for her help in getting the project started; Dr George Sarna for his technical expertise in central drug administration and microdialysis; Dr Mick Harbuz at the Charring Cross Hospital, for performing the in situ hybridization and Gordon Ross and Elizabeth Newbould at St Bart's Hospital for HPLC analysis.

I am also extremely grateful to my fiancé, Nicki, for her constant support over the past few years and for typing the thesis and Dr Nicola Woodroofe and Jane Loughlin for proof-reading.

This study was funded by the Multiple Sclerosis Society, UK.

CHAPTER 1

Interactions between the neural, endocrine and immune systems.

1.1. Introduction.

It has long been recognised by clinicians and epidemiologists that the incidence and severity of various diseases are often linked to psychological factors such as stress. Over the past two decades researchers have begun to piece together the complex interactions which occur between the neuroendocrine and immune systems, that make up a bidirectional communication network. The discovery of cytokines, lymphokines and monokines, which exert effects on the central nervous system (CNS) has helped greatly in the understanding of how the immune system interacts with the brain. There are two pathways by which the nervous system is known to communicate with the immune system: the first is directly through activation of the pituitary-adrenal axis which appears to be under the control of the hypothalamus; the second is by the so called 'hard-wiring' of the immune system to the brain by the sympathetic nervous system (SNS).

1.1.1. Interactions between the pituitary and the immune system.

Most of the interactions between the immune and neuroendocrine systems have been attributed to glucocorticoid hormones, which are released from the adrenal cortex in

response to adrenalcorticotropic hormone (ACTH) produced by the pituitary. The release of ACTH is under the influence of the paraventricular nucleus (PVN) of the hypothalamus and is stimulated by the low molecular weight peptides vasopressin and oxytocin (mw, 1000), but primarily by the higher molecular weight 41-amino acid residue CRF peptide (CRF-41) (mw, 5000). Examination by light and electron microscopic immunohistochemistry has identified a group of parvocellular neurones in the PVN which synthesize both CRF-41 and vasopressin (Tramu et al, 1983; Kiss et al, 1984; Sawchenko et al, 1984). The axons of these cells project to the external zone of the median eminence and release CRF-41 and vasopressin into the hypophyseal portal circulation (Antoni, 1986) which results in pituitary ACTH secretion.

The end result of stimulation of the hypothalamic-pituitary-adrenal axis (HPA-axis) is the release of glucocorticoids which have widespread immunosuppressive properties including the inhibition of lymphocyte proliferation (Wahl et al, 1975), lymphokine secretion (Kelso et al, 1984), T-cell growth factor production (Gillis et al, 1979) and macrophage Ia expression (Snyder et al, 1982).

The immunosuppressive properties of glucocorticoids are counterbalanced by the immunoenhancing effects of prolactin. The secretion of prolactin is also controlled by the hypothalamus through complicated mechanisms involving at least 14 possible factors (McCann et al, 1984). The impairment of antibody-mediated, T-cell mediated and autoimmune reactions in hypophysectomised animals (Nagy and Berezi, 1978) can be restored by treatment with prolactin

(Nagy et al, 1983). Inhibition of prolactin secretion with bromocriptine also impairs contact sensitivity and antibody responses, T-cell proliferation to lectins, secretion of gamma-interferon, and tumoricidal responses by macrophages (Bernton et al, 1988; Nagy et al, 1983).

The opposing actions of ACTH and prolactin on immune function provides a yin-yang mechanism for fine tuning both the magnitude and duration of the immune response.

1.1.2. Production of neuroendocrine hormones by cells of the immune system.

It is now apparent that hormones which are classically associated with neuroendocrine pathways can also be synthesized by cells of the immune system. It was first noted that during the production of IFNα, human leukocytes also expressed an ACTH-like peptide (Blalock and Smith, 1980) which was similar to the pituitary peptide in both biological activity and molecular weight, as well as the retention time on reverse phase HPLC (Smith and Blalock, 1981; Smith et al, 1982; Blalock and Smith, 1985). The presence of pro-opiomelanocortin (POMC) mRNA in resting spleen macrophages (Westley et al, 1986) led to the discovery that newcastle disease virus infected lymphocytes produced immunoreactive (ir) endorphins (Smith and Blalock, 1981). It was later demonstrated that CRF and vasopressin synergistically elicited the de novo production of ACTH and ß-end from human leukocytes (Smith et al, 1986) which could be blocked by the synthetic glucocorticoid dexamethasone. More importantly, virus

infection in hypophysectomised mice resulted in increased circulating corticosterone levels and ACTH production in the spleen. Thus it would appear that a stress response can emanate from the immune system in the absence of the pituitary gland.

1.1.3. Neuroendocrine peptide hormone receptors in the immune system.

Binding studies have demonstrated both high (K_d, 0.11nM) and low (K_d, 4.8nM) affinity receptors for ACTH on mouse spleen cells (Johnson et al, 1982) similar to those found on rat adrenal cells (McIlhinney and Schulster, 1985). Antibody synthesis, B cell proliferation, T cell and macrophage function have all been shown to be modified by ACTH (Johnson et al, 1982; Brooks et al, 1984; Johnson et al, 1984) presumably through a direct action on these ACTH receptors. These studies suggest that ir-peptides produced by cells of the immune system do not have to act through stimulation of the HPA-axis.

Radiolabelled ligand studies have also demonstrated the presence of methionine-enkephalin (met-enk) receptors on mouse splenocytes (Johnson et al, 1982) with a single high affinity binding site of 0.6nM. ß-end binding sites on transformed human lymphocytes have also been reported (Hazum et al, 1979) however, these do not appear to be classic opiate receptors, since binding was not affected by known opiate agonists and antagonists.

The effect of endogenous opioids on immune function

is not clearly understood but appears to be both lymphocyte-subtype specific and variable depending on the peptides studied. ß-end, but not α-end or met-enk enhance the proliferative response of rat splenocytes to Con A and PHA (T-cell mitogens) but not to the ß-cell mitogen PWM (Gilman et al, 1982). This effect was not blocked by naloxone which once again suggests that ß-end is acting at a non-opiate specific receptor. However, ß-end significantly suppressed the proliferative response of human peripheral blood lymphocytes to Con A (McCain et al, 1982) which once again was not effected by naloxone. At present it is not known whether these conflicting effects are due to the different origins of the tissue or lymphocytes or to the concentration of peptide employed in the studies.

1.2. Effects of IL-1 and other cytokines on pituitary function.

One of the major approaches used to study the effects of cytokines on pituitary function is to administer cytokines individually to intact animals and subsequently measure plasma hormone levels. Even these apparently simple experiments have their drawbacks; firstly cytokines have been shown to modulate their own and related molecules production; IL-1 and tumour necrosis factor (TNF) are mutually stimulatory (Le and Vicek, 1987), whilst IL-1 stimulates its own production *in vivo* and in mononuclear cells *in vitro* (Dinarello et al, 1987). IL-1 also stimulates IL-6 and IL-2 which in turn stimulates TNF (Le

and Vicek, 1987; Dinarello, 1989; Mier et al, 1988). Thus it is not unexpected that changes in single cytokines following infection or inflammation are not observed, but what does result is a cascade of cytokine production from immune cells. Such factors should be taken into account when interpreting the in vivo effects of individual cytokines.

1.2.1. The effects of cytokines on ACTH release.

IL-1ß given by intraperitoneal (Ovadia et al, 1989; Berkenbosch et al, 1987; Suda et al, 1989) or intravenous (Katsuura et al, 1988; Uehra et al, 1987a; Uehra et al, 1987b; Naitoh et al, 1989) injection stimulates ACTH secretion. IL-1α has also been shown to have a similar effect in most (Suda et al, 1989; Katsuura et al, 1988; Morimoto et al, 1989; Sapaolsky et al, 1987) but not all studies (Uehra et al, 1987b). It has been suggested that the stimulatory effect on ACTH release is dependent upon fever induction which has been reported following IL-1 administration. However, studies using subpyrogenic doses of IL-1, non-pyrogenic analogs and indomethacin treated animals, have shown that this is not so (Besedovsky et al, 1986; Naitoh et al, 1989; Sapolsky et al, 1987).

The effects of cytokines on the release of other pituitary hormones have also been studied. In general cytokine administration has an inhibitory effect on thyroid stimulating hormone (TSH), species dependant effects on growth hormone (GH) and modest effects on prolactin and gonadotorphs (see table 1.1 for summary). Thus, data obtained from in vivo

Table 1.1.

Changes in serum levels of TSH, GH, prolactin, LH, and FSH following cytokine administration in vivo.

Hormone	Cytokine	Route	Species	Result
TSH	IL-1β	sc	Rat	↓
	IL-1	icv	Rat	↓
	TNFα	iv	Rat	↓
		icv	Rat	↓
	IFNγ	icv	Rat	↓
GH	IL-1β	icv	Rat	↓
		ip	Rat	—
	IL-1	icv	Rat	↑
	TNFα	iv	Human	—
		iv	Bovine	↓
Prolactin	IL-1β	iv,icv	Rat	—
		iv	Rat	—
		iv	Rat	—
	IL-1α	iv,icv	Rat	—
	IL-1	icv	Rat	↑
	TNFα	iv	Rat	—
		iv	Human	↑
	IFNγ	icv	Rat	—
LH	IL-1β	icv	Rat	↓
	IL-1α	icv	Rat	↓
		iv	Rat	—
		icv	Rat	↓
		iv	Rat	—
FSH	IL-1α	icv	Rat	—

Abbreviations: TSH, thyroid-stimulating hormone; GH, growth hormone; LH, leuteinizing hormone; FSH, follicle-stimulating hormone. Taken from Scarborough (Annals of the New York Academy of Science, (1988), Vol 512).

experiments clearly demonstrates that cytokines are able to modify pituitary hormone secretion.

1.2.2. Possible sites of action for cytokine stimulated ACTH release.

A direct central effect of circulating IL-1 at the level of the hypothalamus was at first thought unlikely since it is generally considered that IL-1 does not cross the blood brain barrier in significant amounts (Coceani et al, 1988), although access to the CNS may be achieved via the circumventricular organs (Katsuura and Koves, 1989). An important alternative possibility is that cytokines may be produced from within the hypothalamus or pituitary and thus act as intrinsic modulators of secretion. Such a theory is supported by the findings that IL-1 appears to be made by all major classes of glial cells (astrocytes, oligodendrocytes and microglia) given the appropriate conditions (Malipiero and Fontana, 1989; Merrill and Matsushima, 1988; Heter et al, 1988; Giulian et al, 1988). Immunohistochemical studies have identified IL-1 containing nerve fibres and cell bodies in the human and rat hypothalamus (Breder et al, 1988; Lechan et al, 1989) as well as the rat pituitary (Lechan et al, 1989). Here it has been proposed that IL-1 acts as an intrinsic neuronal protein and suggests a mechanism whereby peripheral signals can act on a signalling system intrinsic to the CNS. IL-1 receptors have been identified in rat brain and hypothalamus (Farrar et al, 1987; Katsuura et al, 1988b) however, it is not known whether the source of the IL-1 which acts at these sites

originates from central or peripheral production.

An important consequence of IL-1 administration, which may effect pituitary function is the alteration in electrical activity (Besedovsky et al, 1977; Besedovsky et al, 1983; Saphier, 1989) and increased NA turnover (Dunn, 1988; Kabiersch et al, 1988) within the hypothalamus. Changes in NA metabolism may be of particular interest since hypothalamic NA has been implicated in the control of CRF and vasopressin secretion (discussed later in this chapter).

The findings outlined above resulted in the hypothalamus being targeted as the possible mediator of IL-1 induced alterations of pituitary function. In a number of studies, prior treatment with antiserum to CRF blocked the ACTH reponse to IL-1 in vivo (Berkenbosch et al, 1987; Uehra et al, 1987; Sapolsky et al, 1987). Similarly, IL-1 has been shown to deplete CRF from the median eminence and increase hypothalamic CRF mRNA and portal blood CRF (Berkenbosch et al, 1987; Suda et al, 1990; Sapolsky et al, 1987). In vitro studies have also shown that IL-1 causes CRF release from rat hypothalami (Tsagarakis et al, 1989) and enhances the CRF response to NA (Widmaier, 1988). All in all there is compelling evidence that IL-1 stimulates hypothalamic CRF secretion.

A direct stimulatory effect of IL-1 at the level of the pituitary cannot be ruled out, although the data obtained from primary cultures of rat anterior pituitary cells or perfusion models have not as yet provided a clear cut answer with reports that both IL-1α and IL-1ß stimulate (Bernton et al, 1987; Beach et al, 1989) or have no stimulatory effect on

ACTH release (Berkenbosch et al, 1987; Tsagarakis et al, 1989; Boyle et al, 1988; Uehra et al, 1987). The conflicting *in vitro* results mean that at best, a direct stimulatory effect of IL-1 on the pituitary *in vivo* remains a distinct but unproven possibility.

1.2.3. IL-1 receptors and second messenger systems.

Work is only at the preliminary stage in trying to classify the IL-1 receptor and possible second messenger systems employed. IL-1α and ß appear to bind to the same class of receptors (Le and Vicek, 1987) although the possibility of two receptors with differing affinities does exist (Dinarello et al, 1989). A number of second messengers have been proposed for IL-1 including cyclic AMP (Shirakawa et al, 1989) and novel phospholipid pathways (Kester et al, 1989; Rosoff et al, 1988)

Prostaglandin mediated effects of IL-1 have also been suggested, based on the fact that prostaglandins have long been known to alter neuroendocrine secretion (Hedge and Hanson, 1972; Ojeda et al, 1980 and 1981). IL-1 has been shown to stimulate prostaglandin pathways in the hypothalamus (Dinarello and Bernheim, 1981) and recent *in vitro* studies have implicated astrocytes as a major source of IL-1 stimulated arachidonate metabolites (Katsuura et al, 1989; Hartung et al, 1989). Although in some cases ACTH responses to IL-1 have been blocked by indomethacin (Coceani et al, 1988; Morimoto et al, 1989; Katsuura et al, 1988) other studies have found no inhibitory effect (Sapolsky et al, 1987;

Bernton et al, 1987; Milenkovic et al, 1989).

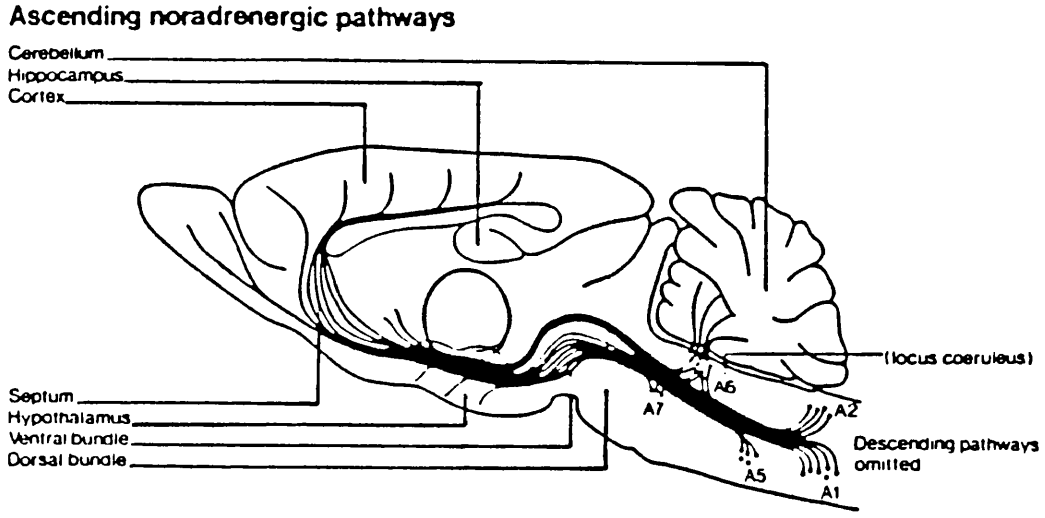
In summary, both in vivo and in vitro studies support the notion that soluble mediators such as IL-1 produced by cells of the immune system are able to alter neuroendocrine responses. The major site of action appears to be at the level of the hypothalamus although a direct effect on the pituitary cannot be ruled out. Such effects may be mediated by circulating IL-1, endogenously produced IL-1 or both. Although the nature of the receptor system and second messenger network employed have not yet been fully elucidated, it is apparent that communication between the immune and neuroendocrine systems is quite extensive and functionally significant.

1.2.4. Adrenergic control of corticotrophin secretion.

Evidence for the involvement of adrenergic neurones in the control of CRF secretion is based on the close anatomical relationship between the central adrenergic and noradrenergic systems and the major hypothalamic peptides that are involved in the regulation of ACTH secretion; CRF-41 and vasopressin. The cell bodies of the catecholaminergic neurones that inervate the parvocellular neurones of the PVN are found in the brain stem (Ungerstedt, 1971; Sawchenko and Swnason, 1982). The majority of neurones arise from the A1 and A2 cell groups (ventrolateral and dorsomedial medulla) with a few originating in the A6 cell group (locus coeruleus) (Fig 1.1). The axons ascend through the ventral noradrenergic

FIG 1.1.

Noradrenaline containing pathways in the rat brain.



The A_6 cell bodies in the locus coeruleus in the floor of the fourth ventricle project to the cortex. These axons travel in the dorsal bundle of fibres; some more caudal cell bodies also contribute to this system. The hypothalamus is innervated by fibres further back in the brain stem (A_1 and A_2) which also travel in the ventral bundle.

bundle (VNAB) and end in close proximity to CRF-41 and vasopressin containing neurones where direct synaptic contacts are apparent (Mezey et al, 1984; Agnati et al, 1985; Liposits et al, 1986a and b).

In addition to this direct innervation, a more distal influence has also been proposed at the external median eminence, where the CRF and catecholamine secreting nerve terminals involved are also in close proximity (Hokfelt et al, 1984). A direct effect at the level of the pituitary through α and ß-adrenergic receptors (Vale et al, 1983; Reisine et al, 1983; De Souza, 1985) following catecholamine secretion into portal vessels is another possibility, although it has not be conclusively shown that adrenaline and noradrenaline are secreted into hypophyseal plasma. It is apparent however, that adrenergic effects are not restricted to the parvocellular neurones in the PVN, which might account at least partly for the persisting controversy surrounding adrenergic control of corticotropic activity.

Early studies proposed that activation of central α-adrenergic receptors inhibited activation of the HPA-axis. In the dog, i.c.v. injection of NA suppressed adrenocortical activity (Van Loon et al, 1971; Ganong et al, 1976) whilst phentolamine, an α_1 and α_2 antagonist, enhanced adrenocortical activity in the rat which was interpreted as demonstrating a tonic inhibitory noradrenergic influence on the HPA-axis (Scapanini and Preziosi, 1973). Depletion of central catecholamine stores by peripheral or i.c.v. administration of catecholamine synthesis inhibitors supported these findings as they too were found to enhance adrenocortical activity

(Scapanini et al, 1970; Van Loon et al, 1971; Scapanini et al, 1972; Cuello et al, 1974; Mezey et al, 1984). An inhibitory action was further implicated since it was shown that i.c.v. administration of agonists and antagonists respectively inhibited and activated corticosterone secretion (Eisenberg, 1975) whilst CRF release from perfused hypothalami was inhibited by NA via α-receptors (Buckingham and Hedges, 1979; Jones et al, 1982).

More recent experimental results tend to favour the opposite theory and indicate that central catecholamines stimulate CRF secretion. Depletion of NA by direct injection of 6-OHDA into the VNAB resulted in a strong inhibition of irCRF-41 release in the PVN and external median eminence (Alonso et al, 1986), which was associated with a 90% reduction in hypophyseal portal CRF-41 (Guillaume et al, 1987; Szafarczyk et al, 1986) and thé obliteration of the circadian ACTH and corticosterone rhythms (Assenmacher et al, 1987).

In intact rats, an i.c.v. injection of NA or Ad increased CRF titers in portal vessels and the median eminence (Plotsky, 1986; Barbanel, 1987) and caused a concomitant rise in plasma ACTH. Pretreatment with α_1 or ß blockers or anti-CRF-41 serum, diminished the ACTH response to NA and Ad as well as that induced by ether stress (Plotsky et al, 1985; Szafarczyk et al, 1986 and 1987).

These studies suggest that central catecholamines play an important part in controlling both diurnal and stress induced modulation of the HPA axis. However, with conflicting results supporting both stimulation and inhibiton of the HPA-axis, it can only be concluded that the role of central

sympathetic pathways remains unresolved.

1.3. Interactions between the sympathetic nervous system (SNS) and the immune system.

Through its extensive innervation of many target tissues throughout the body, the SNS makes up the second major communication pathway between the brain and cells of the immune system. Sympathetic innervation of both primary (thymus and bone marrow) and secondary (spleen and lymph nodes) lymphoid organs has been consistently demonstrated (Galindo and Imaedo, 1962; Felten et al, 1981, 1984, 1985; Williams and Felten, 1981; Joseph and Felten, 1984). Neurochemical and pharmacological techniques have identified NA as the predominant catecholamine in mouse thymus, spleen (Williams and Felten, 1981; Williams et al, 1981) and lymph nodes (Felten et al, 1984). Dopamine and adrenaline are present but only in small amounts, however, studies suggest that Ad secreted from the adrenal medulla (Berecek and Brody, 1982) and serotonin derived from platelets can be taken up into sympathetic nerve terminals and released as classical neurotransmitters following appropriate neural stimulation. The concentration of NA in the spleen is higher than in the circulation, suggesting that it is released from local nerve endings (Felten et al, 1986). This is further supported by the extensive depletion of NA from lymphoid organs following 6-hydroxydopamine (6-OHDA) treatment, a drug that destroys noradrenergic nerve terminals (Jonsson et al, 1979)

SNS nerve endings penetrate the parenchyma of the

lymphoid organs where they make contact with monocytes, T cells and to a lesser extent, B cells (Felten et al, 1987). NA released from sympathetic nerve terminals can diffuse through the local environment (Brown, 1964) and act at a considerable distance from the site of release (Su and Bevan, 1970). Lymphocytes are known to possess adrenergic receptors (discussed below) therefore NA released from sympathetic nerve terminals may bind to receptors on lymphoid cells establishing them as 'mobile targets' innervated by the autonomic nervous system. In rodents, SNS innervation of the spleen develops post-natally, coincident with maturation of the immune system. Innervation is maintained until senescence and then declines (Felten et al, 1987).

1.3.1. Adrenergic receptors.

One criteria for neurotransmission by noradrenergic nerve fibres in lymphoid tissue is the presence of appropriate receptors for the neurotransmitter on target cells. Adrenergic receptors have been widely described on lymphocytes (Aarons and Molinoff, 1982; Bidart et al, 1983; Johnson and Gordon, 1980; Loveland et al, 1981; Miles et al, 1984; Staehelin et al, 1985), macrophages (Abrass et al, 1985; Nowell et al, 1981) and neutrophils (Galant et al, 1978; Davies and Lefkowitz, 1980).

Binding of ß-adrenergic agonists to the receptor stimulates adenylate cyclase activity which results in a rapid increase in cyclic AMP. A transient rise in cAMP is required to trigger the cells of the immune system to move from the

resting (GO) stage of the cell cycle into G1. However in later stages, elevated cAMP levels are associated with decreased cellular proliferation and increased differentiation (O'Dorisio et al, 1981; Sanders and Munson, 1985).

The density of ß-adrenergic receptors varies with cell type, with B cells possessing more receptors than monocytes and monocytes more than T-cells (Khan et al, 1986). In humans, T-cells of the suppressor subset ($CD8^+$, 9.3^-) have 3 times as many ß-adrenergic receptors as cells of the cytotoxic subset ($CD8^+$, 9.3^+) whilst $CD4^+$ cells (helper and effector cells of delayed type hypersensitivity responses) have very few (khan et al, 1986). Such variation in receptor density suggests that SNS control over T cell immune function might be mediated primarily through suppressor cell reponses.

Receptor density is not fixed, the number of receptors on T cells increases following exposure to mitogenic lectins and declines after ß-receptor agonist stimulation which is followed by a period of refractoriness to subsequent ß-adrenergic signalling (Davies and Lefkowitz, 1984; Molinoff and Aarons, 1983; Yu and Hui, 1988). Conversely, deprivation of the neurotransmitter in the target organ following SNS ablation results in an increase in ß-adrenergic receptors on B and T cells, a phenomenon known as denervation hypersensitivity (Miles et al, 1984). In humans with orthostatic hypotension, blood lymphocyte ß-adrenergic density is increased as a result of SNS destruction (Bannister et al, 1981, Hui and Connolly, 1981).

1.3.2. In vitro and In vivo effects of adrenergic agonists and antagonists on immune function.

Studies in which ß-adrenergic agonists were administered prior to or at the initiation of the immune response have produced conflicting data with both stimulatory and inhibitory effects being reported. (Cambell et al, 1985; Felten et al, 1987; Sanders and Manson, 1984a and b; Sanders and Munson, 1985).

During cell proliferation the effects of ß-adrenergic agonists are widespread and undoubtedly inhibitory. IL-2 production and IL-2 receptor expression are both suppressed (Chouaib et al, 1985; Feldman et al, 1987), T and B cell proliferation in response to lectins or IL-2 is inhibited (Beckner and Farrar, 1988; Chouaib et al, 1985; Diamantstein and Ulmer, 1975; Feldman et al, 1987; Hadden et al, 1970; Johnson et al, 1981; Vischer, 1976) as is lymphocyte mediated cytotoxicity (Beckner and Farrar, 1988; Garovay et al, 1975; Henney, 1973). Natural killer cell function is also depressed (Hellstrand et al, 1985, Kamat and Henney, 1976; Katz et al, 1982) whilst antibody secretion, B cell movement and macrophage phagocytic activity are all suppressed (Bourne et al, 1974, Makino and Reed, 1970; Teh and Paetkau, 1976; Shreiner and Unanue, 1975; Lappin and Whaley, 1982).

In general, the effects of ß-adrenergic antagonists have been found to oppose those of the agonists. In vivo administration of NA or isoproterenol results in a rapid release of splenic lymphocytes and granulocytes into the circulation (Ernstrom and Sandberg, 1973) which can be blocked

by prior administration of phentolamine and propranolol respectively, but was still evident in chemically sympathectomized animals suggesting that the agonists are acting directly on the lymphocytes. Preferential flushing of NK cells into the circulation following Ad infusion has also been reported (Crary et al, 1983).

Long term administration of ß-adrenergic agonists results in a down regulation of the receptor which may be responsible for the decline in therapeutic response to ß-agonists observed over time (tachyphylaxis). Alteration in immune function following administration of ß-adrenergic agonists and antagonists may be of great significance given the widespread use of such drugs in medicine.

1.3.3. Sympathetic nervous system ablation.

Most studies have used the neurotoxic drug 6-OHDA to destroy SNS nerve terminals. Administered to newborn animals 6-OHDA produces an almost total and permanent destruction of the SNS (axotomy), since in rodents, the axons of the SNS must contact their target organs during the first week or two after birth or they die. Administered at this stage, when the blood brain barrier (BBB) is incomplete, some of the drug may enter the brain and destroy central catecholaminergic neurons to a greater or lesser extent. Thus, the effects of 6-OHDA treatment in newborn rodents could be the consequence of a loss of SNS neurons, of central neurons, or of both.

6-OHDA administration to older animals also destroys

SNS neurons, however the effect is less complete and since the neuronal cell soma survives, new nerve endings form and function is restored after some weeks (sympathectomy). Because the BBB is now complete, there are no central effects and so alteration in immune function can be ascribed to a compromise of peripheral SNS function alone. The interpretation of experiments following adult sympathectomy are further complicated since immediately following 6-OHDA treatment, NA is released by the damaged nerve terminals which results in a surge of sympathetic overactivity rather than the under activity which is sought. It is also possible that functional recovery may be achieved long before either regrowth of axons, or restoration of catecholamine levels occurs.

Splenic B and T cell proliferative responses to LPS and Con A are increased following adult SNS ablation (Madden et al, 1988; Miles et al, 1981). It has been shown that more cells enter the cell cycle and that these cells move into the growth and DNA-synthesis stages sooner than lymphocytes from controls. (Miles, 1984). Antibody responses to T-cell independent antigens are also enhanced following either 6-OHDA treatment at birth or in adult rats (Miles et al, 1981) a finding that may reflect an over activity of B cells, more effective antigen presentation, an under activity of T suppressor cells, or any combination of the three.

Enhanced antibody responses to T-cell-dependent antigens following surgical sympathectomy have also been reported (Besedovsky et al, 1979). Neonates treated with 6-OHDA also show enhanced antibody responses to SRBC when immunized as adults an effect which was enhanced by α-methyl

tyrosine an inhibitor of catecholamine synthesis (William et al, 1981).

However, both primary and secondary spleen and lymph node responses to thymus-dependent antigens were suppressed immediately following 6-OHDA treatment (Felten et al, 1987; Kasakahra et al, 1977; Hall et al, 1982). This discrepancy may arise from the timing of the 6-OHDA administration and be a consequence of the immunosuppressive effects of the surge of NA from damaged axons. The suppression of the antibody response reported by Felten et al, (1987) was not however blocked by propranolol, a ß-adrenergic antagonist, which argues against such an explanation. The findings to date indicate that depletion of NA augments the antibody response and is greater for T-cell independent, than for T-cell dependent antigens.

SNS ablation at birth has also been shown to decrease the number of B cells and T suppressor cells in adult rats (Miles et al, 1985). When adult mice were axotomized, a reduction in the number of B cells was still apparent, but no alteration in T suppressor cell number was found. These results suggest that SNS innervation during early life is important for the maturation of suppressor cells, which would account for the augmentation of immune responses following SNS ablation.

1.3.4. Conclusions.

It has become increasingly apparent that an extensive

network of bidirectional communication exists between the neuroendocrine and immune systems. Pituitary peptides, which have diverse effects on immune function are produced from within the immune system itself. Furthermore, the neuroendocrine and immune systems share common receptors for these ir-peptides which are capable of altering immune function.

Activation of the hypothalamic-pituitary adrenal axis has been consistently shown after administration of cytokines both in vivo and in vitro, an effect which is thought to be mediated at the level of the hypothalamus, although a direct action on the pituitary is possible. The precise nature of the regulation of the HPA axis by central catecholamine systems has yet to be fully elucidated, however, most recent studies indicate a stimulatory role for both NA and Ad via α_1-adrenergic receptors. IL-1 has also been shown to alter hypothalamic catecholaminergic activity and stimulate the HPA-axis, although the source and site of action of this cytokine are still to be resolved.

The second major regulatory influence is provided by the direct innervation of lymphoid tissue via the sympathetic nervous system. It has been clearly demonstrated that lymphocytes and monocytes possess receptors for SNS transmitters and that immune function can be altered by either the administration of adrenergic agonists, or by the destruction of the sympathetic nerve terminals which innervate lymphoid tissue.

1.4. Experimental Allergic Encephalomyelitis (EAE).

1.4.1. Historical Background.

The occurrence of inflammatory demyelination after the injection of CNS material was first observed in humans treated with rabies vaccine derived from rabbit brain and attenuated rabies virus. (Kortischoner and Shweinberg, 1925). Repeated injections of CNS tissue into monkeys succeeded in inducing neurological signs and CNS lesions, which ruled out the possibility that the attenuated virus was responsible for the observed encephalomyelitis (Rivers et al, 1933 and 1935). However, it was not until it was shown that Freund's mycobacterial adjuvant enhanced the encephalitogenicity of the CNS tissue to such an extent that EAE could be induced experimentally by a single injection, that exploitation of this experimental approach to study demyelinating disease became feasible (Kabot et al, 1946; Morgan, 1946).

1.4.2 Animal models of EAE and induction methods.

EAE has since been induced in mice, rats, guinea pigs, rabbits and monkeys (Paterson, 1959; Paterson, 1966) and may exist in a number of distinct forms, dependant not only upon the species and strain of the animal, but also on the manner of induction.

Spinal cord is usually employed to induce EAE since it is more encephalitogenic than brain, presumably because of

its higher concentration of myelin, in which the encephalitogenic antigen is present (Paterson, 1976). It was shown that this antigen resided in the acid extract of delipidated white matter (Kies and Alvord, 1959) with further purification indicating that the immunogenic activity was localized to myelin basic protein (MBP) (Laatsch et al, 1962). Injection of animals with guinea pig spinal cord (GPSC) or guinea pig MBP (GP-MBP) without Freund's adjuvant rarely results in fully established EAE. Although extensive studies have been carried out, the precise manner in which adjuvant potentiates immunological reactivity is unknown. However, adjuvant has been shown to initiate the proliferation of phagocytic, or macrophage-type cells (Paterson and Bell, 1962) which leads to a marked potentiation by the adjuvant, of the immune response to the administered antigen.

For successful induction of disease, the inoculum needs to be injected into sites in close proximity to draining lymph modes, two major sites being the neck and footpads (Paterson, 1966). Removal of the draining lymph node, or local irradiation of the lymph channels draining the inoculation sites, suppresses the development of EAE in rats (Condie and Good, 1959).

Microscopic examination of CNS lesions reveals focal areas of vascular-perivascular inflammation with varying degrees of demyelination (Paterson, 1966). Lesions are most often found in the subcortical white matter and long tracts of the spinal cord. (Matsumoto and Fujiwara, 1987; Lublin, 1984). The cellular infiltrates consist predominantly of lymphocytes and macrophages which form concentric layers in the

perivascular space forming a cuff (Raine, 1976; Paterson, 1976).

1.4.3. Immunological aspects of EAE.

Early indications that EAE is a cell-mediated immunological disease came from the discovery of delayed-type hypersensitivity reaction to CNS tissue in animals developing EAE (Freund et al, 1947). A direct relationship was observed between the occurrence and intensity of the cutaneous reaction and the production and severity of EAE in the sensitized animals. Delayed type hypersensitivity reactions as classified by Coombs and Gell (1963) take more than 12 hours to develop and cannot be transferred from one animal to another by serum, but can be transferred by T lymphcytes.

Subsequently, Paterson (1960), Stone (1961), and Astrom and Waksman (1962) successfully transferred EAE to naive animals with sensitized lymphoid cells. An attempt to transfer disease with immune serum was unsuccessful (Lisac et al, 1970).

Gonatas and Howard (1974) demonstrated that rats depleted of T-cells, did not develop EAE when inoculated with MBP/CFA. Similarly, T-cell-free lymph node cell preparations transferred to thymectomised, irradiated recipients failed to produce EAE. (Ortiz-Ortiz et al, 1976). Antibodies to the sensitising inoculum were detected in the sera of the recipients, however in the absence of any clinical or histological signs of disease, such a finding only lends further support to the belief that EAE evolves from a cell-

mediated delayed-type hypersensitivity response to CNS antigen.

1.4.4. The role of the adrenals in EAE.

The importance of adrenal steroids in the recovery from EAE was first demonstrated by the high mortality rate in adrenalectomized animals subsequently immunized with CNS/CFA inoculum (Levine et al, 1962; Levine and Wenk, 1965; Levine and Sowinski, 1975) compared to intact rats. The first direct evidence for adrenal activity during EAE came from the discovery of slightly elevated levels of 11-hydroxycorticosteroids at certain times in rats and dogs (Hughes et al, 1966).

Further studies showing the suppression of EAE following administration of exogenous corticosteroids, (Gammon et al, 1953; Greig et al, 1970; Vogel et al, 1972; Elliot et al, 1973) supported a central role for adrenal activation during the course of the disease. Endogenous steroids probably inhibit both the efferent and afferent arms of the immune system since they are equally effective at suppressing adoptively transferred EAE (Levine and Strobel, 1969). Increased corticosterone levels were later found to accompany the severe clinical signs of disease (Levine et al, 1980). Adrenalectomy performed early in the remission of EAE was followed promptly by the disappearance of serum corticosterone and a relapse of clinical signs.

The resistance of the PVG strain of rats to EAE has recently been attributed to adrenal hyperactivity. PVG rats

were found to have higher basal CS levels and a more vigorous steroid response to stress compared to Lewis rats. Furthermore, PVG rats adrenalectomized 8 days after immunization with MBP/CFA, developed severe disease from which they did not recover (Mason et al, 1990). Studies of susceptibility of different rat strains to streptococcal cell wall induced arthritis have also concluded that the neuroendocrine regulation of the immune response in resistant strains is enhanced. (Sternberg et al, 1989). Thus the information to date points to a pivotal role for adrenal activity in the recovery from EAE and in some instances, the susceptibility to disease.

1.4.5 EAE as a model for studying interactions between the neuroendocrine and immune systems.

The fact that EAE is a cell-mediated autoimmune disease which affects the central nervous system makes it an ideal candidate for investigating neuroendocrine-immune interections.The ability to transfer EAE with sensitized splenocytes, representing the effector stage of disease, makes it possible to differentiate between the efferent and afferent arms of the immune response, whilst the injection of adjuvant alone, which produces a potent but non-specific immune response, serves as an appropriate control.

The rich sympathetic innervation of the lymphoid tissue and the presence of ß-adrenergic receptors on lymphocytes, the effector cells of EAE, provides a direct link

through which the SNS may influence the progression of the immune reaction. The production of cytokines during the pre-clinical period where sensitization and proliferation to MBP occurs, and the stress of paralysis during peak clinical signs, may both feedback onto central sympathetic pathways involved in the regulation of the HPA-axis. Due to the nature of the disease itself it is possible that all of the pathways outlined above which make up the complex network of bidirectional communication between the neuroendocrine and immune systems are involved, one way or another, in determining the outcome of the immune response and hence the severity of disease.

CHAPTER 2

Lymphocyte ß-adrenergic and glucocorticoid receptor assays.

2.1. Introduction.

2.1.1 ß-adrenergic receptor binding

Ligand binding to the ß-adrenergic receptor triggers a wide variety of biochemical and physiological reponses. The presence of ß-adrenergic receptors on circulating leukocytes was first indicated by increased adenylate cyclase activity resulting in cyclic adenosine monophosphate (cAMP) production in response to noradrenaline (Bourne et al 1971). More recently ß-adrenergic receptors have been demonstrated on human and murine lymphocytes by direct binding studies using radiolabelled compounds (Williams et al 1976, Johnson et al 1980).

The receptor-adenylate cyclase complex consists of three components: the receptor, G proteins and the catalytic subunit. The G proteins are a family of membrane associated, guanine nucleotide-binding proteins that transduce signals from the receptor to the effector enzyme and thus regulate the turnover of cAMP. Binding of an agonist (noradrenaline, adrenaline or isoproterenol) to the receptor transmits a signal via the G proteins to the catalytic subunit resulting in cAMP production, activation of protein kinase A and phosphorylation of substrates. The amount of cAMP produced following activation of ß-adrenergic receptors and hence the extent of the physiological response, is dependant upon the potency of the agonist binding (isoproterenol > adrenaline >

noradrenaline) and the number of receptors present on the cell. Receptor numbers vary considerably and often reflect the availability of their corresponding transmitters. Continuous administration of ß-adrenergic agonists leads to a down-regulation of receptors and a decline in cAMP production, whilst the reduced circulating noradrenaline levels found in pure autonomic failure, is reflected by an increase in ß-adrenergic receptors on peripheral lymphocytes.

During the early stages of an immune response ß-adrenergic agonists have an inhibitory effect, blocking IL-2 production as well as IL-2 receptor expression on lymphocytes and suppressing the lectin-induced proliferation of T and B cells. Binding studies used to determine specific adrenergic receptor populations might thus reflect changes in sympathetic activity or immune cell activation during the course of experimental allergic encephalomyelitis (EAE).

A wide variability has been reported on leukocyte ß-adrenergic receptor density from control subjects which has been attributed to differences in experimental technique and choice of radioligand (Marinetti et al, 1983). For this reason the aim was to develop a simple and reproducible radioligand binding assay which could be used to determine whether or not lymphocyte ß-adrenergic receptor number was altered during the course of EAE.

2.1.2 Glucocorticoid receptor binding.

Early findings that glucocorticoids at physiological concentrations could reduce the viability (Schrek, 1949), and

inhibit metabolic and mitotic responses (Rauch et al, 1961, Morita et al 1964; Nowell, 1961) of isolated lymphocytes led to the discovery of glucocorticoid receptors (Munk et al, 1967; Schaumburg, 1968). Glucocorticoids have since been implicated in the regulation of a number of immune cell functions including inhibition of lymphocyte proliferation (Wahl et al, 1975), lymphokine secretion (Kelso et al 1984), T cell growth factor production (Gillis et al 1979), and macrophage Ia expression (Synder et al 1982).

Unlike ß-adrenergic receptor-mediated responses which act through the second messenger cAMP, steroid hormones exert their effects directly on the DNA of the target cell. This is brought about by binding of the hormone to a cytoplasmic receptor which rapidly translocates to the nucleus. Translocation is thought to result from conformational changes in the glucocorticoid receptor which take place after binding of the hormone. The rate-limiting step for translocation appears to be the critical temperature-sensitive 'activation' in which the complex acquires high affinity for nuclear sites.

Alterations in circulating corticosterone levels and in glucocorticoid receptor number and affinity may be expected to influence the clinical course of EAE. Transcriptional control of glucocorticoid-sensitive genes and any consequent physiological response to the hormone is mediated by the nuclear glucocorticoid-receptor complex. Thus a nuclear binding site assay was employed.

2.2 Materials and methods.

2.2.1 Isolation of lymphocyte enriched splenocytes.

Female Lewis rats (Bantin and Kingman) were killed by decapitation. The spleens were removed and dissociated through a wire mesh in Hanks Basal Medium Eagle (BME, Gibco UK), washed and spun at 1200 rpm for 5 minutes and resuspended in BME-Earles containing 10% foetal calf serum (FCS, Flow Lab UK) 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco UK). After macrophage depletion on plastic for 1h at 37^{0}C (at a cell concentration of 5 x 10^6/ml) the remaining lymphocyte-enriched population was washed twice with phosphate buffered saline (PBS), pH 7.3 and used for receptor binding studies.

2.2.2 Binding characteristics of (^{3}H)-Dihydroalprenalol to intact lymphocytes.

Splenic lymphocytes were washed with PBS and resuspended at a concentration of 4 x 10^6/ml. The ligand binding assay was carried out in triplicate in round bottom 96 well plates using the ß-adrenergic antagonist ^{3}H-dihydroalprenalol (^{3}H-DHA), specific activity 95 ci/mMol (NEN). Total binding was determined by incubating 200μl aliquots of the cell suspension with increasing concentrations of the ligand. Two different ranges of ligand were used (0.1-30nM and 0.1-12nM). The assay was carried out at 25^{0}C for 30 mins. Non-specific binding was determined over the same concentration ranges of ^{3}H-DHA in the presence of 1μM (-)-

propranolol. All incubations included 1μM phentolamine to exclude any binding to α-adrenergic receptors. After incubation, the cells were harvested onto glass fibre filter mats (Skatron) using a Titertek cell harvester. The mats were dried and dpm counted in toluene (Scintran, BDH) containing 5g/L 2,5-diphenyloxazole (PPO) on a Searle Mark III liquid scintillation counter. Binding kinetics were determined by Scatchard analysis using an EBDA/Ligand computer program (Munson and Rodbard, 1980).

$$\text{Sites/cell} = \frac{\text{Bmax (M)} \times (6.023 \times 10^{23})}{\text{no. of cells/L}}$$

2.2.3 Kinetics for ^{3}H-DHA binding.

The time course of binding was determined by incubating 4 x 10^{6} cells with 10nM ^{3}H-DHA in the absence and presence of 1μM (-)-propanolol. At various times after the addition of the ^{3}H-DHA the cells were harvested and specific binding determined and expressed as disintegrations per minute (dpm).

2.2.4 Competition for ^{3}H-DHA binding by (-) and (+) propranolol and phentolamine.

Splenic lymphocytes (4 x 10^{6}/ml) were incubated for 30 min at 25^{0}C with 10nM ^{3}H-DHA in the presence of increasing concentrations of unlabelled competitors. Each concentration was carried out in triplicate and the amount of bound ^{3}H-DHA

expressed in dpm.

2.2.5 Binding of ^{3}H-DHA to isolated monocytes and red blood cells (r.b.c.).

The spleen was dissociated and the cells resuspended at a concentration of 10 x 10^{6}/ml and layered over Percoll (density = 1.077g/ml). The tubes were then spun for 20min at 1500 rpm. Isolated monocytes were washed in BME-Earles and macrophages depleted on plastic for 1 hour. The r.b.c. were washed with PBS and resuspended at a concentration of 10 x 10^{6}/ml (approximate concentration found in the assay). Binding assays for both cell populations were carried out as described above using ^{3}H-DHA over a concentration range of 0.1-12nM.

2.2.6 Binding of ^{125}I-cyanopindolol (^{125}I-CYP) to splenic lymphocytes.

In later experiments ^{125}I-CYP was used to determine ß-adrenergic density. This ligand provided two advantages over ^{3}H-DHA: firstly it is a more selective ligand, binding to ß-adrenergic receptors only, and secondly, as it is an iodinated ligand, scintilation counting in toluene is not required. The binding assay was similar to that used for ^{3}H-DHA except phentolamine was omitted from the PBS. ^{125}I-CYP was used over a concentration range of 0.1-300 pM. The assay was carried out in 96 well round bottom plates in triplicate and incubated at 30^{0}C for 90 mins. The cells were harvested onto glass

fibre filter mats and the discs counted in a mini gamma counter (LKB) at 80% efficiency. Scatchard analysis was performed using the EBDA/Ligand computer program.

2.2.7 Kinetics for ^{125}I-CYP binding

The time course for binding was determined by incubating 4 x 10^6 cells with 25pM ^{125}I-CYP in the absence and presence of 1μM (-)-propranolol. Specific binding was determined in triplicate at various times after the addition of the ^{125}I-CYP and expressed as cpm.

2.2.8 Competition for ^{125}I-CYP binding with (-) and (+) propranolol, and phentolamine

Splenic lymphocytes (4 x 10^6/ml) were incubated for 90min at 30^{0}C with 25pM ^{125}I-CYP in the presence of increasing concentrations of the unlabelled competitors. Each concentration was carried out in triplicate and the amount of ^{125}I-CYP bound expressed as counts per minute (cpm).

2.2.9 Binding of ^{3}H-dexamethasone (^{3}H-Dex) to intact splenic lymphocytes.

Splenic lymphocytes were isolated as described above. After depletion of macrophages on plastic for 1 hour the cells were washed with PBS and resuspended at a concentration of 50 x 10^6/ml. The assay was carried out in 1.5ml conical polypropylene centrifuge tubes at 37^{0}C for 30 mins. 50μl of

the cell suspension was incubated with ^{3}H-Dex (Amersham, specific activity 95.7 ci/mMol) at eight concentrations ranging from 0.1-30nM. Non-specific binding was determined over the same concentration range of ^{3}H-Dex in the presence of 2μM unlabelled dexamethasone. At the end of the incubation period the cells were lysed by a 30 fold dilution in ice-cold $MgCl_2$ (1.5mM) followed by vigorous mixing for 15sec and incubation on ice for a further 15 mins. The nuclei were then pelleted at 12,000 x g and the supernatant aspirated. The tips containing the pellet were cut off and placed in toluene and the dpm counted in a scintillation counter. Binding kinetics were determined by EBDA/Ligand analysis.

2.2.10 Cytoplasmic to nuclear translocation of the ^{3}H-Dex receptor complex.

Cell suspensions (50 x 10^{6}/ml) were incubated with 20nM ^{3}H-Dex with and without 2μM unlabelled dexamethasone at 3^{0}C for 180 min. Aliquots were removed and the amount of ^{3}H-DEX bound to nuclear sites expressed as dpm. The remaining cells were then transferred to a water bath at 37^{0}C and nuclear binding determined after a further 30 mins. Binding to nuclear receptors at 3 and 37^{0}C was carried out in triplicate.

2.2.11. ^{3}H-Dex binding to mitogen stimulated lymphocytes.

Splenic lymphocytes (2 x 10^{6}/ml, 40ml) were incubated in BME-Earles with 10% FCS, 100 U/ml penicillin and 100μg/ml

streptomycin. At time 0, 1μg/ml Concanavalin A (Con A, Sigma) was added to the flasks and incubated at 37°C. 200μl aliquots of the cells with and without Con A were transferred to round bottom 96 well plates and incubated at 37°C to determine lymphoproliferation rates. ^{3}H-Dex binding assays were carried out at 0, 48 and 72 hours after the addition of Con A. Proliferation at these time points was determined by pulsing the 96-well plates with 1μci ^{3}H-Thymidine. The plates were then incubated for 6 hours at 37°C and harvested onto glass fibre filter mats. Incorporation of the ^{3}H-Thy was determined by counting the dpm from the cells with and without Con A.

2.3 Results.

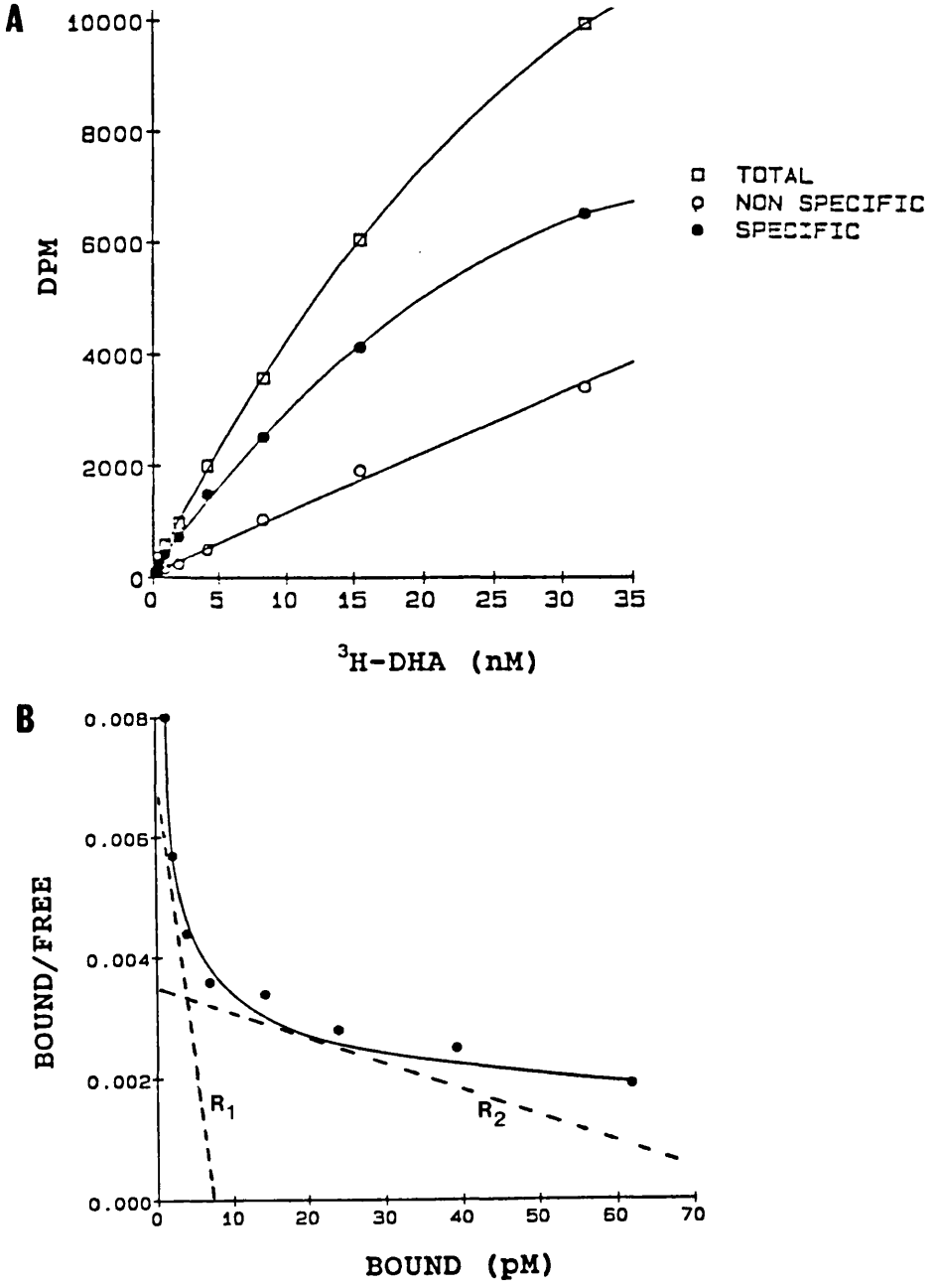
2.3.1 ^{3}H-DHA binding to intact splenic lymphocytes.

Lymphocyte viability prior to the ß-adrenergic receptor assay was greater than 85% as shown by eosin dye exclusion. Specific binding of ^{3}H-DHA over a range of 0.1-35nM did not appear to reach saturation (fig 2.3.1.). When the data was transformed by Scatchard analysis, a curvilinear plot was produced indicating two apparent receptor populations, one with a high affinity (R_1, K_d=3nM) and a low number of binding sites (approximately 3,000 sites/cell) and one with a low affinity (R_2, K_d=45nM) but with a much larger receptor population (approximately 35,000 sites/cell).

When ^{3}H-DHA was used over a lower concentration range (0.1-12nM) Scatchard analysis revealed a linear plot indicating a single receptor population with a K_d of 7nM and

FIG 2.3.1.

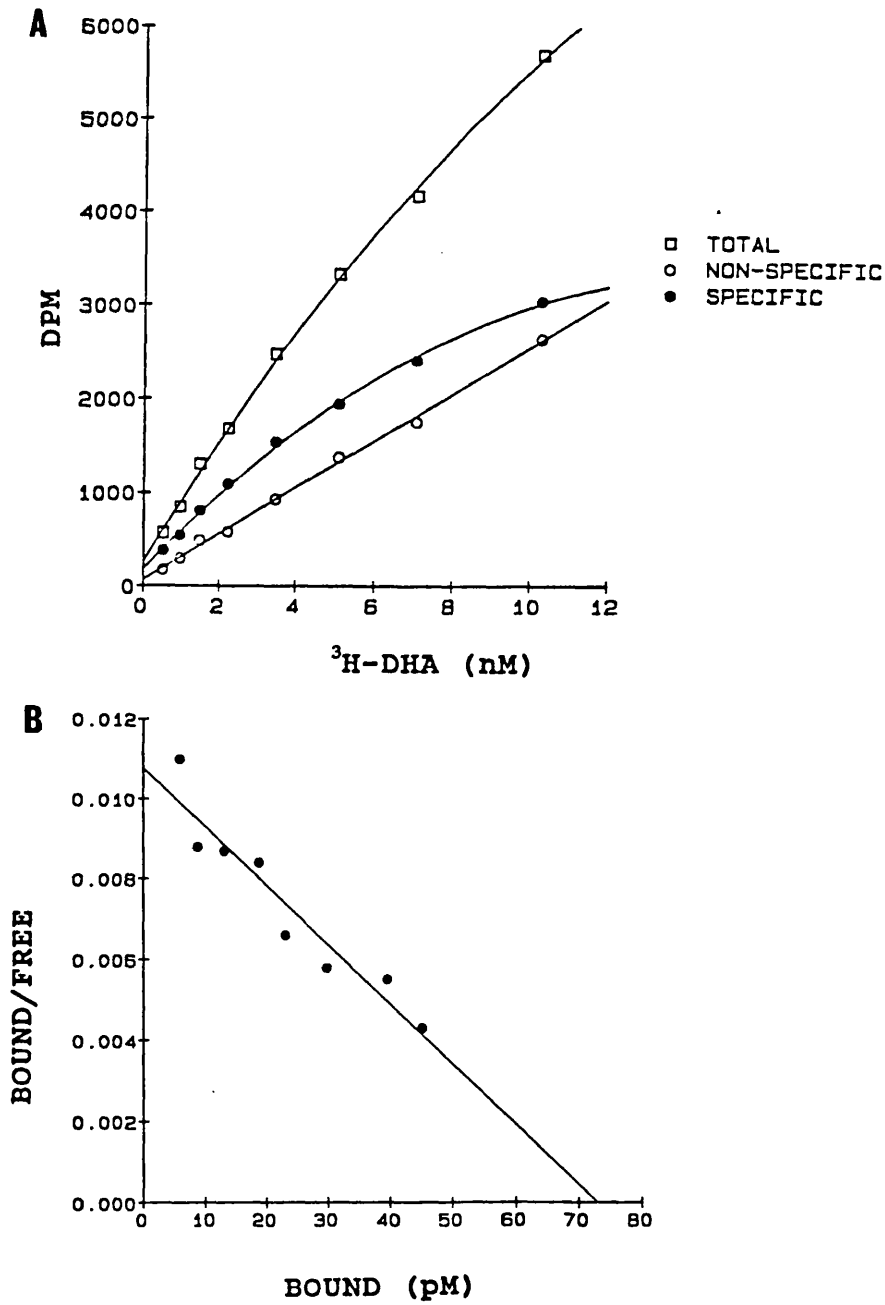
Binding of ^{3}H-DHA (0.1-35nM) to intact splenic lymphocytes.



(A) Binding characteristics of ^{3}H-DHA (0.1-35nM) to splenic lymphocytes (4 x 10^6/ml). The assay was carried out at 25^0C for 30 mins. Non-specific binding was determined in the presence of 1μM (-)-propranolol. **(B)** Scatchard analysis revealing a curvilinear plot indicating two apparent receptor populations one with a low K_d (R_1, 3nM) and approx. 3000 sites per cell, the other with a high k_d (R_2, 45nM) and approx. 35,000 sites.

FIG 2.3.2.

Binding of ^{3}H-DHA (0.1-12nM) to intact splenic lymphocytes.



(A) Binding characteristics of ^{3}H-DHA (0.1-12nM) to splenic lymphocytes (4 x 10^{6}/ml). The assay was carried out at 25^{0}C for 30 mins. Non-specific binding was determined in the presence of 1μM (-)-propranolol. **(B)** Scatchard analysis revealing a linear plot indicating a single receptor population with a K_d of 7nM and approximately 6000 receptors per cell.

approximately 6,000 receptors per cell (fig 2.3.2.).

2.3.2 Time course of ^{3}H-DHA binding to splenic lymphocytes and displacement with (+) and (-)-propranolol and phentolamine.

Maximum specific binding of ^{3}H-DHA (10nM) achieved equilibrium over a 25-45 minute period (fig 2.3.3.). Displacement by the (-)-isomer of propranolol was greater than that of the (+)-isomer although at the highest concentration of unlabelled ligand (10^{-4}M) maximum displacement of ^{3}H-DHA was achieved by both isomers. Phentolamine, an α_1 and α_2 antagonist displaced 15-20% of the total ^{3}H-DHA binding to splenic lymphocytes (fig 2.3.4.).

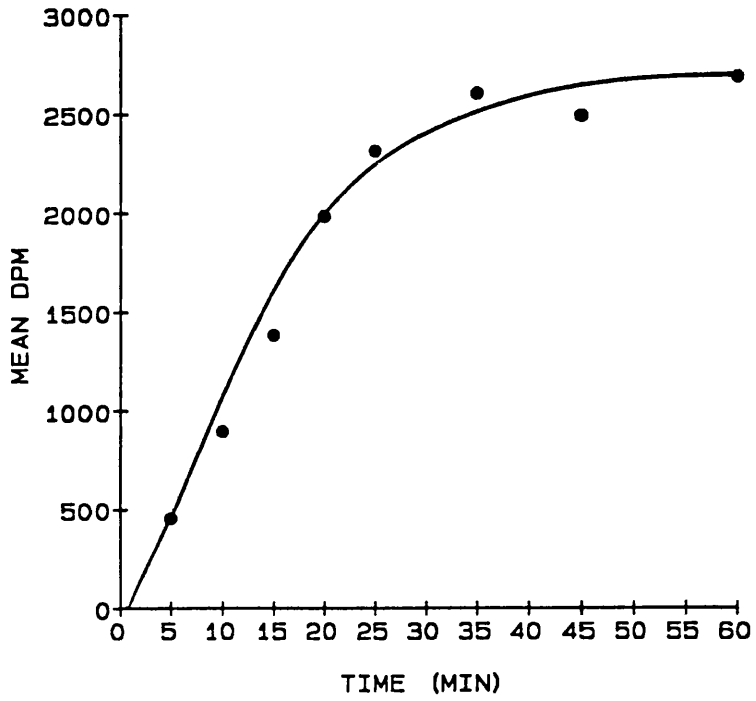
2.3.3 Binding of ^{3}H-DHA to isolated lymphocytes and r.b.c.

Separation from r.b.c. over a Percoll gradient did not change the binding characteristics of ^{3}H-DHA to splenic lymphocytes. Non-specific binding was linear over the range of ligand used. Specific binding accounted for approximately 80% of the total binding (5000 dpm).

In order to determine whether or not r.b.c. which contaminate the final splenic lymphocyte cell suspension accounted for any of the ^{3}H-DHA binding, the assay was repeated on isolated r.b.c. The cell concentration used was 10 x 10^6cells/ml which was greater than that contaminating the splenic lymphocyte preparation prior to separation on Percoll. Total and non-specific binding of ^{3}H-DHA to isolated r.b.c.

FIG 2.3.3.

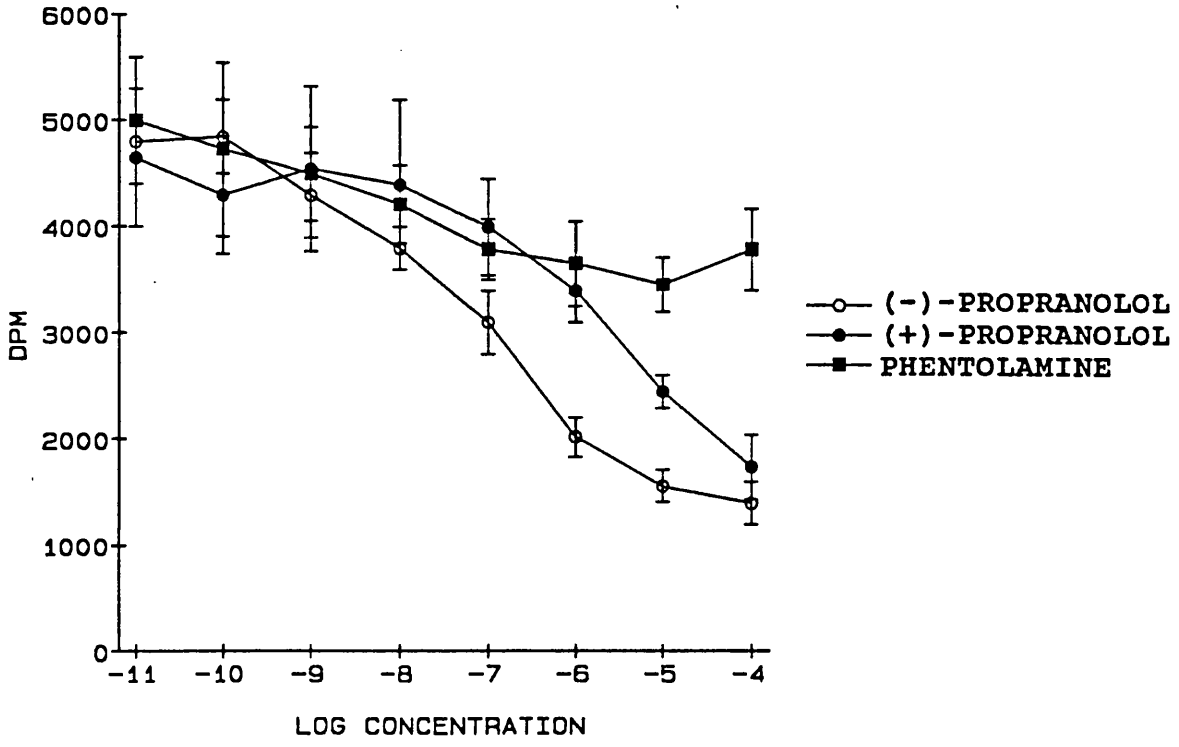
Time course for specific binding of ^{3}H-DHA to intact splenic lymphocytes.



Splenic lymphocytes (4×10^{6}) were incubated at 25^{0}C in round bottom 96 well plates with 10nM ^{3}H-DHA with and without 1μM (-)-propranolol. At the times indicated, the cells were harvested and the mean specific binding from triplicate wells expressed as disintegrations per minute (DPM).

FIG 2.3.4.

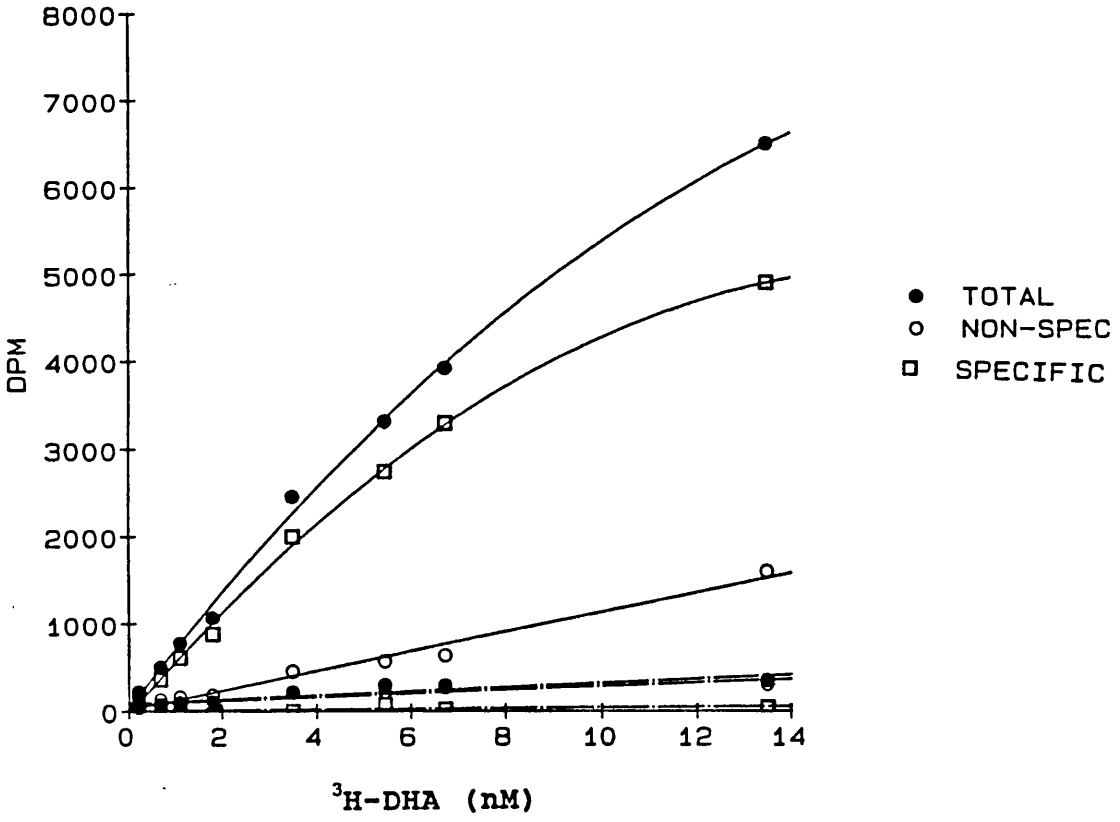
Displacement of ^{3}H-DHA binding by (-) and (+)-propranolol and phentolamine.



Splenic lymphocytes (4 x 10^{6}/ml) were incubated for 30 min at 25^{0}C with 10nM ^{3}H-DHA in the presence of increasing concentrations of unlabelled competitors . Each point was carried out in triplicate and the amount of ^{3}H-DHA bound expressed as disintegrations per minute (DPM).

FIG 2.3.5.

Binding of ^{3}H-DHA to isolated monocytes and red blood cells (r.b.c.).



Splenic monocytes were isloated from r.b.c. over a Percoll gradient, washed in BME-Earles and depleted of macrophages. Separated r.b.c. were washed with PBS and resuspended at a concentration of 10 x 10^{6} /ml. Binding assays for both cell populations were carried out as described in materials and methods using ^{3}H-DHA over a concentration range of 0.1-12nM.

was almost identical, demonstrating no specific binding of ^{3}H-DHA (fig 2.3.5.).

2.3.4 Binding of ^{125}I-CYP to intact splenic lymphocytes.

^{125}I-CYP used over a range of 0.1-300pM resulted in saturation of the receptor (fig 2.3.6.). Non-specific binding increased linearly over the range of ligand used. Transformation of the data by Scatchard analysis resulted in a linear plot indicating a single receptor poulation with a K_d of 7pM and approximately 600 sites/cell.

2.3.5 Time course of ^{125}I-CYP binding to intact lymphocytes and displacement with (+) and (-)-propranolol and phentolamine.

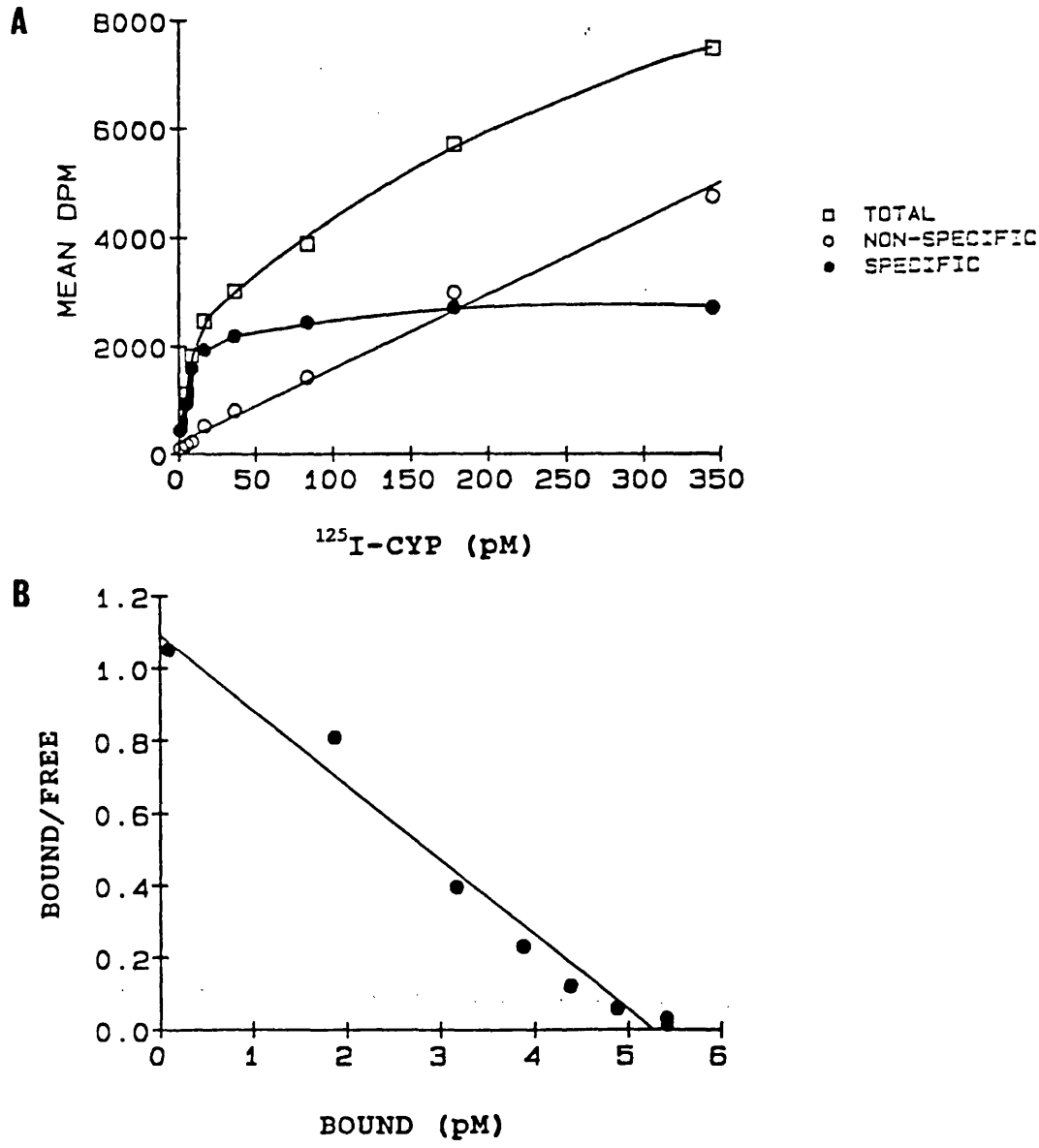
Maximum specific binding of ^{125}I-CYP (25pM) at 30^{0}C achieved equilibrium over a 100-120 minute period (fig 2.3.7.). Displacement profiles produced by (-) and (+)-propranolol were similar to those seen for ^{3}H-DHA binding, with the (-)-isomer having the greater effect (fig 2.3.8.). Phentolamine caused very little displacement of ^{125}I-CYP up to a concentration of 10^{-6}M. However, at the highest concentration used (10^{-4}M), total binding was reduced slightly.

2.3.6 Binding of ^{3}H-Dex to intact splenic lymphocytes.

Over a concentration range of 0.1-30nM ^{3}H-Dex binding to intact splenic lymphocytes was saturable (fig 2.3.9.).

FIG 2.3.6.

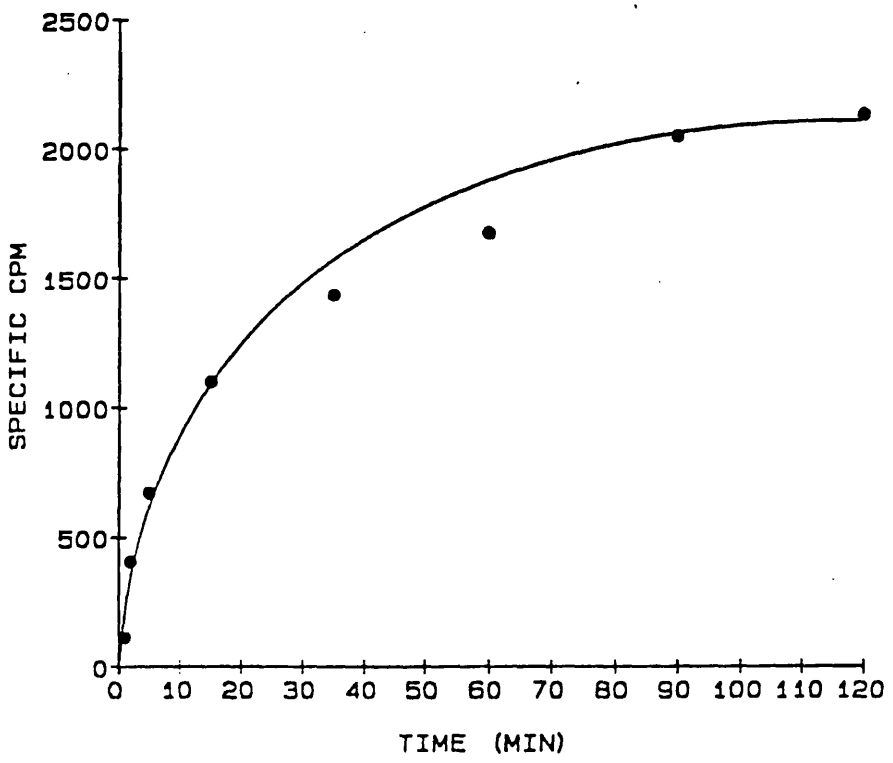
Binding of ^{125}I-CYP to intact splenic lymphocytes.



(A) Binding characteristics of ^{125}I-CYP to splenic lymphocytes (5 x 10^6/ml). The assay was carried out at 30^{0}C for 90 mins in 96 well plates. Non-specific binding was determined in the presence of 1μM (-)-propranolol. **(B)** Scatchard analysis revealing a linear plot indicating a single receptor population with a K_d of 7pM and approximately 600 receptors per cell.

FIG 2.3.7.

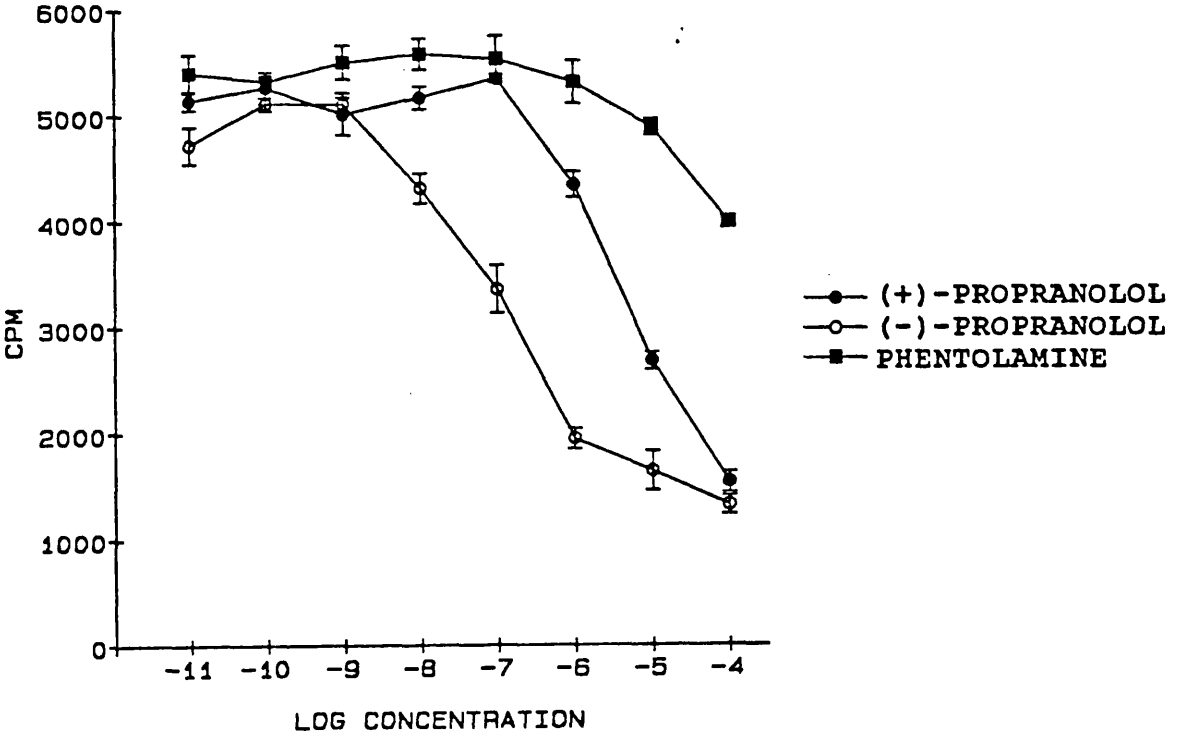
Time course for specific binding of ^{125}I-CYP to intact splenic lymphocytes.



Splenic lymphocytes (4 x 10^6) were incubated at 30^{0}C in round bottom 96 well plates with 25pM ^{125}I-CYP with and without 1μM (-)-propranolol. At the times indicated, the cells were harvested and the mean specific binding from triplicate wells expressed as counts per minute (CPM).

FIG 2.3.8.

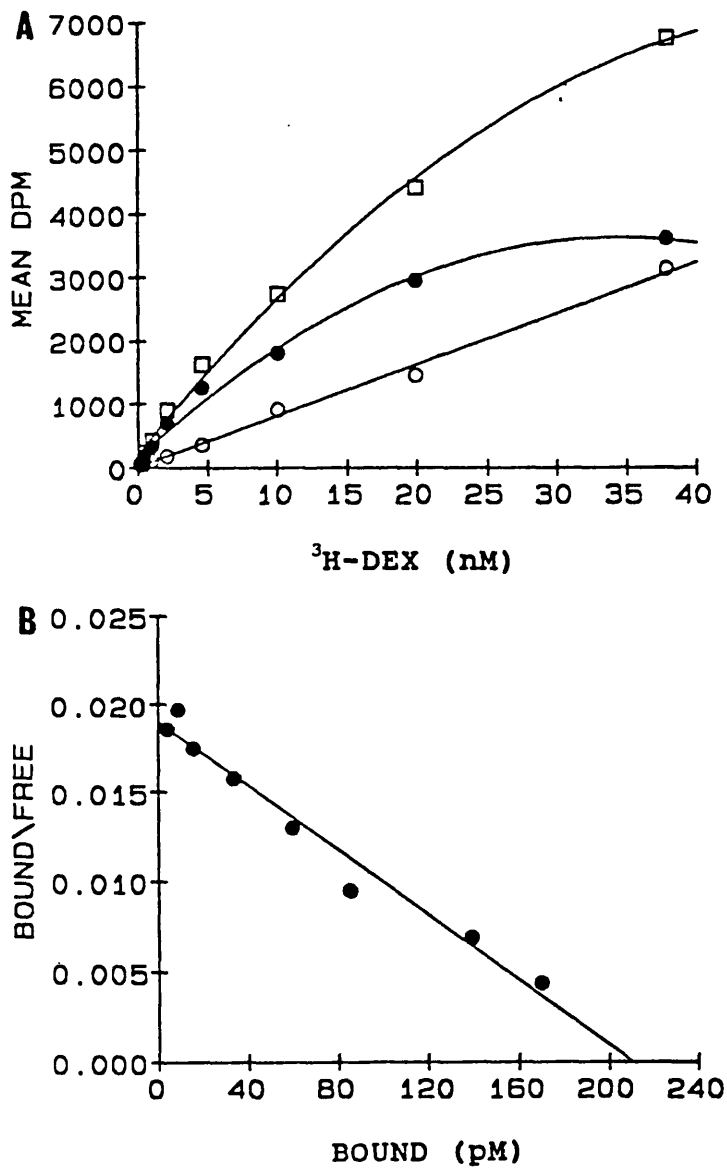
Displacement of ^{125}I-CYP binding to splenic lymphocytes by (-) and (+)-propranolol and phentolamine.



Splenic lymphocytes (4 x 10^6/ml) were incubated for 90 min at 35^{0}C with 25pM ^{125}I-CYP in the presence of increasing concentrations of unlabelled competitors . Each point was carried out in triplicate and the amount of ^{125}I-CYP bound expressed as counts per minute (CPM).

FIG 2.3.9.

Binding of ^{3}H-DEX (0.1-30nM) to intact splenic lymphocytes.



(A) Binding characteristics of ^{3}H-DEX to splenic lymphocytes (50 x 10^6/ml). The assay was carried out at 37^{0}C for 30 mins. Non-specific binding was determined in the presence of 2μM unlabelled DEX. Cells were lysed with $Mgcl_2$ (1.5 mM), spun at 12,000 x g and the dpm from the pellets counted. **(B)** Scatchard analysis revealing a linear plot indicating a single receptor population with a K_d of 15nM and approximately 2,500 nuclear receptors per cell.

Non-specific binding in the presence of 2μM unlabelled dexamethasone increased linearly with increasing ligand concentration. Transformation of the data using Scatchard analysis indicated a single population of receptors with a K_d of 15nM and approximately 3200 sites/cell.

2.3.7. Cytoplasmic to nuclear translocation of the glucocorticoid receptor.

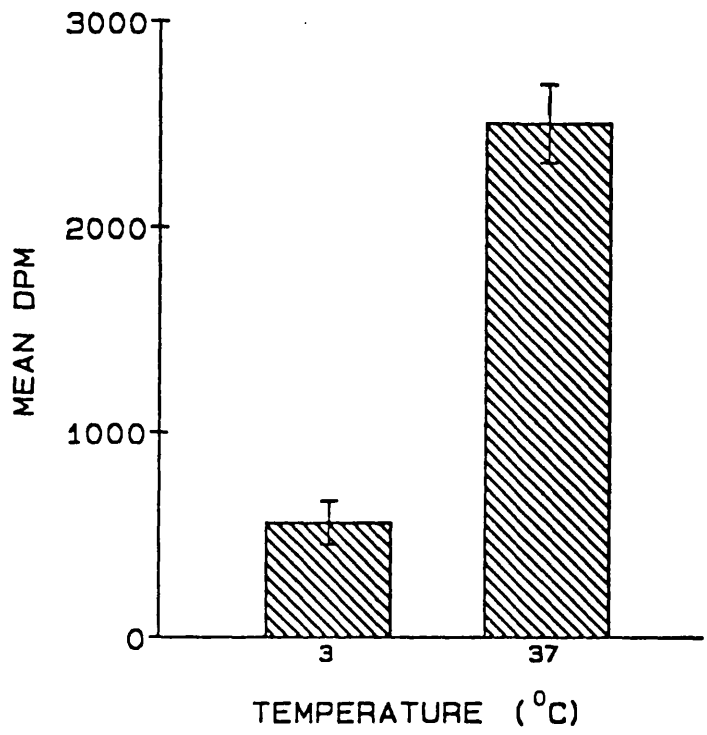
Translocation of the steroid receptor complex is illustrated in fig 2.3.10. Cell suspensions were incubated with 20nM ^{3}H-Dex for 180 min at 3^{0}C with and without 2μM unlabelled dexamethasone to allow maximum binding to cytoplasmic receptors. At this temperature specific binding of ^{3}H-Dex to the nuclear receptor resulted in approximately 500 dpm. When the cell suspensions were transferred to a water bath at 37^{0}C specific nuclear binding increased to approximately 2500 dpm.

2.3.8. ^{3}H-Dex binding to mitogen stimulated lymphocytes.

Incubation of splenic lymphocytes with the mitogen Con A caused a marked increase in cell proliferation as indicated by the incorporation of ^{3}H-Thymidine. The stimulation index (SI = dpm from stimulated cells/dpm from non stimulated cells) was increased at both 48 and 72 hrs (fig 2.3.11.) and was accompanied by an increase in the number of nuclear receptors which were approximately 3-fold higher after 72 hrs incubation with Con A.

FIG 2.3.10.

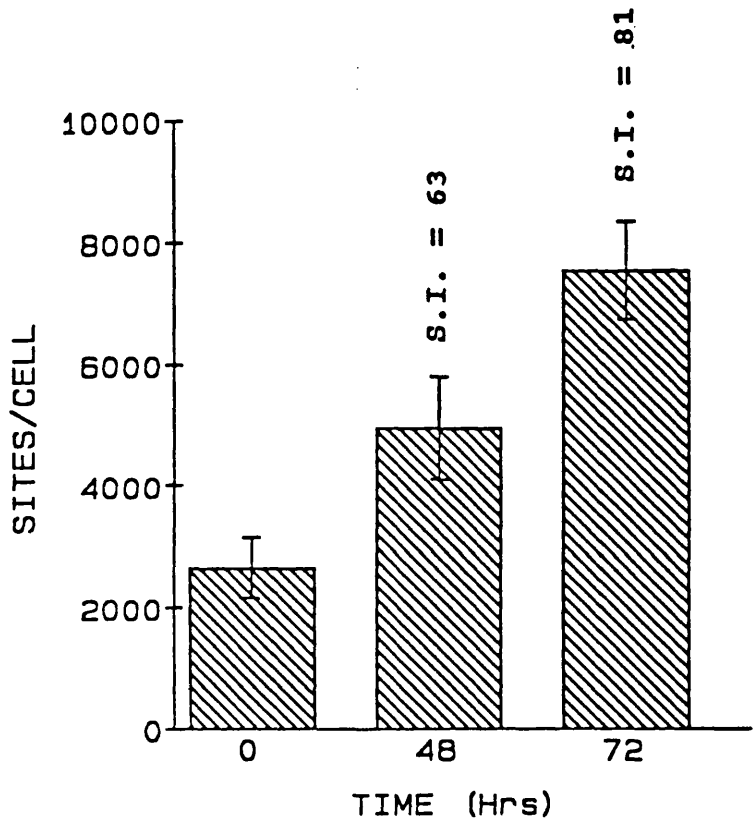
Cytoplasmic to nuclear translocation of the ^{3}H-DEX receptor complex.



Cell suspensions (50 x 10^{6} cells/ml) were incubated with 20 nM ^{3}H-DEX with and without 2 μM unlabelled DEX at 3^{0}C for 180 min. Aliquots were removed and the amount of ^{3}H-DEX bound to nuclear sites expressed as dpm ± SEM. The remaining cells were transferred to a water bath at 37^{0}C and nuclear binding determined after a further 30min incubation.

FIG 2.3.11.

The effect of mitogen stimulation on lymphocyte nuclear glucocorticoid receptor number.



Splenic lymphocytes (2 x 10^6/ml) were incubated with Con A (1 μg/ml) in 250 ml tissue culture flasks. At 0, 48 and 72 hrs glucocorticoid nuclear receptor assays were carried out as described in materials and methods. Results are expressed as sites/cell ± s.e.m. Proliferation was determined by measuring the incorporation of ^{3}H-Thy at 48 and 72 hrs and expressed as the stimulation index (S.I. = dpm from stimulated cells / dpm from non-stimulated cells).

2.4 Discussion.

The method for the ß-adrenergic receptor assay was adapted from a previous assay system used to determine ß-adrenergic receptors on splenic lymphocytes from mice (Miles et al, 1984). Due to the small volume of the assay (250µl total) it was possible to carry out the whole assay in round bottom 96 well plates. This reduced both the preparation and harvesting times allowing up to eight assays to be run at once.

When ^{3}H-DHA was used over a concentration range of 0.1-30nM it was not possible to demonstrate stereospecific binding. Transformation of the data revealed a biphasic Scatchard plot indicating two receptor populations. Similar problems have been reported previously using ^{3}H-DHA to determine ß-adrenergic receptors on viable murine splenocytes (Fuchs et al, 1988) and human polymorphonuclear (PMN) leukocytes (Dulis et al, 1980). At high concentrations of the drug it is thought that the lipophilic antagonist ^{3}H-DHA diffuses into the lysosomes where it is protonated in the acidic environment and trapped. This is reflected in the binding curves in which saturation of the receptor at high concentrations does not appear to be achieved. Uptake of ^{3}H-DHA into lysosomes can be reduced by the addition of chloroquine to the assay buffer which has been shown to eliminate a large component of ligand binding to human PMN cells (Marinetti et al 1983). However, since the cell population in these studies was predominantly lymphocytes which possess few cytoplasmic granules (Zucker-franklin,

1969), it would appear that non-specific uptake of the ligand is not solely responsible for the biphasic Scatchard plots.

In order to achieve a linear Scatchard plot it was found necessary to reduce the highest concentration of ^{3}H-DHA to 12nM which was similar to that used by Miles et al (0.5 - 15nM). Transformation of the data by Scatchard analysis revealed a single receptor population with an affinity (K_d) of 7nM and approximately 6000 sites/cell.

In the competition studies, maximum binding of 10nM ^{3}H-DHA was reduced by approximately 20% when phentolamine (10^{-6}-10^{-4}M), an α-adrenergic antagonist was used as the competing ligand, indicating that ^{3}H-DHA has a weak affinity for α-adrenergic receptors. For this reason, 1μM phentolamine was routinely included in the assay buffer to restrict the binding of ^{3}H-DHA to ß-adrenergic receptors.

Displacement of ^{3}H-DHA binding by (-)-isomer of propranolol was greater than for the (+)-isomer at the lower concentrations of ligand used, although at 10^{-4}M both displaced approximately 85% of the bound ^{3}H-DHA. This <u>in vitro</u> response to the (+) and (-)-isomers of propranolol relects the order of potency of (+) and (-)-isomers of adrenergic agonists and antogonists <u>in vivo</u>, with the (-)-isomers considerably more potent than the (+)-isomers observed in physiological ß-adrenergic responses.

Unlike avian and amphibian erythrocytes (Lekfowitz et al, 1974; Mukherjee et al, 1975) no specific binding of ^{3}H-DHA was found on isolated rat r.b.c. when assayed at a concentration of $10x10^{6}$/ml. Similarly no difference in binding characteristics were seen between splenic lymphocytes

isolated over a Percoll gradient and assays where lymphocytes were not separated from r.b.c. For this reason separation of lymphocytes from r.b.c. prior to the ß-adrenergic receptor assay was not carried out.

In later experiments in this thesis ^{125}I-CYP was used instead of ^{3}H-DHA. CYP is a more selective ligand with virtually no affinity for α-adrenergic receptors. This was evident in the competition studies where displacement profiles for the two stereoisomers of propranolol were similar to those seen for ^{3}H-DHA, however phentolamine was only capable of displacing ^{125}I-CYP binding when the drug was used at the highest concentration (10^{-4}M). Binding curves for ^{125}I-CYP to viable intact splenic lymphocytes revealed a saturable single site receptor population with a K_d of 6pM and approximately 550 sites per cell. This difference in the number of ß-adrenergic sites can be attributed to the greater affinity and specificity of ^{125}I-CYP.

Binding of ^{3}H-Dex to nuclear glucocorticoid receptors at 37^{0}C was saturable over the concentration of ligand used indicating a single receptor population with a K_d of 17nM and approximately 3000 sites per cell, values which are similar to those reported previously (Crabtree et al, 1980). Cytoplasmic to nuclear translocation of the receptor was demonstrated by carrying out nuclear binding assays at different temperatures. When ^{3}H-Dex binding was carried out at 3^{0}C, at which temperature the conformational changes required for translocation are inhibited, very little ^{3}H-Dex was found binding to nuclear sites. However, when the cell suspensions were transferred to a water bath at 37^{0}C, nuclear binding of

^{3}H-Dex increased rapidly. No assays were carried out on cytoplasmic glucocorticoid receptors, however, it has been reported (Crabtree et al 1980) that at 37^{0}C, when nuclear glucocorticoid receptor binding sites are saturated, some ligand is still bound in the cytoplasm. Since it is the binding of the receptor complex to nuclear sites that leads to the physiological response and that under optimal conditions for nuclear binding (37^{0}C) not all the cytoplasmic bound ^{3}H-Dex translocates to the nucleus, it was decided that changes in nuclear binding sites were of primary importance.

To ensure that the assay system was sensitive enough to detect changes in nuclear glucocorticoid receptors, splenic lymphocytes were incubated <u>in vitro</u> with Con A which has been shown to increase nuclear receptor binding in human lymphocytes (Smith et al, 1987). An approximate 3-fold increase in receptor sites was observed after 72hrs incubation with Con A which coincided with the increased lymphocyte proliferation as shown by ^{3}H-thymidine incorporation, thus confirming previous studies and the validity of the assay used.

CHAPTER 3

Evidence for interactions between the neural, endocrine and immune systems during the course of experimental allergic encephalomyelitis.

3.1 Introduction.

It is well established that the endocrine and nervous systems, triggered by signals from an activated immune system, exert feedback control on immunological reactivity (Cavagnaro, 1988). The sympathetic innervation of the spleen includes direct synapse-like links with lymphocytes which possess adrenergic receptors (Felten and Olschowka, 1987) the activation of which can inhibit components of the immune response. A role for such bidirectional communication in EAE was demonstrated by the increased disease severity in animals which had undergone neurotoxic destruction of the splenic noradrenergic sympathetic nerve terminals, prior to disease induction (Chelmicka-Schorr, et al, 1988).

Axotomy has been shown to deplete noradrenaline stores in the spleen and increase ß-adrenergic receptors on splenic lymphocytes (Miles et al, 1984). The possibility of ß-adrenergic receptor-mediated modulation of EAE was strongly suggested by the suppression of clinical signs of disease in animals treated with the ß-adrenergic receptor agonist isoproterenol (Chelmicka-Schorr et al, 1989).

Lymphocytes also possess steroid receptors which mediate a number of immunoregulatory actions including the

inhibition of lymphokine secretion (Kelso et al, 1984) and T-cell growth factor production (Gillis et al, 1979). Changes in circulating levels of corticosterone during EAE and its role in the recovery from disease is well established (Levine et al, 1980; MacPhee et al, 1989) and could well be reflected by changes in lymphocyte steroid receptor number.

In order to investigate neuroendocrine and neural responses to immune stimulation, changes in lymphocyte ß-adrenergic and nuclear glucocorticoid receptors and their relation to splenic NA and circulating CS levels were examined during the pre-clinical and clinical stages of EAE. Two methods of disease induction were used which enable differentiation between the efferent and afferent arms of the immune response; (1) active induction, by the injection of guinea pig spinal cord in adjuvant, where sensitization and proliferation to the antigen (MBP) occurs <u>in vivo</u> and (2) passive transfer, where cells are sensitized <u>in vitro</u> with MBP, representing the effector stage of the disease and then transferred to naive animals. In each case either the injection of adjuvant alone, or the transfer of PPD sensitized cells, which both provide a non-specific immune challenge, served as controls.

3.2 Materials and Methods.

3.2.1. Preparation of crude guinea pig myelin basic protein (MBP).

Crude guinea pig MBP was prepared by the method of

Dunkley and Canegie. Adult Duncan Hartley or strain 13 guinea pigs were sacrificed and the brain and spinal cord removed. To eliminate degradation of basic protein by brain acid proteinases, defatting was performed by adding chloroform-methanol (2:1, v/v) previously chilled to -10^{0}C, to the fresh tissue (19ml/g tissue) and homogenised in an ultramix blender for 5min. The suspension was then filtered under vacuum using a sintered glass funnel. The defatted tissue was homogenised for a further 5min with cold water (2ml/g original tissue) and the pH carefully decreased to 3 by dropwise addition of 1M HCl. This pH was maintained by the further addition of HCl while stirring on ice and the suspension was then filtered through a sintered glass funnel and the supernatant retained. The residue was reextracted at pH 3 for a further 30min and the two supernatants pooled and dialysed against three changes of distilled water and then freeze dried. The crude extract was resuspended in PBS and the protein content determined by the method of Lowry (1951).

3.2.2. Preparation of guinea pig spinal cord (GPSC) and MBP inoculum.

GPSC inoculum was made from fresh guinea pig spinal cord (500mg/ml) homogenised in complete Freund's adjuvant (CFA) containing 10mg/ml *Myocobacterium tuberculosis* (Difco).

Guinea pig MBP inoculum was made from crude MBP mixed with CFA containing 10mg/ml *Mycobacterium tuberculosis* to give a final concentration of 0.1% MBP.

3.2.3 Immunization protocol for active EAE.

Female Lewis rats were immunized with 50µl of the GPSC inoculum in each hind foot. Control animals received an equal volume of an emulsion containing 10mg/ml *Mycobacterium tuberculosis* (CFA) only. Rats were kept in pairs and handled and weighed on a daily basis for at least one week prior to immunization and afterwards until the day before the experiment. Non-immunized controls were also handled daily. Clinical disease was assessed as follows:

(1) Flaccid tail and weight loss.
(2) Hind limb hypotonia with further weight loss.
(3) Partial or complete hind limb paralysis.

Free access to food and water was ensured throughout the experiment.

3.2.4. Immunization protocol for the adoptive transfer of EAE.

Female Lewis rats were immunized with 50µl of the GPSC or guinea pig MBP inoculum in each hind foot. Control animals received an equal volume of CFA alone. 11 days after immunization the animals were sacrificed and the spleens removed. The cells were dispersed through a wire mesh and washed three times and then resuspended at a concentration of 2 x 10^6/ml in BME-Earles containing 10% FCS, 100U/ml

penicillin, 100μg/ml streptomycin, 1mg/ml indomethacin, 20μM 2-mercaptoethanol with either guinea pig MBP or purified protein derivative (PPD), both at 1μg/ml, as the stimulating antigen. After 3 days in culture at 37°C the cells were spun down, washed twice with fresh BME-Earles and 40 x 10^6 cells transferred to each naive recipient by intraperitoneal (i.p.) injection. Animals were weighed daily and scored as described in section 3.2.3.

3.2.5. Collection of spinal cord and brain for histological examination during the course of EAE.

Rats were anaesthetized with an i.p. injection of sagatal and perfused with 60ml of 10% formalin in PBS by inserting a needle into the left ventricle and cutting the return supply to the right auricle. After the perfusion was complete the brain was removed and stored in buffered formalin. In order to assist the removal of the spinal cord, the whole of the spinal column was removed and left in buffered formalin overnight before the cord was removed.

3.2.6. Collection of samples and cell preparation.

On appropriate days post-immunization (d.p.i.) or post-transfer (d.p.t.) animals were removed quietly from the holding room and decapitated immediately in an adjacent room. Trunk blood was collected, spun at 2000 r.p.m. at 3°C for 10 mins and the serum stored at -20°C for subsequent corticosterone (CS) analysis. The spleen was removed and

weighed whole, smaller samples for determination of NA content were taken by two different methods: initially, small samples of red and white pulp were removed from a cross-section of frozen spleen using a liver biopsy needle (40mm x 1mm, Downs Surgical Ltd, UK) and stored at -70^{0}C. In later experiments a larger sample of spleen containing both red and white pulp was removed, weighed and stored at -70^{0}C. In both cases the remainder of the spleen was dissociated through a wire mesh in BME-Hanks, spun down at 1200 rpm for 5 mins and washed three times with fresh medium. The cells were then resuspended at a concentration of 3 x 10^{6}/ml in BME-Earles containing 10% foetal calf serum (FCS) 100U/ml penicillin and 100 μg/ml streptomycin (Gibco UK). Macrophages were depleted on plastic for 90min at 37^{0}C. The remaining lymphocyte enriched population was washed twice with PBS and used for receptor assays. These were carried out as described in materials and methods Chapter 2.

3.2.7. Preparation of spleen samples for high performance liquid chromatography (HPLC).

Frozen spleen samples were homogenised in 200 μl of 0.1M $HClO_4$ containing 50pM 3,4-dihydroxybenzylamine (DHBA) as an internal standard (Sigma). The small samples of red and white pulp could be disrupted by sonication, however, this was not possible for the larger samples of spleen which were homogenised by passing the samples in $HCLO_4$ through consecutively smaller gauge needles (19-25G). This procedure

was found to be time consuming and so a purpose built homogeniser was made by moulding dental acrylic in an Eppendorf ultracentrifuge tube to a piece of stainless steel. This could then be attached to a mounted drill and enabled rapid and successful homogenisation of the larger spleen samples. After disruption of the tissue, the tubes were spun for 5 mins in an Eppendorf ultra-centrifuge. The supernatants were removed and the pellets stored for protein determination. NA and DHBA were absorbed onto Type WA-4 alumina (Sigma Co) in 1ml of 1M tris buffer containing 2g/L EDTA and 10mg/L sodium metabisulphite adjusted to pH 8.8 with concentrated HCl. 150μl of 0.5M NaOH was added to maintain a favourable alkaline pH for absorption which took place over 10 min on a Spiramix (Denley).

The supernatant was then discarded and the alumina washed three times with 1ml distilled water. After the last wash the water was aspirated and desorption of the NA and DHBA effected over 5 mins using 200μl of 0.5M H_3PO_4. 175μl of the supernatant was frozen and stored at -70^{0}C for future HPLC analysis.

3.2.8. HPLC Analysis.

NA was quantified by HPLC with electrochemical detection using an LKB 2150 pump with pulse dampener, a Gilson 231 sampler and diluter, a stainless steel column (100 x 4.6mm) packed with 3μm ODS hypersil and a waters M460 electrochemical detector set at +0.69V and 2nA range. Data analysis against the DHBA internal standard was by a Hewlett-

Packard 3390A integrator. The mobile phase consisted of aqueous KH_2PO_4 and citric acid (both 40mM) containing 0.5mM 1-octanesulphonic acid and 4mM EDTA with 10% methanol. This was filtered then degassed and pumped at 1.2ml/min.

3.2.9. Protein determination (Lowry Method).

Reagents

(a) 1% $CuSo_4$

(b) 2% Na-K-tartrate

(c) 4% Na_2CO_3

Just before use Folin Solution A was prepared by mixing 2ml (a) + 2ml (b) + 96ml (c).

(d) Folin & Ciocalteau's phenol reagent (Sigma) was diluted x 5 with distilled H_2O to prepare Folin B solution.

(e) Bovine serum albumin (BSA Sigma) 1mg/ml.

(f) 0.1M NaOH containing 0.1% SDS.

A standard curve for BSA was made by pipetting increasing volumes of (e) into 10ml non-sterile conical based tubes. 1ml of 10% SDS was added to the frozen spleen pellets and heated to 90°C for 30 mins to solubilize the pellets. 50μl of the supsension was pipetted into 10ml tubes and made up to 1ml with solution (f). 2ml of Folin A was then added to the standard curve and unknown samples, vortexed and allowed to react at room temperature for 10min. 1ml of Folin B was then added to all the tubes, vortexed and placed in a water bath at 60°C for 10min. The tubes were allowed to cool and

the absorption read at A_{750}. Protein content of the unknown samples were read off the standard curve.

3.2.10. Serum Corticosterone determination.

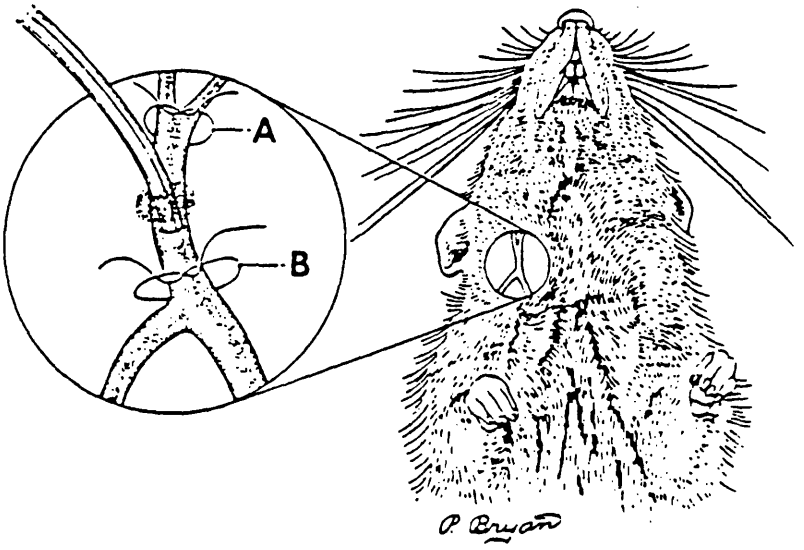
Frozen serum samples were thawed and corticosterone determined using a ^{125}I-labelled corticosterone radioimmunoassay kit (Immunodiagnostics). Corticosterone was calibrated over a 25-1000 ng/ml range.

3.2.11. Implantation of Jugular vein Cannula for repeated blood sampling.

Adult female Lewis rats (200-250g) were anaesthetized with sagatal and a 3cm incision made on the ventral-lateral aspect of the neck. The right jugular vein was exposed using blunt dissection and the surrounding connective tissue cleaned from the vein. Two pieces of suture were used as ligatures and positioned as shown in Fig 3.2.1. The superior ligature (A) was tied first to prevent blood flow from the cranium and a small incision was made on the lateral aspect of the vein. The cannula containing 100U/ml heparin in 0.9% NaCl (silastic tubing 0.64 mm i.d x 1.19mm o.d) was then inserted approximately 2.5 cm into the incision at which point the inferior ligature (B) was secured around both the vein and the cannula. To help anchorage, the cannula was secured to the superior ligature and then flushed with heparinized saline and externalized through a small incision (0.5cm) on the dorsal side of the neck. It was then secured between a piece

FIG 3.2.1.

Insertion of jugular vein cannula.



Ventral view of right jugular vein. (A) superior ligature, (B) inferior ligature. Taken from Axelson and Bruot. (Physiology and Behaviour, 1982, 28, p949-951).

of velcro which was sutured to the animal at the point where the cannula was externalized. The tube was sealed by insrting a blocked needle which could then be removed for blood sampling.

3.3. Results.

3.3.1. Preparation of Crude Guinea Pig MBP.

The crude MBP was run on 15% sodium dodecyl sulphate (SDS) polyacrylamide gel. Samples were prepared in SDS sample buffer (10% SDS, 10% sucrose, 100mM DTT, 0.05% bromophenol blue, 5% 2-mercaptoethanol) and run using a 5% acrylamide stacking gel and 15% separating gel. The gel was stained with 0.25% Coomassie Blue in 10% acetic acid (50% methanol) and destained with 10% acetic acid (50% methanol). The MBP eluted between the 20 and 30KD MW protein markers (fig 3.3.1).

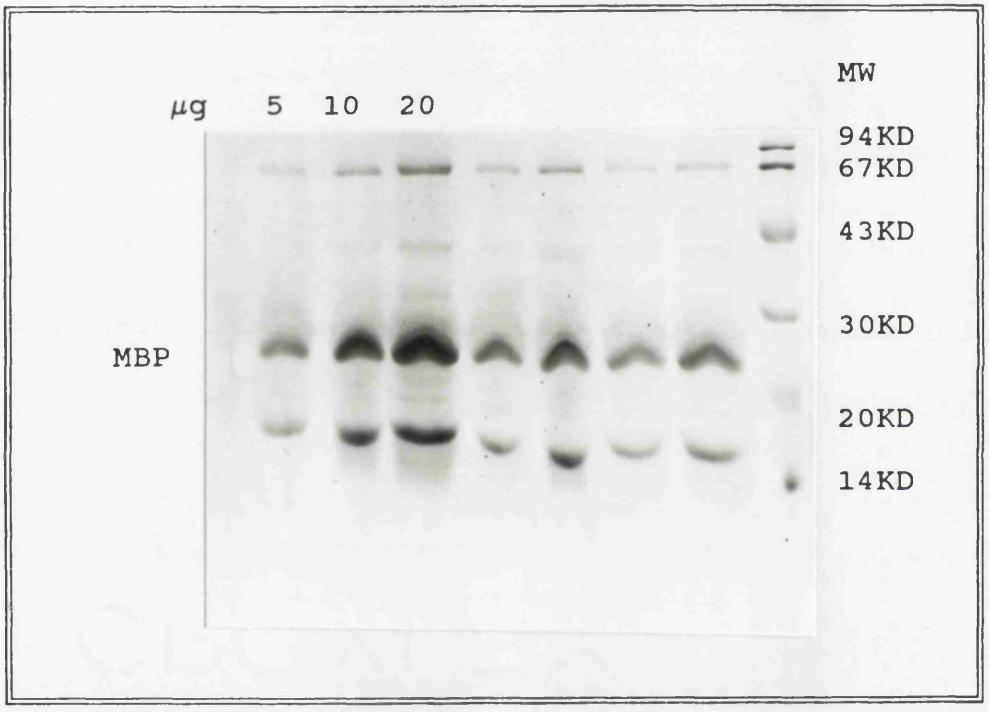
3.3.2. Clinical course of active EAE.

The first clinical signs of EAE appeared on average 11 days after immunization with the GPSC or GP-MBP inoculum and reached a maximum score on about day 13. Recovery from EAE was usually complete by 18dpi (fig 3.3.2a.). With the onset of clinical signs, the animals consistently lost weight with maximum weight loss coinciding with maximum clinical disease (fig 3.3.2b.).

During the early stage of EAE, the total spleen weight and mononuclear cell (MNC) count remained unchanged

Fig 3.3.1.

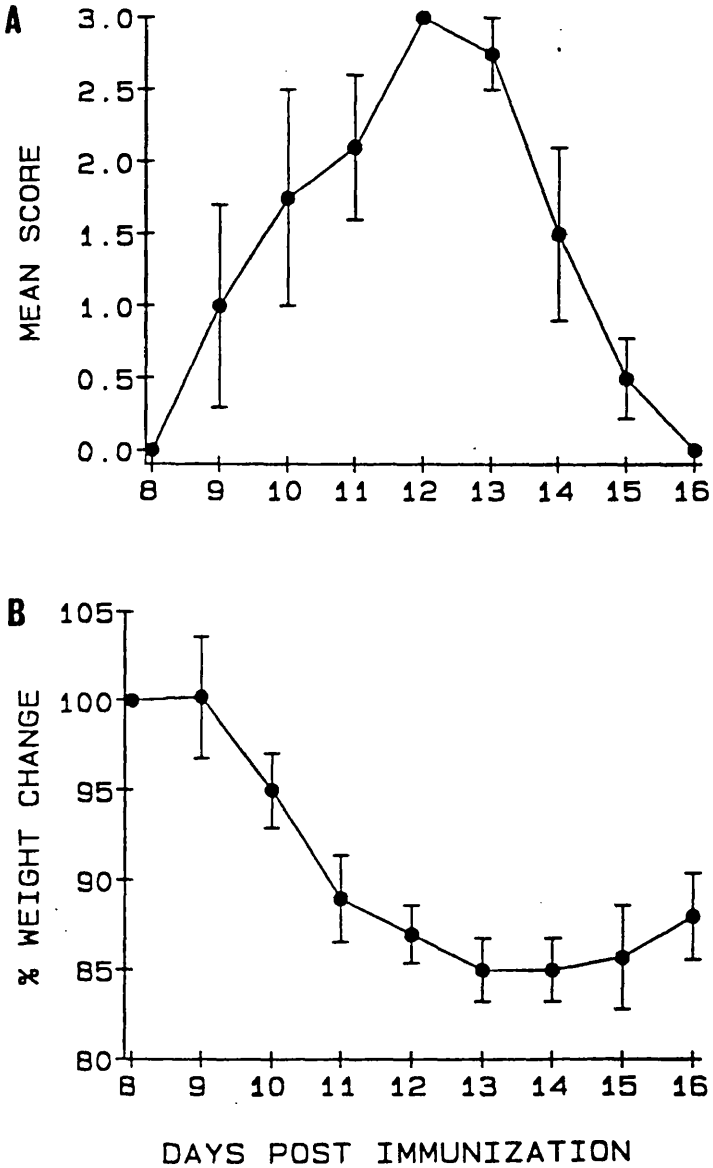
15% polyacrylamide gel of the crude MBP extract.



The crude MBP extract was prepared in SDS sample buffer and run on a 15% SDS polyacrylamide gel. The gel was stained with Coomassie blue and destained with acetic acid. The MBP (5, 10 and 20μg protein) eluted between the 20 and 30KD MW protein markers.

FIG 3.3.2.

Clinical course of active EAE.



(A) Typical time course of EAE following immunization with either GPSC or MBP inoculum. **(B)** Weight loss associated with the onset of disease expressed as percentage change in body weight from day 8. Each point represents the mean ± s.e.m. from a group of 4 animals.

TABLE 3.3.1

Changes in spleen weight and mononuclear cell count (MNC) during the clinical course of EAE.

Immunization protocol	n	spleen weight (mg)	MNC x 10^{-6} [a]
Unimmunized	11	632 ± 32	381 ± 48
CFA 3 d.p.i.	8	591 ± 42	412 ± 37
GPSC 3 d.p.i.	11	610 ± 40	396 ± 38
CFA 7 d.p.i.	8	647 ± 37	394 ± 28
GPSC 7 d.p.i.	7	635 ± 37	410 ± 30
CFA 11-13 d.p.i.	5	674 ± 40	379 ± 35
GPSC 11-15 d.p.i.[b]	5	457 ± 38 *+	196 ± 19 *+

Animals were sacrificed on the days post immunization (d.p.i.) indicated and the spleens removed and weighed.

[a] Total splenic MNC count.

[b] Animals with clinical scores > 2

Difference significant at $P < 0.01$ (*) compared to unimmunized animals (unpaired t-test).

Difference significant at $P < 0.01$ (+) compared to CFA animals at 11-13 d.p.i. (unpaired t-test).

(table 3.3.1) but with the onset of clinical signs, both fell significantly. No change in spleen weight or MNC count was seen in animals immunized with CFA alone at any of the time points studied.

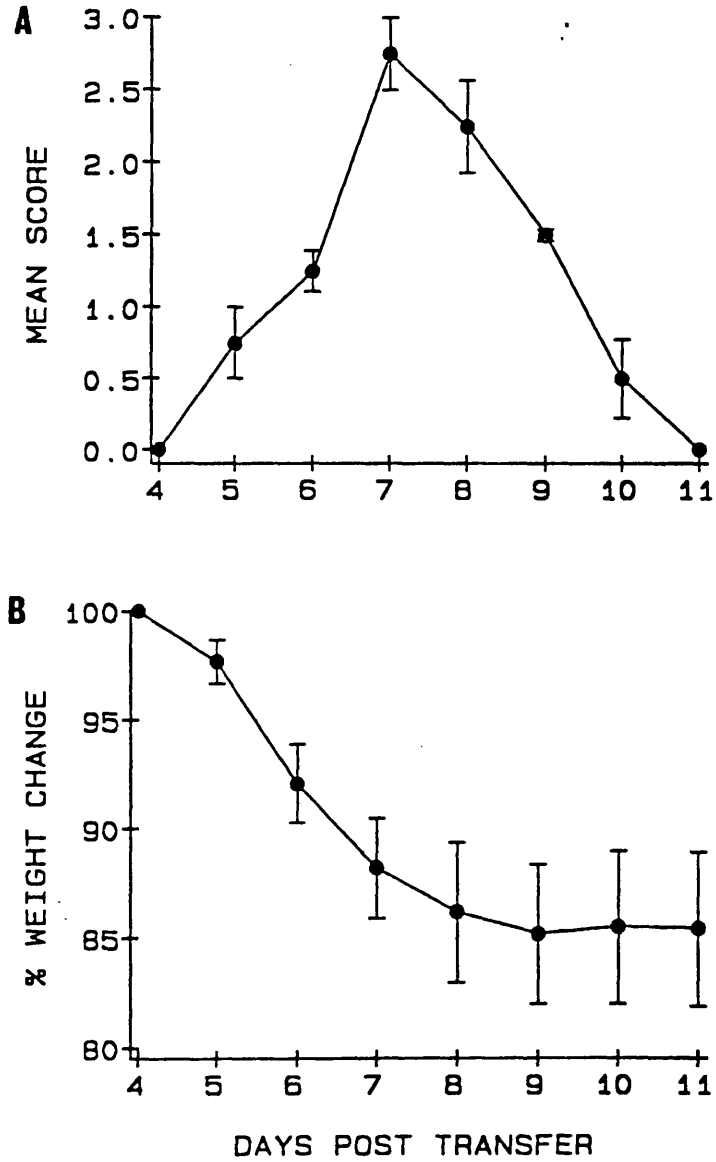
3.3.3. Clinical course of adoptively transferred EAE.

The first clinical signs of EAE appeared on average 5 days after the transfer of MBP-sensitised T-cells reaching a peak clinical score of 3 by day 7. Full recovery from disease was usually complete by day 10 (fig 3.3.3a.). As with the active model of EAE, onset of clinical signs was accompanied by dramatic weight loss, however, no reduction in spleen weight or total MNC count was seen during the course of disease.

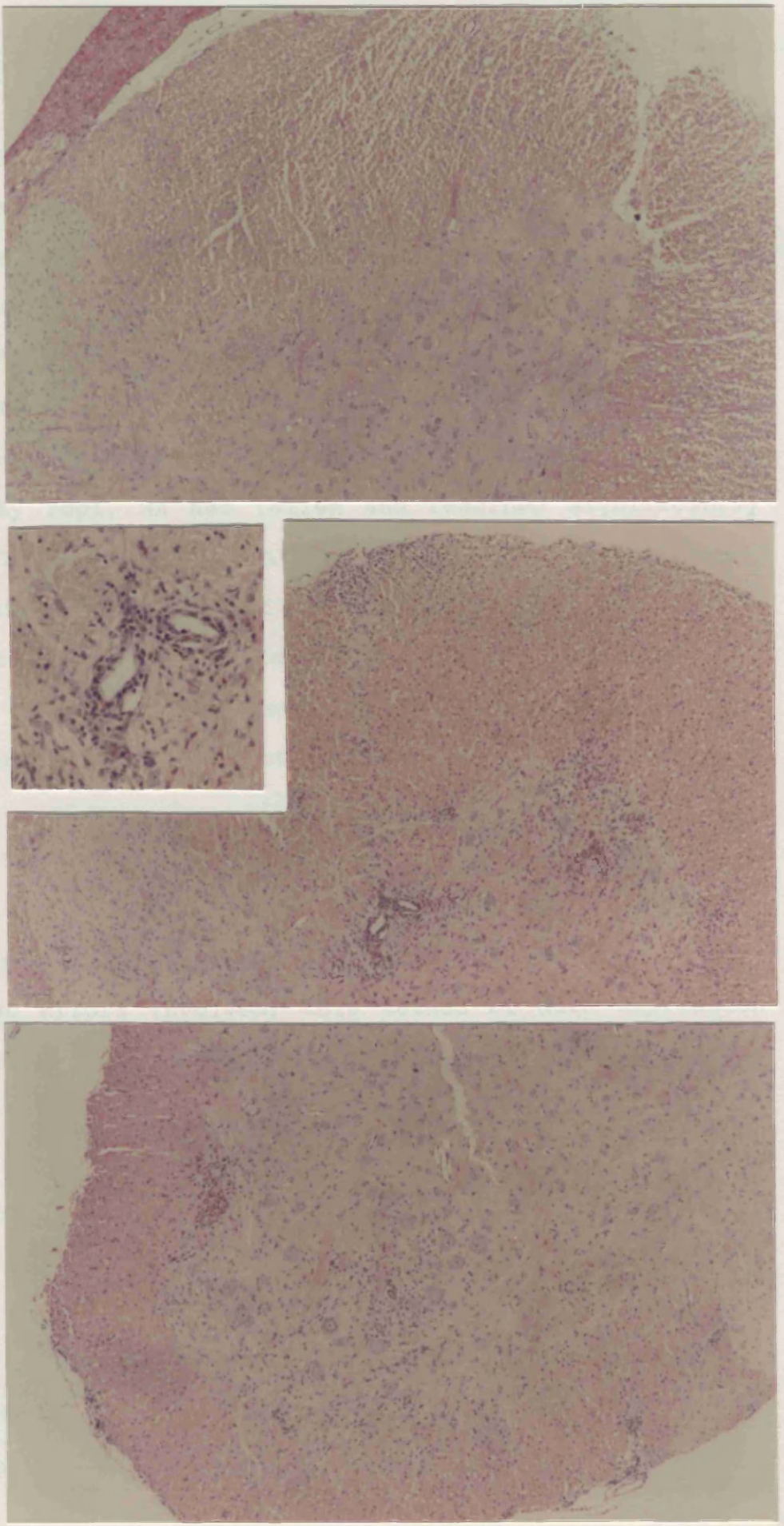
During maximum clinical disease histological examination of sections of brain and spinal cord stained with haematoxylin and eosin revealed extensive perivascular cuffing at the border of the white and gray matter (fig 3.3.4.). Active induction with GPSC in adjuvant resulted in a wide distribution of perivascular cuffs which were evident throughout the brain as well as spinal cord. In comparison, cell infiltration in the transferred model was restricted to the spinal cord. In both instances the number of perivascular cuffs was greatest in the cervical region of the spinal cord. No perivascular cuffs were found in the control group.

FIG 3.3.3.

Clinical course of adoptively transferred EAE.



(A) Typical time course of EAE following the transfer of MBP-sensitized lymphocytes. **(B)** Weight loss associated with the onset of disease expressed as percentage change in body weight from day 4. Each point represents the mean ± s.e.m. from a group of 4 animals.



(A), Control **(B)**, Active EAE (13 d.p.i) and **(C)** Transferred EAE (7 d.p.t.).

3.3.4. Changes in splenic NA during active EAE.

Fig 3.3.5. shows typical HPLC traces for 1pMol mixed catecholamine standards and extracted catecholamines from a rat spleen sample. The extraction method onto alumina (Bouloux et al, 1985) resulted in approximately 40% recovery of the internal standard DHBA.

Changes in splenic NA levels in EAE and CFA animals at various time points after immunization are shown in table 3.3.2. By 3dpi, NA had fallen and remained significantly lower at 7dpi in both the EAE and CFA animals. Animals given an injection of paraffin oil showed no change in NA content (data not shown). In animals with clinical signs of EAE scored 2 or greater, NA levels were found to be increased significantly compared to both unimmunized animals and the CFA controls.

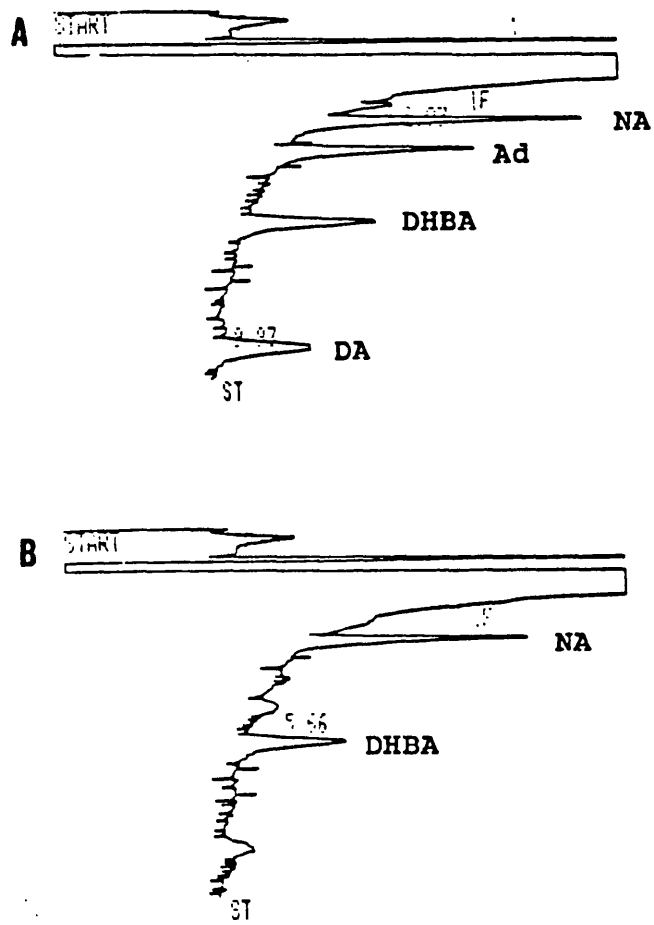
The same trend in splenic NA changes were seen when the data was expressed as NA/10^6 cells, however, because of the smaller number of splenic samples with cell counts and the cumulative errors involved, this method of data expression did not show the same levels of significance.

3.3.5. Changes in Lymphocyte ß-adrenergic receptor number during active EAE.

Three days after immunization with either GPSC inoculum or CFA, the number of splenic lymphocyte ß-adrenergic receptors had almost doubled (fig 3.3.6.). This increase was still present at 7dpi in both groups (table 3.3.3) and was not

FIG 3.3.5.

Typical HPLC traces from mixed catecholamine standards and extracted spleen samples.



(A) HPLC trace from mixed catecholamine standards (1 pMol) of noradrenaline (NA), adrenaline (Ad), 3,4-dihydroxybenzylamine (DHBA) and dopamine (DA) dissolved in 0.5M phosphoric acid.
(B) HPLC trace from an extracted spleen sample in 0.5M phosphoric acid.
Injection volume was 50μl.

TABLE 3.3.2

Changes in splenic noradrenaline content in the pre-clinical and clinical stages of EAE.

Immunization protocol	n	NA (pMol/mg protein)	NA (pMol/ 10^6 cells)
Unimmunized	16	101 ± 9	18.1 ± 3.2
CFA 3 d.p.i.	6	85.5 ± 8 *	13.2 ± 1.2
GPSC 3 d.p.i.	8	77.0 ± 8 *	15.1 ± 1.8
CFA 7 d.p.i.	5	67.6 ± 13 **	13.6 ± 2.7
GPSC 7 d.p.i.	8	79.0 ± 9 **	16.0 ± 1.9
CFA 11-13 d.p.i.	6	58.0 ± 13 **	11.3[b]
GPSC 11-15 d.p.i.[a]	5	196 ± 36 *+	38.6 ± 9 *

Animals were sacrificed on the d.p.i. indicated and the spleens removed and weighed. NA was extracted onto alumina as described in materials and methods and expressed as either NA/pMol mg protein or NA/10^6 cells.

[a] Animals with clinical scores > 2

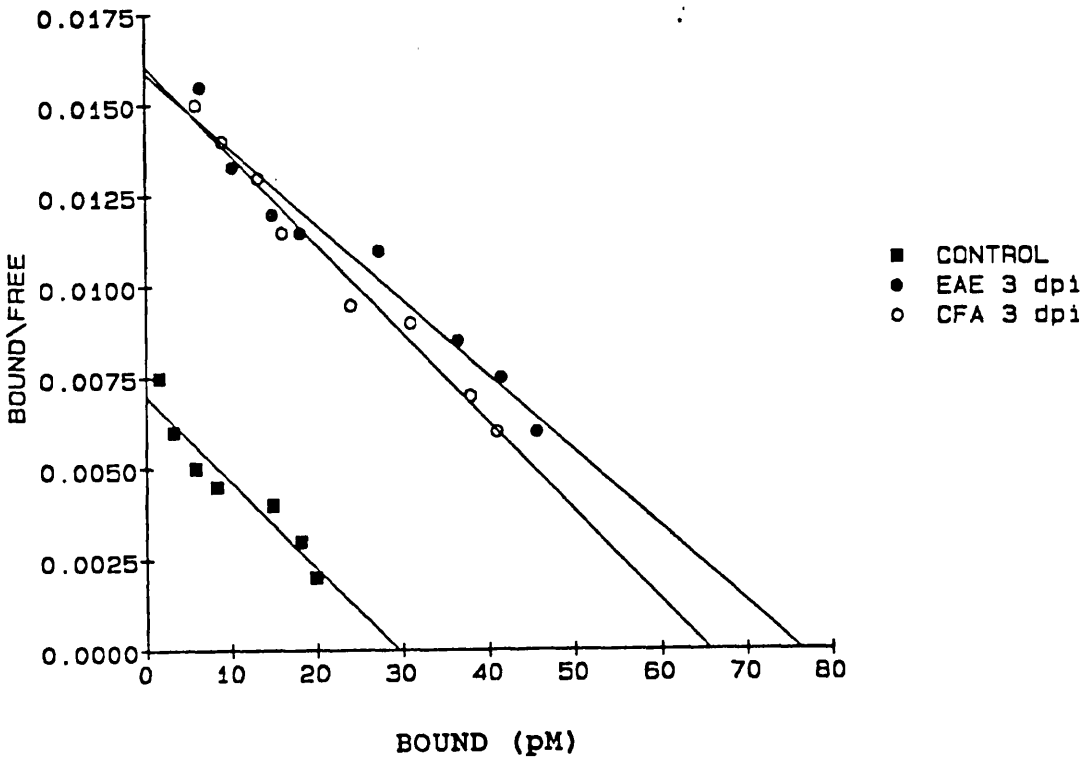
[b] Only two samples, 12.1 and 10.6 respectively, for which cell counts were available.

Difference significant at $P<0.05$ (*) and $P<0.01$ (**) compared to unimmunized animals (unpaired t-test).

Difference significant at $P < 0.01$ (+) compared to CFA animals at 11-13 d.p.i. (unpaired t-test).

FIG 3.3.6.

Representative Scatchard plots of ^{3}H-DHA binding to control and experimental animals 3 days post immunization (d.p.i.).



Rats were immunized with either GPSC inoculum or CFA alone. splenic lymphocyte ß-adrenergic receptor assays were carried out 3 d.p.i and compared to non-immunized controls. Mean figures for the K_d and Bmax are given in table 3.3.3.

TABLE 3.3.3

Changes in splenic lymphocyte ß-adrenergic receptor number in the preclinical and clinical stages of EAE.

Immunization protocol	n	sites/cell[a,b]
Unimmunized	10	5,984 ± 571
CFA 3 d.p.i.	4	10,318 ± 1,381 *
GPSC 3 d.p.i.	6	11,128 ± 1,793 *
CFA 7 d.p.i.	6	12,959 ± 1,792 **
GPSC 7 d.p.i.	7	10,261 ± 1,578 *
CFA 11-13 d.p.i.	4	8,546 ± 2,231
GPSC 11-15 d.p.i.[c]	3	6,191 ± 850

Animals were sacrificed on the d.p.i. indicated and splenic lymphocytes isolated as described in materials and methods. Receptor assays were carried out using the ß-adrenergic antagonist ^{3}H-DHA with a cell concentration of 4 x 10^{6} cells/ml.

[a] Maximum receptor number expressed as sites/cell

[b] The affinity (K_d) was unchanged between control and experimental animals (7.1 ± 1 nM and 8.0 ± 1 nM respectively)

[c] Animals with clinical scores > 2

Difference significant at $P < 0.05$ (*) and $P < 0.01$ (**) compared to unimmunized animals (unpaired t-test).

due to alterations in affinity for the receptor which was unchanged between the control and experimental groups (7.1±1nM and 8.0±1nM respectively). With the onset of clinical signs, the increased receptor number on splenic lymphocytes was no longer evident, however at the equivalent time point in the CFA group (11-13 dpi) ß-adrenergic receptors were still elevated, although the difference was no longer significant.

3.3.6. Plasma corticosterone levels and lymphocyte nuclear glucocorticoid receptors.

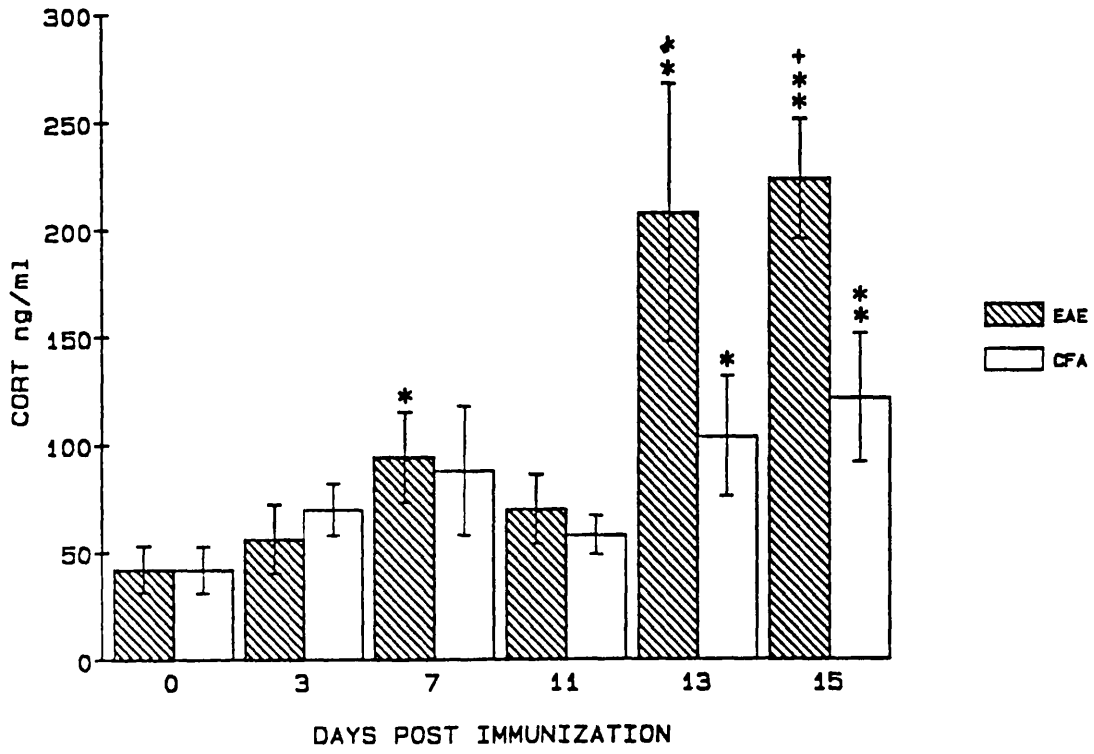
Basal serum corticosterone levels were obtained from non-immunized rats which had undergone the same handling procedure as the experimental animals. All animals were sacrificed at the same time in the morning when corticosterone levels are at the nadir of their circadian variation (De Beer and Van der Gugten, 1987).

Corticosterone levels had risen slightly by 7 dpi (fig 3.3.7.), but only reached significantly higher values in the EAE group. By 13 and 15 dpi serum corticosterone levels were significantly elevated from basal levels in both groups, however at each time point the significance level was greater in the EAE animals than the CFA group and at 15 dpi corticosterone levels in the EAE group were significantly higher than the CFA controls.

Despite these changes seen in serum corticosterone levels, no change in either the affinity or the number of splenic nuclear glucocorticoid receptors could be detected in either the CFA or EAE groups at any of the time points

FIG 3.3.7.

Changes in serum corticosterone[a] during the course of active (EAE).



Animals were immunized with either MBP inoculum or CFA alone. Trunk blood was collected on the days indicated and serum CS determined using a ^{125}I-labelled radioimmunoassay kit.

[a] ± s.e.m.

Difference significant at P<0.05 (*) and P<0.02 (**) compared to unimmunized (day 0) animals.

Difference significant at P<0.02 (+) compared to CFA animals.

studied.

3.3.7. Changes in splenic NA during adoptively transferred EAE.

Three days after the transfer of PPD-sensitized T-cells, splenic NA levels had fallen considerably compared to the unimmunized group and remained lower at 7 dpt (table 3.3.4). No changes preclinically (1 or 3 dpt) were seen in the EAE group, however, by day 7 at the time of maximum clinical score, splenic NA levels were increased significantly. These changes were comparable whether NA was expressed as pMol/mg protein or pMol/mg wet weight. No significant change in plasma NA levels were found during the course of EAE (table 3.3.5.).

3.3.8 Changes in lymphocyte ß-adrenergic receptor number during adoptively transferred EAE.

Increases in lymphocyte ß-adrenergic receptor number of 67% and 50% were detected three days after the transfer of either MBP or PPD sensitized cells respectively, which in the latter group persisted for up to 7 days (table 3.3.6.). With the onset of clinical signs, receptor density returned to within the range of control values. Once again, the affinity of the ligand for the receptor in the control and experimental groups was not significantly different (10.2 ± 1nM and 9.8 ± 0.4 nM respectively).

TABLE 3.3.4

Changes in splenic noradrenaline content in the preclinical and clinical stages of adoptively transferred EAE.

Immunization protocol	n	NA (pMol/mg protein)	NA (pMol/ mg tissue)
Unimmunized	4	115.2 ± 13.6	11.5 ± 1.0
CFA[a]/PPD[b] 3 d.p.t	6	74.4 ± 7.48 **	8.2 ± 0.6 **
MBP /MBP 3 d.p.t.	6	127.2 ± 14.0	12.4 ± 0.9
CFA /PPD 7 d.p.t.	6	87.6 ± 7.00 *	10.2 ± 1.0
MBP /MBP 7 d.p.t.	6	162.7 ± 18.1 **	21.9 ± 2.3 **
CFA /PPD 13 d.p.t.	4	121.6 ± 6.00	10.5 ± 1.3
MBP /MBP 13 d.p.t.	4	129.2 ± 7.60	12.4 ± 1.3

Animals were sacrificed on the d.p.t. indicated and the spleens removed and weighed. NA was extracted onto alumina as described in materials and methods and expressed as either NA/pMol mg protein or NA/mg tissue (± s.e.m.).

[a] Spleen cells were obtained from rats immunized with the indicated inoculum.

[b] Spleen cells were stimulated *in vitro* with the indicated antigen.

Difference significant at $P < 0.05$ (*) and $P < 0.01$ (**) compared to unimmunized animals (unpaired t-test).

TABLE 3.3.5.

Changes in plasma noradrenaline during the preclinical and clinical stages of adoptively transferred EAE.

Collection time	n	NA pg/ml
Day -2	3	152 ± 62[a]
Day 0	3	272 ± 67
Day 1	3	304 ± 61
Day 2	2	220
Day 4	2	234
Day 7[b]	3	324 ± 62[c]

A jugular vein cannula was inserted 1 week prior to the transfer of MBP-primed lymphocytes. Blood samples were taken first thing in the morning between 09-00 and 10-30 hrs.

[a] ± s.e.m.

[b] Samples taken under anaesthesia from animals with clinical signs.

[c] corresponding values for samples taken from control animals under anaesthesia are 248 and 353 pg/ml.

TABLE 3.3.6

Changes in splenic lymphocyte ß-adrenergic receptor number[a] during the preclinical and clinical stages of adoptively transferred EAE.

Immunization protocol	n	sites/cell	
Unimmunized	4	9,777 ± 378	
CFA[b]/PPD[c] 3 d.p.t	6	14,670 ± 478	*
MBP /MBP 3 d.p.t.	6	16,338 ± 1,874	*
CFA /PPD 7 d.p.t.	6	12,451 ± 705	*
MBP /MBP 7 d.p.t.	6	8,746 ± 283	
CFA /PPD 13 d.p.t.	4	8,841 ± 261	
MBP /MBP 13 d.p.t.	4	8,759 ± 386	

Animals were sacrificed on the d.p.t. indicated and splenic lymphocytes isolated as described in materials and methods. Receptor assays were carried out using the ß-adrenergic antagonist ^{3}H-DHA with a cell concentration of 4 x 10^{6} cells/ml.

[a] ± SEM.

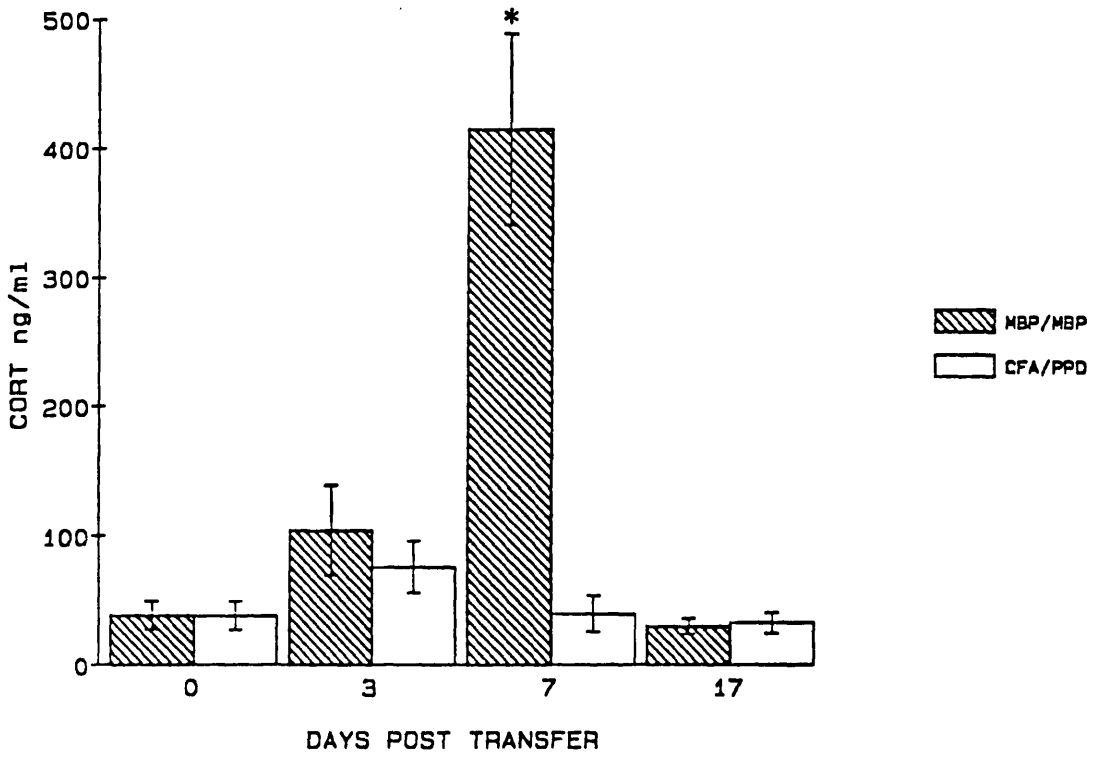
[b] Spleen cells were obtained from rats immunized with the indicated antigen.

[c] Spleen cells were stimulated in vitro with the indicated antigen.

Difference significant at $P<0.01$ (*) compared to unimmunized animals (unpaired t-test).

FIG 3.3.8.

Changes in serum corticosterone[a] during the course of adoptively transferred EAE.



Animals were injected i.p. with either MBP or PPD-sensitized lymphocytes. Trunk blood was collected on the days indicated and serum CS determined using a ^{125}I-labelled radioimmunoassay kit (n = 6-8).

[a] ± s.e.m.

Difference significant at $P<0.01$ (*) compared to unimmunized (day 0) animals.

3.3.9. Changes in serum corticosterone during adoptively transferred EAE.

Similar to the preclinical response in active EAE, an increase in serum CS was detectable in animals receiving either MBP or PPD-sensitized cells (fig 3.3.8.) but did not reach significance.

In the EAE animals showing maximum clinical signs (7 dpt) serum CS levels were found to be approximately 4-fold higher than both the unimmunized animals, and the corresponding controls (PPD-sensitized cells) at the equivalent time point.

3.4 Discussion.

Changes in neural and endocrine parameters which could have a regulatory influence on the progression of the disease were found during the pre-clinical and clinical stages of EAE. Splenic NA levels fell significantly during the period preceeding and coincident with the lymphoproliferative stage of the cell mediated immune response to the mycobacterial components of the Freund's adjuvant which was present in both the GPSC and CFA inoculum (Manie et al, 1985). When rats were given a sham injection of paraffin oil no change in splenic NA was found, ruling out the possibility of injection-induced stress. The time scale of the response was similar to that reported in mice immunized with sheep red blood cells in which the decrease in splenic NA was found to precede the rise in antibody producing cells (Besedovsky et

al, 1979). Both the absolute values and the percentage drop (35-40%), 3-7 days after immunization with GPSC/CFA or CFA alone, are in agreement with the data of Besedovsky et al, indicating that the immunological signals to the sympathetic innervation of the spleen are comparable in humoral and cell mediated reactions. Wesselman et al (1987) found no significant change in splenic NA during the course of EAE in comparison to CFA-treated rats, but did not include pre-inoculation data.

In animals with acute symptoms of EAE, splenic NA levels rose significantly above control values which may be of great relevance since in vitro studies have demonstrated a variety of effects of adrenomimetics on mediators of the immune response (Felten et al, 1987). NA was found to inhibit the gamma-interferon induced major histocompatability complex anitgen class II expression on cultured brain astrocytes in a dose dependent fashion (Frohman et al, 1988). ß-adrenergic agonists down regulate interleukin-2 receptors on human peripheral lymphocytes and inhibit mitogen induced T-cell proliferation (Feldman et al, 1987) and propranolol, a ß-adrenergic antagonist exacerbates EAE in the rat (Brosnan et al, 1985). Thus, the increased concentration of NA in lymphoid organs found during peak clinical signs could provide a local immunosuppressive environment and contribute to recovery from disease.

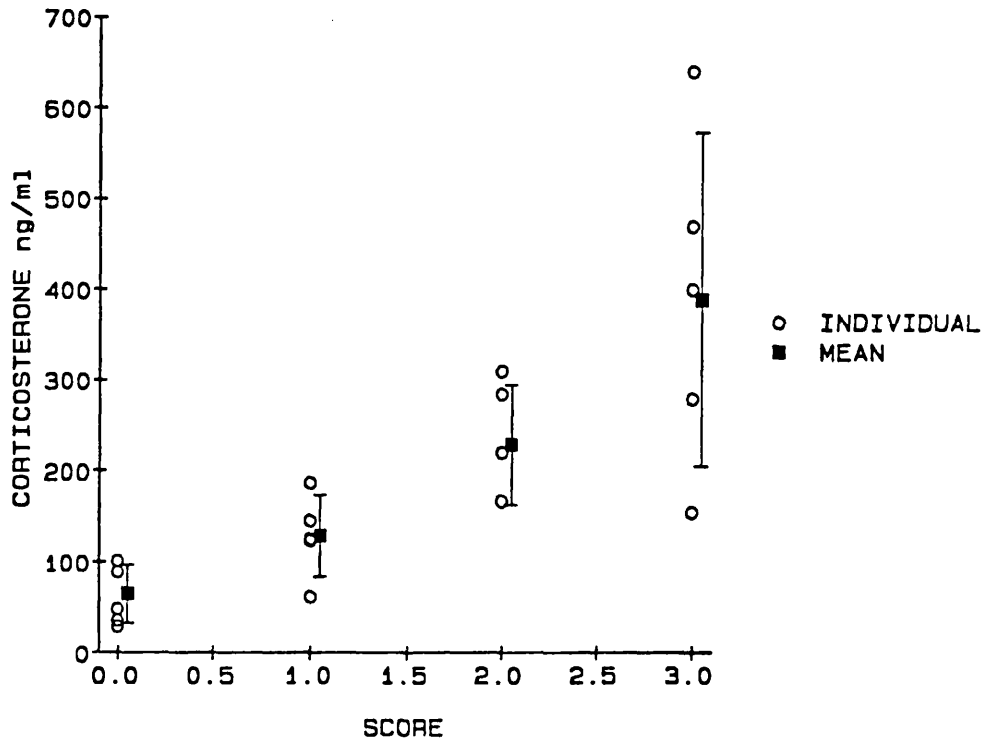
Activation of the pituitary-adrenal axis in response to immune challenge was detectable in both experimental groups 3-7 days after immunization. The observed rise in serum corticosterone was comparable in both magnitude and time scale

to that provoked by antigenic stimuli in other models (Besedovsky, 1975; Schaunstein et al, 1987). Since both antigenic challenge or injection of IL-1 leads to a transient increase in glucocorticoid levels (Bezedovsky and Del Rey, 1987) the present data may well reflect the release of lymphokines associated with lymphoproliferation during the pre-clincial period. It has been proposed that this increase in circulating glucocorticoids during the early stages of an immune response may have a role in limiting the scope of the antigenic challenge (Munk et al, 1984) and may thus be of particular interest in an autoimmune disease model such as EAE. Certainly, the serum glucocorticoid response to immune challenge was found to be absent in an autoimmune strain of chicken (Schauenstein et al, 1987). The stressful symptoms of clinical EAE induce immunosuppressive levels of plasma CS (Levine, 1975) which show a marked correlation with the severity of clinical signs of disease in individual animals (fig 3.4.1.).

The decrease in spleen weight and cellularity in animals immunized with GPSC/CFA probably reflects the trafficking of sensitised lymphocytes out of the spleen, as a reduction in spleen weight was not seen in animals where EAE was induced by the transfer of MBP-sensitised splenocytes. In the animals immunised with CFA alone, serum CS levels were also elevated at the equivalent time period of clinical signs although they were significantly lower than in the EAE group. This is more than likely due to the occurrence of adjuvant induced arthritis in these animals which can be induced by a similar protocol and has been shown to result in increased

FIG 3.4.1.

Correlation between serum corticosterone levels and clinical score in actively induced EAE.



Animals were immunized with MBP/CFA inoculum and sacrificed 13-15 d.p.i. Trunk blood was collected and serum corticosterone measured. The graph shows corticosterone levels in relation to clinical score for individual animals and also the mean ± S.D. for each score.

levels of pituitary proopiomelanocortin mRNA at this time point (Harbuz et al, 1989).

Despite the significant increase in circulating CS, no change in the number of glucocorticoid receptors was detected in either group of animals at any of the time points studied. The sensitivity of the pituitary-adrenal axis to lymphokine secretion may be such that the time frame in which an increase in receptor density could be detected is very limited. A 50% increase in the number of glucocorticoid receptor sites in lymph node cells of adrenalectomised rats immunized with the potent immunogen, keyhole limpet haemocyanin has been reported which was attributed to an increase in the number of lymphoblasts (Crabtree et al, 1980). However, most studies have found it difficult to demonstrate a direct correlation between glucocorticoid sensitivity and either steroid receptor number or affinity (Munck et al, 1979; Naray et al, 1980).

Neural and hormonal modulation of the cell mediated immune response during preclinical and clincial stages of EAE following the the injection of GPSC/CFA appears to operate on two levels. The first represents the response to the general immune challenge and may have a regulatory influence on the extent and duration of lymphocyte proliferation in both EAE and CFA animals, this stage is followed by the onset of disease, the stress of which causes further hormonal and neurotransmitter changes possibly constituting a recovery mechanism (see table 3.4.1. for a comparison of neural and endocrine changes in the two groups).

In order to examine more accurately how interactions

TABLE 3.4.1.

Changes in corticosterone (CS), splenic noradrenaline (NA) and lymphocyte ß-adrenergic receptor number (ß-AR) in EAE and CFA animals.

Time (d.p.i.)		GROUP	
		EAE	CFA
3	CS	↑	↑
	ß-AR	↑↑	↑↑
	NA	↓↓	↓↓
7	CS	↑↑	↑
	ß-AR	↑↑	↑↑↑
	NA	↓↓↓	↓↓↓
11-15	CS	↑↑↑	↑↑
	ß-AR	-	-
	NA	↑↑	↓↓↓

KEY. One arrow: Change but not significant.
Two arrows: $P<0.05$ (unpaired t-test) compared to unimmunized control.
Three arrows: $P<0.01$ (unpaired t-test) compared to unimmunized control.

between the immune, nervous and endocrine systems could influence the immunopathology of disease, EAE was induced by the adoptive transfer of MBP-sensitised spleen cells, where the most immunogenic component of the inoculum, *mycobacterium tuberclulosis* was absent. The splenic noradrenergic and adrenocortical responses during adoptively transferred EAE mirrored in many respects those that occur following sensitisation with GPSC and CFA, despite the absence of the potent primary immune challenge. An increase in serum CS was seen at 3dpt in both the MBP and PPD groups although significance was not reached. It is unlikely that the in vivo production of IL-1 following the transfer of antigen-specific splenocytes, typifying the effector phase of the immune response, will reach systemic levels as high as the administered subpyrogenic dose of recombinant IL-1 which has been shown to significantly elevate CS levels (Kabiersch et al, 1988).

Unlike the early splenic noradrenergic response which was seen in both the GPSC/CFA and CFA animals, an early drop in splenic NA was observed only after the transfer of PPD-primed cells. This may be a result of the greater immunogenic properties of CFA resulting in persisting lymphokine production and enhanced proliferation to PPD in vitro. Certainly the changes in splenic NA and lymphocyte ß-adrenergic receptor number in the animals immunized with CFA alone were longer lasting than in the animals immunized with GPSC/CFA where the preclinical noradrenergic responses may have been curtailed with the onset of clinical signs.

Despite the unchanged NA content of the spleen following the adoptive transfer of MBP-primed cells the density of ß-adrenergic receptors on splenic lymphocytes was increased in both experimental groups within 3 days of inoculation. As this event was dissociated from any changes in splenic NA in the MBP-primed group it would appear that it was not due to a classical upregulation of receptors in response to a fall in neurotransmitter, but more likely a reflection of the expansion of antigen specific cells which at least in vitro results in enhanced expression of ß-adrenergic receptors (Felten et al, 1987).

In summary, changes in neural and endocrine parameters which were seen during the preclinical and clinical stages of EAE induced by GPSC/CFA were still present after the transfer of MBP-primed cells. However in the absence of the highly immunogenic *mycobacterium tuberculosis,* it is unlikely that the primary immune challenge will be as strong, this resulted in reduced splenic noradrenergic and CS responses during the pre-clinical period following the transfer of PPD or MBP primed splenocytes which probably reflects a reduction in lymphokine secretion. However, the neuroendocrine responses associated with the stressful clinical signs were still evident, indicating that these changes are associated specifically with the disease itself and are not dependant on the method of induction.

CHAPTER 4

The effect of in vitro stimulation of lymphocytes on adrenergic receptor number and cytokine production.

4.1 Introduction.

During the early stages of the immune response ß-adrenergic agonists are observed to have an inhibitory effect, the extent of this inhibition may vary depending on the number of receptors on the immune cells. ß-adrenergic receptor density increases on T cells driven to proliferate in vitro by mitogenic lectins. Target organs deprived of normal innervation often up-regulate receptors to compensate for the reduced levels of neurotransmitter, a phenomenon known as denervation hypersensitivity. Conversely, exposure to ß-adrenergic agonists is rapidly followed by a decrease in receptor number and a relative refractoriness to further stimulation (Molinoff and Aarons, 1983; Yu and Hui, 1988).

It is becoming increasingly clear that signals from both the immune and endocrine systems can also influence ß-adrenergic receptor density. Thus both IL-1 and cortisol have been shown to increase ß-adrenergic receptors on cultured human A549 lung tumour cells (Nakane et al, 1990). During the course of EAE an increase in splenic lymphocyte ß-adrenergic receptor number was found during the expected period of lymphoproliferation following immunization with either MBP/CFA or CFA alone, this was also evident following the transfer of MBP or PPD-

sensitized splenocytes. The fact that the increase occurred in all groups suggests that it is a general response to immune challenge and is not specific to the induction of EAE.

The experiments in this chapter were aimed at identifying possible mechanisms responsible for the upregulation of ß-adrenergic receptors and determining their role in EAE by comparing the immunosuppressive properties of corticosterone and isoproterenol on T-cell proliferation and cytokine production.

4.2 Materials and Methods.

4.2.1 Effects of mitogen/antigen or endotoxin stimulation on lymphocyte ß-adrenergic receptor number.

Splenic lymphocytes from unimmunized female Lewis rats were isolated as described in Chapter 2 and incubated at a concentration of 2×10^6/ml in RPMI 1640 Dutch modified medium. Concanavalin A, a T-cell mitogen and the endotoxin lipopolysaccharide (LPS) were added to the flasks at a concentration of 1µg/ml. Immediately prior to and 48hrs after the addition of Con A, ß-adrenergic receptor assays were carried out using the ligand ^{125}I-CYP and the extent of proliferation determined by measuring the incorporation of ^{3}H-Thy into the stimulated cells.

In separate experiments animals were immunized

with 50μl of CFA containing 10mg/ml *Mycobacterium tuberculosis* and 0.1% guinea pig MBP. Control animals received 50μl of CFA alone. After 11 days, both groups of animals were sacrificed and the spleens removed. Splenic lymphocytes were cultured as described above with either 1 or 10μg/ml of guinea-pig MBP or PPD respectively. ß-adrenergic receptor assays were carried out prior to and 48hrs after antigen stimulation.

4.2.2 Effects of mitogen and antigen stimulation on lymphocyte proliferation.

Splenic lymphocytes from animals immunized with CFA/MBP or CFA alone were incubated in 96 well round bottom plates in RPMI 1640 Dutch modified medium at a concentration of 1 x 10^6/ml containing either guinea pig MBP or PPD respectively at concentrations of 1, 10 and 100μg/ml. Splenic lymphocytes from unimmunized animals were incubated under the same conditions with Con A (1μg/ml). Twenty four, 48, 72 and 96 hrs after the addition of the stimulating antigen/mitogen the plates were pulsed with 1μci of ^{3}H-Thy and incubated for a further 6 hrs. The plates were then harvested onto glass fibre filter mats and the dpm determined from each well.

Lymphocyte proliferation assays were carried out as above in the presence of either isoproterenol (ISO), methoxamine (METH), or dexamethasone (10^{-8}-10^{-4}M). The stimulating antigens (MBP or PPD) were added at a

concentration of 10μg/ml, whilst Con A was used at 1 μg/ml. ^{3}H-Thy incorporation was determined after 48hrs in culture.

4.2.3 Production of IL-1 and IL-6 from stimulated splenocytes.

Splenocytes from three control and appropriately immunized animals were pooled and incubated in RPMI 1640 Dutch modified medium at a concentration of 2 x 10^{6}/ml. Con A or LPS (1μg/ml) were added to the controls, MBP or PPD (1 or 10μg/ml) to the pooled cells from immunized animals. 200 μl aliquots were incubated in triplicate in 96 well flat bottom plates for 48 hrs after which time the plates were spun at 900 rpm for 5 mins and the supernatants from the triplicate wells pooled and frozen for subsequent IL-1 and IL-6 determination.

The assay was repeated under the same conditions but with the addition of DEX or ISO (10^{-8}-10^{-4}M) to the Con A or LPS stimulated cell suspensions.

4.2.4 Bioassays for IL-1 and IL-6.

Il-1 levels were determined using the NOB-1 cell line (Gearing et al, 1987) a subclone of the murine thymoma line EL4.6.1. This line secretes IL-2 in response to IL-1 which is then measured using the IL-2-dependent cytotoxic T cell line, CTLL-2, which proliferates in response to IL-2. Proliferation was assessed by ^{3}H-thymidine incorporation

into DNA and the radioactivity measured by scintillation counting. A titration of the internal standard for IL-1 (86/632), in which 1 unit corresponds to 10pg of human IL-1 was included in each assay.

IL-6 levels were determined using the murine hybridoma cell line, B9 (Brakenhoff et al, 1987). This line proliferates in response to IL-6 which is measured by uptake of the tetrazolium salt, MTT (3-(4, 5-dimethylthiazol-2-ys)-2, 5-diphenyl tetrazolium bromide. The formazan dye produced is quantified using a microelisa reader at a wavelength of 620 nm. A titration of the NIBSC reference reagent for IL-6 (88/514) where 1 unit corresponsds to approximately 200pg of human rIL-6 was included in each assay.

4.2.5 Effect of corticosterone and isoproterenol on the adoptive transfer of EAE.

Splenic lymphocytes from donor rats were prepared as described in chapter 3. The three day *in vitro* incubation with guinea-pig MBP was carried out with the addition of either 750ng/ml CS, or 10^{-6}M ISO. After 72hrs in culture the cells were washed and 40 x 10^6 cells transferred to naive recipients which were monitored for clinical signs of disease.

4.3 Results.

4.3.1 Effect of mitogen and antigen stimulation on lymphocyte adrenergic receptor number.

Incubation of splenic lymphocytes *in vitro* with Con A resulted in a doubling of the β-adrenergic receptor number after 48 hrs, which coincided with an increase in cell proliferation. The addition of LPS also resulted in an increase in receptor number but had little effect on the stimulation index (S.I.) (fig 4.3.1.). When splenic lymphocytes from animals immunized with either MBP or CFA were incubated *in vitro* with 1 or 10 μg/ml MBP or PPD respectively, no significant change in receptor number was seen (fig 4.3.2.).

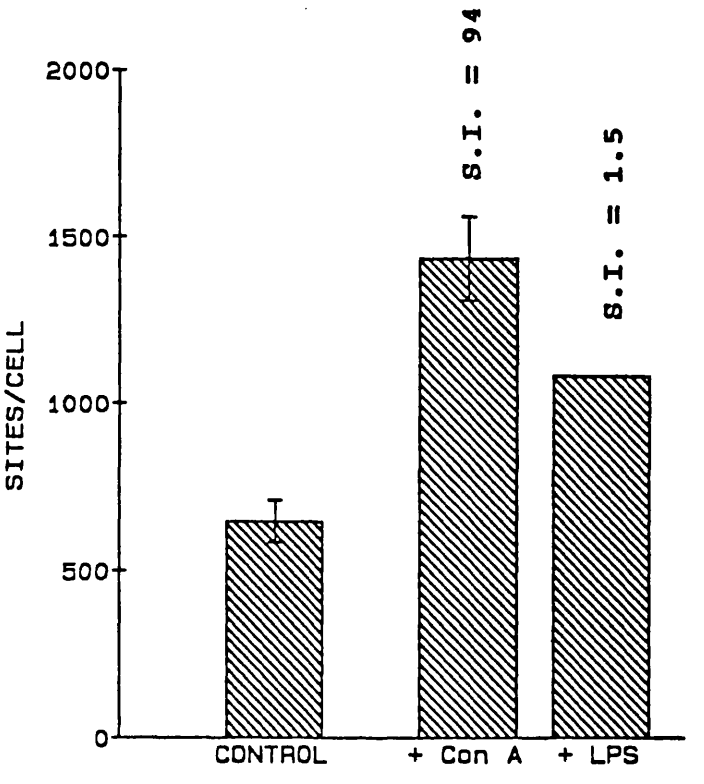
4.3.2 Effect of antigen stimulation on lymphocyte proliferation.

Incubation of lymphocytes sensitized to MBP with either 1, 10 or 100 μg/ml MBP *in vitro* produced very little lymphocyte proliferation at any of the time points studied (table 4.3.1). The S.I. was highest after 48 hrs incubation with 100 μg/ml MBP but still did not reach a value of 2.

Proliferation of cells in response to PPD was greater than for MBP but was still only modest. Again the S.I. was highest after 48 hrs and increased with increasing concentration of stimulating antigen. Incubation with the T-cell mitogen, Con A, produced considerable proliferation

FIG 4.3.1.

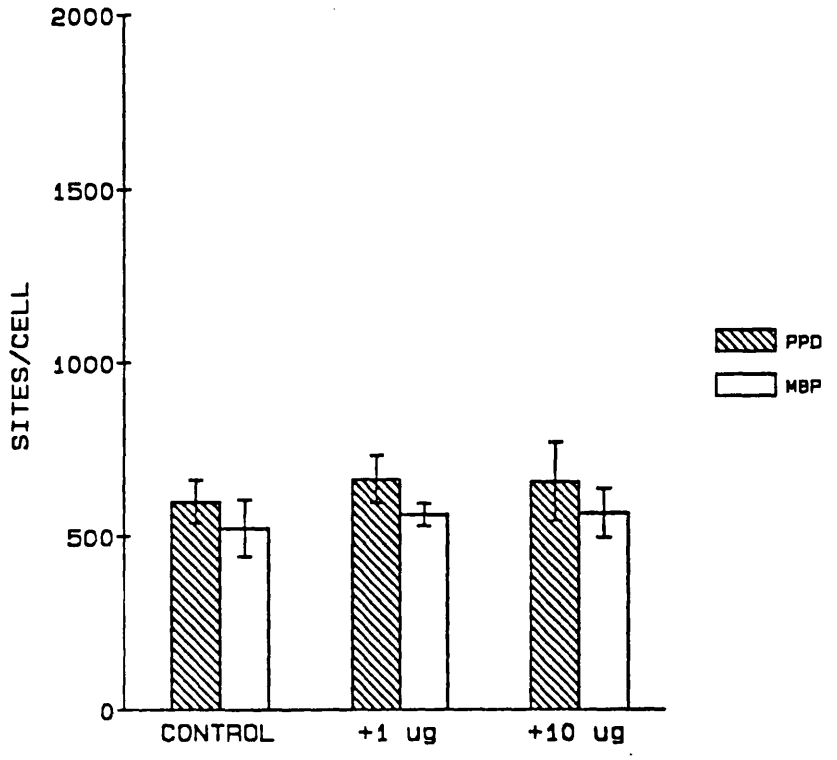
The effect of Con A and LPS stimulation on lymphocyte ß-adrenergic receptor number.



Splenic lymphocytes (2 x 10^6/ml) from unimmunized rats were incubated with either Con A or LPS (1µg/ml) for 48hrs. ß-adrenergic receptor assays were carried out prior to and following the 48hr incubation using the ligand ^{125}I-CYP. The results are expressed as sites/cell ± s.e.m. for the control and Con A groups (n = 3) and the mean of two experiments for LPS. The S.I. for Con A and LPS are shown above each bar.

FIG 4.3.2.

The effect of antigen stimulation on lymphocyte ß-adrenergic receptor number.



Splenic lymphocytes (2 x 10^6/ml) from rats immunized with either MBP inoculum or CFA alone were incubated for 48hrs in vitro with MBP or PPD (1 or 10 μg/ml) respectively. Receptor assays were carried out prior to and following the incubation with the specific antigen using the ligand ^{125}I-CYP. The results are expressed as sites/cell ± s.e.m. (n = 3).

TABLE 4.3.1

The effect of antigen or mitogen stimulation in vitro on lymphocyte proliferation (expressed as stimulation index).

Time (hrs)	Conc.[a] (μg/ml)	PPD[b]	MBP[c]	Con A
24	1	1.2	-	6.46
	10	1.3	1.29	nd[d]
	100	1.55	1.04	nd
48	1	1.26	1.3	91
	10	2.32	1.35	nd
	100	4.59	1.94	nd
72	1	1.05	1.07	123
	10	-	1.16	nd
	100	1.06	1.05	nd
96	1	2.0	1.12	129
	10	1.14	1.24	nd
	100	1.96	-	nd

Splenic lymphocytes (1 x 10^6/ml) from appropriately immunized rats were incubated in vitro in 96 well plates with either MBP, PPD or Con A. After 24, 48, 72 and 96 hrs the plates were pulsed with 1μci of ^{3}H-Thy and incubated for a further 6 hrs after which time the dpm from each well was determined and the stimulation index calculated. (n=3).

[a] Concentration of stimulating antigen/mitogen.

[b] Animals were immunized with CFA and stimulated in vitro with PPD.

[c] Animals were immunized with MBP and stimulated in vitro with MBP.

[d] Not done.

which was detectable after 24 hrs and persisted up to 96 hrs where the S.I. was highest.

Stimulation of splenic lymphocytes by Con A in the presence of CS or ISO (10^{-8}-10^{-4}M) was reduced (table 4.3.2). CS was more effective at blocking proliferation than ISO. The α_1-adrenergic agonist methoxamine had no effect on Con A induced proliferation.

4.3.3 IL-1 and IL-6 production from stimulated lymphocytes.

Basal release of IL-1 from unstimulated cells was undetectable in the assay system employed. The addition of LPS or Con A (1μg/ml) to the cell suspension stimulated the release of both IL-1 and IL-6 (tables 4.3.3 and 4.3.4.). CS was able to inhibit the release of both cytokines in a dose dependent manner, although it had little effect at the lowest concentration used (10^{-8}M).

A clear cut action of ISO on cytokine release was hard to detect. Both IL-1 and IL-6 release appeared to be slightly reduced by ISO however this effect was not obvious until high concentrations of the drug (10^{-4}M) were added to the cell cultures.

IL-1 release from cells incubated with MBP or PPD was undetectable at the original cell concentration used ($2x10^6$/ml). The assay was repeated at a higher cell concentration ($10x10^6$/ml) which enabled basal release of IL-1 to be measured. The addition of either antigen resulted in a dose dependent increase in IL-1 release with PPD

TABLE 4.3.2

The effect of corticosterone (CS), isoproterenol (ISO) and methoxamine (METH) on Con A induced proliferation.

Con A	Competing drug		
+	CS	ISO	METH
0[a]	52	52	22
10^{-8}M	38	50	22
10^{-7}M	30	48	20
10^{-6}M	28	44	21
10^{-5}M	23	36	23
10^{-4}M	13	31	24

Splenic lymphocytes (1 x 10^6/ml) were incubated in vitro in 96 well plates with Con A (1μg/ml) for 48hrs with increasing concentrations of competing drug. Each point for CS, ISO (ß-adrenergic agonist) and METH (α_1-adrenergic agonist) represnts the mean S.I. from four wells. (n=3).

[a] Maximum proliferation for cells incubated with Con A expressed as stimulation index.

TABLE 4.3.3

The effect of corticosterone (CS) and isoproterenol (ISO) on LPS and Con A induced IL-1 secretion (pg/ml).

	LPS		Con A	
	+CS	+ISO	+CS	+ISO
Cells	-[a]	-	-	-
1μg/ml[b]	27	28	35	29
$+10^{-8}$M	21	18	29	19
$+10^{-7}$M	15	25	7	16
$+10^{-6}$M	-	16	-	13
$+10^{-5}$M	-	22	-	20
$+10^{-4}$M	-	10	-	-

Splenocytes from three unimmunized rats were pooled and 2 x 10^6 cells/ml incubated in vitro in 96 well plates with LPS or Con A (1μg/ml) for 48hrs with increasing concentrations of CS or ISO.

[a] Release of IL-1 was undetectable.

[b] Release of IL-1 from cells stimulated with either Con A or LPS (1μg/ml).

TABLE 4.3.4

The effect of corticosterone (CS) and isoproterenol (ISO) on LPS and Con A induced IL-6 secretion (pg/ml).

	LPS		Con A	
	+CS	+ISO	+CS	+ISO
Cells	90	90	70	70
1μg/ml[a]	750	750	450	450
$+10^{-8}$M	350	420	320	350
$+10^{-7}$M	112	550	120	260
$+10^{-6}$M	80	600	60	350
$+10^{-5}$M	-[b]	350	-	350
$+10^{-4}$M	-	90	-	100

Splenocytes from three unimmunized rats were pooled and 2 x 10^6 cells/ml incubated in vitro in 96 well plates with LPS or Con A (1μg/ml) for 48hrs with increasing concentrations of CS or ISO.

[a] Release of IL-6 from cells stimulated with either Con A or LPS (1μg/ml).

[b] IL-1 release was undetectable.

TABLE 4.3.5

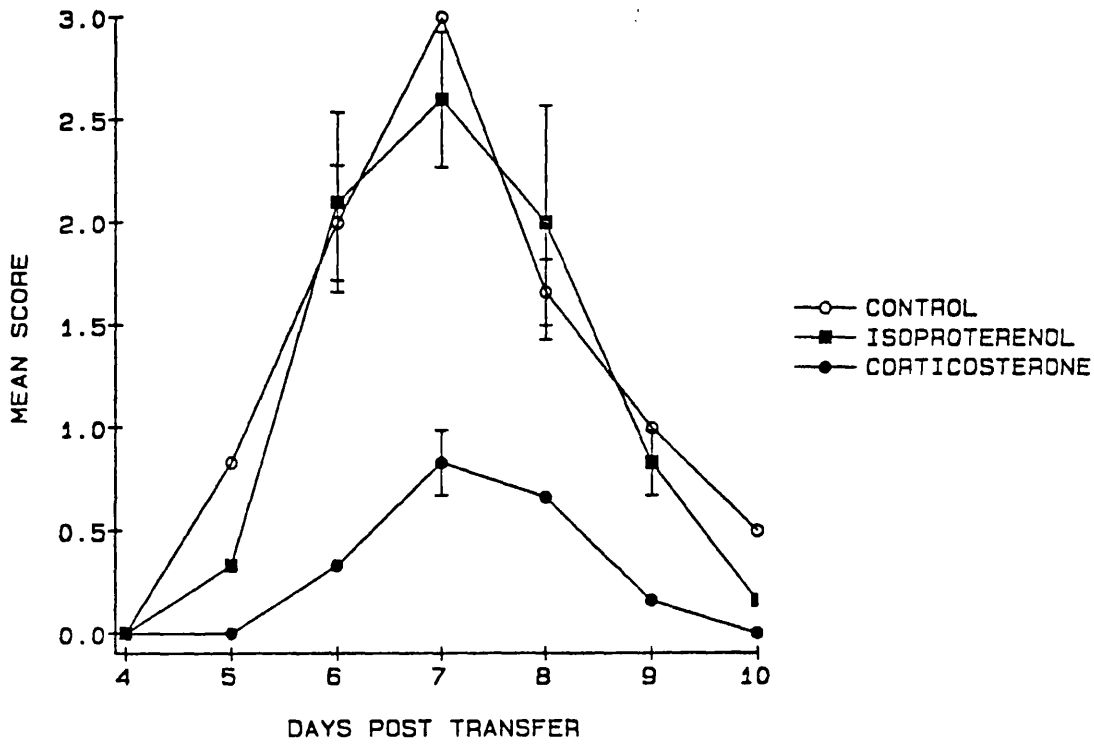
The effect of in vitro MBP and PPD stimulation on splenocyte IL-1 release.

Antigen	Conc.[a] (μg/ml)	IL-1 (pg/ml)
MBP	0	2.0
	1	4.5
	10	8.5
PPD	0	1.5
	1	6.2
	10	14.0

Splenocytes from appropriately immunized animals were pooled and incubated in vitro ($10x10^6$ cells/ml) in 96 well flat bottom plates with either MBP or PPD (1 or 10 μg/ml). 48 hrs after antigen stimulation the plates were spun (900 rpm) and the supernatants from triplicate wells pooled and frozen for subsequent IL-1 determination.

FIG 4.3.3.

The effect of CS and ISO in vitro on the adoptive transfer of EAE.



Splenocytes (2 x 10^6/ml) from rats immunized with MBP inoculum were incubated for 72hrs *in vitro* with MBP (1 μg/ml) plus either CS (2 x 10^{-6}M) or ISO (1 x 10^{-6}M) and the effect on the subsequent clinical course of disease following the transfer of cells studied. The results represent the mean clinical score (± s.e.m.) for each group of rats (n = 3).

having a greater stimulatory effect than MBP (table 4.3.5.). However, taking into account the higher cell concentrations required to detect IL-1, their effects were mild compared to Con A or LPS stimulation. IL-6 was not determined for this set of experiments.

4.3.4 Effects of CS and isoproterenol on the adoptive transfer of EAE

The addition of CS (2×10^{-6}M) during the three day incubation of splenic lymphocytes with guinea pig MBP suppressed the subsequent clinical signs of EAE transfer of cells (40×10^{6}) to naive recipients (fig 4.3.3.). The addition of 1×10^{-6}M isoproterenol to the three day *in vitro* incubation had no effect on the clinical course of disease.

4.4 Discussion

Incubation of splenic lymphocytes with the T-cell mitogen Con A resulted in a doubling of the ß-adrenergic receptor number which was detectable 48 hrs after stimulation. This coincided with a marked increase in cell proliferation where the S.I. was greater than 100. In two experiments it was found that the addition of LPS (1µg/ml) also increased receptor number in the absence of a proliferative response. Thus it would appear that

proliferation is not a prerequisite for the changes seen in receptor number, although upregulation of receptors may accompany proliferation. A likely alternative candidate is the cytokine IL-1 which is produced mainly by macrophages and is associated with the first steps of the immune response (Dinarello et al, 1987). Both Con A and LPS stimulated the release of IL-1 and IL-6 from the mixed splenocyte cell suspension, where macrophages were present during the incubation period but depleted prior to the receptor assay.

As it has been shown that the addition of IL-1 to cultured human A549 lung tumour cells results in a 2-3 fold increase in the number of ß-adrenergic receptors (Nakane et al, 1990), the enhanced production of IL-1 after Con A or LPS stimulation of splenocytes may be responsible for the observed increases. Since the interaction of resting T-cells with antigen-presenting macrophages leads to IL-1 production prior to IL-2 expression and proliferation the change in ß-adrenergic receptor number may be an early reponse to activation.

It is clear from the studies using MBP and PPD stimulated lymphocytes that the pre-clinical change in receptor number is not a result of antigen stimulation <u>in vitro</u> since neither MBP or PPD had any effect on receptor number. However, if IL-1 production and proliferation are responsible for changes in receptor number this finding is not unexpected since PPD and MBP had little stimulatory effect on cell proliferation and only stimulated measurable IL-1 production when high cell and antigen concentrations

were used.

Much is known about the in vitro conditioning steps required for the successful transfer of EAE, for example, the need for macrophages and IL-2 (Panitch, 1980; Oritz-Oritz and Weigle, 1982) and that replication of cells usually accompanies but is not essential for the transfer of disease (Richart et al, 1979; Peters and Hinrich, 1982). Unknown is the fate of the activated cells following transfer to naive animals. There is still a latent period of 4-6 days between the injection of cells and the development of clinical signs which may point to the need for cell replication, differentiation or recruitment of recipient cells. It is during this latent pre-clinical period that an increase in ß-adrenergic receptor number was detected and thus could be the result of local production of IL-1 or the expansion of the sensitized cell population in vivo.

The elevated circulating CS levels seen pre-clinically might also influence ß-adrenergic receptor number. In general, corticosteroids have been shown to potentiate the effect of adrenergic stimulation in several tissues including bronchial smooth muscle, heart, vascular smooth muscle, liver (Foster et al, 1983; Kaumann, 1972; Besse and Bass 1966 and Exton et al, 1972) and also in lymphocytes (Lee and Reed, 1977; Parker et al, 1973). More recently an increase in ß-adrenergic receptors on human lymphocytes and A549 lung tumour cells following hydrocortisone treatment has been reported (Cotecchia and De Blasi, 1984; Nakane et al, 1990). Preliminary

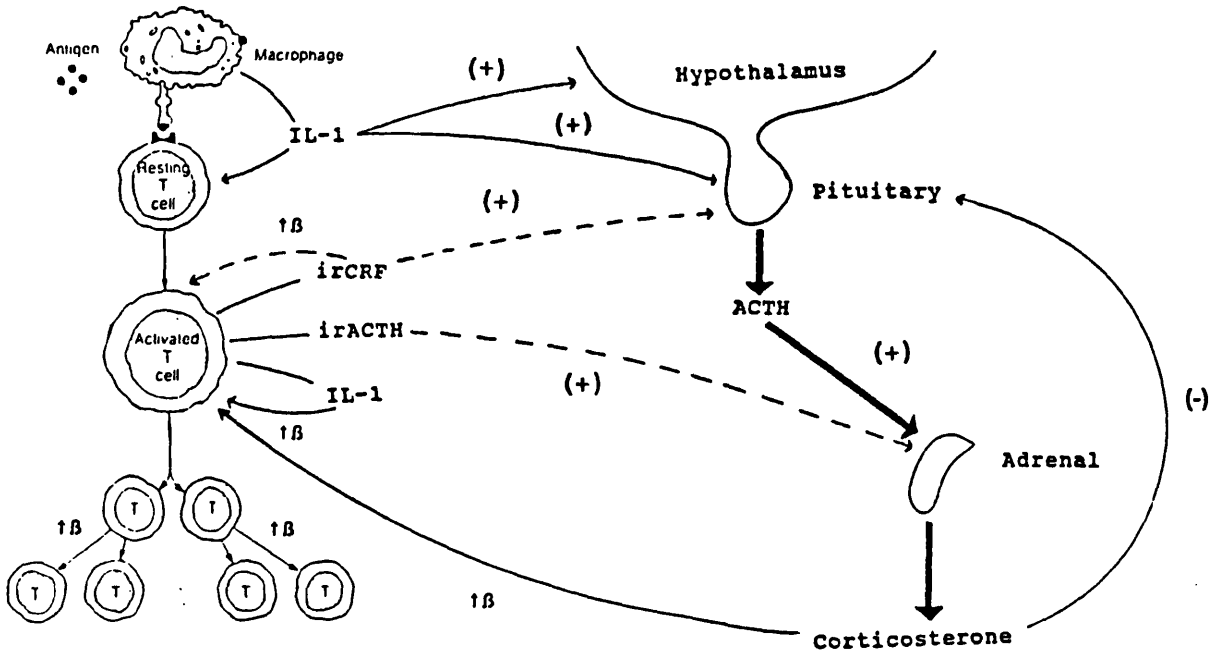
experimental results from this laboratory support this finding with a doubling of ß-adrenergic receptors noted 24hrs after the addition of 10^{-5}M hydrocortisone (Zoukos, unpublished finding).

The search for mediators responsible for the upregulation of lymphocyte ß-adrenergic receptors is further complicated by the finding that lymphocytes themselves can produce immunoreactive POMC and CRF peptides (Smith et al, 1986; Stephanou et al, 1990) which may act either directly on lymphocytes or via the production of CS following activation of the HPA-axis to alter receptor number. The possible factors which may influence ß-adrenergic receptor number during the preclinical stage of disease are summarised in fig 4.4.1.

A comparison of the effects of ISO and CS on cell proliferation and IL-1 production indicated that both have immunosuppressive properties. Glucocorticoids have been shown to block the production of a number of cytokines including IL-1 (Snyder and Unanue, 1982; Staruch and Wood, 1985), IL-2 (Arya et al, 1984; Grabstein et al, 1986) and IL-3 (Culpepper and Lee, 1985). Since these cytokines are essential for cell proliferation it is not surprising that CS effectively blocked mitogen induced proliferation. It is notable that glucocorticoids are less effective if administered after the initiation of an immune response than if given prior to stimulation. Since the actions of cytokines are only partially (Grabstein et al, 1986; Schaffner, 1985) or not at all (Girard et al, 1987; Shen et al, 1986) inhibited by CS, suppression of the immune

FIG 4.4.1.

Possible factors contributing to the elevated ß-adrenergic receptor number found during the pre-clinical stages of EAE.



Lymphoproliferation during the early stages of the immune response results in IL-1 production, activation of T cells and clonal expansion. Activation of the HPA-axis occurs in response to cytokine production and possibly the release of immunoreactive (ir) peptides. Broken lines indicate hypothetical pathways, solid lines represent established pathways which might affect ß-adrenergic receptor number.

response can be attributed to the inhibition of cytokine production.

The effect of ISO on IL-1 and IL-6 production was not obvious, with only a mild inhibitory effect apparent at high concentrations of the drug. The primary source of IL-1 in these cultures would be the resident splenic macrophage population which possess ß-adrenergic receptors (Abrass et al, 1985) and thus might be expected to respond to adrenergic agonists. There is some evidence for a cAMP-dependent inhibitory pathway (Knudson et al, 1986; Brandwein, 1986) but as the addition of cAMP to peritoneal macrophages cultured with LPS did not decrease IL-1 synthesis or secretion (Gilbert and Hoffmann, 1985; Hayari et al, 1985) it's precise role remains to be determined.

ISO was however able to block the proliferation of T-cells to Con A in a dose dependent manner. T-cell activation requires two initial signals that first lead to the expression of IL-2 receptors and then to the initiation of IL-2 synthesis which is followed by proliferation. cAMP has been shown to inhibit IL-2 synthesis by human T-cells (Mary et al, 1987) as well as increasing the rate of IL-2 receptor internalization and decreasing the rate of expression of new IL-2 receptors (Johnson and Smith, 1990). By inhibiting IL-2 receptor expression and IL-2 synthesis ISO was able to block the proliferative response to Con A. This inhibitory effect appears to be mediated through ß-adrenergic receptor activation since methoxamine, an α_1-adrenergic agonist, had no effect on Con A induced proliferation.

The contrasting effects of ISO and CS on IL-1 production and T-cell proliferation may well explain their ability to alter the course of adoptively transferred EAE. Since measurable proliferation to MBP in vitro is not required for the successful transfer of the disease it is not unexpected that ISO, which blocks the progression of T cell proliferation (Lingk et al, 1990), but does not appear to inhibit the production of IL-1 required for the initiation of the immune response, had no effect on the subsequent course of EAE. On the other hand, CS which blocked both IL-1 and IL-6 production as well as proliferation was able to reduce the severity of the disease.

The net effect of the upregulated lymphocyte ß-adrenergic receptor may well be to limit the extent of the immune reaction through the immunuosupressive effects of NA which is elevated in the spleen during the clinical stage of the disease. Preliminary data indicates that the receptors are functional, with an approximate 3-fold increase in cAMP production in response to ISO (10^{-4}M) stimulation in splenic lymphocytes isolated 7 days after immunization with CFA, compared to unimmunized controls (2.1 and 0.75 pmol/10^6 cells respectively). A role for limiting rather than blocking the induction of disease was implicated by the finding that ISO treatment was more effective at reducing the severity of EAE when given 8-14 days after immunization, than when given for the first 7 days of disease (Chelmicka-Schorr et al, 1989).

CHAPTER 5

Alteration in the course of EAE by chemical sympathectomy and adrenalectomy.

5.1 Introduction

The results in Chapter 3 indicate that adoptively transferred EAE provides a well delineated model of autoimmune disease for investigating the immunomodulatory role of the neural and endocrine systems. Activation of the HPA axis pre-clinically may have a regulatory influence on the lymphoproliferative stage and progression of the disease, while the highly significant increases in circulating corticosterone and splenic NA content, both immunosuppressive, during peak clinical signs represents a recovery mechanism.

Recent studies have demonstrated that NA release in the hypothalamus stimulates the production of glucocorticoids. This response can be triggered by a variety of stressors as well as by the cytokine IL-1 (see Chapter 1). Thus there are two periods during the course of EAE when noradrenergic activation of the HPA axis might be expected, pre-clinically as a result of the production of soluble immunological mediators such as IL-1 and clinically when stress induced release of glucocorticoids is known to occur.

Disruption of central or peripheral sympathetic pathways have been shown to alter the clinical course of EAE (Karpus et al 1988; Chelmicka-Schorr et al, 1988) and these effects have been attributed to alterations in ß-adrenergic receptor number or suppressor function of lymphocytes (Miles

et al, 1984; Cross and Roszman, 1988). However, the effect of sympathectomy on the HPA axis was not studied despite the fact that the adrenocortical response is of paramount importance in determining the outcome of EAE since adrenalectomised Lewis rats fail to recover (Levine et al, 1980). Because of the immunomodulatory actions of glucocorticoids and the ability of the central noradrenergic system to activate the HPA axis the relationship between neuroendocrine status and the clinical course of EAE was investigated following adrenalectomy and depletion of noradrenaline either centrally or peripherally.

5.2 Methods and Materials

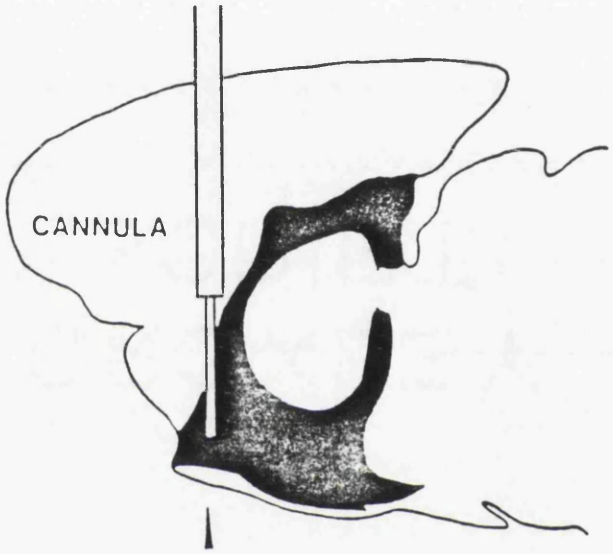
Female Lewis rats 170-220g were used for all the experiments. Animals were housed in pairs, allowed free access to food and water and maintained on a twelve hour light/dark cycle.

5.2.1 Intracerebroventricular (i.c.v.) administration of 6-OHDA.

A 15mg/ml solution of 6-OHDA hydrobromide (Sigma co St Louis) was prepared in 0.9% saline containing 0.1mg/ml ascorbic acid (BDH) as an anti-oxidant. The 6-OHDA was infused into the 3rd ventricle under sagatal anaesthesia (Bregma -0.3mm, ventral = skull surface, -8.5mm; fig 5.2.1) at 3μl/min for a 10min period. This delivered a total of 450μg 6-OHDA into the 3rd ventricle which has been shown to deplete central NA (Nicholson et al, 1986). Control animals received

FIG 5.2.1.

Administration of 6-Hydroxydopamine (6-OHDA) into the anterior 3rd ventricle.



6-OHDA was prepared in 0.9% saline containing 0.1 mg/ml ascorbic acid. The drug was perfused into the anterior 3rd ventricle (bregma -0.3mm, ventral = skull surface -8.5mm) at 3μl/min for 10 min (450μg total). Control animals received an equal volume of saline only.

an equal volume of vehicle injected into the same site. The rats were allowed 10 days to recover before induction of EAE.

5.2.2 Intraperitoneal (i.p.) administration of 6-OHDA (adult).

Adult Lewis rats were injected i.p. daily for 7 days with 6-OHDA dissolved in 0.9% saline containing 1mg/ml ascorbic acid at a dose of 150mg/kg/day. Control animals received i.p. injections of saline and ascorbic acid. MBP sensitised spleen cells were transferred 10 days after the last injection of 6-OHDA.

5.2.3 i.p. administration of 6-OHDA (neonate).

Newborn Lewis rats were injected i.p. for the first 5 days of life with 150 μg/g/day of 6-OHDA containing 1mg/ml ascorbic acid . Control animals were injected i.p. with vehicle only. The rats were housed in pairs for 6-8 weeks before induction of EAE.

5.2.4 Immunization protocol.

MBP-sensitised lymphocytes for the transfer of EAE were prepared as described in Chapter 3. Animals were sacrificed on the appropriate d.p.t., sample collection, cell preparation and receptor assays were as described in Chapters 2 and 3. In addition to this, the hypothalamus was dissected out and stored at -80^{0}C for subsequent HPLC analysis of NA.

5.3 Results.

Maximum clinical signs of EAE were apparent 7 days after the transfer of MBP sensitised splenocytes, at which time point a direct correlation could be made between the clinical score and estimates of tissue and plasma metabolites in individual animals. Direct correlation analysis could not be made in the pre-clinical stage (3dpt) and are thus based on the cumulative score over the whole period of disease (table 5.3.1). Administration of 6-OHDA i.p. to either neonate or adult Lewis rats resulted in greater disease severity which was reflected by the higher cumulative scores when compared to either the relevant sham controls or untreated animals. Depletion of central NA alone following i.c.v. injection of 6-OHDA reduced disease severity (fig 5.3.1). The effect of administration of 6-OHDA either centrally or peripherally was less dramatic than that of bilateral adrenalectomy which resulted in fatal disease.

5.3.1 Sham treatments.

The changes in hypothalamic NA, splenic NA and serum CS during the course of EAE are presented in fig 5.3.2. A similar pattern was noted in all but the peripheral 6-OHDA group. No change in splenic NA was found during the preclinical stage of disease (3dpt) but a significant rise, coinciding with the onset of clinical signs occurred 7dpt.

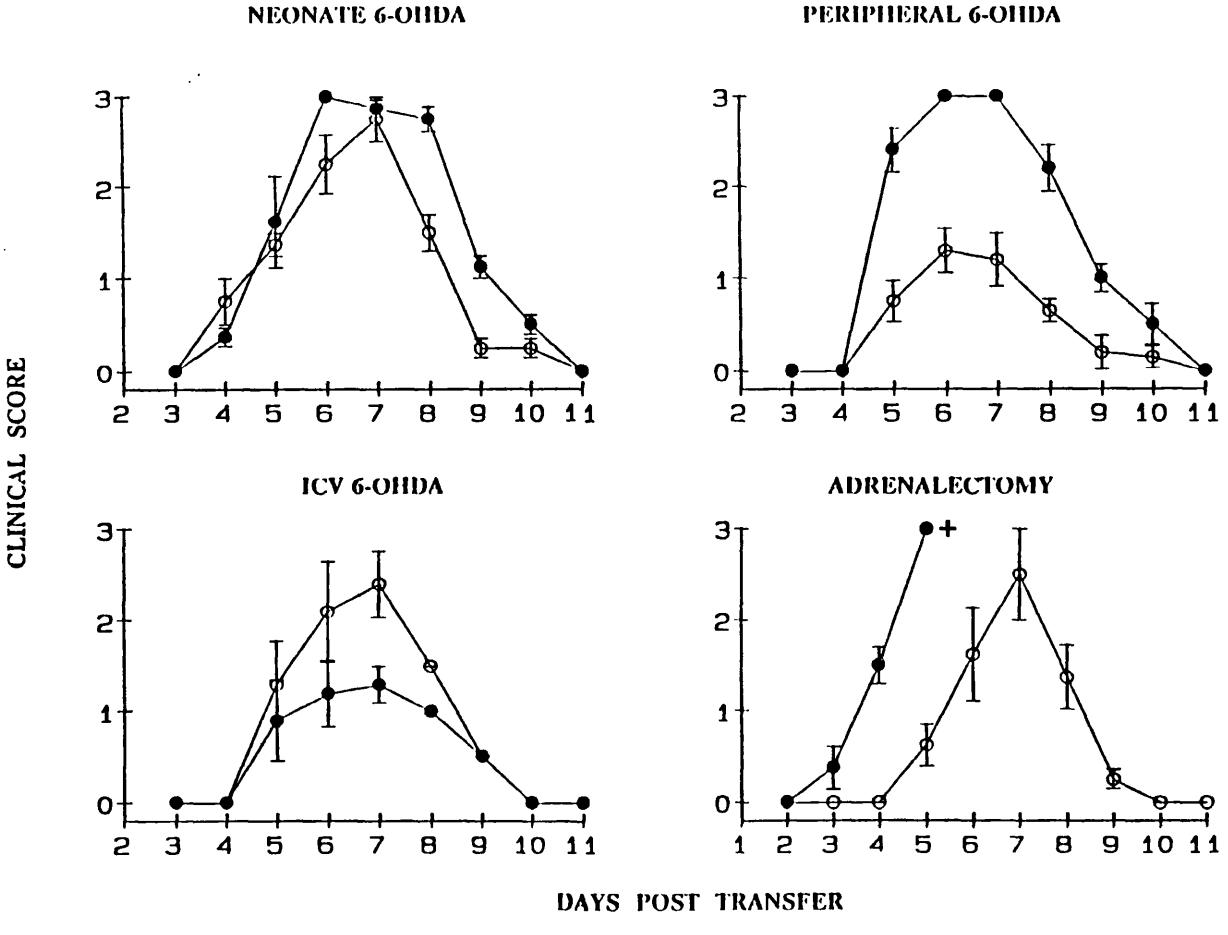
TABLE 5.3.1.

Mean cumulative clinical scores (± s.e.m.) during EAE following adrenalectomy or administration of 6-OHDA prior to disease induction.

Treatment	Mean cumulative clinical score[a]
Untreated controls	8.3 ± 0.65
6-OHDA i.p. (adult)	13.0 ± 1.0*+
Sham control	4.8 ± 2.5
6-OHDA i.p. (neonate)	11.9 ± 0.5*+
Sham control	8.5 ± 0.75
6-OHDA i.c.v.	4.9 ± 0.96*+
Sham control	9.0 ± 0.54
Adrenalectomy	-
Sham control	7.8 ± 0.24

[a] The cumulative clinical scores were calculated by adding up the daily score for individual animals over the whole time period when clinical signs were evident.
Difference significant at $P<0.02$ (*) compared to untreated controls.
Difference significant at $P<0.02$ (+) compared to corresponding sham controls (unpaired t-test, $n = 4-6$).

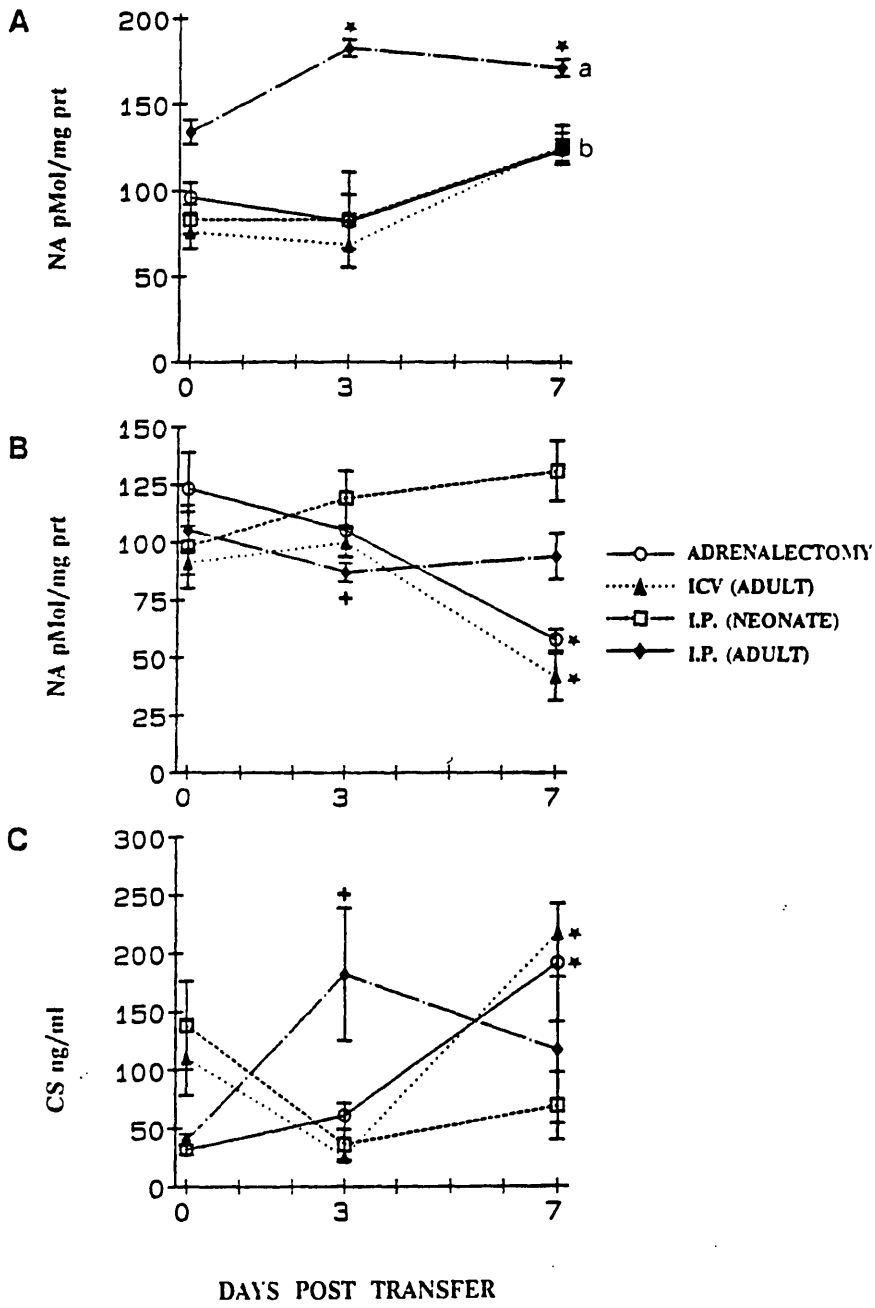
Fig 5.3.1 Clinical course of adoptively transferred EAE following adrenalectomy and i.c.v or i.p. (adult or neonate) administration of 6-OHDA.



6-OHDA (150mg/kg) was administered i.p. for the first 5 days of life (neonate) or daily for 7 days (adult). Depletion of central NA was achieved by a single i.c.v. injection of 6-OHDA (450μg total) into the 3rd ventricle. Bilateral adrenalectomy was performed under Sagatal anaesthesia.

FIG 5.3.2.

Changes in splenic NA (A), hypothalamic NA (B) and serum CS (C) during the course of EAE in the four sham groups.



[a] Difference significant at P<0.01 compared to the other sham groups at 0, 3 and 7 dpt.

[b] Difference significant at P<0.01 compared to day 0 in all three groups.

Difference significant at P<0.05 (+) and P<0.01 (*) compared to day 0 (unpaired t-test).

There was an inverse correlation between hypothalamic NA and serum CS which was most evident at peak clinical signs where serum CS was elevated. Although baseline CS levels prior to cell transfer were variable, at 3dpt all three groups had similar hypothalamic NA content and serum CS levels. The cumulative scores in these sham groups were not significantly different from untreated animals.

5.3.2 Splenic ß-adrenergic receptor number.

Binding of ^{125}I-CYP to intact splenic lymphocytes was saturable over the concentration of ligand used. The k_d was 6pMol which gave a Bmax of approximately 500-600 sites/cell in untreated animals. Splenic lymphocyte ß-adrenergic receptor number was increased at 3dpt in all but the adrenalectomised and sham i.c.v. groups. (table 5.3.2). No correlation between receptor number and splenic NA or serum CS could be found at this time point, indicating that the changes seen were most likely due to early events in the immune response and not a result of neuroendocrine activation. Administration of 6-OHDA i.p. to neonatal rats resulted in almost complete depletion of splenic NA when measured 6-8 weeks later, which caused a significant increase in the baseline receptor number compared to the sham group. Administration of an equivalent dose of 6-OHDA to adult Lewis rats was not as effective at depleting splenic NA and did not produce any significant alteration in receptor number. In the adrenalectomised animals, no change was seen pre-clinically, however, with the onset of clinical signs both splenic NA and ß-adrenergic

TABLE 5.3.2.

Changes in splenic lymphocyte ß-adrenergic receptor number in the preclinical and clinical stages of EAE following adrenalectomy or treatment with 6-OHDA prior to induction.

	Bmax (sites/cell)		
Treatment	0 dpt[a]	3 dpt	7 dpt
6-OHDA i.p. (adult)	657 ± 106	1325 ± 60*	583 ± 60
Sham control	406 ± 86	1169 ± 30*	583 ± 30
6-OHDA i.p. (neonate)	901 ± 70+	2278 ± 246*+	769 ± 104+
Sham control	601 ± 83	1208 ± 125*	393 ± 30
6-OHDA i.c.v.	674 ± 33	1099 ± 122*	1030 ± 120
Sham control	625 ± 70	844 ± 138	843 ± 102
Adrenalectomy	749 ± 60	849 ± 51	1628 ± 92*
Sham control	729 ± 41	1942 ± 92*	1140 ± 51

Lymphocyte ß-adrenergic receptor assays were carried out as described in materials and methods (ch. 2) using the ligand ^{125}I-CYP. The K_d was unchanged between groups (mean = 6.1 pMol).

[a] Days post transfer.

Difference significant at P<0.01 (*) compared to day 0.

Difference significant at P<0.05 (+) compared to sham group (unpaired t-test). n = 4-6.

receptor number increased significantly. This atypical response in receptor changes at 3dpt may be due to the lack of circulating CS which has been shown to increase adrenergic receptors in vitro (Nakane et al, 1990).

5.3.3 Adult and neonatal i.p. administration of 6-OHDA.

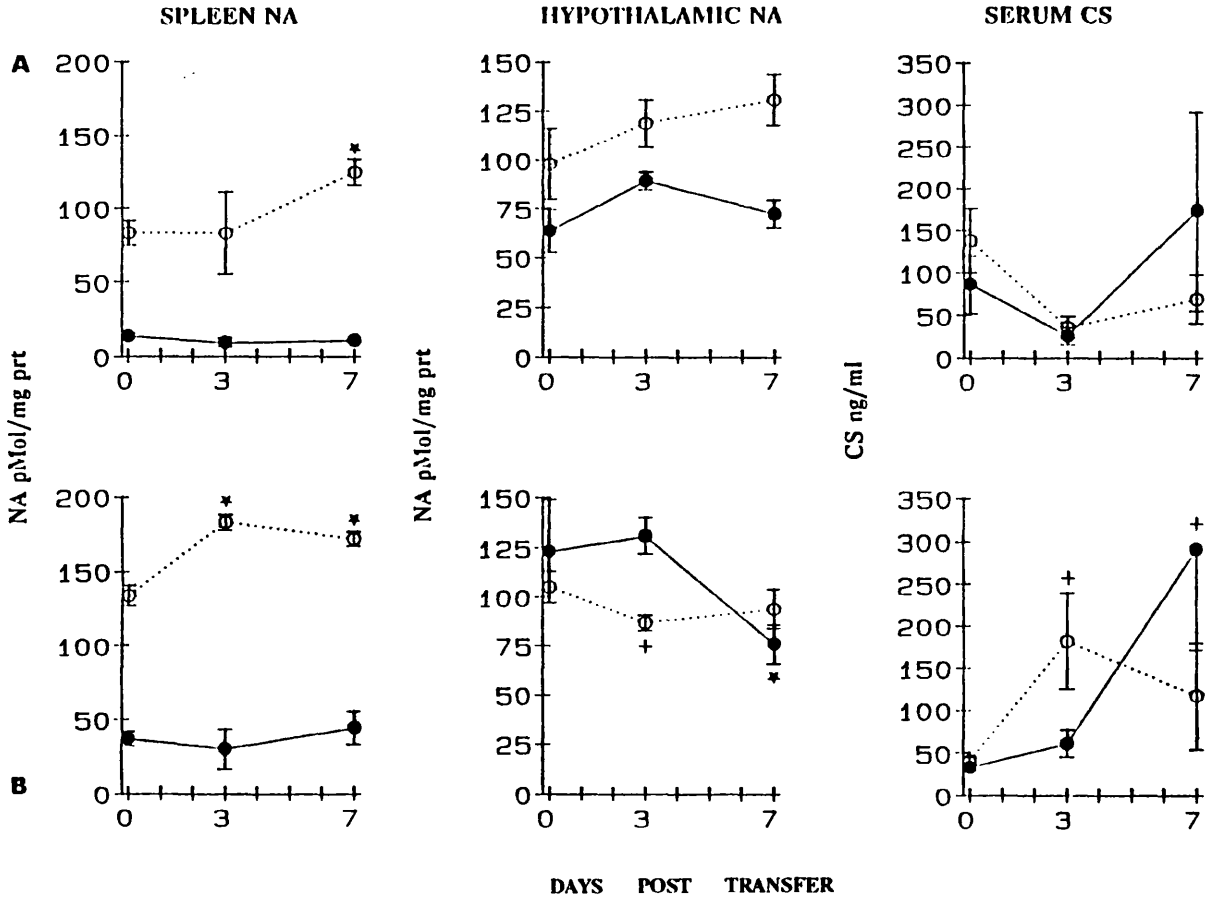
Splenic NA remained depleted and unchanged from day 0 throughout the course of EAE and the cumulative scores, which were higher in both groups compared to shams, were similar. 6-OHDA administration to neonatal rats, where the blood brain barrier is incomplete, did not significantly reduce hypothalamic NA prior to cell transfer, although levels tended to be lower than the sham controls. There was no evidence of depletion of central NA in the adult group given 6-OHDA peripherally.

Serum CS levels prior to cell transfer were higher in the neonate 6-OHDA animals than the adult group, however by 3dpt both groups showed a similar CS response. With the onset of the clinical signs serum CS levels rose which once again coincided with inverse changes in hypothalamic NA (fig 5.3.3) Significance was not reached in the neonate 6-OHDA group although a fall in hypothalamic NA and a rise in serum CS was seen.

5.3.4 i.c.v. administration of 6-OHDA.

A single i.c.v. injection of 6-OHDA into the 3rd ventricle significantly reduced hypothalamic NA but had no

Fig 5.3.3. Changes in splenic NA, hypothalamic NA and serum CS during the course of EAE after prior treatment with 6-OHDA i.p. to neonates (A) or adult rats (B).



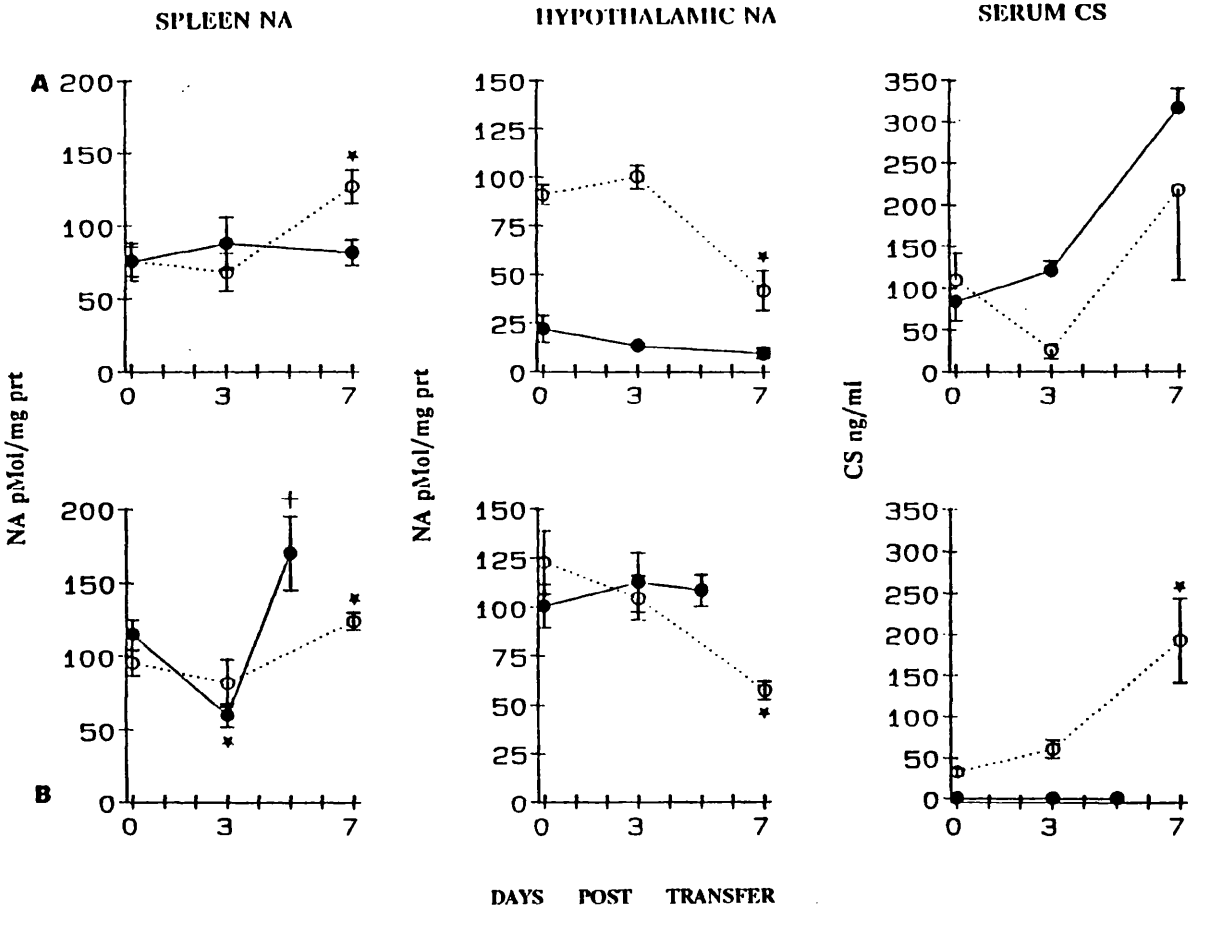
6-OHDA (150mg/kg) was administered i.p. for the first 5 days of life (neonate) or daily for 7 days (adult). Closed circles represent animals treated with 6-OHDA, open circles represent corresponding controls (n = 4-6). Difference significant at P<0.01 (*) and P<0.05 (+) compared to day 0 (unpaired t-test).

effect on peripheral sympathetic nerve terminals in the spleen (fig 5.3.4a). Serum CS levels prior to cell transfer were not significantly different compared to the sham controls although both were higher than normal basal values obtained from untreated animals, which was probably due to the stress of surgery. Hypothalamic NA, although depleted prior to cell transfer, tended to decrease further still during the course of disease and was almost significant at 7dpt (P= 0.06, fig 5.3.5). Serum CS rose during both the pre-clinical and clinical periods of disease reaching higher values at 7dpt than seen in the sham controls, even though clinical signs were milder. No change was seen in splenic NA over the 7 day time period.

5.3.5 Adrenalectomy.

Serum CS levels were below the detection limit of the assay in animals which had undergone bilateral adrenalectomy (fig 5.3.4b). Since the animals did not survive beyond day 6, they were sacrificed at day 5 when all were showing maximum clinical signs. At 3dpt splenic NA fell significantly, this was reversed with the onset of severe paralysis reaching levels significantly higher than either the 0 or day 3 time points. These changes in splenic NA were not due to alterations in spleen weight which remained constant throughout the period of study. Hypothalamic NA was not altered by adrenalectomy and remained unchanged during the clinical course of disease.

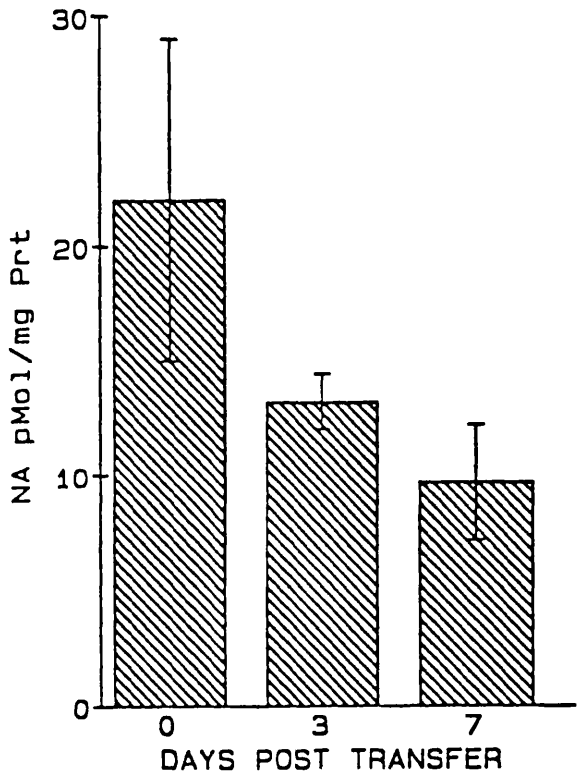
Fig 5.3.4. Changes in splenic NA, hypothalamic NA and serum CS during the course of EAE after prior i.c.v. administration of 6-OHDA (A) and adrenalectomy (B) in adult rats.



6-OHDA (450μg total) was administered by i.c.v. injection. Bilateral adrenalectomy was performed under Sagatal anaesthesia. Closed circles represent treated animals, open circles represent corresponding controls (n = 4-6). Difference significant at P<0.01 (*) and P<0.05 (+) compared to day 0 (unpaired t-test).

FIG 5.3.5.

Changes in hypothalamic NA during EAE following i.c.v. administration of 6-OHDA.



Central NA was depleted by a single i.c.v. injection of 6-OHDA into the 3rd ventricle. The data represents the mean ± s.e.m. of hypothalamic NA after the transfer of MBP-sensitized lymphocytes (n = 4). At 7 dpt P = 0.06.

5.4 Discussion.

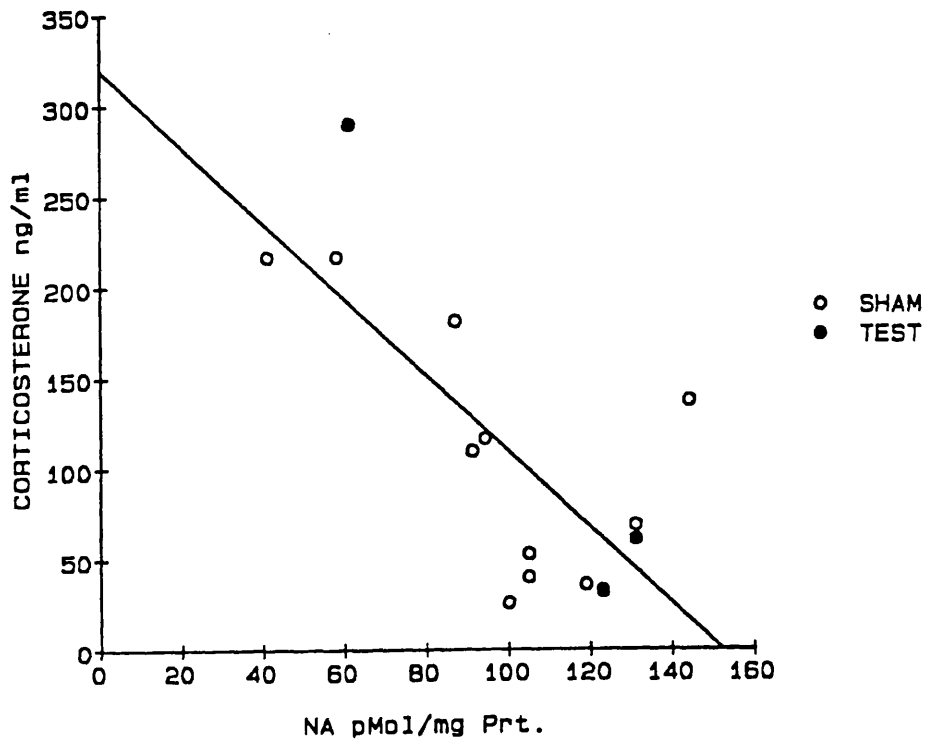
The results in this chapter show a significant correlation between hypothalamic NA content and circulating CS during peak clinical signs of EAE in all the sham control groups and when NA had been depleted only in the peripheral nervous system (fig 5.4.1.). As reported previously in Chapter 3, a positive correlation was found between serum CS and disease severity (fig 5.4.2.). In all experimental animals with intact peripheral and/or central noradrenergic pathways a uniformly increased splenic NA content was also observed at peak disease.

In Chapter 3, a pre-clinical increase in CS was consistently observed during the expected period of lymphokine production in both the actively induced and adoptively transferred models of EAE, as well as their corresponding controls. This early rise in CS was not always apparent and may have been inhibited due to the negative feedback of CS, which was elevated as a result of the stress of the treatments employed. Despite the raised CS levels prior to cell transfer, these animals were not protected from subsequent immunopathology. This is not unexpected since PVG rats which are resistant to EAE (Ben-nun et al, 1982), although having higher basal CS levels than Lewis rats, still develop full clinical signs of disease if adrenalectomised 8 days after immunization (Mason et al, 1990). Thus it would appear that sensitization and proliferation to MBP *in vivo* does occur despite the higher basal CS levels.

Increased hypothalamic NA turnover and activation of

FIG 5.4.1.

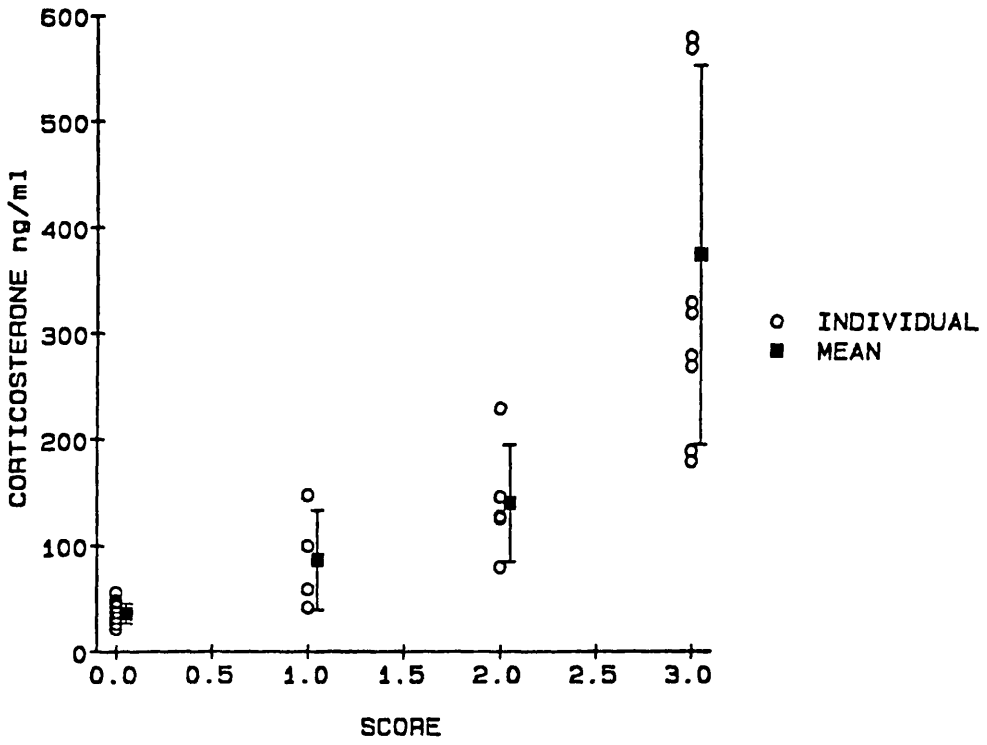
Relationship between hypothalamic NA content and serum CS during the course of EAE.



Correlation between hypothalamic NA and serum CS at 0, 3 and 7 dpt. Open circles represent sham treated animals, closed represent the adult peripheral 6-OHDA group where central noradrenergic pathways were not depleted.
The Pearson product moment correlation coefficient = -0.745 which is significant at $P<0.01$ ($n = 15$).

FIG 5.4.2.

Correlation between serum corticosterone levels and clinical score following the adoptive transfer of EAE.



Trunk blood was collected on various d.p.t. and serum corticosterone measured. The graph shows corticosterone levels in relation to clinical score for individual animals and also the mean ± S.D. for each score.

the HPA axis following administration of IL-1 either intravenously or by i.c.v. injection is well documented (Dunn, 1988 ; Kabiersh et al, 1988). However, in only two of the sham groups (adrenalectomy and peripheral sympathectomy) could a drop in hypothalamic NA, which coincided with increased circulating CS, be detected during the pre-clinical period when IL-1 production would be expected to be at its highest. This may well reflect the discrepancy between data recorded during the local production of cytokines in response to an immune stimulus and that following the pulsed delivery of such mediators. Although a reduction in tissue NA does not always indicate release of transmitter, a reduced NA content and increased MHPG, the primary metabolite of NA, has been reported in the hypothalamus following i.c.v administration of IL-1 (Dunn, 1988).

The results on the effect of sympathectomy are in agreement with previous findings. Depletion of NA in the peripheral or central nervous systems augments or ameliorates the clinical signs of disease respectively (Chelmicka-Shcorr et al, 1988; Karpus et al, 1988). These alterations in the course of EAE were less marked than those resulting from bilateral adrenalectomy (Levine et al, 1980).

Administration of 6-OHDA to neonatal or adult Lewis rats produced a significant depletion of splenic NA alone, although in the former group, hypothalamic NA tended to be lower than controls. In both instances disease severity was increased despite the fact that CS was in a range that suppressed IL-1 production and lymphocyte proliferation <u>in vitro</u> (Chapter 4). Thus it would appear that the rise in

splenic NA levels at peak clinical signs which was found in the sham groups makes a significant contribution to recovery. This is supported by the observation that the immunosuppressive events which follow IL-1 infusion into the CNS were more marked in splenic lymphocytes, where there is a rich sympathetic input, than in circulating blood lymphocytes. Furthermore, although IL-1 infusion caused an increase in circulating CS, suppression of immune responses were still evident after IL-1 infusion into adrenalectomised rats (Sundar et al, 1989). Since NA and other ß-adrenergic agonists suppress lymphocyte stimulation (Li et al, 1990) and chemical sympathectomy reduces the number of splenic T-suppressor cells (Cross and Roszman, 1988), the increased disease severity seen after peripheral sympathectomy may be attributable to removal of the direct immunosuppressive influence of the sympathetic nervous system and not to a reduction in circulating CS.

A single i.c.v. injection of 6-OHDA into the 3rd ventricle resulted in an 80% reduction of hypothalamic NA which modified the subsequent clinical severity of EAE. There is supporting evidence for both a stimulatory and inhibitory effect of central NA depletion on circulating glucocorticoid levels (Szatarzyk et al, 1985; Cross et al, 1980). A comparison of CS levels after i.c.v. administration of 6-OHDA or saline in these experiments showed no significant difference between the two groups. Serum CS levels rose pre-clinically in the treated group following cell transfer reaching a peak at day 7, which was higher than that seen in the sham group, despite the milder clinical disease and the 80% reduction in hypothalamic NA content. Whether or not the

remaining 20% of intact noradrenergic nerve fibres are able to maintain extracellular fluid (ECF) levels of NA and respond to stimulation is not known. It has however been shown by microdialysis that ECF concentrations of dopamine are maintained after extensive 6-OHDA depletion of the transmitter and that given the same stimulus the dopaminergic response from intact and depleted tissues are similar (Touchet and Bennett Jr, 1989). An increase in tyrosine hydroxylase activity and DOPAC content following 6-OHDA administration has also been reported (Onn et al, 1986) suggesting an increase in synthesis and release of DA from residual terminals that may serve to compensate for the damage to dopaminergic neurons. Compensatory mechanisms are also implicated in Parkinson's disease where it is generally accepted that loss of dopaminergic neurons needs to reach a critical level (70-80%) before Parkinsonian symptoms become apparent. (Bernheimer et al, 1973; Hornykiowicz, 1982 Birkmayer and Riederer, 1983).

Compensatory adrenergic hyperactivity has also been demonstrated in the sympathetic nervous system after extensive 6-OHDA induced sympathectomy (Mueller and Thoenen, 1969). Similarly increased catecholamine turnover has been demonstrated in central noradrenergic systems after 6-OHDA lesions (Agid et al, 1973; Johnson et al, 1979, Acheson et al, 1980).

Alpha-adrenergic receptors have been strongly implicated in activation of the HPA-axis (for review see Al-Damluji. 1988) however, there is also evidence for stimulation through ß-adrenergic receptor activation (Morton et al, 1990), the density of which increases in the hypothalamus after

i.c.v. adminstration of 6-OHDA (Johnson et al, 1989). If depleted tissue is able to maintain ECF levels of NA and respond to stimulation, it is possible that due to upregulated post-synaptic receptors, activation of the HPA-axis will be enhanced. Certainly, pretreatment with 6-OHDA i.c.v. has been shown to facillitate the action of a subsequent injection of NA on the stimulation of CS release suggesting the development of denervation hypersensitivity (Kawa et al, 1978).

It is surprising that most studies on sympathetically mediated CS release have concentrated on NA since i.c.v. infusion of adrenaline (Ad), which has a greater affinity for α-adrenergic receptors, results in a 10-fold increase in hypothalamic CRF-41 release and a long lasting stimulatory effect on ACTH secretion (Barbanel et al, 1989). The lack of information on the role of central adrenergic pathways may be due in part to the low levels of the transmitter in the brain. However, the terminals capable of producing Ad have been mapped using antibodies prepared against the enzyme that converts NA to Ad, phenylethanolamine-N-methyltransferase (PNMT) (Koslow and Schlump, 1974). These studies demonstrated that Ad containing neurones project upwards from the brainstem into the hypothalamus (Hokfelt et al 1974). The determination of a functional role is further complicated by problems in finding pharmacological agents selective for adrenergic neurones, since with the exception of PNMT inhibitors, drugs affecting the adrenergic neurone also affect noradrenergic neurones (Routledge and Marsden, 1987).

In a separate experiment, hypothalamic Ad, as well as NA was found to be reduced during the stressful period of

clinical paralysis where circulating CS levels were elevated. The changes in hypothalamic transmitter levels appear to be specific for NA and Ad since no change in hypothalamic DA was found during the course of EAE (tab 5.4.1.). Injections i.c.v. of 6-OHDA at a similar dose to that used in these studies seemed to spare adrenergic neurones since adrenaline levels were only depleted by 46% compared to NA which was depleted by 84% (Nicholson et al, 1986). Thus it is possible that activation of the HPA axis which occurs following central administration of 6-OHDA is due to the remaining adrenergic neurones in the hypothalamus.

Despite the wealth of information available, controversy still surrounds the role of central catecholamines in the regulation of the HPA axis. The experiments in this chapter were not aimed at resolving this issue. Since reduced tissue levels cannot be assumed indicative of transmitter release an inhibitory role for sympathetic neurones cannot be ruled out. However, the significant correlation between hypothalamic NA, serum CS and disease severity suggests a regulatory role for hypothalamic sympathetic neurones during the course of EAE which may affect the outcome of the disease.

The overriding immunoregulatory influence of glucocorticoids was demonstrated by the rapid onset of clinical EAE and morbidity in adrenalectomised animals. However, splenic NA and lymphocyte ß-adrenergic receptor number were both increased during the peak clinical stage prior to death, possibly a compensatory mechanism for the loss of CS. A lack of a noradrenergic response in the hypothalamus is puzzling as continuous stimulation of the HPA-axis would be

TABLE 5.4.1.

Changes in hypothalamic transmitter content during the course of adoptively transferred EAE.

	Transmitter		
Time	**Noradrenaline**	**Adrenaline**	**Dopamine**
Untreated	123 ± 26	8.5 ± 1.5	38.9 ± 5.0
EAE 3dpt	131 ± 9.0	8.4 ± 0.9	40.1 ± 4.5
EAE 7dpt	75 ± 8.2*	2.8 ± 0.8*	41.6 ± 3

Hypothalamic transmitter levels were determined by HPLC in control (untreated) and EAE animals during the pre-clinical and clinical stages of disease. (n=4).
Difference significant at $P<0.01$ compared to untreated animals (unpaired t-test).
Mean clinical score at 7 dpt was 2.75.

predicted if feedback inhibition is mediated via circulating CS. However, since both CRF and POMC mRNA are increased approximately 7 days after adrenalectomy (Beyer et al, 1988), it is possible that the regulation of central NA release is mediated higher up the HPA axis and already inhibitied prior to cell transfer.

In summary the results in this chapter demonstrate a pivotal role for hypothalamic noradrenergic pathways in regulation of the HPA axis at the time of diease induced stress in EAE and also a possible involvement during the early stages of immune activation. The important immunomodulatory role of both noradrenaline and glucocorticoids is highlighted by examination of the mild disease course in the sham group for the animals dosed peripherally with 6-OHDA. In this group splenic NA was significantly elevated at all time points whilst at 3dpt, hypothalamic NA had fallen, which coincided with elevated circulating CS. There is no explanation for these atypical responses but it does demonstrate how neural and endocrine parameters can interact to alter the course of an immune reaction. Although sympathectomy had significant bearing on the clinical course of the autoimmune mediated diease EAE, the immunoregulatory properties of glucocorticoids clearly outweigh those of catecholamines. However, the good correlation demonstrated between hypothlamic NA and circulating CS indicates that immunosuppression may ultimately be controlled by central sympathetic regulation of the HPA-axis.

CHAPTER 6

Activation of the HPA-axis by central sympathetic pathways.

6.1 Introduction.

An increase in circulating corticosterone was evident during both the pre-clinical and clinical stages of EAE although the level at which activation of the HPA-axis occurs was not resolved. The data presented in this chapter is from preliminary experiments designed to try and confirm these changes and determine the site of activation by measuring hypothalamic CRF and pituitary proopiomelanocortin (POMC) mRNA during the course of EAE using *in situ* hybridization.

In Chapter 5 a strong correlation between changes in hypothalamic NA content and serum CS was found especially during the stressful clinical stage of disease when circulating levels of CS were elevated. Although the results were not able to confirm a precise role for NA, the significant correlation seen implicated hypothalamic NA/Ad metabolism as a possible factor in regulating the severity of disease. For this reason centrally acting α_2-adrenergic drugs were administered i.p. to animals with EAE to see if the course of disease was altered. Clonidine which is primarily an α_2-agonist was used to block central release of NA/Ad by its action on presynaptic receptors whilst yohimbine, an antagonist of the same receptor was used to increase transmitter release (Rossetti et al, 1989). The advantage of using these drugs is that they do not differentiate between adrenergic and noradrenergic neurons (Routledge and Marsden,

1987) and so equally affect both central NA and Ad release. As a preliminary test into the role of central sympathetic pathways in EAE this is beneficial since the drugs will not be selective for specific post-synaptic receptors (α or ß).

Finally, the feasibility of using *in vivo* microdialysis to study hypothalamic NA release has been explored in freely moving rats.

6.2. Analysis of hypothalamic CRF and pituitary POMC mRNA by in situ hybridization.

6.2.1 Materials and methods.

The transfer of MBP and PPD primed splenocytes was identical to that described in Chapter 3. After the transfer of appropriately primed cells, animals were killed by decapitation on days 3 (pre-clinical), 7 (clinical symptoms) and day 14 (recovery). Trunk blood was collected for corticosterone analysis and the pituitary and brain rapidly removed and frozen on dry ice for subsequent determination of POMC and CRF mRNA by *in situ* hybridization.

In situ hyridization histochemistry was performed by Dr M. Harbuz of the Neuroendocrinolgy Unit, Charing Cross Hospital. Briefly, the sections were warmed to room temperature, fixed in 4% (v/v) formaldehyde, washed in phosphate-buffered saline and placed in 0.25% (v/v) acetic anhydride in 0.1 mol triethanolamine/1-0.9% (w/v) NaCl to reduce non-specific binding and passed through 70, 80, 95 and 100% ethanol, 100% chloroform and 100 and 95% ethanol, before

drying in air. The probes were synthetic 48-base oligonucleotides, complementary to part of the exonic mRNA sequences coding for CRF and POMC. The probes were labelled using terminal deoxytransferase to add a ^{35}S-labelled deoxyATP (1000 Ci/mmol) tail to the 3' end of the probe. The specific activities of the probes were 1.17×10^{15} and 8.79×10^{14} d.p.m./mol for CRF and POMC respectively. All control and experimental sections were hybridized in the same incubation reaction. The probe (in 45 μl hybridization buffer) was applied to each section and left overnight at 37^{0}C for the hybridization. The sections were then washed in four 15-min changes of NaCl (0.15 mol/l)-sodium citrate (0.015 mol/l) buffer (SSC; pH 7.2) at 55^{0}C followed by two changes of SSC for 1h each at room temperature to remove non-specifically bound probe. The sections were then rinsed in water, dried and apposed to Hyperfilm MP autoradiography film (Amersham International plc, Amersham, Bucks, UK) for an appropriate exposure time. The optical density of the autoradiographic images was measured using an image analysis system (Kontron/Zeiss Electronics Ltd, Watford, Herts, UK). The optical densities were converted to copies of probe hybridized/μm^3 tissue using ^{35}S-labelled standards and the minimum number of copies of probe calculated.

The results are presented as means ± s.e.m. and are expressed as minimum copies of probe hybridized per section. Statistical analysis was performed by comparing control and treatment means using Dunnett's test following one-way analysis of variance. Values of $P<0.05$ were considered significant.

6.2.2. Results.

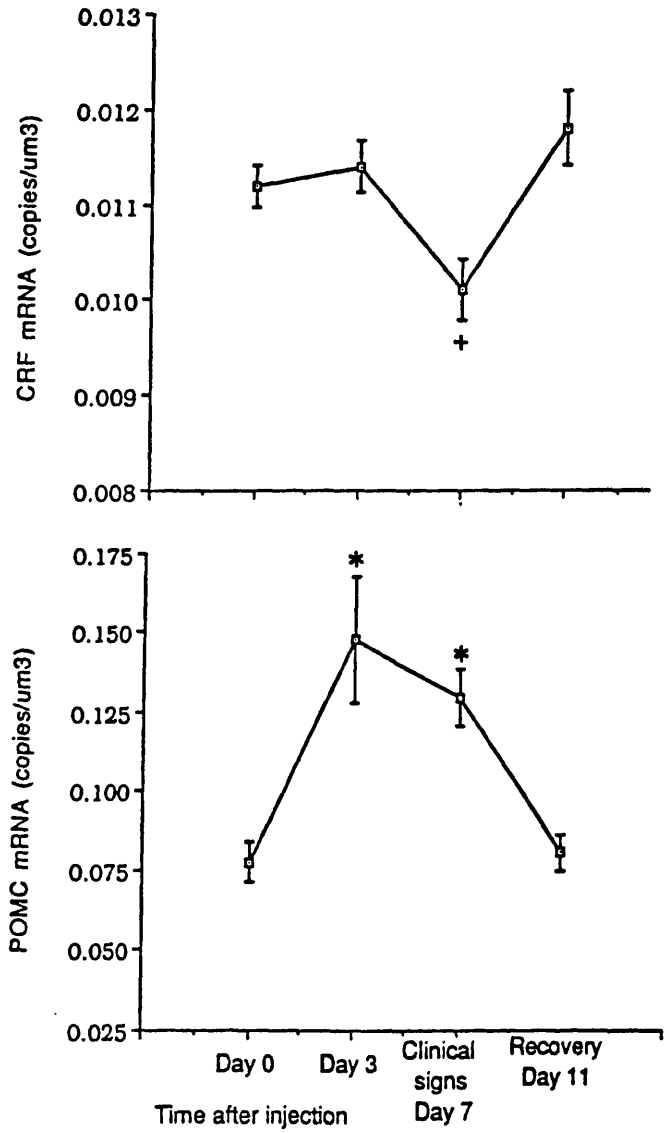
Three days after the transfer of MBP-primed splenocytes pituitary POMC mRNA and serum CS levels were significantly increased (figs 6.2.1 and 6.2.2 respectively). However, no change in hypothalamic CRF mRNA was found. POMC mRNA and circulating CS remained elevated with the onset of clinical disease at which point hypothalamic CRF mRNA was found to be significantly reduced compared to pretransfer levels.

6.2.3. Discussion.

Following the adoptive transfer of MBP-sensitized splenocytes a pre-clinical rise in pituitary POMC mRNA was found which coincided with elevated circulating CS during the expected period of lymphoproliferation and IL-1 production . Despite the large standard deviation in the CS values the increase was significant compared to unimmunized control animals. Changes in pituitary POMC mRNA were not accompanied by increases in hypothalamic CRF which suggests that the increase in POMC is either driven by a different hypothalamic peptide such as vasopressin or that the CS response is initiated at the level of the pituitary. It has been suggested that IL-1 may bind directly to anterior pituitary cells and cause ACTH release by a direct action (Woloski et al, 1985; Bernton et al, 1987; Kehrer et al, 1988) although other studies have not supported these findings (Berkenbosch et al, 1987; Sapolsky et al, 1987). Dense IL-1ß

FIG 6.2.1.

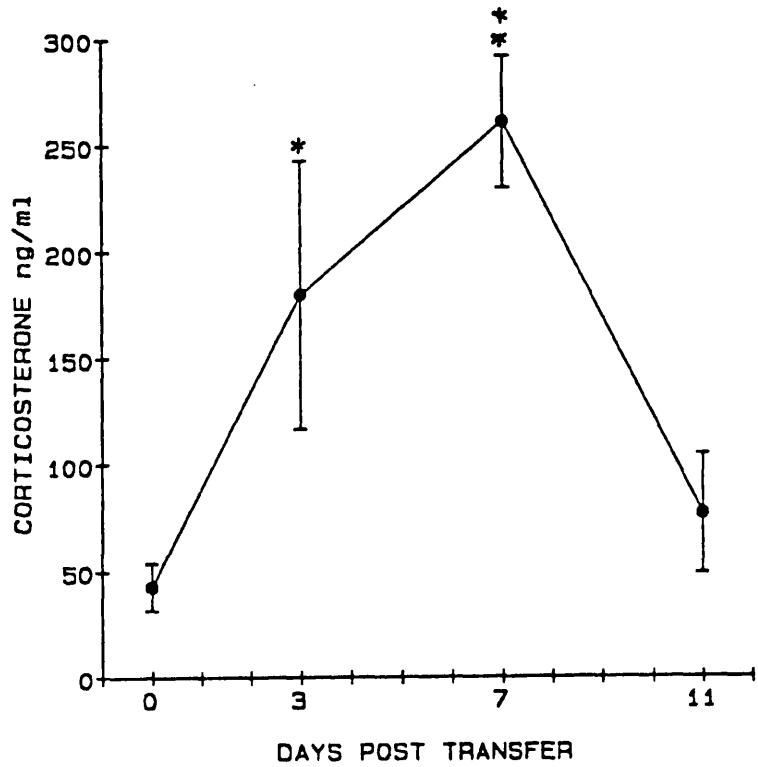
Changes in hypothalamic CRF and pituitary POMC mRNA during the course of adoptively transferred EAE..



Hypothalamic and pituitary samples were taken at pre-clinical (day 3), clinical and recovery time points following the induction of disease. In situ hybridization was performed by Dr M Harbuz of the Westminster Medical School. The results are expressed as copies/μm^3 ± s.e.m. (n = 8). Difference significant at P<0.01 (*) and P<0.05 (+).

FIG 6.2.2.

Changes in serum corticosterone during the course of EAE from the in situ hybridization studies.



Serum corticosterone levels were determined during the course of EAE. At all time points the changes reflected similar changes in pituitary POMC mRNA levels (fig 6.3.1b.).
Difference significant at $P<0.05$ (*).
Difference significant at $P<0.01$ (**).

immunoreactive innervation of the median eminence has been reported (Breder et al, 1988) leading to speculation that IL-1 may be secreted directly into the hypophyseal portal vessels, which may then act directly on the anterior pituitary. However, administration of either IL-1α or IL-1ß has been shown to stimulate both hypothalamic CRF and pituitary POMC mRNA in a dose dependent fashion (Suda et al, 1990) although the changes seen in POMC levels were greater than CRF. It may be that due to amplification of the signals moving down the HPA-axis, preclinical changes in CRF mRNA do not occur because the release of CRF from hypothalamic neurones is not sufficient to trigger the synthesis of new peptide.

With the onset of clinical signs both pituitary POMC mRNA and circulating CS remained elevated. Paradoxically however, there was a fall in hypothalamic CRF mRNA content. Similar changes have been reported with the onset of adjuvant-induced arthritis (Harbuz et al, 1989) where the reduction of CRF mRNA was accompanied by a fall in hypophyseal portal blood CRF-41 immunoreactivity. Both experimental results suggest that immunologically induced stress changes in POMC mRNA are not mediated by CRF.

It is during this stressful period of clinical paralysis that changes in hypothalamic NA and Ad were found which correlated well with increased circulating CS (Chapter 5). Alpha-1 adrenergic stimulation of ACTH release by the agonist methoxamine was reduced by the vasopressin antagonist dPTyrMeAVP but not by an equipotent dose of the CRF-41 antagonist Alpha-helical CRF 9-41 suggesting that vasopressin

and not CRF-41 is responsible for the α-adrenergic stimulated ACTH release (Thomas et al, 1989). This, along with the finding that stress stimulates hypothalamic NA release (Yokoo et al, 1990) as well as selectively activating the vasopressin containing subset of CRF neurones in the PVN (Whitnall, 1989), suggests that POMC and CS might well be elevated as a result of central catecholamine release and vasopressin secretion during the clinical stage of EAE . Such a hypothesis could account for the discordant response of CRF and POMC mRNA during the course of disease.

6.3. The effect of clonidine and yohimbine on the course of EAE.

6.3.1. Materials and methods.

Clonidine and yohimbine were dissolved in saline and injected i.p. at a dose of 0.3 mg/kg and 4mg/kg respectively. EAE was induced by a footpad injection of GP-MBP/CFA inoculum. The animals were then left for 8 days before the administration of the drugs which was carried out daily at mid-day. Injections of saline were used as a control. The dosing was maintained throughout the period where the animals were displaying clinical signs until they were fully recovered.

After recovery, the animals were dosed once more and killed 4 hrs later by decapitation. Hypothalamus and spleen samples were taken for determination of catecholamine content

and trunk blood collected for CS determination.

6.3.2 Results.

Both the α_2-adrenergic agonist clonidine and the antagonist yohimbine suppressed the clinical signs of EAE when given daily prior to and during the expected period of paralysis (fig 6.3.1). The animals treated with clonidine were sluggish in their behaviour after dosing with the drug, an effect which lasted for approximately 6 hrs and was not seen in either the yohimbine or saline treated animals. The reduced clinical severity was reflected in the significantly lower cumulative scores (table 6.3.1) in the yohimbine and clonidine groups compared to the saline treated animals.

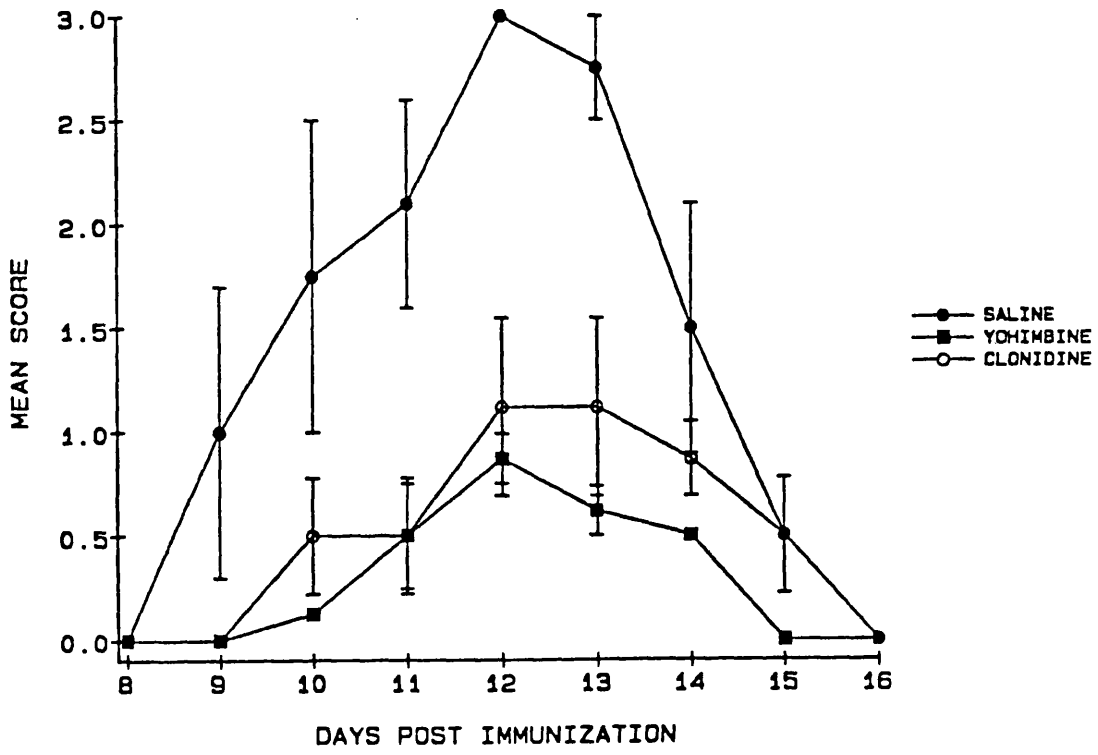
Four hours after the administration of clonidine and yohimbine, hypothalamic and splenic catecholamine content were reduced compared to the saline treated animals (table 6.3.2.). Both drugs elevated serum CS levels compared to the control group (fig 6.3.2.).

6.3.3. Discussion.

Both clonidine and yohimbine, when administered prior to and during the expected period of clinical manifestations, suppressed the severity of EAE compared to saline treated controls. By inhibiting central NA release via presynaptic α_2-adrenergic receptors, and so preventing the possible sympathetically mediated CS response, it was thought that

FIG 6.3.1.

The effect of clonidine and yohimbine on the clinical course of actively induced EAE.



Clonidine (α_2-agonist) and yohimbine (α_2-antagonist) were dissolved in saline and injected i.p. at a dose of 0.3 mg/kg and 4 mg/kg respectively immediately prior to (8 dpi) and during the period of clinical disease. Control animals were injected with saline only. The results are expressed as mean clinical scores ± s.e.m. (n = 4).

TABLE 6.3.1.

The effect of clonidine and yohimbine on the mean cumulative clinical scores (± s.e.m.) of actively induced EAE.

Treatment	Mean cumulative clinical score[a]
Saline	13.0 ± 0.84
Clonidine	4.6 ± 2.0*
Yohimbine	2.75 ± 0.96*

[a] The cumulative clinical scores were calculated by adding up the daily score for individual animals over the whole time period when clinical signs were evident.
Difference significant at $P<0.01$ (*) compared to saline controls (unpaired t-test, $n = 4$).

TABLE 6.3.2.

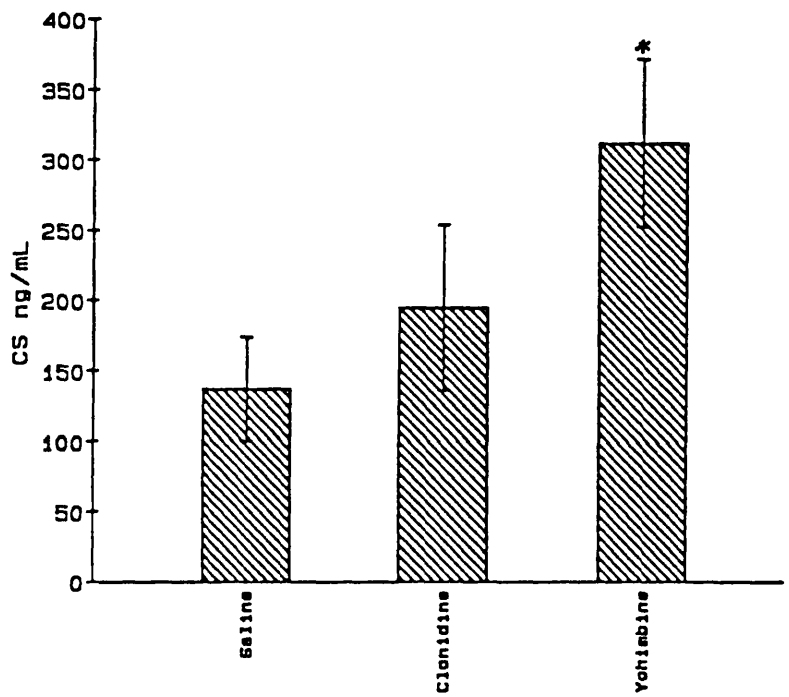
Changes in hypothalamic and splenic catecholamines following clonidine and yohimbine administration.

	Hypothalamus		Spleen
	NA (pMol/ mg wt)	Ad (pMol/ mg wt)	NA (pMol/ mg wt)
Saline	5.1 ± 0.40	0.31 ± 0.037	11.7 ± 1.28
Clonidine	4.0 ± 0.48	0.25 ± 0.051	3.06 ± 0.78*
Yohimbine	2.8 ± 0.45*	0.21 ± 0.055	3.99 ± 0.76*

Hypothalamic and splenic catecholamine levels were determined by HPLC 4hrs after an i.p. injection of clonidine (0.4mg/kg), yohimbine (4mg/kg), or Saline (n=4).
Difference significant at $P<0.01$ compared to saline controls.

FIG 6.3.2.

The effect of clonidine and yohimbine i.p. on corticosterone levels.



Clonidine (α_2-agonist) and yohimbine (α_2-antagonist) were dissolved in saline and injected i.p. at a dose of 0.3 mg/kg and 4 mg/kg respectively after recovery from disease. Trunk blood was collected for determination of serum corticosterone 4hrs later. Control animals were injected with saline only. Difference significant at $P<0.05$ (*) compared to saline injected animals (unpaired t-test). Error bars indicate s.e.m. ($n = 4$).

clonidine would exacerbate the clinical signs of EAE. This was clearly not the case. The rats treated with clonidine showed a distinct sedative or hypoactive response characteristic of the drug (Drew et al, 1979) which is known to be result of central presynaptic α_2-adrenergic receptor stimulation (Heal et al, 1988) indicating that the dose of clonidine used was sufficient to inhibit NA release. Tissue levels of NA and Ad were slightly reduced in both the hypothalamus and spleen in animals treated with clonidine. This too was an unexpected result as it was predicted that preventing transmitter release would lead to an accumulation of NA and Ad in the tissue. The most likely explanation for this finding is an inhibitory effect of clonidine on catecholamine synthesis due to chronic administration of the drug. Similar discrepancies have been reported following long term treatment with other α_2-adrenergic drugs (Sugrue, 1981). The fact that clonidine had a marked sedative effect on the animals does suggest that catecholamine release was inhibited; however, despite this, serum CS levels were elevated 4hrs after the final injection of clonidine. The affinitiy of clonidine for the α_1-adrenergic receptor has been shown to be approximately 10-15 x less than for the α_2 receptor (Brown et al, 1980; Summers et al, 1980). It is possible therefore that the dose used was sufficient to activate α_1 receptors resulting in CS release and a reduction in the severity of disease.

Treatment with the α_2-adrenergic receptor antagonist yohimbine, which has been shown to increase NA release (Rossetti et al, 1989) also suppressed the clinical signs of

EAE. Tissue levels of hypothalamic NA and Ad were both reduced following administration of the drug and serum CS significantly elevated. It would be unwise to conclude from this preliminary data that CS release is mediated through activation of post-synaptic α_1-receptors following central NA/Ad release since both drugs also acted on peripheral sympathetic nerve terminals. However, the fact that the administration of drugs which are known to modify central NA/Ad release did influence the severity of the disease lends support to the conclusion in Chapter 5 that the strong inverse correlation between hypothalamic NA/Ad and serum CS, which was most evident during the stressful period of clinical paralysis, may ultimately play a role in the regulation of CS release and hence the severity of disease.

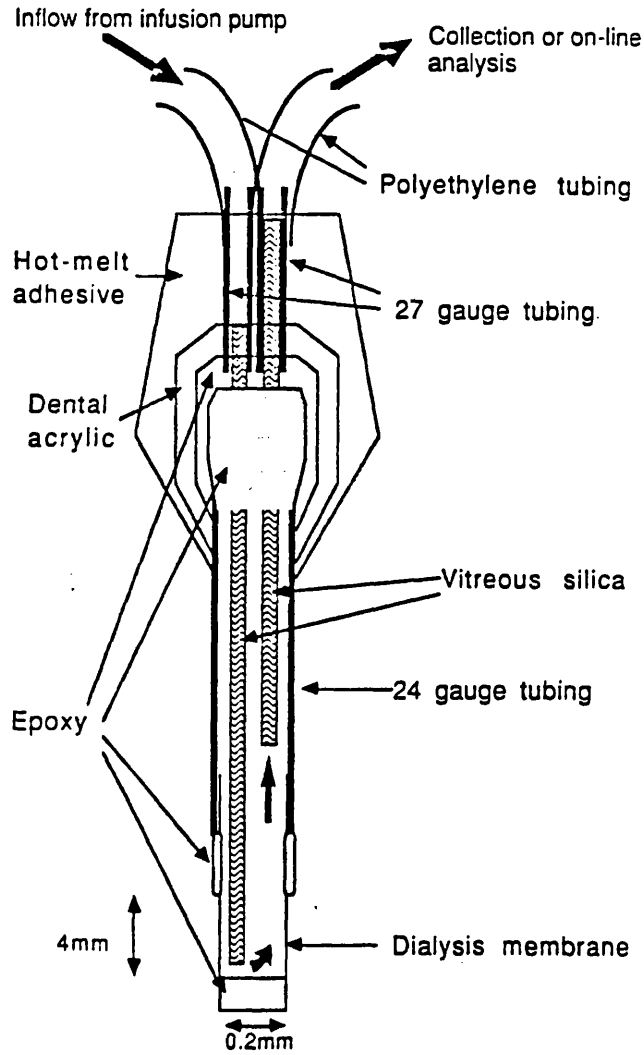
6.4. Determination of hypothalamic NA release using in vivo microdialysis.

6.4.1. Materials and methods.

The microdialysis probe was constructed using 24 gauge outer tubing with two pieces of vitrious silica inserted for infusion and collection of the artificial cerebral spinal fluid (ACSF) (125mM NaCl, 2.5mM KCl, 1.18mM $mgCl_2$ 1.26mM $CaCl_2$). The dialysis membrane was sealed with epoxy glue leaving 4mm of membrane free for implantation. The silicon was connected to polyethylene tubing using two small pieces of 27 gauge tubing and sealed with epoxy glue, dental acrylic and a hot-melt adhesive (see fig 6.4.1 for probe

FIG 6.4.1.

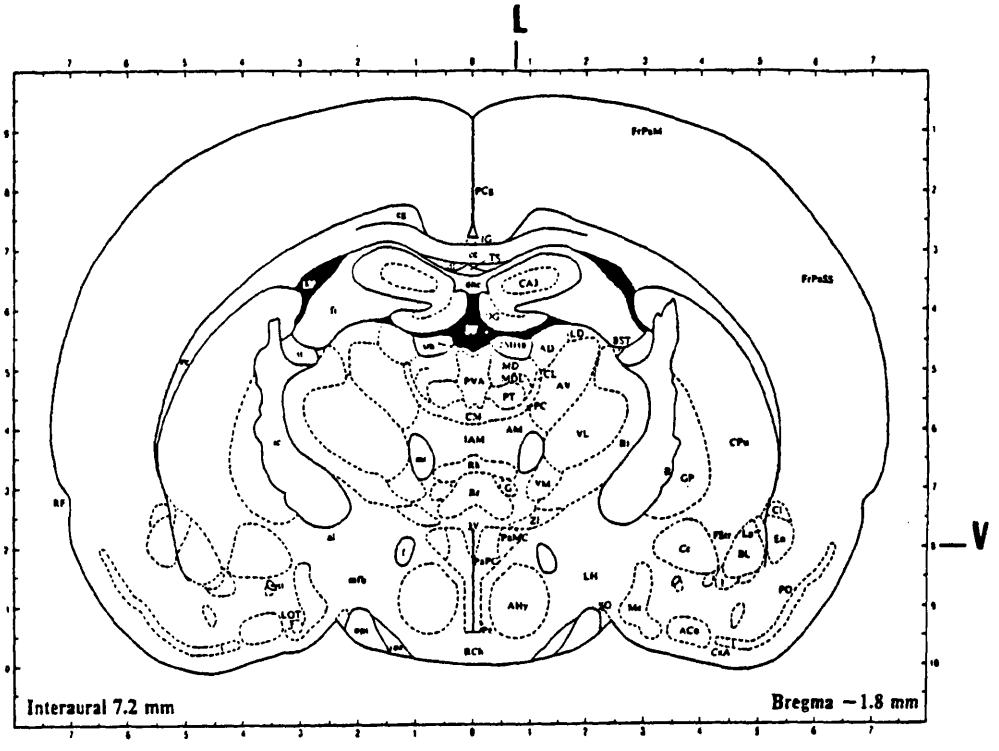
Construction of the microdialysis probe used for in vivo determination of NA release.



The microdialysis probe was constructed according to the method of Sarna and Hutson. Taken from 'Construction details for a concentric dialysis probe' by George Sarna and Peter Hutson.

FIG 6.4.2.

Coordinates for implantation of microdialysis probe into the paraventricular nucleus of the hypothalamus.



The microdialysis probe was implanted into the hypothalamus adjacent to the paraventricular nucleus, bregma = -1.8mm, lateral (L) = 0.7mm and ventral (V) = skull surface, -8.0mm. Animals were left for 24hrs after the insertion of the probe before perfusion with ACSF. (Taken from Paxinos and Watson; The rat brain in stereotaxic coordinates).

construction). The probe was then inserted into the hypothalamus adjacent to the paraventricular nucleus (coordinates = Bregma, -1.8mm, lateral = 0.7mm, and ventral = skull surface, -8mm, fig 6.4.2). Two small screws were secured to the skull next to the probe which was then held in place with dental acrylic. The animals were left for 24 hrs after implantation to recover and then connected up to a Carnegi pump and perfused with ACSF at a rate of 2μl/min for 30 min. The sample was injected directly into the HPLC system for analysis of NA. To determine whether or not the probe was measuring neuronal release it was perfused with high K^+ ACSF which causes depolarisation and transmitter release.

6.4.2. Results.

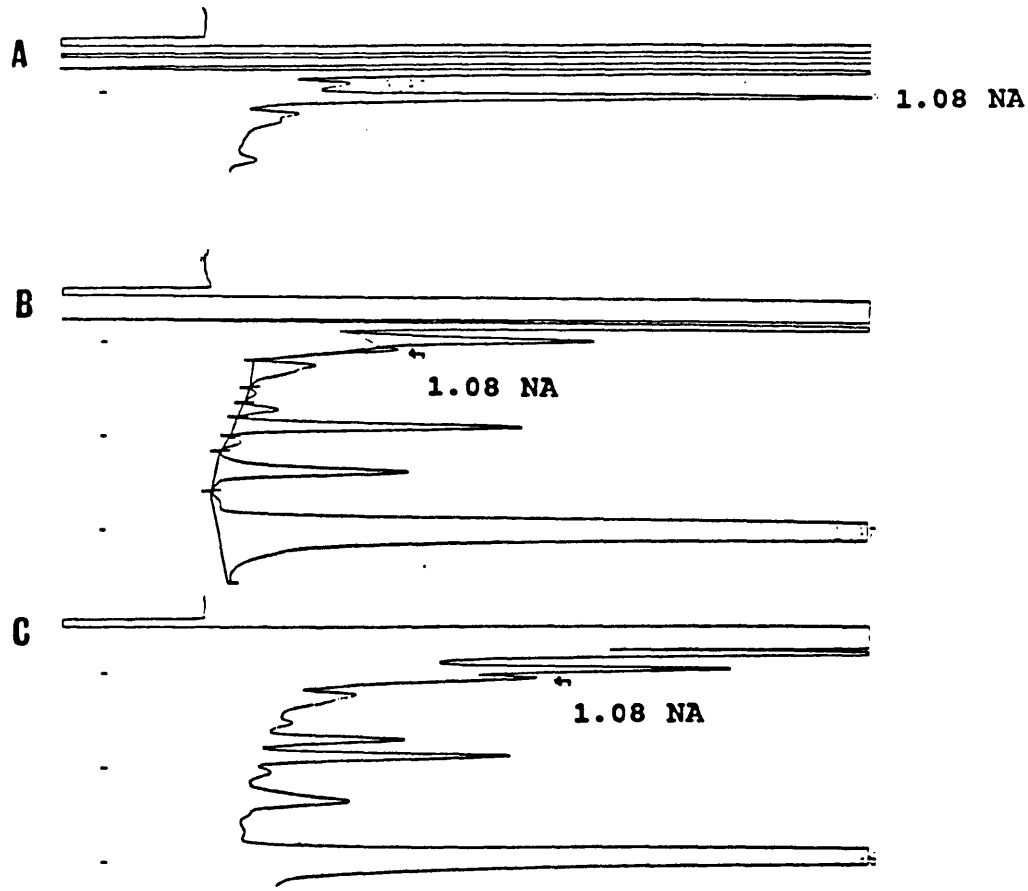
Basal release of NA could be detected using in vivo microdialysis although the levels measured were very low (approximately 0.1pmol/60μl dialysate sample). When the probe was perfused with high K^+ ACSF a noticeable increase in NA release was detected (fig 6.4.3.).

6.4.3. Discussion.

Work is underway to determine whether or not the reduced hypothalamic tissue levels of NA and Ad actually reflect transmitter release. In vivo microdialysis is now a well established method which enables quantification of the release of a number of transmitters including NA. Preliminary

FIG 6.4.3.

HPLC trace from in vivo microdialysis for determination of NA release .



The microdialysis probe was inserted adjacent to the paraventricular nucleus of the hypothalamus and perfused with ACSF at a flow rate of 2μl/min over a 30min period. Samples were injected directly into the HPLC system.

(A) 1pMol NA standard. (Retention time = 1.08min).

(B) Basal release of NA (approx. 20pg/60μl sample).

(C) High K^+ ACSF stimulated NA release (approx. 50pg/60μl sample).

data from a microdialysis probe implanted adjacent to the paraventricular nucleus of the hypothalamus demonstrated basal NA release in the region of 20pg/60μl sample which is in agreement with previous findings from studies in both the hypothalamus and hippocampus (Kapoor and Chalmers, 1987; Abercrombie et al, 1988). Perfusion of the probe with high K^+ ACSF resulted in a 2-3 fold increase in NA release compared to basal values, which is also in agreement with other studies (Kapoor and Chalmers, 1987). Microdialysis has recently been used to demonstrate a stress-induced two-fold increase in NA release in the hypothalamus (Yokoo et al, 1990).

The results so far are only preliminary; optimising the size of the probe and flow rates through it for collection of samples is underway. However, the data is in agreement with the results from other workers and indicates that the technique is suitable for measuring NA release in the hypothalamus. Used in conjuction with intracerebral and jugular vein cannulas for sympathetic drug delivery and repeated blood sampling respectively, it should be possible to elucidate the role of central sympathetic pathways involved in the activation of the HPA-axis following systemic administration of IL-1 and other cytokines, as well as the response to stress.

6.5. GENERAL CONCLUSIONS.

The data in this thesis provides experimental evidence for interactions between the neural, endocrine and immune systems during the course of experimental allergic encephalomyelitis which may influence the progression of the disease. An increase in lymphocyte ß-adrenergic receptor number following the induction of disease appears to be a general response to immune challenge as similar increases in receptor number were seen in control animals injected with adjuvant alone. This may well be the result of either IL-1 release, lymphoproliferation or elevated levels of circulating corticosterone during the early stages of the immune response, all of which have been shown to increase ß-adrenergic receptor number *in vitro*.

Studies on the inhibitory effect of isoproterenol on cytokine release and lymphocyte proliferation *in vitro* provide support for a role for noradrenaline in limiting the progression of the immune response. Such a hypothesis fits well with the changes seen *in vivo* where a pre-clinical rise in lymphocyte ß-adrenergic receptor number and an elevated splenic noradrenaline content during the clinical period of disease would be expected to contribute to the recovery. Adrenergic receptor-mediated immunosuppression was further implicated by the increased disease severity in animals where the splenic sympathetic innervation was destroyed.

Serum corticosterone levels were consistently found to be elevated during the pre-clinical period of disease reflecting activation of the hypothalamic-pituitary-adrenal

axis. In situ hybridization studies confirmed these findings with elevated levels of pituitary POMC mRNA detectable at this time point but no increase in hypothalamic CRF mRNA. This suggests that the elevated corticosterone is either the result of vasopressin stimulated release of ACTH or a direct effect of IL-1 or possibly lymphocyte derived immunoreactive peptides at the level of the pituitary.

A strong inverse correlation between hypothalamic noradrenaline and serum corticosterone was found during the stressful clinical stage of disease. Disruption of central sympathetic function with either 6-OHDA or adrenergic agonists and antagonists affected circulating corticosterone levels and altered the course of EAE. Hypothalamic CRF mRNA was significantly reduced during peak clinical signs which is consistent with the reported findings of central α_1-adrenergic receptor-mediated and stress-induced vasopressin release. However, activation of the HPA-axis by noradrenaline or adrenaline can not be assumed as it has yet to be proven that these transmitters are released.

The overriding regulatory influence of corticosterone was demonstrated by the rapid onset and fatal disease in adrenalectomized animals. However, the significant correlation between hypothalamic noradrenaline and adrenaline levels and circulating corticosterone, indicates that regulation of the HPA-axis during EAE may ultimately be controlled by central sympathetic pathways.

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