MODULATION OF CELL-MEDIATED

HYPERSENSITIVITY IN EXPERIMENTAL

ALLERGIC ENCEPHALOMYELITIS

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STATEMENT

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With these exceptions, the experiments and techniques reported in this thesis are my own work.

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ABBREVIATIONS

BSA	Bovine serum albumin
CFA	Complete Freunds Adjuvant
CNS	Central Nervous System
Con A	Concanavalin A
CSF	Cerebrospinal fluid
DFO	Desferrioxamine
DMEM	Dulbeccos modified Eagle medium
DTH	Delayed-type hypersensitivity
EAE	Experimental allergic encephalomyelitis
FITC	Fluorescein isothiocyanate
GC	Galactocerebroside
GPMBP	Guinea pig myelin basic protein
GPSCH	Guinea pig spinal cord homogenate
GPSCH-CFA	Guinea pig spinal cord homogenate + CFA
HIFCS	Heat-inactivated foetal calf serum
IFA	Incomplete Freund's adjuvant
IL-1	Interleukin 1
IL-2	Interleukin 2
LNC	Lymph node cells
MAb	Monoclonal antibody
MBP	Myelin basic protein
MPCA	Macrophage procoagulant assay
MS	Multiple Sclerosis
PCF	Procoagulant factor
PCIF	Procoagulant-inducing factor
PEC	Peritoneal exudate cells
PNS	Peripheral nervous system
RR	Ribonucleotide reductase

SUMMARY

Lewis rats that are primed with guinea pig spinal cord homogenised in Complete Freund's adjuvant develop overt symptoms of experimental allergic encephalomyelitis (EAE). Treatment with the iron-chelating agent, Desferrioxamine B (DFO) at various times prior to the onset of EAE markedly reduced both the severity and duration of disease. When DFO was administered to rats soon after the development of neurological signs a rapid recovery occurred, though mild, transient symptoms were often seen about one week after withdrawal of the drug. Treatment with DFO was accompanied by a diminution of T cell responses, and, on histological examination, an absence of inflammatory cells from lesions.

In vitro experiments confirmed that DFO, at concentrations effective in vivo, totally inhibited the proliferation of Concanavalin A-stimulated lymphocytes. This inhibition was reversed by adding iron, but not other metal salts to the cultures. DFO was more effective at suppressing T lymphocytes than B lymphocytes, and had no detectable effect on macrophage function or production of lymphokines.

Using alternate in vivo models, it was shown that DFO suppressed the expression of delayed hypersensitivity, and altered the "homing" of normal T lymphocytes (but not B lymphocytes) to their dependent areas of the spleen.

Thus, DFO appears to suppress EAE by inhibiting the sensitization and proliferation of T effector cells, altering the migration of these cells to sites of inflammation and, as a result, the expression of delayed hypersensitivity.

PUBLICATIONS

The work contained in this thesis has been published in the following papers:

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CHAPTER 1. INTRODUCTION.

1.1 Experimental Allergic Encephalomyelitis (EAE).

Experimental allergic encephalomyelitis (EAE), which serves as a model for multiple sclerosis (MS), originated in early investigations into the adverse effects of rabies vaccination, which often resulted in "paralytic accidents" associated with acute disseminated sclerosis. It was thought at the time that the cause of the severe reactions may have been the brain material in which the virus was grown, rather than the virus itself. This was shown to be the case in 1933 by Rivers, Sprunt and Berry who injected normal rabbit brain into 8 monkeys, three times a week over a period of months. Two of the monkeys developed ataxia and weakness, accompanied by demyelination and mononuclear cell infiltration into the brain and spinal cord.

Induction of EAE without adjuvant required multiple injections of central nervous system (CNS) material, but with the introduction of Freund's adjuvant, supplemented with *Mycobacteria*, (Freund and McDermott, 1942) EAE became practical as a laboratory disease model. Rivers et al (1933) used as many as 94 injections to achieve symptoms in 2 out of 8 monkeys, whereas a single injection of CNS material in complete Freund's adjuvant produced symptoms in monkeys in approximately two weeks (Morgan, 1947; Kabat, Wolf and Bezer, 1947).

The possibility that MS might have an "allergic" origin, together with the failure to find a viral cause for

MS, resulted in an experimental boom for EAE in the early 1960s. The factor responsible for producing symptoms was shown to reside in white matter rather than grey matter (Morgan 1947, Alvord 1948). Furthermore, the encephalitogenic activity of the CNS material was shown to parallel the ontogenic development of myelin, which is poorly developed at birth, reaching sufficient quantity by 2 weeks of age to induce EAE in guinea pigs (Schwenkter and Rivers, 1934). In 1962, Laatsch et al showed by differential centrifugation of brain homogenate that the encephalitogenic activity was associated with the greatly enriched myelin fraction. The isolation by Kies et al in 1958 of the encephalitogenic polypeptide myelin basic protein (MBP), obtained by acid extraction of the water-soluble fraction of myelin, led to the search for a similar "antigen" for multiple sclerosis.

The cause of MS is still unknown; epidemiological trends suggested that infection during chilhood (or a chronic infection in the CNS, by a virus as yet unidentified) could result in immune sensitization to a sequestered CNS antigen, and that reactivation of the immune response occurred following a second encounter with the virus or another precipitating event such as pregnancy. However, despite much searching, neither virus nor antigen has been found, although the virus model still enjoys considerable support (Wege, Watanabe and ter Meulen, 1984; Tardieu et al, 1984). Several viruses have been isolated from the brains of patients with MS, including measles, herpes simplex, cytomegalovirus, rubella, coronavirus and lymphocytic choriomeningitis (Bauer,

Poser and Ritter, 1980; Boese, 1980) but no common etiology has emerged from these studies.

EAE has had variable acceptance as a model for MS. The introduction of a chronic, relapsing form in strain 13 guinea pigs (Stone and Lerner, 1965; Wisniewski and Keith, 1977) and SJL/J mice (Brown and McFarlin, 1981; Lublin et al, 1981), which has many clinical and pathological similarities to MS (McKay, Carnegie and Coates, 1973), provided a valuable tool for investigation into the relapsing/remitting nature of MS. Recent research has focussed on immune regulation in EAE and in MS, since alterations in the ratios of T cell subsets have been found to occur in both diseases concurrent with relapses (Reinherz, Weiner and Hauser, 1980; Brinkman, Nilleson and Hommes, 1983; Weiner and Hauser, 1983; Antel et al, 1984). The CNS has always been considered an immunologically privileged site, because it is shielded from the immune system by the blood-brain barrier (BBB), and lacks an organised lymphatic system. Early studies showed that it was possible to transplant allogeneic, and even xenogeneic grafts into the CNS (Murphy and Sturm, 1923). Generally, antigens introduced into the CNS do not induce an immune response in the periphery, unless the BBB is damaged (Leibowitz and Hughes, 1983). Furthermore, Santos (1982) has shown that introduction of T dependent antigens into the CNS failed to activate peripheral T suppressor cells. However, Scheinberg, Edelman and Levy (1964) found that tissue grafts in the brain are rejected in a similar fashion to those in the periphery (albeit slower), and evidence

is now accumulating that the cells of the CNS themselves are associated with immune responses which were previously thought to be restricted to cells of the peripheral lymphoreticular system (Fontana et al, 1981a, 1981b, 1982, 1983; Merrill et al, 1983; Fontana, Fierz and Wekerle, 1984). In addition, the CNS undoubtedly plays a part in the regulation of the peripheral immune system through the action of various neuropeptides (Faith et al, 1984; Blalock, 1984: Renoux, 1984).

The complex relationship between the CNS and the immune system is currently an area of active research (Ader, 1981; Leibowitz and Hughes, 1983; Behan and Spreafico, 1984), and is of special relevance to MS, where abberations of immune regulation, once thought to be causative, may prove instead to be a result of the disease process. EAE is an appropriate model in which to study such neuroimmunological relationships.

1.2 Induction of EAE.

EAE can be induced in a wide variety of species, including mice, rats, guinea pigs, rabbits, dogs, sheep, monkeys and chickens. Susceptibility varies among inbred strains; amongst the most commonly used species, EAE is most readily induced in the Strain 13 guinea pig, the SJL/J mouse, and the Lewis rat. Susceptibility in the rat is thought to be associated with an immune response gene closely linked to the major histocompatability complex (MHC), RT1.B, which is thought to control Class II (Ia) responses (Williams and Moore, 1973; Gasser et al, 1975; Wettstein, Frelinger and Hood, 1981). However, a resistant Lewis strain (Le-R) colony

has been described in which resistance is not linked to RT1.B; incubation of donor cells with lipopolysaccharide restored their ability to transfer EAE to naive Le-R rats (Driscoll, Kies and Alvord, 1985).

The usual inoculum consists of brain and spinal cord white matter, whole spinal cord or purified myelin basic protein (0.5-1.25mg/animal), homogenized in saline and emulsified 1:1 in Complete Freund's Adjuvant to which Mycobacterium tuberculosis has been added (60ug-2.5mg/animal). The use of adjuvant is thought to result in better antigen presentation, to boost both DTH and antibody responses, and to be virtually obligatory for rapid, reproducible disease induction for laboratory studies. Encephalitogenicity depends upon the species of neural antigen used, the amount of Mycobacteria, and the proportions of water and oil in the adjuvant emulsion (Lee and Schneider, 1962; Paterson and Bell, 1962). In particular, the amount and type of Mycobacteria are important. M. butyricum is known to boost DTH responses markedly, while having little effect on antibody production (Bomford, 1980a and 1980b). It also induces a more severe form of EAE in rats than M. tuberculosis H37Ra (Hempel et al, 1984), which is usually used for the induction of chronic relapsing EAE. It has been reported that EAE can be induced in the rat without Mycobacteria (Paterson and Bell, 1962; Beck, Whitehouse & Pearson, 1976) and without adjuvant (Levine and Wenk (1965a).

Macrophage activation and presentation of MBP in an

immunogenic form is essential for stimulation of effector lymphocytes. Soluble MBP is not immunogenic, but MBP bound to microspheres can sensitize rats, and lymph node cells taken from these animals can induce disease in naive recipients (Carbone, Ovadia and Paterson, 1983). Naive thymocytes can be sensitized in vitro by incubation with macrophages which have been pulsed with MBP (Steinman et al, 1977; Ben-Nun, Otmy and Cohen, 1981).

Bordatella pertussis vaccine is necessary for induction of acute EAE in the mouse (Lee and Olitsky, 1955). Two intravenous injections, 48 hours apart, are given at the time of injection with CNS material. This has the effect of increasing blood-brain permeability in certain mouse strains with genetically determined sensitivity to vasoactive amines (Linthicum and Frelinger, 1982; Linthicum, Munoz and Blaskett, 1982). The active factor is the toxin pertussigen (Bergman, Munoz and Portis, 1978), which has several effects, including lymphocytosis, the ability to increase vascular permeability, and increased sensitivity to vasoactive amines. Its effects can be blocked by serotonin antagonists (e.g. methysergide) (Linthicum, Munoz and Blaskett, 1982), indicating a role for serotonin in the induction phase.

B. pertussis can be substituted for Freund's adjuvant in guinea pigs, although the dose is critical (Weiner, Tinker and Bradford, 1959). In rats, the addition of B. pertussis to guinea pig spinal cord and CFA induces a severe, fatal form of EAE termed hyperacute EAE (Levine and Wenk, 1965b; Levine and Sowinski, 1973; Levine, 1974; Lennon, Westall, Thompson and

Ward, 1976). EAE has been induced in genetically resistant strains of mice by means of pertussigen (Munoz and McKay, 1984).

1.3 Clinical course of the disease.

The clinical course of EAE is similar for all susceptible species; approximately two weeks after injection of spinal cord or MBP in CFA, animals exhibit symptoms of ascending paralysis, beginning with a flaccid tail (in caudate species), and involving hindlimb weakness or paralysis, incontinence, loss of righting reflex, and sometimes forelimb paralysis and death. In adult guinea pigs EAE is a severe fatal disease with onset of signs 9-14 days after sensitization. The relapsing models in the guinea pig and mouse are usually of delayed onset with more variation in the duration and severity of symptoms, and with a relapsing/remitting or a chronic progressive disease course (Stone and Lerner, 1965; Brown and McFarlin, 1981).

EAE in the Lewis rat is an acute, monophasic disease, (described by Paterson et al, 1970). Animals exhibit a weight loss of 5-10% on about day 8 after injection, followed by the onset of paralysis on days 10-12, lasting an average of about 8 days, with full recovery by about day 28 (Fig. 1.1). Forelimb paralysis, chronic disease and death are seldom seen in the Lewis rat, occurring more frequently in older (more than 16 month) animals. In about 10% of animals there is a second episode of paralysis, usually mild, occurring between days

Figure 1.1 Lewis rat, showing signs of EAE.

Lewis rat, 16 days after injection of spinal cord homogenised in Complete Freund's adjuvant, showing typical signs of flaccid tail and hindlimb paralysis.

19-25 (McFarlin, Blank and Kibler, 1974).

Correlation between flaccid paralysis and the number of lesions in the CNS is poor (Hoffman et al, 1973; Raine et al, 1980; Paterson, 1982; Simmons et al, 1982 and 1984). Damage associated with large, chronic lesions and necrotic tissue damage generally causes long-term spastic paralysis (Stone and Lerner, 1965; Lassman, Kitz and Wisniewski, 1980). The flaccid paralysis which occurs in acute EAE may resolve rapidly, occurring in the presence of severe inflammatory lesions, and other mechanisms have been proposed to explain loss of nerve transmission which leads to paralysis. Several authors have suggested that oedema, resulting from a decrease in effectiveness of the blood-brain barrier, is the major factor responsible for clinical signs in acute EAE (Daniel, Lam and Pratt, 1981; Leibowitz and Kennedy, 1972; Simmons et al, 1982, 1984). Considerable swelling of the spinal cord occurs during EAE (Levine, Simon and Wenk, 1966), and pressure on the nerve fibres of the cauda equina, and oedema associated with fibrin deposition in EAE lesions (Paterson, 1976) have both been implicated as a cause of cumulative loss of conduction as impulses travel down nerve fibres. Paterson (1976) showed that fibrinogen-depleted rats did not develop paralysis although the extent of cellular infiltration was the same as in EAE controls.

Other metabolic changes which may lead to paralysis include a decrease in noradrenaline and 5-hydroxytryptamine (5HT) in the lumbar-sacral region (Lycke and Roos, 1973; Honeggar and Isler, 1984). Those areas of the spinal cord which are rich in 5-HT terminals, i.e the lumbar and sacral

regions, are also sites of predilection for lesions of EAE (Lennon and Carnegie, 1971), and it has been suggested that interference with neurotransmission in this area may account for the localization of clinical symptoms of EAE. Synthesis of 5HT is not reduced, but there is a greater turnover (Honeggar and Isler, 1984). MBP binds 5HT (Weinstock et al, 1977) and this may be the reason for decreased levels during EAE. MBP itself causes a long-lasting depolarization of neurones in isolated frog spinal cord, leading to loss of transmission (Honegger et al, 1977; Gahwiler and Honegger, 1979; Isler and Honegger 1983). Guinea pigs with symptoms of EAE showed depletion of 5HT receptors and, in severely affected animals, there was a total loss of receptors (Weinstock et al, 1977; White, 1979). An increase in monoamine oxidase activity has been reported in guinea pigs with EAE. (Saregea et al, 1965). This reflects increased metabolism of serotonin, and may partially account for the fall in 5-HT levels.

Disturbances in neuro-electrical transmission have been demonstrated in rabbits injected with whole brain and adjuvant. Such animals exhibited marked disturbance in the electroencephalogram before onset of clinical signs (Feldman, Tal and Behar, 1969). Sera from animals with EAE, and from patients with MS, block the intraneuronal electrical activity of cultures of mammalian brain (Ross and Bornstein, 1969). Bieger and White (1981) reported an impairment of axoplasmic flow in EAE, with extensive damage to catecholamine axons, which are involved in regulation of the immune response by the

hypothalamus (Besedovsky et al, 1983).

Many attempts have been made to explain the rostral progression of paralysis, with recovery proceeding in the reverse order, which occurs in all forms of EAE. It is not related to the injection site (which is usually the footpad), since the same pattern occurs when other routes of sensitization are used, including transfer of sensitized cells (Oldendorf and Towner, 1974). Simmons et al (1982) found increased oedema and lactacidosis in the sacrococcygeal region compared to lumbar (less) and thoracic (least) regions, which correlated well with the appearance and abatement of symptoms. Simmons et al (1982) suggested that progressive metabolic disturbance of the peripheral nodes of Ranvier in nerve root myelinated fibres could account for the ascending nature of the paralysis. White, Bhatnagar and Bardo (1983) found norepinephrine depletion in the spinal cords of rats with EAE, in a rostrocaudal gradient with most severe depletion in the lumbar region, and suggested this as a reason for the rostral progression of paralysis.

1.4 Myelin and Myelin Basic Protein.

The myelin sheath, which encircles and insulates axons greater than 2 um in diameter, is formed by oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system. Each oligodendrocyte sends out a number of processes, ensheathing up to 40 axons (Peters and Vaughn, 1970). Myelin is characterized by its high lipid content (Table 1.1), its relative stability and low enzyme activity. Its structure

Table 1.1 Composition of rat myelin.

	% dry weight (approx.)	
Cholesterol	27.7	70-80%
Galactolipids	27.5	
cerebrosides*	22.7	
sulfatides*	3.8	
Phosphoglycerides	43.1	
Sphingomyelin	7.9	
Protein		20-30%
proteolipid protein	20.0	
myelin basic protein	10.0	

* not found in peripheral nerve myelin.

(shown in Figure 1.3) is essentially a lipid bilayer. The basic protein forms the major dense line, which can be seen on electron micrographs (Figure 1.2). In both EAE and MS, it is the myelin sheath which is destroyed, while axons are spared. For this reason, the "antigen" of EAE and of MS was thought to reside in myelin.

Lumsden, Robertson and Blight (1966) were the first to show that a dialysable small molecular weight component of bovine myelin was able to induce the encephalitis of EAE. This was found to be a peptide with a molecular weight of 18,000 daltons (Carnegie and Lumsden, 1966 and 1967). Subsequent sequencing of GPMBP by Westall et al (1971) and Carnegie (1971a, 1971b), revealed that a small portion only of the MBP molecule was responsible for encephalitogenic activity, that the configuration was important, and that encephalitogenicity resided in the tryptophan residue (115), since blocking this residue abolished activity. Carnegie (1974) also recognised the complementarity in shape between the encephalitogenic fragment containing the tryptophan residue (111-121) and serotonin (5-hydroxytryptamine).

The complete amino acid sequences are now known for bovine (Eylar et al, 1971; Brostoff et al, 1974), human (Carnegie, 1971b), rabbit (Martensen et al, 1981), rat (Dunkley and Carnegie, 1974), guinea pig (Diebler, Nomura and Kies, 1982), mouse (Zeller et al, 1984), chicken (Eylar et al, 1974) chimpanzee (Westall et al, 1975) and monkey (Shapira et al, 1971) myelin basic proteins. The sequence of MBP shows marked

Figure 1.2 The structure of myelin.

(Drawn from Chapman, 1975; Davison and Cuzner, 1977)

homology between species, with the exception of rat MBP which has two encephalitogenic basic proteins, one small and one large molecular weight (Martenson et al, 1972).

Although the sequence of the molecule shows little variation between species, the portion which induces encephalitis may vary, for example bovine MBP sequence 113-121 is encephalitogenic for guinea pigs, but Lewis rats respond to sequence 75-84 (Hashim, Carvalho and Sharpe, 1978).

Guinea pig MBP is the most encephalitogenic of all species in the Lewis rat, being 25 times more active than bovine, human or rabbit MBP and 10 times more active than rat MBP (McFarlin et al, 1973). This is due to the substitution of serine for threonine at position 82 (Kibler et al, 1977). The sequence for guinea pig MBP, shown in figure 1.3, contains a major encephalitogenic fragment 71-85, with a minor determinant 44-48 (Alvord, 1984). The shortest effective peptide is 74-87, but additional residues (63-91) increase encephalitogenicity, 45-91 being equivalent to the whole molecule in encephalitogenic activity (Alvord, 1984)

All T lymphocyte functions (T help, T cell induction of EAE and lymphoproliferative responses) in the rat are associated with residues 71-85 of GPMBP (Chou et al 1979a). Sequence 44-48, the minor encephalitogenic fragment, also elicits DTH (Alvord, 1984). This does not necessarily imply that encephalitogenicity depends upon DTH, since in some species the peptide sequences for DTH and encephalitogenicity are located in different regions (Spitler et al, 1972), and

N-Ac-Ala-Ser-Gln-Lys-Arg-Pro-Ser-Gln-Arg-His-Gly-Ser-Lys-Tyr-Leu-Ala-Thr-Ala-Ser-Thr-Met-Asp-His-Ala-Arg-His-
10 20

-Gly-Phe-Leu-Pro-Arg-His-Arg-Asp-Thr-Gly-Ile-Leu-Asp-Ser-Ile-Gly-Arg-[Phe-Phe-Gly-Ser-Asp]-Arg-Gly-Ala-Pro-Lys-Arg-
30 40 50

-Gly-Ser-Gly-Lys-Asp-Ser-His-His-Ala-Ala-Arg-Thr-Thr-His-Tyr-Gly-[Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser*-Gln-Asp-Glu-]
60 70 80

-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys-Gly-Arg-Gly-Leu-Ser-Leu-Ser-
90 100 110

-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Pro-Gly-Phe-Gly-Tyr-Gly-Gly-Arg-Ala-Asp-Tyr-Lys-Ser-Lys-Gly-Phe-Lys-Gly-Ala-
120 130 140

-His-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys-Ile-Phe-Lys-Leu-Gly-Gly-Arg-Asp-Ser-Arg-Ser-Gly-Ser-Pro-Met-Ala-Arg-Arg-COOH
150 160 170

Figure 1.3 Guinea pig Basic protein showing the peptide sequences for ☐ all T cell functions and encephalitis, ⁘ antibody formation, [ ] minor encephalitogenic sequence, * peptide responsible for encephalitogenic activity in the Lewis rat.

Sources: Chou, Jen-Chou, Fritz and Kibler (1979); Alvord (1984); Martensen (1984).

alteration of a single peptide to abrogate encephalitogenicity does not alter DTH (Hashim and Sharpe, 1974). That is, lymphocytes can still recognise and respond to the fragment, but it does not cause disease. The sequence which induces antibody is 85-88 (Chou et al, 1979a), and Chou, et al (1979b) have shown that giving antiserum raised against residues 68-79 to rats will protect against induction of EAE, but antiserum to residues 79-88 will not.

Chronic EAE has been produced in rabbits (Cambi et al, 1983) and guinea pigs (Hashim, Wood and Moscarello, 1980) by proteolipid protein. Attempts to induce EAE with other purified myelin antigens have been unsuccessful. Galactocerebroside, which has been implicated in demyelination by an antibody/complement dependent mechanism, not only fails to induce disease, but does not protect against subsequent challenge, as is the case with MBP (Hughes and Leibowitz, 1975).

1.5. The lesion of EAE. Early changes.

One of the first measurable changes in the CNS is an increase in vascular permeability on day 6 in the rat preceding cellular infiltration by 2-3 days (Oldstone and Dixon, 1968; Daniel, Lam and Pratt, 1981 and 1983; Suckling et al, 1983; Sobel, Blanchette and Colvin, 1984). This may not be related to antigen sensitization alone, however, since CFA itself can increase vascular permeability to serum albumin (Suckling, 1984), a maximum level being reached on day 7, and

declining to day 0 levels by day 13. In contrast, GPSCH-CFA results in a long-lasting increase in permeability which peaks on day 14 and remains at that level for at least 50 days. Similar results have been obtained by Stohl and Gonatas (1978) who demonstrated chronic blood-brain barrier-permeability to specifically sensitized lymphocytes in rats lasting at least 60 days after injection of GPSCH-CFA. This was not antigen-specific, since MBP-immune, CFA-immune and normal cells all infiltrated to the same degree. Rumjanek, Leyton and Morley (1984) measured cellular infiltration and plasma protein extravasation into the brain and spinal cord of Lewis rats with EAE and found that cellular infiltration occurred earlier, was of short duration in the spinal cord, and was accompanied by plasma protein extravasation which correlated well with severity of clinical symptoms. Upon re-challenge, cellular infiltration occurred in the brain but not the spinal cord, and was not accompanied by plasma protein extravasation. They concluded that, in re-challenged animals, the reaction is more specific, and that cellular infiltration is not dependent upon gross vascular damage. This view is supported by Rose and Parrott (1977), who have shown that lymphocyte migration and vascular permeability are not interdependent. Vascular permeability is a serotonin-dependent phenomenon (Schwartz, Askenase and Gershon, 1977), resulting in plasma protein extravasation. Lymphocytes, however, can migrate independently of serotonin-dependent vascular permeability. Several authors have demonstrated a good correlation between plasma protein extravasation and severity of symptoms in the Lewis rat

(Daniel, Lam and Pratt, 1981 and 1983; Leibowitz and Kennedy, 1972; Simmons et al, 1982), and have suggested that the resulting oedema is the major factor responsible for the clinical signs in acute EAE, since extravasation leads to leakage of fibrinogen and deposition of fibrin, which has been correlated with paralytic signs in EAE (Paterson, 1976; Kristensen and Wisniewski, 1977). Daniel, Lam and Pratt (1983) found that permeability to mannitol in rats with EAE, began in the lower spinal cord where it reached its highest level during the acute phase of the attack. From 15 days onwards, it slowly returned to normal, starting from the caudal end of the spinal cord.

Blood-brain barrier-permeability appears to be related to the amount of Mycobacteria used in the inoculum. Simon and Anzil (1974) found that higher doses of Mycobacteria resulted in increased vascular permeability to anti-MBP antibody, but this was not related to levels of circulating antibody to MBP.

Some areas of the blood-brain barrier, for example the spinal and trigeminal ganglia, spinal roots, area postrema, hypothalamic structures and optic chiasm, do not exhibit the tight junctions found elsewhere in the CNS, having fenestrated junctions similar to vascular endothelium elsewhere, Juhler et al (1984) have postulated that permeability is effected by a "loosening" of these junctions rather than damage to vascular endothelium, suggesting that a chemical mediator is responsible for the initial blood-brain barrier changes.

During the first few days after injection of antigen,

there is local proliferation in the lymph node draining the injection site. Removal of the draining lymph node 2-10 days after injection of spinal cord in rabbits, prevents development of disease (Condie et al, 1957). Effector cells then leave the lymph node and enter the blood circulation via the thoracic duct (Stohl and Gonatas, 1980). How they get into the CNS is not known at present, but has been the subject of much investigation. The fact that lesions of EAE begin around capillaries of the CNS suggests that events on the vascular endothelium are responsible for the cellular infiltrate. Lymphocytes are normally excluded from the CNS, however MBP-sensitized cells can enter the CNS with apparent ease, in the absence of gross damage to vascular endothelium (Stohl and Gonatas, 1978). The interesting question of what it is that lymphocytes "see" raises several possibilities. Simon and Anzil (1974) found evidence of perivascular localization of MBP as early as 5 days after injection of guinea pigs with syngeneic MBP or brain material. This was thought to be of endogenous origin, and occurred in the absence of apparent inflammatory histology. Smith (1969) also found altered turnover of myelin components before histological changes became apparent. Class II major histocompatability complex (MHC) glycoproteins (Ia antigens) are also expressed on vascular endothelium in the pre-clinical stage of EAE (by day 7), in both resistant and susceptible strains (Sobel et al, 1984b).

Ia antigen certainly appears to play a role in EAE, since treatment of antigen-presenting cells with anti-Ia antibody

blocks T cell proliferation to MBP in vitro, and prevents relapses in SJL/J mice in vivo (Steinman et al, 1981; Sriram and Steinman, 1983). Ia presented in conjunction with antigen (MBP) could represent the first "target" of EAE, resulting in the activation of T helper cells, with release of lymphokines and amplification of the immune response, thus facilitating entry into the CNS. Recently, McCarron et al (1985) have reported presentation of GPMBP on vascular endothelial cells from SJL/J mice.

Other pre-clinical changes include enhanced astrocyte reactivity, as measured by increased GFAP turnover (Linington, Suckling and Cuzner, 1983), fibrillary astrogliosis (Field, 1961; Bubis and Luse, 1964; Lampert, 1967) and expression of fibronectin on vascular endothelium from day 5 after injection of Strain 13 guinea pigs with GPSCH-CFA (Sobel, Blanchette and Colvin, 1984). Fibronectin is a 440 kilodalton glycoprotein produced by endothelial cells and macrophages, and is deposited in inflammatory sites during DTH reactions (Clark, Dvorak and Colvin, 1981). It has been implicated in cell migration and macrophage activation, and can be produced by glial cells in culture (Hynes and Yamada, 1982), although its functions in vivo are not known.

1.6 The nature of the cellular infiltrate.

The first cells to enter the perivascular space in the CNS are lymphocytes, which have been detected as early as 5 days after injection of neural antigen (Waksman and Adams,

1962; Rauch and Einstein, 1974; Traugott, Stone and Raine, 1978; Traugott et al, 1981). The earliest infiltrating cells in Lewis rats with EAE were found in the spinal root entry zone and sub-pial area (Hickey et al, 1983). The general pattern of cellular distribution is similar for all models of EAE (Traugott et al, 1981, 1982a, 1982b; Sriram et al, 1982; Hickey et al, 1983; Hickey and Gonatas, 1984; Traugott, 1985). In early CNS lesions (5 days after injection of antigen), T cells of the DTH/helper subset are found in the perivascular space, together with albumin and (non-specific) immunoglobulin. By day 8, B cells and macrophages enter the lesion, and immunoglobulin and complement deposits are also evident at this stage. At the height of clinical signs, the typical lesion of EAE contains approximately 60-80% T cells, of which 40-60% are W3/25+ (DTH/helper) and 18-20% are Ox 8+ (cytotoxic/suppressor), 30% macrophages, and 12-15% B cells.

Staining with the W3/13 marker for mature T cells has revealed that only a small proportion (18%) are positive, suggesting that the majority of T cells in lesions are recently activated (Hickey, 1983). In all forms of EAE studied (and interestingly, in MS), DTH/helper T cells can be found scattered throughout the normal white matter parenchyma; other cell types are confined to the perivascular lesion (Traugott, 1985). Lewis rats differ from the guinea pig and SJL mouse in that during recovery there are fewer total infiltrating cells, most of these are Ox8+ cells, and they are found in the lesion and scattered throughout the white matter parenchyma (Hickey and Gonatas, 1984).

Early lesions contain approximately 30% macrophages, with the proportion increasing to about 50% as the lesion progresses. Although there is some evidence for activation of microglia (Field, 1961), most of the phagocytes appear to be blood-derived (Kosunen et al, 1963; Waksman and Adams, 1962). In MS this may not be the case, since most Ia+ cells do not stain with the monoclonal antibody OKM1, which stains peripheral blood monocytes, indicating either a microglial or astroglial origin (Traugott, Reinherz and Raine, 1983b; Traugott, Scheinberg and Raine, 1985).

Polymorphonuclear cells are not usually seen in lesions of EAE. Exceptions are the acute phase in rabbits (du Clos et al, 1981), where pertussis has been used in the sensitizing injection, e.g. in mice (Traugott, 1985), and hyperacute EAE in rats (Levine and Wenk, 1965b).

It has been argued that the predominance of DTH/helper cells implied a major role for them in the pathology of the lesion. However, Trotter and Steinman (1984a) have shown that both T cell subsets (Lyt-1+ and Lyt-23+) home equally well to CNS lesions in the SJL/J mouse, and the ratios are the same as those found in blood, that is, DTH/helper:cytotoxic/suppressor of approximately 3-3.5:1. (McKenzie and Potter, 1979). Furthermore, the homing pattern was not antigen-specific. Werdelin and McCluskey (1971) found a similar lack of antigen-specificity in passive transfer of ^{3}H-thymidine-labelled, senstized lymph node cells in Lewis rats. Less than 5% of cells accumulating in CNS lesions were of donor origin, and

antigen specific. This does not necessarily negate a role for T cells in the pathology of the lesion, however. Normal T cells bind to myelin (Kuttner and Woodruff, 1979), and T cells with a wide range of antigen specificities may accumulate in lesions where they encounter previously sequestered antigens, thus broadening the specificity of the immune response.

In acute EAE, there is a fall in the numbers of DTH/helper cells in the blood at the time of onset of symptoms (Hauser et al, 1984b; van Lanbalgen et al, 1984): this coincides with the arrival of lymphocytes in lesions. Dissociation of the brains of guinea pigs with chronic relapsing EAE yielded a population of lymphocytes sufficient to account for the fall in peripheral blood lymphocytes (Schuller-Levis et al, 1984).

In summary, although migration of T lymphocytes to the CNS can be demonstrated, and the cellular distribution in CNS lesions is well documented, the way in which T lymphocytes cause CNS damage in EAE has yet to be fully elucidated.

1.7 Demyelination.

Chronic relapsing EAE, like MS, is characterised by extensive demyelination in the region of inflammatory lesions. In contrast, MBP-induced EAE produces an inflammatory disease with very little demyelination (Hoffman, Gaston and Spitler 1973). Incorporation of any one of the myelin lipids sulfatide, galactocerebroside or ethanolamine phosphoglyceride in the inoculum, enhances demyelination in lesions of guinea pigs with EAE (Moore et al, 1984), and it

is generally thought that, while MBP is necessary for induction of the inflammatory response, an additional immune response against one or more of the lipid myelin surface antigens is essential for demyelination.

Demyelination is preceded by axonal swelling (Condie and Good, 1959; Bubis and Luse, 1964; Field and Raine, 1966), the mechanism of which is unclear. A calcium-activated neutral protease which degrades axonal proteins, MBP and proteolipid protein has been found in the CNS (Guroff, 1964; Zimmerman and Schlaepfer, 1982; Banik et al, 1983). An increase in intracellular Ca++ could theoretically activate such an enzyme, initiating the degradation of myelin from within, with resultant widening of the intraperiod spaces between the myelin lamellae. Such changes are often seen prior to demyelination, both _in vivo_ and _in vitro_. This is followed by vesicular lysis of the major dense lines by macrophages. Ultrastructural analysis has shown macrophages apparently actively "stripping" myelin lamellae from the axon, by insertion of processes under the sheath (Lampert and Carpenter, 1965; Lampert, 1967), and some authors have observed that damage only occurs where there is intimate contact between inflammatory cells and myelin (Kosunen et al, 1963; Bubis and Luse, 1964; Lampert, 1965).

Activated macrophages secrete several enzymes, including phospholipases (Trotter et al, 1982) and proteinases (Cammer et al, 1978), sometimes on a continuous basis for several days (Werb and Dingle, 1976). Inhibitors of acid proteinases

(Boehme et al, 1978) and neutral proteinases (Sibley, Kiernat and Laguna, 1978; Brosnan et al, 1980) have been shown to protect animals against EAE. While proteolipid is resistant to proteolytic attack, MBP and the acidic proteins which make up about half of the protein in myelin are particularly susceptible to protease activity (Einstein and Czejtey, 1968; Brosnan, Bornstein and Bloom, 1981). Banik, Hogan and McAlhaney (1984) have suggested that, since the proteins MBP and proteolipid protein are buried in the lipid bi-layer, phospholipases might allow the exposure of proteins for proteolysis to take place, i.e. swelling of the myelin membrane might not be a problem unless activated macrophages are also present. Banik et al (1976) have shown that susceptibility of MBP to the action of trypsin is enhanced by prior degradation of myelin lipids with phospholipase.

Recently, Trotter and Smith (1984) have demonstrated that opsonization of myelin liposomes with antisera to rat myelin increased uptake and degradation of myelin by macrophage monolayers. This could mean that oligoclonal and circulating immunoglobulin may potentiate demyelination by macrophages.

Demyelination can be demonstrated *in vitro* by incubation of spinal explants or cerebellar cultures with sera from patients with MS, or animals with EAE. EAE sera will demyelinate cerebellar cultures only if disease is induced with whole CNS tissue (Bornstein and Appel, 1961) but not if MBP is used (Seil, Falk, Kies and Alvord, 1968).

Cerebellar cultures exposed to antiserum from rabbits

sensitised with whole CNS tissue show demyelination by 1-3 hours, which is complete by 6 hours (Raine and Bornstein, 1976). Demyelination *in vitro* appears to involve two separate processes. One is a complement-dependent myelinolysis, the other, observed when complement is inactivated before addition of the sera to the cultures, is a swelling of myelin with a doubling of the intraperiod space, similar to that observed *in vivo*. Raine and Bornstein (1976) showed that the factor responsible for both activities was anti-galactocrebroside antibody. Subequently, Brosnan et al (1977), and Brosnan, Traugott and Raine (1983) have used the rabbit eye model to elucidate the roles of cellular and humoral events in demyelination. Injection of anti-GC serum into the vitreous has no pathologic effect. However, the addition of lymphokine obtained from Con A-stimulated lymphocytes, or induction of an inflammatory infiltrate in response to sensitization by rabbit spinal cord or an unrelated antigen (PPD or lecithin), resulted in primary demyelination in the vicinity of the cellular reaction.

Thus, two mechanisms of primary demyelination in EAE have been demonstrated in vivo; bystander demyelination and antibody-dependent cell-mediated demyelination.

Bystander demyelination occurs as the result of a cell-mediated response to non-neural antigens in the immediate vicinity of myelinated nerve fibres. Wisniewski and Lassman (1983) have suggested that the small amount of demyelination which does occur in MBP-sensitized animals is a result of such

a response to the MBP pool present in the extracellular space (Simon and Anzil, 1974). In MS, however, the cellular infiltrate may not necessarily be in response to CNS antigen(s) but could possibly be directed against viral antigens on the surface of persistently infected cells.

There are several reports of direct cytotoxic effects of lymphocytes upon myelinated cultures *in vitro* (Arnason, Winkler and Hadler, 1969; Bornstein and Iwanami, 1971). Recently, Lyman et al (1985) have reported that continuous T cell lines mediating EAE, obtained from SJL/J mice, demyelinated syngeneic spinal cord cultures, and that both helper and cytotoxic/suppressor subsets possessed demyelinating activity.

1.8 Contribution of CNS cells to the pathology of EAE.

The idea that cells of the CNS can act in an immunological way is a relatively new idea. However, the increased activity and proliferation of astrocytes which is seen in lesions of MS and EAE (Field, 1961; Adams, 1983), and the presence of lymphoid cells in lesions, has suggested that some interaction between these cells or their products may contribute to demyelination.

It is now known that astrocytes possess some macrophage-like properties; following incubation with lipopolysaccharide (LPS) astrocytes release prostaglandin E and an interleukin 1

(IL-1)-like mediator which is capable of stimulating lymphocytes, as measured by increased thymidine uptake (Fontana et al, 1982). This has interesting implications, since glucocerebroside has been shown to induce both the formation and release of IL-1 (Gery and Lepe-Zuniga, 1984) and one of the effects of IL-1 is to increase the viscosity of Lyt 1+ lymphocyte membranes, thus increasing their capacity to bind antigens (Puri, Schinitzky and Lonai, 1980). Astrocytes can also present antigen (MBP) to cloned T cell lines (Fontana, Fierz and Wekerle, 1984). In addition, although cells of the CNS do not normally express Ia antigens, astrocytes may be induced to do so by gamma interferon (Wong et al 1984a, 1984b, 1985). Astrocytes contain lysosomal enzymes capable of degrading myelin, which are activated in EAE lesions (Arstila et al, 1973; Allen, 1983), and can phagocytose myelin. Thus it is possible that their activation by immune cells, or their products, may perpetuate myelinolysis within the lesion.

Astrocytes can be stimulated to proliferate by lymphokines released by T and B cells of human (Barna et al, 1984) and rat (Merrill et al, 1983; Fontana et al, 1981a, 1981b) origin in response to mitogens. Astrocytes also bind IgG; Barna et al (1984) found that exposure of human astrocytoma cells to purified human IgG results in enhanced DNA synthesis, cytoplasmic process formation, and intensity of immunohistochemical staining for acid phosphatase and GFAP. By immunoperoxidase analysis, fibronectin, which was virtually absent

from untreated astrocytes, appeared to be slightly enhanced by prior incubation with IgG (Barna et al, 1984). Further implication of glial cells in the pathology of EAE was suggested by Bigner, Pitts and Wikstrand (1981), who induced clinical and histological EAE in guinea pigs and monkeys by injection of a human glioblastoma. Thus it is possible that glial cells can produce factor(s) which can induce symptoms of EAE.

The role of microglia, if any, in EAE is not known. The majority of macrophages and monocytes found in lesions of EAE are derived from the peripheral blood circulation (Werdelin and McCluskey, 1970), although Field (1961) has reported activation of microglia, demonstrated by Hortega's silver carbonate stain.

While the CNS does not possess organized lymphatic tissue, Prineas (1979) has described the presence of thin-walled channels, containing lymphocytes and macrophages, occurring in the perivascular space, in CNS tissue from patients with various neurological diseases, including multiple sclerosis, which may serve an immunological function. Large molecular weight antigens introduced into the brain drain into the deep cervical lymph nodes of the neck (Oehmichen et al, 1983), where, presumably, sensitization of lymphocytes could occur. Smaller molecules are probably transported out of the CNS via astrocytes directly into the blood, or diffuse into the bloodstream through the cribriform

plate (Bradbury, 1981). Thus, it would seem that the brain has an efficient system for drainage of waste molecules OUT, but that transport of both molecules and cells INTO the brain is much more selective (Oehmichen et al, 1983).

1.9 The resolving lesion: recovery mechanisms.

Acute EAE is a self-limiting disease, and animals recovering from disease are resistant to further attempts to induce EAE for at least 8 weeks (Willenborg, 1979). Such resistance can be demonstrated in both cell transfer (Ben-Nun and Cohen, 1981) and CFA-induced models (Welch, Holda and Swanborg, 1980; Swierkosz and Swanborg, 1975). Recovery appears to depend upon inhibition of effector cell activity; although antigen-reactive cells can be found in recovered animals, their activity is suppressed (Waxman, Fritz and Hinrichs, 1980). This is not due to lack of antigen stimulation, since MBP is found in the antigen depot for at least a year after injection (Tabira et al, 1984). Lewis rats are resistant to re-induction after about day 18 (Welch, Holda and Swanborg, 1980), and lymph node cells taken from rats at this time will transfer resistance to naive animals (Willenborg, 1979; Welch, Holda and Swanborg, 1980). Soluble factors extracted from T suppressor cells, obtained from mice protected against EAE by injection of mouse spinal cord in IFA, also transfer resistance (Arnon, 1981; Beraud et al, 1982). The inhibition of T cell responses is not simply due to

repair of the blood-brain barrier, since the CNS remains permeable to T cells for at least 60 days after injection (Stohl and Gonatas, 1978).

Thus it appears that T suppressor cells are probably responsible for recovery in acute EAE, and may be involved in remissions of chronic relapsing EAE and MS. Manipulations which lower the numbers of suppressor cells, such as cyclophosphamide treatment (Lando, Teitelbaum and Arnon, 1979; Miyazaki et al, 1985) or irradiation (Willenborg, 1982) allow reinduction of disease in animals with EAE. Similarly, a lowering of the numbers of suppressor cells, as seen during relapses of MS (Bach, 1985), may upset the peripheral immunoregulatory balance, allowing reactivation of T helper cells.

Complete remyelination is common in acute EAE, probably because of the cessation of production of the factor(s) which cause demyelination. It has been shown in vitro that removal of demyelinating sera results in remyelination of cerebellar cultures (Bornstein and Appel, 1961; Raine and Bornstein, 1970; Seil, 1977). However if cells are left in contact with demyelinating sera, oligodendrocytes eventually die. This could presumably happen in vivo.

1.10 The role of antibody.

Antibody to MBP can be detected in serum by day 6 after antigen challenge (Gonatas et al, 1974). Serum from recovered animals can protect if given early, from days 0-8 (Hughes, 1974), however Richert et al (1982) found that antibody did not prevent cellular transfer of disease in rats.

There is no direct evidence that antibody plays a role in the pathogenesis of acute EAE. Antibody production does not correlate well either with time of onset, severity of disease or with relapses (Lisak et al, 1969; Lennon et al, 1971; Tabira and Endoh, 1985). Attempts to transfer disease to normal animals with serum have been unsuccessful (Kabat et al, 1947; Chase, 1959; Bernard et al, 1976), except by direct injection of serum into the brain (Jankovic, Draskoci and Jancic, 1965; Simon and Simon, 1975), which results in symptomless lesions which were, albeit, typical of EAE.

Further evidence that antibody is not directly involved in the induction of EAE was provided by Bernard et al (1976), who showed that transfer of purified T cells into mice induced disease without detectable antibody production. Conversely, transfer of purified B cells induced antibody to MBP, without symptoms or CNS lesions. Similarly, Ortiz-Ortiz and Weigle (1976) showed that suicide of specific T cells by ^{125}I-MBP inhibited induction of EAE, however suicide of B cells did not affect disease production, even though no antibody could be detected.

Interest in antibody arose as a result of the discovery of oligoclonal bands of IgG in the serum and CSF of patients with MS. However, most of this antibody has no known antigenic counterpart. Oligoclonal bands have been described in both chronic (Olssen et al, 1984) and acute EAE (Whitacre et al, 1981; Whittaker and Whitacre, 1984). Much of this Ig appears to

* Furthermore, since EAE can be prevented by thymectomy, but not bursectomy, of chickens (Jankovic and Ivaneski, 1963; Blaw et al, 1967), these results require further elucidation.

be directed against the Mycobacteria in the inoculum (Glynn et al, 1982; Olssen, 1984), However, Mehta, Patrick and Wisniewski (1985) recently reported that oligoclonal IgG from guinea pigs with chronic relapsing EAE, obtained during subsequent relapses, was largely specific for MBP.

About 10-12% of inflammatory cells in EAE lesions are B cells (Sriram et al, 1982), and cells producing antibody to MBP have been detected in EAE lesions (Lennon et al, 1972). Serum from animals injected with whole spinal cord contains antibody to a wide range of neural antigens (Bornstein and Appel, 1961; Rauch and Raffel, 1964; Bornstein and Crain, 1965).

Treatment of rats from birth with anti mu chain antibody inhibits B cell differentiation and prevents antibody production (Bazin, 1978; Cooper et al, 1980). Gausas et al (1982) found that anti-mu-treated rats failed to develop clinical signs or antibodies to MBP when challenged with GPSCH-CFA, but not with MBP-CFA. All rats had cellular infiltrates, but fibrin deposition was almost absent. Willenborg and Prowse (1983) showed that treatment of neonatal rats with antiserum to IgM inhibited both clinical and histological EAE. T cell responses, as measured by PHA stimulation, and allograft rejection, were not affected. However, there is some evidence that T helper function may be impaired by anti-mu treatment (Kim et al, 1984).*

Aclacinomycin A is an antibiotic which inhibits RNA (but not DNA) production, and is effective against B cell leukaemias and certain B cell-mediated autoimmune diseases. It has no

effect against T cell leukaemias or the generation of allogeneic cytotoxic effectors, but it suppresses antibody plaque formation and in vivo antibody production to sheep red blood cells. Treatment of Lewis rats with EAE suppressed production of anti-MBP antibody, however all T cell responses were normal, and the treated animals developed clinical and histological EAE (Dickneite et al, 1984).

Antibody may play a role in demyelination within inflammatory lesions, and it may be important both during recovery and for resistance to re-induction of EAE (Paterson and Harwin, 1963; Willenborg 1979, 1980). The relevance of antibody in MS is less certain, mainly because the nature of the antigen against which the oligoclonal Ig is directed is unknown.

1.11 Delayed hypersensitivity.

The general pathology of EAE is typical of delayed-type hypersensitivity (DTH) reactions: the disease can be transferred with DTH/helper T cells but not with serum; the inflammatory lesions contain a predominance of DTH/helper lymphocytes in the early stages, while macrophages are at a higher frequency in older lesions; there are few polymorphonuclear cells, (which are a feature of antigen-antibody complex mediated hypersensitivity reactions); there is oedema and fibrin deposition; DTH can be demonstrated as early as 3-4 days after sensitization, before antibody can be detected.

There is little doubt that T lymphocytes are required for the induction of EAE. Depletion of T cell precursors *in vivo* by neonatal thymectomy (Wick, 1972; Bernard, Leydon and McKay, 1976), thoracic duct drainage (Gonatas and Howard, 1974) or anti-thymocyte serum (Ortiz-Ortiz and Weigle, 1976) results in a failure to induce disease. Susceptibility to EAE is restored by injection of normal thymocytes (Ortiz-Ortiz and Weigle, 1976). Bursectomy of chickens (Blaw et al, 1967), on the other hand, has no effect on disease induction.

EAE can be transferred with lymphoid cells from animals with EAE to naive recipients (Paterson, 1960; Stone, 1961; Astrom and Waksman, 1962), the optimum time being 9 days after sensitization (Stone, 1961). Manipulation of the transferred cell population revealed that induction of disease depended upon the Lyt 1+23$^-$ (mouse), W3/25+ (rat) T cell subset which is responsible for helper and delayed hypersensitivity functions (Bernard and McKay, 1983; Pettinelli and McFarlin, 1981; Holda and Swanborg, 1982; Bernard and McKay, 1983). MBP-specific T cell lines isolated from Lewis rats with EAE all transferred disease to naive recipients; these were shown to be W3/25+ (Ben-Nun, Wekerle and Cohen, 1981; Hauser et al, 1984b). Relapsing EAE has been induced in SJL/J mice by transfer of lymph node cells (Mokhtarian, McFarlin and Raine, 1984).

Transfer of disease requires prior activation of cells by incubation with MBP (Holda, Welch and Swanborg, 1980; Hinrichs, Roberts and Waxman, 1981; Richert, Kies and Alvord, 1981), Con A (Panitch and McFarlin, 1977; Killen and Swanborg, 1982a;

Ortiz-Ortiz and Weigle, 1982), interleukin 2 (Ortiz-Ortiz and Weigle, 1982) or activation by mixed lymphocyte culture (Hayosh, Simon and Swanborg, 1984), procedures which presumably expand clones of effector cells. Incubation of EAE effector spleen cells with monoclonal antibodies to T helper cells (W3/25) before transfer, abrogates their activity (Swanborg, 1983). However, removal of B cells from transferred cell populations has no effect on the course of the disease (Richert, Kies and Alvord, 1981).

Macrophages appear to be necessary for the successful transfer of EAE, since depletion of either donor or recipient macrophages results in failure to induce disease (Panitch and Ciccone, 1981; Brosnan, Bornstein and Bloom, 1981; Killen and Swanborg, 1982a). EAE has been transferred with peritoneal cells from which non-adherent cells had been removed (Driscoll, Kies and Alvord, 1979), with macrophages primed with MBP *in vitro* (Carbone, Ovadia and Paterson, 1983) and with small numbers of dendritic cells obtained from lymph nodes or spleens of Lewis rats with EAE (Knight et al, 1983).

It appears, therefore, that induction of EAE requires an antigen-processing cell and an immune T cell. This does not necessarily mean that the cell that finally induces the disease process is the donor T cell: other recipient-derived cells may also be involved in mediating CNS damage. One point that is established is that recipient T cells are not required for disease expression, since transfer of spleen cells into the

Lewis nude rnu/rnu rat induced disease with similar severity to that found for immunocompetent Lewis rats (Hinrichs, Wegmann and Humphres, 1984). Similar results have been obtained using the nude SJL/J mouse (Bernard, 1984).

Skin reactivity to intradermal injection of MBP has been detected as early as 3 days after sensitization in guinea pigs (Shaw et al, 1965). Peak levels are reached at approximately day 9-10, that is, 3-4 days before the appearance of clinical signs (Shaw et al, 1965; Field, 1975) and reactivity reduces to recovery levels by day 18, where it remains for a relatively long period (at least 40 days) after injection (Swanborg and Holda, 1980), and long after symptoms have cleared and remyelination has begun (Stohl and Gonatas, 1978). The persistence of DTH in the face of clinical recovery is in contrast to MS, where at least one report has suggested that clinical exacerbations and remissions are accompanied by a rise and fall in MBP-specific DTH (Colby et al, 1977).

In EAE, early skin reactivity to MBP correlates well with the severity of subsequent disease (Waksman, 1956; Shaw et al, 1965; Hughes and Field, 1968; Vadas et al, 1975). During the acute attack there is a fall in DTH skin responses. This may be due to migration of sensitized T cells to the CNS (Traugott, Stone and Raine, 1978) or to the initiation of a suppressor T cell response to MBP (Ben-Nun, Eisenstein and Cohen, 1982). Skin reactivity has been demonstrated to peptides containing the encephalitogenic fragment; the response parallels that induced by MBP (Hashim et al, 1973; Hashim and Sharpe, 1974 and 1975).

The presence of sensitized T cells in the peripheral blood, lymph nodes, spleen and peritoneal exudates has been demontrated *in vitro* by macrophage migration inhibition (David and Paterson, 1965; Hughes and Field, 1968; Vandenbark and Hinrichs, 1974) and by lymphocyte proliferation in response to MBP (Brockman et al, 1968; Dau and Peterson, 1969; Bartfield and Atoynatan, 1970; Lisak and Zweiman, 1974) and to whole spinal cord (David and Paterson, 1965). Cellular hypersensitivity to whole spinal cord was found to be directed against the MBP component (David and Paterson, 1965). However, subsequent investigators failed to show the presence of cellular hypersensitivity to the highly encephalitogenic peptide 112-126, indicating a dissociation between encephalitogenicity and cellular immunity to MBP (Eylar et al, 1970; Bergstrand, 1972; Bailey, 1972; Spitler et al, 1972).

Proliferation has been observed *in vivo* in blood lymphocytes of rabbits with EAE. Dowling and Cook (1968), using incorporation of radiolabel in peripheral blood leukocytes, observed an increased percentage of leukocytes actively synthesizing DNA between 6 and 11 days after sensitization with neural antigen.

The expression of DTH requires the T-dependent release of vasoactive amines such as histamine and serotonin from mast cells, which results in an increase in vascular permeability (Gershon, Askenase and Gershon, 1975). Susceptibility to EAE in the mouse has been associated with VAA sensitivity (Linthicum

and Frelinger, 1982; Linthicum, Munoz and Blaskett, 1982). Mast cells are found in the meninges and post-capillary venules of the CNS, occurring in greater numbers in the guinea pig and man than in rats or mice. Depletion of serotonin by the drug Reserpine inhibits DTH responses, and has been shown to abrogate the T-cell-mediated inflammatory resonse induced in mice by Sindbis virus (Mokhtarian and Griffin, 1984). This may be of relevance in relapses of EAE and MS, which are associated with severe meningeal inflammation and macrophage activation (Suckling et al, 1984a).

Fibrin is deposited in lesions of EAE (Oldstone and Dixon, 1968; Rauch, Einstein and Csejtey, 1978), being first detected 2-3 days before cellular infiltration (Paterson, 1976). Activation of the clotting system and deposition of fibrin in sites of inflammation is a feature of DTH reactions (reviewed by Dvorak, Galli and Dvorak, 1980). It is presumed to occur as a consequence of increased vascular permeability (Colvin and Dvorak, 1975), which allows extravasation of plasma proteins into the perivascular space where fibrinogen is converted to fibrin. Two additional sources of fibrin are worthy of consideration. Firstly, neural cells are a rich source of thromboplastin; any damage within the CNS may thus result in release of thromboplastin, leading to additional fibrin formation. This can be measured quantitively in vitro as procoagulant activity, and is a sensitive indicator of CNS trauma (Graebar and Stuart, 1978). Secondly, fibrin deposition could result from infiltrating monocytes, which produce cell-

bound procoagulant factor in response to immune stimulants such as interleukin 2 (Geczy and Hopper, 1981) or endotoxin (Farram, Geczy, Moon and Hopper, 1983). Geczy et al, (1984) showed that guinea pig peritoneal cells produced procoagulant on incubation with MBP, and that activity was maximum at the height of disease, i.e. 12-14 days after sensitization.

Ackerman Ulrich and Heitz (1981) have observed that fibrinogen was restricted to vessels containing cellular infiltrates, and was not found elsewhere in histological sections of spinal cord from Lewis rats with acute EAE. This would suggest that fibrin deposition does not occur as a result of a general increase in vascular permeability, leading to plasma protein extravasation, but is produced by infiltrating cells. Fibrinogen is a much larger molecule than albumin (340,000 : 66,000 daltons), and it may be that fibrinogen can only enter lesions where vascular damage has occurred.

A large proportion of the cells in sites of DTH inflammation are blood-derived monocytes (Gershon, Askenase and Gershon, 1975). In EAE, these cells comprise about 50% of the cellular infiltrate at the time of clinical symptoms (Traugott et al, 1982b; Sriram et al, 1982), and are thought to play a prominent role in the pathology of the lesion. A monocyte chemotactic factor has been described in the CSF of guinea pigs with chronic relapsing EAE (Kirby, Suckling and Rumsby, 1983), and macrophage chemotactic and activating factors have been

demonstrated _in vitro_ by stimulation of sensitized T lymphocytes with MBP (Hughes and Newman, 1968; Vandenbark and Hinrichs, 1974). Activated macrophages are theoretically capable of causing considerable damage and demyelination within CNS lesions; apart from directly binding to (Epstein, Prineas and Raine, 1983) and phagocytosing (Lampert, 1978) the myelin sheath, they secrete several factors which have been shown to damage myelin, including neutral proteases (Cammer et al, 1978; Brosnan et al, 1980), superoxide (Chia, Thompson and Moscarello, 1983) and hydrogen peroxide (Freund and Pick, 1985; Willmore et al, 1983). In addition, rat IL-1 has been shown to possess protease activity (Dessaint et al, 1979, Katz et al, 1980), and secretion of IL-1 by activated macrophages could contribute to myelin damage as well as activating T lymphocytes.

As discussed previously, the primary targets of neutral proteases, myelin basic protein and proteolipid protein, are not readily accessible to proteolytic attack; the former is buried deep in the lipid layer, while the latter is resistant to proteolysis. However, disruption of the lipid layer by the products of superoxide or hydrogen peroxide, could expose these proteins. Thus, the process of demyelination might reflect a combination of oxidative damage by macrophages, and a protein-generated immune response.

1.12 Lipid peroxidation and CNS damage.

The toxicity of oxygen and the reactive intermediates which are produced during its reduction to water are now well documented (Fridovich, 1983; Di Guiseppi and Fridovich, 1984; Halliwell and Gutteridge, 1984 and 1985b). The action of oxygen radicals on the nervous system has been reviewed recently by Halliwell and Gutteridge (1985a). The complete reduction of one molecule of oxygen to water requires four electrons, and during this sequential process, several intermediates are produced:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} HO\cdot \xrightarrow{e^- + H^+} H_2O$$

(each of the last two steps also yields H_2O)

These intermediates, particularly the hydroxyl radical ($\cdot OH$), are highly reactive. Protective mechanisms which exist for scavenging radicals (usually before $\cdot OH$ can be formed) are listed below.

$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$	superoxide dismutase
$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$	catalases
$H_2O_2 + RH_2 \longrightarrow 2H_2O + R$	peroxidases

Activated macrophages produce both superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These products by themselves are poorly reactive, however in the presence of small amounts of catalytic iron (Fe II), the highly toxic hydroxyl radical

may be formed by the reaction described by Haber and Weiss (1934):

$$Fe^{3+} + O_2 \longrightarrow Fe^{2+} + O_2 \quad \text{(reduction of iron)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad \text{(Fenton reaction)}$$

$$O_2^{-} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe-salt}} O_2 + OH^{\cdot} + OH^{-} \quad \text{(Haber-Weiss reaction)}$$

The hydroxyl radical is an unstable and highly reactive chemical species which oxidizes virtually any organic compound it encounters. Although its life span is measured in microseconds, and its concentration may be extremely small, it is capable of a great amount of damage, particularly in lipid-rich environments, where damage to fatty acid side chains may start a chain-reaction of lipid peroxidation. Furthermore, the toxic effects may be perpetuated due to altered lipids, lipid peroxides, altered proteins and peptides, some recirculating, long-lived and potentially harmful (Esterbauer, 1982). The products of lipid peroxidation are also chemotactic for macrophages (Petrone et al, 1980) and cytotoxic (Slater, 1984).

Myelin, the main component of white matter, is approximately 70% lipid. In addition the brain is rich in iron (Stocks et al, 1974; Floyd and Zaleska, 1984) and ascorbic acid (Spector, 1977). Iron (II) salts are involved in several neurotransmitter functions, including degradation of catecholamines (Dillman, Johnson and Martin, 1979), and the

binding of serotonin to its receptors (Tamir and Lin, 1982).

Nearly all non-haem iron is bound to the transport proteins transferrin and ferritin and reacts very slowly, if at all; however, in the presence of a reducing agent such as ascorbate, ferric iron may be reduced to the catalytic ferrous form. In healthy tissue these reactants are insulated from each other, however in sites of cellular damage they can interact, thus greatly increasing the potential for free radical generation and oxidative damage (Dormandy 1983). In EAE, initial damage to myelin appears to be as a result of macrophage contact (Lampert, 1968), and macrophages activated by lymphokine are known to release H_2O_2 (Freund and Pick, 1985). Lesions of EAE also contain additional iron from extravasation and breakdown of red blood cells, and catalytic iron is present in CSF (Gutteridge et al, 1982; Halliwell and Gutteridge, 1984). Several enzymes (e.g. monoamine oxidase) release H_2O_2 directly, and production of H_2O_2 by rat brain in vivo has been demonstrated (Sinet, Heikkila and Cohen, 1980).

The two main scavengers of H_2O_2 in blood are catalase and glutathione peroxidase. Levels of catalase are low in the CNS (Hartz et al, 1973), being 1/75th that of other lipidic (adipose) tissue, and the small amount that is present in the brain is concentrated in the hypothalamus and substantia nigra, two areas of high iron content. Therefore, the most likely radical scavengers present in the brain are probably glutathione peroxidase, ceruloplasmin, and α-tocopherol (vitamin E). Glutathione peroxidase removes H_2O_2 by using it to oxidize the tripeptide glutathione (glutamyl-cysteinyl-

glycine), a process requiring selenium for its action (Wickstrom, Westermarck and Palo, 1976). Ceruloplasmin is found in appreciable quantities in the CNS (Al-Timini and Dormandy, 1977), and acts by transporting ferric iron out of tissue, where it combines with transferrin in the blood. Free radicals are also scavenged by 5-hydroxytryptamine (5HT or serotonin); this may be another reason for the loss of this transmitter in EAE.

Lipid peroxidation causes loss of membrane potential and integrity, increases permeability to ions (e.g. Ca^{++}) and inactivates membrane-bound enzymes and receptors. The binding of serotonin to its receptors in rat cortical membranes is decreased by lipid peroxidation (Muakkassah-Kelly et al, 1983). Free radicals can also affect proteins as well as lipids, for example by rearrangement of immunoglobulin molecules, with resultant alteration of immunological properties (Wickens et al, 1983). In addition, free radicals cause an increase in vascular permeability, an effect which can be inhibited by superoxide dismutase and catalase (Del Maestro et al, 1980).

Thus, it is possible that lymphoid cells liberating O_2^-, or H_2O_2, could cause the initial damage to the myelin membrane in EAE, exposing previously sequestered proteins. Both normal lymphocytes (Kuttner and Woodruff, 1979) and macrophages (Epstein, Prineas and Raine, 1983) have been shown to bind to myelin. It may be that activation by any means may be sufficient to cause

"bystander" demyelination, once the appropriate cells are attracted into the CNS. Lipid damage could result in chemotaxis, further damage, and activation of macrophages, while exposure of the myelin proteins may perpetuate the specific immune response.

1.13 Aim of the present investigation.

The macrophage procoagulant assay, developed by Geczy and Meyer (1982) afforded a new sensitive technique for the quantitation of delayed hypersensitivity (DTH) *in vitro*. It was hoped to establish whether the procoagulant assay was a reliable measure of DTH in EAE, and whether DTH responses contributed to the pathology of EAE.

In addition, Lewis rats were treated with the iron-chelating agent desferrioxamine, to determine the effect of depletion of catalytic iron on the clinical course of EAE, on the immune response in EAE, and on DTH responses in general.

Finally, the role of other protective mechanisms, in particular, the acute phase response, was examined in EAE.

* Unless otherwise stated, rats were used at 5 per group.

CHAPTER 2 MATERIALS AND METHODS

2.1 Animals.

All animals were bred at the Animal Breeding Establishment at the John Curtin School of Medical Research, A.N.U. Female Lewis/JC rats were bred from pairs obtained from CSIRO, Brisbane in 1972. Due to contamination in Brisbane, the Lewis strain rats bred at the JCSMR carry the AA allele; most other Lewis rats are aa. This strain, now known as Lewis/JC, is fully susceptible to EAE. However the disease is not as severe as in the original strain, and deaths are rare. They were used at 8-10 weeks of age and weighed approximately 120-150g.*

CBA/H and BALB/c xSJL/J mice were used at 6-8 weeks of age, and weighed approximately 25g.

Adult female Belgian lop-eared rabbits weighed between 1.6 and 2.8 kg.

An iron-deficient diet, and a control diet which had been replenished with iron, were obtained from ICN Nutritional biochemicals (Cleveland, Ohio). All other animals were fed "Barastoc" animal pellets (Barastoc Products, Sth Yarra, Vic) and water *ad libitum*.

2.2 Statistics.

Statistical significance of data was evaluated by means of the student's t-test. Unless otherwise shown, standard deviations were less than 10%.

Purified protein derivative (PPD) was obtained from the State Serum Institute, Copenhagen, Denmark.

The 5E9 monoclonal antibody against the human transferrin receptor was a generous gift from Dr. D. Fayle, Dept. Medicine and Clinical Science, JCSMR.

2.3 Chemicals and media.

Concanavalin A (Con A) was obtained from Pharmacia; phytohaemagglutitin (PHA) from DIFCO (Detroit, Michigan, USA). Lipopolysaccharide (LPS), pokeweed mitogen (PWM), ovalbumin (OA) Grade VI, Carrageenan (type IV lambda), rat apotransferrin, human transferrin and human ceruloplasmin (Type X, 3,200 units/ml) were obtained from Sigma Chemical Co. (St. Louis, USA).

Fluorescein-conjugated Concanavalin A (FLK-2100 Fluorescein lectin kit) was obtained from Vector Laboratories Inc. (Burlingame, Ca., USA). Thy 1.2 antibody, Clone F7D5, was obtained from OLAC, (Blackthorn, U.K.)

All other chemicals were of Analar grade from BDH Chemicals (Aust).

Media for use in tissue culture were obtained from Grand Island Biological Company, Grand Island, New York. Dulbecco's Modified Eagle Medium was supplemented with 10% heat-inactivated foetal calf serum (HIFCS), 10^{-4} M L-asparagine and antibiotics (penicillin 100U/ml, streptomycin 100U/ml, neomycin 100ug/ml) for culture of PU5-1.8 cells. RPMI-1640 medium, supplemented with HIFCS and antibiotics as above, was used for all in vitro assays.

Bacto fluid thioglycollate medium (DIFCO, Michigan, USA) was made up at 59.6 g/L in distilled water and sterilized by autoclaving. To induce peritoneal macrophages, 2 ml of this solution was injected intraperitoneally into rats.

T cell growth factor (TCGF) was prepared by pulsing rat spleen cells ($2x10^6$/ml in RPMI + 10% HIFCS) with 4 ug/ml Con

A for 4 hr. The medium was replaced and the cells incubated for a further 18 hr. The supernatant was harvested and concentrated 10X in an ultrafiltration unit (Amicon Corp. Mass. USA).

<u>Desferrioxamine B mesylate</u> (Desferal) was kindly donated by Ciba-Geigy Aust. Ltd. Its properties are outlined in detail in section 4.2. MW 656.8. The LD50 in mice is 1240 mg/kg i.p. and 10g/kg p.o. For in vitro studies, saturated DFOM was prepared by mixing equimolar amounts of DFOM with $FeCl_3$, since DFOM binds Fe (III) in a 1:1 ratio.

<u>Hydroxyurea</u> (Hydrea) was obtained from the Royal Canberra Hospital. MW 76.06. Hydroxyurea is readily absorbed from the gastrointestinal tract. Peak blood levels are reached in 2 hr, and the drug is not detectable at 12 hr after ingestion.

<u>Butylated hydroxyanisole</u> (BHA) was kindly made by Dr. W. Cowden. BHA is a commercial food antioxidant. LD50 in mice: 2000, 2200 mg/kg. MW 180.24

Butylated hydroxyanisole (BHA)

OH

$C(CH_3)_3$

OCH_3

HBED (N,N'-di(2-hydroxybenzyl)ethylenediamin-N,N'-diacetic acid dihydrochloride (MW 496.0) was kindly made by Dr. W. Cowden, Dept. Medical Chemistry, John Curtin School of Medical Research, ANU.

N,N'-di(2-hydroxybenzyl)ethylenediamin-N,N'-diacetic acid dihydrochloride (HBED)

Pyridine-2 carboxaldehyde thiosemicarbazone (PATS) and the equivalent compound bound to FeCl (FePATS) were made by Dr. W.B. Cowden.

Pyridine-2 carboxaldehyde thiosemicarbazone (PATS)

Fe PATS

Pyridoxal isonicotinoyl hydrazone (PIH) was also made by Dr. W.B. Cowden. It is readily absorbed from the gastrointestinal tract, and diffuses readily into the CSF. It is stable for 48 hr in water.

Pyridoxal isonicotinoyl hydrazone (PIH)

Dihydroergotoxine mesylate (Hydergine) was obtained from Sandoz. Hydergine is composed of the ergot alkaloids ergocornine, ergocristine, α-ergokryptine and β-ergokryptine in the ratio 3:3:3:1. MW 696.5. LD50 in mice 33 mg/kg, in rats 40 mg/kg.

Dihydroergotoxine mesylate (Hydergine)

CH_3SO_3H

dihydroergocomine	R = $-CH(CH_3)_2$
dihydroergocristine	R = $-CH_2-C_6H_5$
dihydro-α-ergocryptine	R = $-CH\ CH(CH_3)_2$
dihydro-β-ergocryptine	R = $-CH(CH_3)CH_2CH_3$

2.4 Preparation of cell suspensions.

Peritoneal exudate cells (PEC) were induced by i.p. injection of thioglycollate medium three days prior to sampling. The cells were obtained from anaesthetised animals by washing out the peritoneal cavity with 20 ml cold Hanks Balanced Salt Solution. The cells were centrifuged at 200g for 10 min at 4°C, and resuspended in RPMI-1640 medium. This procedure allowed repeated sampling of individual rats. For use in the procoagulant assay, erythrocytes were removed by layering the cell suspension on Isopaque FICOLL (Pharmacia, Uppsala, Sweden), and centrifuging at 1,250g for 20 min at 4°C. Less than 1% red cells remained after separation.

Spleen and lymph node cell (LNC) suspensions were prepared by gently pressing organs through a 60 gauge wire mesh into RPMI-1640 medium. Aggregates were removed by passing the suspensions through nylon gauze (20u mesh, Simons Ltd, Sydney). Cells were washed and resuspended in RPMI-1640 medium containing 5% heat-inactivated fetal calf serum and antibiotics.

2.5 Preparation of Myelin Basic Protein.

Myelin basic protein was prepared from guinea pig spinal cord by the method of Diebler, Martensen and Kies (1972). Briefly, spinal cords were defatted by homogenization in chloroform/methanol, 2:1 (v/v), and the residue extracted with 0.01N HCl. Purified MBP was then obtained by ion-exchange chromatography on CM-52 resin. The yield of MBP from 50g of original spinal cord material was

50mg. The purified MBP was lyophilized and stored at $-70^{o}C$.

2.6 Induction of EAE.

Spinal cords were excised from adult Hartley guinea pigs, meninges and vasculature removed, and a 20% w/w suspension prepared by homogenization in 0.9% saline, using a MulsiJet syringe (MulsiJet, Elmhurst, Ill. USA), and aliquots were stored at $-70^{o}C$. For use, the homogenate was emulsified in an equal volume of Freund's Incomplete Adjuvant (CSL, Melbourne, Aust.) to which had been added 4 mg/ml *Mycobacterium butyricum* (Difco, Detroit, USA). This inoculum is designated GPSCH-FCA. Rats were injected with 0.2 ml, divided between the footpads of all four feet.

2.7 Clinical assessment of EAE.

Rats were weighed daily, and assessed for clinical symptoms using a scale of 0-5, as follows:

0 = asymptomatic
1 = weight loss, flaccid tail
2 = ataxia, slow righting reflex
3 = paraparesis, incontinence
4 = paraplegia or quadriplegia, no voluntary limb movement
5 = moribund or death.

2.8 Histology.

Animals were anaesthetised with Avertin, and perfused via the left ventricle with saline, followed by fixation/ perfusion with formol saline. The brain and spinal cord were then removed into formal saline and embedded in paraffin. Mid-sagittal sections were cut at 6u thickness, and stained with haemato xylin and eosin to demonstrate cellular infiltrates in lesions or with toluidine blue for areas of demyelination. Sections were scored as follows: (based on the system described by Levine and Wenk, 1963)

0 = no lesions

1 = 1-5 lesions in an entire section (mild)

2 = 1-5 lesions in 10 fields

3 = more than 5 lesions in 10 fields

4 = many lesions in almost all fields (severe)

Cell suspensions and pieces of tissue for electron microscopy were fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.2M sodium cacodylate buffer, pH7.3. After 3 hr, the tissues were postfixed in osmium tetroxide and embedded in Spurr's resin.

2.9 Administration of Desferrioxamine.

Desferrioxamine is excreted rapidly in vivo, with a half-life of approximately 30 min (Keberle, 1964). For this reason, it was administered via an osmotic pump (type 2ML1 for rats and type 2001 for mice, from ALZA Corp., Palo Alto, USA) which was implanted subcutaneously on the back. The

* The dosage of 70 mg/rat/day (584mg/kg) was chosen as the maximum soluble amount with which pumps could be filled, and was well below the lethal limit of 1240mg/kg.

drug was reconstituted in water at 250 mg/ml, and the pumps, which operated for a period of seven days, delivered 70mg DFOM/rat/day or 9 mg DFOM/mouse/day at a constant rate. In all experiments, EAE controls inoculated with GPSCH-FCA were sham operated. At the end of the treatment, the pumps were removed and checked to make sure they had emptied completely.

2.10 Measurement of Desferrioxamine in plasma.

The method used was that of Summers et al (1979). To a 10 ml sample of heparinized blood was added 100ul of 10mM ferric nitriloacetate. The plasma was separated by centrifugation, and 50ul of 1M $MgSO_4$ was added. The plasma was then filtered through a Centriflo ultrafiltration unit (Amicon, Sydney) to remove proteins larger than 25,000 Daltons, and the optical density was measured in a Hitachi model 101 spectrophotometer at 440 nm, using the same subject's ultrafiltrate obtained before injection of DFOM as a blank. Ferrioxamine standards were prepared in 0.9% NaCl. The recovery of ferrioxamine and desferrioxamine added to serum is 97-100%. A scaled-down version of this method was used for CSF determinations, which were read in a Dynatech MR 600 plate reader.

2.11 Measurement of CNS permeability to ^{125}I-BSA.

Iodination of Bovine Serum Albumin (BSA) was performed according to the method of Fraker and Speck (1978). The inside of a tube was coated with 25 ul of Iodogen, which was

* The dissociation rate of ^{125}I from BSA has not been determined. As discussed in Nossal and Ada (1971), nearly all (greater than 99.9%) of ^{125}I forms a stable complex with tyrosine. The breakdown products of iodo-labelled proteins are thought to result from proteolysis rather than deiodination of the protein. Furthermore, there is no reutilization of radioactive iodide. Thus, any "leakage" of unbound ^{125}I would be negligible, as indicated by CNS levels of ^{125}I found in uninjected controls.

dried by aspiration with N_2 gas; 100ug of BSA in 100ul was added to 5ul of ^{125}I (Specific activity 5 mCi/mg, Amersham, England), and reacted for 5 min on ice, with gentle shaking. The mixture was diluted to 1 ml in PBS, and dialysed for 24hr in 3 changes of PBS, at 4^0C, to remove unbound ^{125}I. * At various times after sensitization, rats were injected with 5 uCi/100g body weight intraperitoneally. After 16hr, the animals were perfused with saline via the left ventricle for 5 min, the brain and spinal cord were removed and counted in a Beckman Gamma 4000 counter.

2.12 Measurement of mononuclear infiltrate by [^{125}I]UdR labelling.

This procedure, based upon that described by Vadas et al, (1975) depends upon the incorporation of the DNA precursor deoxyuridine into rapidly dividing mononuclear cells which localise in inflammatory lesions. Rats were injected i.p. with 1 ml of 10^{-4} M 5-fluorodeoxyuridine (Sigma, St. Louis, USA) in saline; 30 min later, 15 uCi (0.6 ml) of [^{125}I]5-iodo-2'-deoxyuridine ([^{125}I]UdR) (specific activity 5 Ci/mg, Radiochemical Centre, Amersham) was given by i.p. injection. The animals were perfused with saline 18-24 hr later, and the brain and spinal cord was counted as for ^{125}I-BSA.

2.13 Measurement of DTH by footpad swelling.

Mice and rats were primed as described for individual experiments. An eliciting dose of 10^8 sheep red blood cells, Con A (0.5 mg/ml) or Carrageenan (Type I, Sigma C-1013) was administered in 50 ul using a 30-gauge needle. The degree of swelling was measured at 4 hr, 24 hr and 48 hr using a dial-gauge caliper ("Schnelltaster", H.C. Kroplin, Hessen, Germany). Each "unit" represents an increase in thickness of 0.1mm ($\pm$ 0.05mm).

2.14 Macrophage procoagulant assay (MPCA).

The MPCA assay is based upon the fact that specifically sensitised T cells recognise antigen presented on appropriate stimulator cells, and react by producing lymphokine(s) which, in turn, cause the production of procoagulant on the surface of macrophages. It is this procoagulant which is measured by the capacity to reduce the clotting time of normal plasma. All media used in this assay are first filtered through a Zetapor filter (AMP, Chatswood, Australia) to remove endotoxin. Peritoneal cells ($2x10^6$) were incubated with or without Myelin Basic Protein (MBP) at 5, 10, or 20 ug/ml, in 0.5ml RPMI containing 5% heat-inactivated foetal calf serum. After 16 hr incubation at 37 ^oC, 5% CO_2, the cells were washed twice and resuspended in 200ul of Hanks Balanced Salt Solution. 100ul of cell suspension was mixed with an equal volume of rat plasma (prepared as described by Geczy and Meyer (1982)), and, upon

addition of 100ul of $CaCl_2$ (0.03M), the clotting time was determined using a BBL Fibrinometer (Becton Dickinson, Sunnyvale, Ca. USA). The percent reduction in clotting time (RCT) was extimated as follows:

$$\% \text{ RCT} = \frac{(\text{CT without antigen}) - (\text{CT with antigen})}{(\text{CT without antigen})} \times 100$$

Lymph node cells were incubated with Myelin Basic Protein (10ug/ml) at 2×10^6/ml in RPMI + 5% heat-inactivated foetal calf serum + 10^{-4} M 2-mercaptoethanol. After 72 hr, the lymphokine-rich supernatant was removed and diluted 1/2, 1/4 and 1/8. A 0.3ml aliquot was then incubated for 16 hr with 4×10^5 PU5 cells, a continuous macrophage line derived from BALB/c mice, which produces procoagulant upon stimulation with lymphokine (Geczy et al, 1983), and the cells were assayed in triplicate as above.

sensitized peritoneal exudate cells + antigen

($2x10^6$ cells/0.5ml) (MBP, 10ug/ml)

OR

lymphokine from sensitized LNC or + normal macrophages

spleen cells incubated with MBP or PU5 cell line

(final dilution 1/2, 1/4, 1/8) ($2x10^5$ cells/0.5ml)

↓ 12 hr, 37^oC

Wash cell pellet three times

↓

Resuspend in 0.5ml

↓

0.1ml cell suspension

0.1ml rat plasma

0.1ml 0.03M $CaCl_2$

↓

Record recalcification time

Fig 2.1 The macrophage procoagulant assay. Outline of the method.

2.15 Depletion of Thy 1+ cells.

Thy 1+ cells were eliminated from mouse spleen cell suspensions by treatment with antibody and complement. Anti-thy.1.2 antibody (Clone F7D5, OLAC, Blackthorn, UK) was diluted 1:10 in PBS, and 25 ul was added to $5x10^6$ cells in 1 ml PBS. After 30 min at room temperature, the cells were washed by centrifugation at 400g, and guinea pig complement (CSL Laboratories, Melbourne, Aust.) was added to give a final concentration of 1:20. The cells were incubated at 37^0C for 30 min, and washed again. Approximately 30% of spleen cells were depleted by this method.

2.16 Rosetting of Ig+ cells.

Coupling immunoglobulin to sheep red blood cells (SRBC).

SRBC were washed four times in 0.9% NaCl and resuspended at 0.25 ml packed cells to 4 ml saline in a 15 ml tube. To this tube was added 30ul sheep anti-mouse Ig (120 mg/ml), followed by 0.4 ml $CrCl_3$ (0.1%), with mixing. After 5 min at room temperature, The cells were centrifuged gently, resuspended in 5 ml PBS, centrifuged again, and finally resuspended in 2.25 ml RPMI containing 5% HIFCS.

Rosetting of Ig+ cells.

A suspension of CBA spleen cells was prepared as in section 2.4, and suspended at $4x10^7$/ml; 2.5 ml was added to 2.5 ml Ig-coupled SRBC at 4^0C, 20ul 25% NaN_3 was added, and the cells were centrifuged gently. The pellet was resuspended and layered onto Isopaque FICOLL to remove

* Human PBL were obtained from normal blood donors at the Royal Canberra Hospital. Whole blood was centrifuged at 1,000g for 10 min. The buffy coat was removed, layered onto Isopaque-FICOLL and centrifuged at 1,250g for 20 min at 4°C.

rosetted cells, which go to the bottom of the tube. Non-rosetted (Ig^-) cells, which form a band on top of the FICOLL, were recovered, washed, and resuspended in RPMI+10% HIFCS.

2.17 Measurement of T cell proliferation by thymidine uptake.

Spleen cells, lymph node cells, or human peripheral blood lymphocytes* (obtained as a buffy coat of blood taken from normal individuals) were suspended in RPMI-1640 medium containing 5% heat-inactivated foetal calf serum and antibiotics, and incubated at 37^{0}C in 5% CO_2 in air for 48 hr, in 96-well Linbro plates at 2x10^5 cells/0.2ml/well. Reagents were added at the following concentrations: Con A, 1ug/ml; A23187, 1uM; PHA, PWM and LPS, all at 50ug/ml; PPD, 1 µg/ml MBP, 5ug/ml; OA and DFOM, 10ug/ml. During the last 4 hr of culture, ^{3}H-thymidine (0.2uCi in 25ul, Specific activity 102 mCi/ml, Radiochemical centre, Amersham, U.K.) was added to each well. Cells were harvested onto filter paper using a Titertek cell harvester, transferred to vials containing 5ml scintillation fluid (Xylene/PPO) and counted in a Packard Tri-carb liquid scintillation spectrometer.

2.18 Labelling of spleen cells with H33342 dye.

Hoechst 33342 is a bisbenzimide derivative which binds to DNA, and once bound becomes strongly fluorescent. It is taken up by viable cells, and remains intracellular, being diluted only by division. The method used was that of Brenan and Parish (1984). A stock solution of H33342, 600ug/ml

ditilled water, was stored at 4 °C. For use, 6 ug/ml was added to 5 10^7 spleen cells in 1 ml PBS. The cells were incubated for 15 min at 37 °C. Labelling was stopped by adding cold PBS. Cells were washed twice and resuspended in PBS for i.v. injection (10^7 cells in 0.2 ml). After one hour, mice were killed, the spleens removed into cold sucrose and snap frozen in liquid nitrogen. A longitudinal face was cut using two razor blades mounted in a perspex holder, and the whole spleen placed in a pe tri dish containing 0.9% NaCl. A coverslip was placed over the cut face of the section, and the whole spleen was examined on an Olympus (BH series) microscope with an HBO 100W mercury vapour lamp and 365 nm excitation and >435 emission filters.

2.19 Interleukin 1 (IL-1)assay.

Augmentation of PHA-stimulated C3H/HeJ mouse thymocytes was used as a measure of IL-1 activity (Mizel, Oppenheim and Rosenstreich, 1978). The macrophage cell line PU5-1.8 produces IL-1 on stimulation with lipopolysaccharide (LPS). Cells were seeded at 1 x 10^6/ml in RPMI-1640 medium containing 10% HIFCS and 20ug/ml LPS, and incubated for 48 hr at 37 °C, 10% CO_2 in air. Both supernatants and cells were assayed for IL-1 activity. Cells were gently resuspended in the supernatants, transferred to centrifuge tubes and spun at 100g for 5 min. Supernatants were removed and the cell pellet was resuspended in a equal volume of RPMI-1640 medium, and frozen and thawed three times to release intra-

cellular Il-1. Cell debris was removed by centrifugation. Twofold dilutions of material to be assayed were added to cultures of mouse thymocytes obtained from 5-7 week-old C3H/HeJ mice, at 10^6 cells/well in a 96-well microtitre plate, in RPMI-1640 medium containing 10% heat-inactivated foetal calf serum, $2x10^{-5}$ M 2-mercaptoethanol and 1 ug/ml PHA. Tritiated thymidine (luCi/well) was added at 48 hr, the cells were harvested at 72 hr onto filter paper using a Titertek cell harvester and counted in a Packard Tri-carb scintillation counter.

2.20 Interleukin 2 (IL-2) Assay.

The IL-2 assay depends upon the ability of IL-2-containing supernatants to support growth of Con A blasts (Lafferty et al, 1980). Con A blast cells were prepared by stimulating spleen cells ($1x10^6$/ml) with Con A at 1 ug/ml, in RPMI + 10% HIFCS, for 3 days at 37 °C. Doubling dilutions of supernatants containing IL-2 were placed in a 96-well tray, and Con A blasts (2×10^4/well) were added in 200 ul. Trays were incubated for 16 hr at 37°C and then ^{3}H-thymidine was added for 5 hr (2 uCi/well in 25 ul). Cells were harvested and counted as above.

2.21 Mixed lymphocyte culture.

Mixed lymphocyte cultures were prepared by mixing 1 ml of stimulator cells (CBA spleen cells irradiated with 1000R from ^{60}Co source) at $2x10^6$ cells/ml with an equal number of F_1 responder cells (BALB/c x SJL/J lymph node cells) in 24-well

Linbro plates. The culture trays were placed at 37°C in a gassed (5% CO_2-air) incubator. Cultures were harvested either at 3 days for cell proliferation assay or 5 days for cytotoxic T cell assay. For measurement of proliferation, cultures were dispensed in 0.2 ml volumes in Linbro 96-well trays, 2 uCi of ^{3}H-thymidine was added and the cells harvested 6 hours later.

Cytotoxic T cell activity was measured using an automated colorimetric assay described by Parish and Mullbacher (1983). Briefly, L929 (H-2^k) target cells were seeded in flat-bottomed 96-well Linbro plates at $5x10^4$ cells/well in 0.1ml of RPMI 1640 medium plus 10% foetal calf serum. The Linbro plates were first coated with Poly-L-lysine (50ug/ml) for 1 hr and then washed in PBS to enhance cell attachment. Target cells were allowed to adhere to the plates for 1 hr before the addition of effector cells. Effector and target cells were incubated together for 4 hr, the medium was then discarded and viable target cells stained for 20 min at 37°C with 0.1ml/well of warm 0.035% neutral red in Hanks Balanced Salt Solution. The stain was discarded and the target cells washed twice by submerging the plates in PBS and tipping off the excess buffer. Neutral red was released by adding 100ul of 50mM acetic Acid/ethanol mixture and the optical density of each well was then measured by a Titertek MR600 plate reader. Per cent lysis of target cells at each K/T ratio was calculated using the following formula:

Culture of astrocytes and oligodendrocytes.

Primary astrocyte and oigodendrocyte cultures were established by the method of McCarthy and de Vellis (1980).Brains were removed from 3-4 newborn rats, cut into small pieces and placed in 10 ml DME containing 0.1% trypsin and 10 ug/ml deoxyribonuclease 1 (Calbiochem, La Jolla, CA) at 37°C, with occasional shaking. After 20 min, the suspension was filtered through nylon gauze (20μ, Simons, Sydney). The cells were deposited by centrifugation at 100g, resuspended in 30 ml DME containing DNase, 10% HIFCS, antibiotics, and supplemented with Eagles NEAA (CSL, Melbourne). After 7 days culture in 75cm flasks (Nunc, Roskilde, Denmark) monolayers of astrocytes could be seen, with rounded, refractile oligodendrocytes resting on top of the monolayer. At this stage, the flasks were placed on an orbital shaker at 250 rpm for 12 hr, after which the oligodendrocyte-rich supernatant was removed to a fresh flask and astrocyte monolayers were subcultured by trypsinization. This procedure was repeated three times. The resulting astrocyte- and oligodendrocyte-rich cultures were more than 90% pure as determined by anti-GFAP and anti-galactocerebroside staining respectively, and by electron microscopy.

$$\% \text{ lysis} = \frac{C-T}{C} \times 100$$

where C = mean OD of control wells

T = mean OD of wells containing effector cells

2.22 Assay for anti-MBP antibody.

Guinea pig MBP was added to a 96-well microtitre plate (Dynatech) at 2ug/well in 200ul. Plates were incubated overnight at 4^{0}C, and then washed three times with phosphate-buffered saline (PBS). To reduce the amount of non-specific binding, 200ul of 2% BSA in PBS was added to each well for 30 min at room temperature, and the plates were washed three times with 0.2% gelatin, 0.5% Tween 20 in PBS (G/T). Serum dilutions were made in 2% BSA, 0.5% Tween 20 in PBS, and added at 200ul/well. After 90 min at room temperature, plates were washed 5 times with G/T and 200ul/well of peroxidase-labelled rabbit anti-rat immunoglobulin (DAKO, Denmark) diluted 1:2,000 in G/T was added. After 1 hr at room temperature, plates were washed 5 times with G/T. The substrate (0.3% w/v o-phenylene diamine, 0.04% H_2O_2 in 10 ml citrate phosphate buffer [15mM Na citrate, 50mM $NaHPO_4$, pH 5.4]) was added at 200 ul/well, for 15-30 min at room temperature, and the reaction was stopped by adding 50ul 2N HCl to each well. Plates were then read in a Dynatech plate reader at 540nm.

The profiles obtained from FACS analysis represent light scatter, which essentially measures cell size (horizontal axis) and intensity of fluorescence (vertical axis). In this way, it is possible to quantitate the numbers of brightly fluorescent (positively stained) cells. The results are expressed as a percentage of the total cell population.

2.23 Flow cytometry.

Cell sorting. The lymphocytes that respond in the procoagulant assay were characterised using monoclonal antibodies specific for rat T cell subsets. The W3/25 (helper/DTH) and OX8 (cytotoxic) monoclones were kindly supplied by Dr. Alan Williams, Sir William Dunn School of Pathology, Oxford. Lymph node cells were incubated (30 min at 4°C) with these monoclonal antibodies, washed 3 times in PBS, incubated with fluorescein-conjugated goat anti-mouse IgG (CSL, Melbourne) and washed again 3 times in PBS. They were then separated on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson) using a 488u excitation wavelength.

DNA analysis. Mouse spleen cells were suspended in RPMI 1640 medium with 5% heat-inactivated foetal calf serum and plated at $4x10^6$ cells/well in Linbro 24-well trays. Con A was added at 2 ug/ml, and DFOM at concentrations ranging from 1.5uM to 150uM. After 48 hr incubation, the cells were centrifuged at 1,000 rpm, and the pellet resuspended in 1 ml of phosphate-buffered saline, pH 7.0. The DNA was stained by the method of Taylor (1980). To each sample, 80ul of Mithramycin solution (125 ug/ml in 75mM $MgCl_2$, 1% Triton X-100; Pfizer, Inc.) was added, followed 1 min later by 40ul of ethidium bromide (250ug/ml in 1% Triton X-100; Sigma Chemical Co.). The sample was immediately filtered through 20um nylon gauze and analysed in the fluorescence-activated cell sorter using a 457nm excitation band and a 520nm long-pass emission filter.

The spectrometric assay for aldehydes (Jacobsen and Dickinson, 1974) is based on the formation of intense magenta and violet coloured 6-mercapto-3-substituted-s-triazolo(4,3- b)-s-tetrazine derivatives from 4-amino-3-hydrazino-5-mercapto- 1,2,4-triazole.

The bleomycin assay for catalytic ("free") iron was as described by Gutteridge, Rowley and Halliwell (1981) and modified by Buffinton et al (1986). Briefly, bleomycin in the presence of iron (II) degrades DNA to form a thiobarbituric acid-reactive product. This provides a sensitive assay for "free" iron which would be available to participate in the formation of hydroxyl radical <u>in vivo</u>. The bleomycin assay does not detect iron bound to transport proteins or to enzymes. Although other metal ions can bind to bleomycin, they do not result in DNA degradation. Thus, the assay is highly specific for iron (II).

2.24 Estimation of fibrinogen in plasma.

Fibrinogen levels in plasma were determined by the method of Wycoff (1970). An aliquot of plasma (50 ul) was diluted in 3 ml McIlvaine's buffer (0.2M Na_2HPO_4, 0.1M citric acid, pH 5.2). After incubation at 55°C for 20 min, the turbidity was measured at 400 nm in a Hitachi 101 spectrophotometer.

2.25 Measurement of serum caeruloplasmin.

Caeruloplasmin can function in vitro as an oxidase, converting the substrate p-phenylenediamine hydrochloride into a purple oxidation product (Wurster's Red). The assay, which is based on that of Ravin (1961) is essentially the same for rat and human serum, except that the optimum pH for rat is 5.6 and for human 5.4 (Smith and Wright, 1974). Serum was diluted 1:80 in acetate buffer (pH 5.5), and 160ul was added to each of 8 wells in a 96-well Linbro round-bottomed plate. The substrate p-phenylenediamine hydrochloride, had been recrystallized, stored dessicated and made up immediately before use (0.5% in distilled water). 20ul was added to each well, and the plate was incubated for 15 min at 37°C. Sodium Azide (20ul of a 100 mM solution) was added to 4 of the 8 wells to act as a control. At the end of the incubation, 20 ul was added to the rest of the wells to inhibit further oxidation, and the plate was read at 540nM on a Dynatech MR600 plate reader. A standard sample at 32 units/ml was prepared from Sigma human caeruloplasmin Type 1 (3200 units/mg/ ml).

Purification of α_2M.

Briefly, an acute phase response was produced in male Sprague Dawley rats aged between 21 and 28 weeks by subcutaneous injection with commercial grade mineral turpentine. Protease was removed by treatment with aprotinin (Sigma) and the a-globulin fraction extracted on CM Affigel Blue followed by separation on a Sepharose CL6B (Pharmacia) column. The α_2M was eluted using a gradient of 0-0.25 M NaCl in 0.05 M Tris HCl buffer, pH 8.0. The purity of the preparation was determined by immunoelectrophoresis and by isoelectric focusing, and was shown to consist of a single band in the free (unbound) form. The protein was stored at 4°C in Dulbecco's phosphate-buffered saline (PBS).

2.26 Measurement of $\alpha_2$macroglobulin. (α_2M)

Purification and measurement of serum α_2M was performed by N. Hunter and K. Weston, as described in Hunter et al (1985). Briefly, serum α_2M was meaured by rocket electrophoresis against goat anti-human α_2M (Cappel), at a concentration of 150ul per 12 ml 1% agarose M per glass plate. Electrophoresis was carried out for 16 hr at 6 V cm^{-1} in 0.025M sodium barbitone, pH 8.6. The plates were dried and stained with 0.5% Coomassie blue G 250.

2.26 Measurement of Ca++ uptake by quin2 acetoxymethyl ester.

The method of Tsien, Pozzan and Rink (1982) was used to detect Ca++ uptake by the fluorescent indicator quin2. Quin2 acetoxymethyl ester (50 uM) was added to a suspension of CBA/H thymocytes at 10^8cells/ml and incubated at 37 ^{0}C for 20 min. The suspension was then diluted tenfold and incubated for a further 40-60 min. The cells were then centrifuged at 1,000g for 3-4 min, resuspended in fresh RPMI-1640 medium at 10^7/ml and kept at room temperature. For measurement of fluorescence, 1-2ml of cells were centrifuged at 14,000g for a few seconds, and the cells were resuspended in HEPES-buffered balanced salt solution without phenol red. Quin2 fluorescence was read on a Perkin-Elmer LS-5 Luminescence spectrometer in cuvettes kept at 37^{0}C. Excitation and emission wavelengths were 339 and 492 nm with 4 and 10 nm bandwidths. Recordings were made from cells containing 1 uM A23187 with or without the addition of 10 uM DFO.

CHAPTER 3 PROCOAGULANT AS A MEASURE OF DELAYED HYPERSENSITIVITY IN EAE.

3.1 Introduction.

The production of procoagulant factor on the surface of cells of the macrophage lineage is thought to be responsible for their immobilization, adherence to endothelium and the deposition of fibrin which are the hallmarks of DTH reactions (recently reviewed by Geczy, 1983). As such it has been used as the basis of a sensitive *in vitro* test for delayed hypersensitivity in the mouse (Geczy et al, 1983; Farram et al, 1983) and in man (Geczy and Meyer, 1982) termed the macrophage procoagulant assay (MPCA). Sources of macrophages used in the MPCA include blood monocytes, peritoneal macrophages, and several cell lines, including PU5-1.8 (obtained from BALB/c mice) which was used in this study.

The nature of the procoagulant factor is unknown. It is similar to thromboplastin in that it can activate the extrinsic clotting pathway (Geczy and Hopper, 1981). However the mechanism may depend on the type of inducer used. Lipopolysaccharide-induced PCF appears, for example, to be different from lymphokine induced PCF, since activation of the clotting cascade occurs at two separate points, possibly by two different factors; one acting on factor VII and the other on factor X (see figure 3.1), (Geczy, 1983).

PCF can be induced directly on the surface of macrophages by several agents, including endotoxin, or indirectly by the action of a lymphokine, procoagulant inducing factor (PCIF),

which is liberated by T cells of the W3/25 (helper/DTH) type following contact with mitogens such as Con A, or an antigen to which they have been sensitized. Release of PCIF requires the presence of one or more accessory cells, which have yet to be fully characterised (Geczy et al, 1983). The PCIF appears to be very similar to migration inhibition factor (MIF) (Geczy, 1983), however, its exact nature is also unknown.

Fig. 3.1. Proposed activation of the clotting cascade, by macrophages activated with either lymphokine (L) or endotoxin (E). From Geczy, (1983).

3.2 Clinical course of EAE.

Lewis rats injected with whole guinea pig spinal cord invariably showed a sudden weight loss of some 8-10% on day 8 or 9 (see fig 3.3). This was followed in 2-4 days by neurological signs which ranged from a flaccid tail to severe paralysis. Clinical signs (assessed as described in section 2.7) showed a peak on day 14, and animals usually recovered by day 21-23. However a second, milder incidence of disease during the next 7 days occurred in approximately 10% of animals. Both mortality (2%) and the occurrence of chronic disease (less than 1%) were rare.

Histological sections of medulla and spinal cord taken from groups of 5 rats, on days 4, 7, 10, 14, 18, 22 and 28 after sensitization, showed perivascular infiltrates of mononuclear cells, which were first evident on day 10 after injection, and were fully developed by day 14 (Figure 3.2). By day 28, lesions were still evident, (containing predominantly macrophages), although all animals had recovered by this time.

NEXT PAGE:

Figure 3.2 Histological sections from rat spinal cord showing, a) normal,

b) moderately severe lesions, 14d after injection with GPSCH-CFA

c) severe, confluent lesions, 22d after injection with GPSCH-CFA

a

b

c

Fig 3.3 Clinical course of EAE in a group of 10 rats showing

TOP: weight of rats with EAE (●—●) and normal controls (o·····o)

BOTTOM: concomitant clinical scores.

3.3. Kinetics of the procoagulant assay.

Spleen cells, lymph node cells (LNC) and peritoneal exudate cells (PEC), taken from rats 10 days after injection of GPSCH-CFA, all reacted to myelin basic protein in a similar manner (figure 3.4). The clotting time of normal rat plasma was reduced by approximately 50% at the optimum incubation time of 12 hr. In initial experiments, 50 rats were tested for non-specific PCA reactivity one week before use, and all were found to be negative.

The concentrations of guinea pig MBP routinely used to

stimulate these cultures were 5, 10 and 20 ug/ml. The optimum concentration was found to be 10 ug/ml, and all results shown are at that concentration.

All cell suspensions obtained from animals for use in the procoagulant assay were routinely purified on Isopaque-FICOLL to remove erythrocytes, since it was found that as few as 10^5 per 2×10^6 incubated cells were sufficient to reduce the clotting time of normal rat plasma by 80%.

Figure 3.4 Kinetics of the procoagulant assay.

Spleen (●—●) and lymph node cells (o--o), incubated with PU5-1.8 cells, and peritoneal exudate cells (■--■) were incubated in the presence of 10 ug/ml GPMBP. Results are shown as the % reduction in clotting time compared with normal plasma. (o····o) normal LNC incubated with 10 ug/ml MBP.

3.4 Correlation of PCA activity with lymphoproliferation.

One of the most widely used *in vitro* tests for cell-mediated immunity is the proliferative response of lymphocytes to specific antigen, which results in the release of interleukin-2 (IL-2). A comparison was made between the lymphoproliferative and procoagulant response to GPMBP. Popliteal lymph node cells, taken from rats 10 days after injection of GPSCH-CFA, were incubated with 5, 10 and 20 ug/ml GPMBP for 48hr. The supernatants were removed, and tested for induction of procoagulant on PU5-1.8 cells, and the remaining cells were pulsed for a further 4 hr with ^{3}H-thymidine. The results, (figure 3.4), show that the correlation between procoagulant activity and lymphoproliferation as *in vitro* indicators of delayed hypersensitivity was close, as indicated by the line of best fit.

The PCA has been reported to be more sensitive than the lymphoproliferative assay for the demonstration of DTH to some antigens, notably PPD in man (Geczy and Meyer, 1982). However it was found that the response of rat LNC to MBP was negative below 5 ug/ml MBP when measured by both lymphoproliferation and PCA (figure 3.4), indicating that in EAE, at least, both methods appear to be of equal sensitivity.

stimulation index

0 10 20 30

0 20 40 60

% reduction in clotting time

Fig. 3.4 Correlation of procoagulant activity with lymphoproliferation.

LNC taken from rats injected with GPSCH-CFA 10 days previously, were incubated with GPMBP at ▲ 5ug/ml, ● 10ug/ml, ■ 20ug/ml. Open and closed symbols represent 2 separate experiments. ▩ unstimulated cells.

3.5 Specificity of the procoagulant assay.

LNC from animals sensitized to GPSCH-CFA responded to GPMBP but not to ovalbumin in the MPCA (Table 3.1). Conversely, animals sensitized to ovalbumin in CFA, or to CFA alone, did not react to MBP *in vitro*. All rats which had received CFA in the inoculum responded to Purified Protein Derivative (PPD), since CFA contains *Mycobacterium tuberculosis*, from which PPD is derived.

Table 3.1 Specificity of the MPCA assay for myelin basic protein.

Sensitization in vivo		MPCA response (% reduction in clotting time) on *in vitro* challenge with:			
Antigen	Day	MBP	OA	NIL	PPD
GPSCH-CFA	7	23.4	2.5	3.7	27.8
GPSCH-CFA	14	45.9	3.0	2.5	54.6
GPSCH-CFA	21	25.2	3.5	3.6	46.4
NIL	-	3.4	-	2.3	5.5
CFA	14	4.8	3.8	3.3	49.5

* These results clearly show that the cells which produce the lymphokine, now termed procoagulant-inducing factor (PCIF), are W3/25+, OX8-. This table represents a single experiment.

3.6 Characterization of effectors in procoagulant assay.

The cells which produce lymphokine in response to MBP are W3/25+ (helper/DTH) cells (Table 3.2). LNC taken from rats which had been sensitized with GPSCH-FCA 10 days previously were stained with the monoclonal antibody W3/25 or Ox 8, and separated on a Fluorescence-activated cell sorter (as described in section 2.23) into stained and unstained populations. These were then incubated with normal rat peritoneal cells at a ratio of LNC:PEC 1:10, for 72 hr, in the presence of MBP, and supernatants were assayed for procoagulant activity using the PU5-1.8 cell line. *

Table 3.2 FACS separation of T cells responding in the procoagulant assay.

Lymph node cells (LNC) sorted for:	% of cells stained	% reduction in clotting time
W3/25 (helper/DTH)	25.3	35.4±2.1
Unstained	0	5.0
Ox 8 (cytotoxic/suppressor)	7.2	5.0
Unstained	0	38.2±3.5
Unseparated	0	48.0±4.4

3.7 Time course of MPCA in vivo.

Delayed hypersensitivity was measured by reactivity to MBP in the MPCA, over a period of 60 days. Cells taken from the draining (popliteal) lymph nodes, spleen and peritoneal cavity showed earliest activity at 4-6 days (figure 3.5), with a peak at 8 days, and a decline to normal levels by day 20, with the exception of LNC, which continued to show reactivity until day 60.

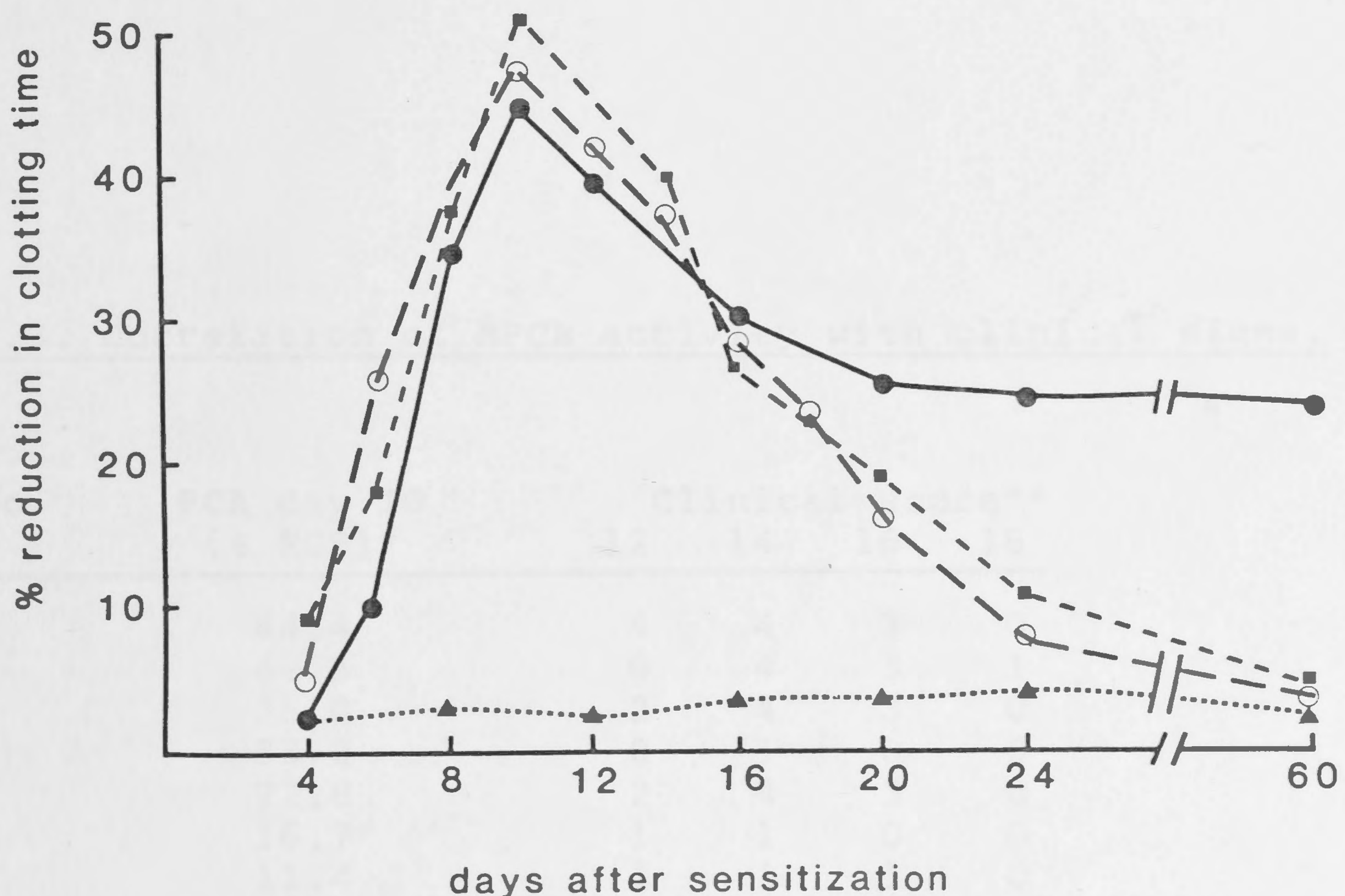

Figure 3.5 Time course of MPCA in vivo, showing the response to GPMBP of ■---■ spleen cells, ●—● lymph node cells and ○--○ PEC, taken from rats sensitized with GPMBP.

▲.....▲ LNC from rats sensitized with ovalbumin in CFA.

3.8 Correlation of MPCA with clinical signs.

The procoagulant activity of PEC taken on day 10 was compared with the subsequent clinical picture. Generally, rats which had high PCA on day 10 also developed high clinical scores, (Table 3.3). However there were exceptions to this, for example rat #4 had high PCA but mild symptoms, and rat#9 had low PCA on day 10, but developed severe symptoms by day 14, which lasted for 7 days. It was concluded that the MPCA activity of PE cells did not correlate well with either length or severity of disease.

Table 3.3. Correlation of MPCA activity with clinical signs.

Rat No.	PCA day 10 (% RCT)*	Clinical score** 12	14	16	18
1	44.4	4	4	3	0
2	42.5	0	4	3	1
3	33.0	2	4	3	0
4	33.0	0	1	0	0
5	22.8	2	4	3	0
6	16.7	1	1	0	0
7	11.4	1	2	1	0
8	10.6	0	0	0	0
9	9.3	2	4	2	2
10	7.3	0	1	2	3

* Procoagulant activity was measured from PEC. Animals were then observed daily until fully recovered.

** Clinical score is given from day 12-18 after injection with GPSCH-CFA. No symptoms were present before day 12.

3.9 Correlation of MPA with cellular infiltrate into the CNS.

Twenty rats were injected with GPSCH-CFA on day 0, and PEC were tested for PCA in response to GPMBP on days 7 and 14. On day 14, they were killed, and sections of medulla and spinal cord were graded according to severity of histological lesions (section 2.8). Table 3.4 shows that the severity of lesions in the CNS and procoagulant activity of PEC correlated well; rats with low histological scores had reduced procoagulant activity compared with those with severe infiltration.

Table 3.4 Correlation of PCA with cellular infiltration.

Histological score (no. animals)	Procoagulant activity of PEC (% reduction in clotting time)		clinical score.
day 14	day 7	day 14	day 14
1 (3)	10.3±1.7	34.5±3.2	1,2,3
2 (6)	26.2±2.4	56.1±4.7	1,2,2,3,3,4
3 (8)	30.5±2.8	61.4±5.9	2,2,3,3,3,3,4,4
4 (3)	34.7±5.6	65.9±6.0	3,4,4

*

This experiment consists of 2 parts:

1) To determine the cellular source of coagulant activity found in CNS damage, thought to be thromboplastin.

2) To determine whether CNS cells could be induced to form a procoagulant factor, using LPS or IL-2 as the stimulus, which could contribute to the high coagulant activity found in CNS damage.

3.10 Attempted induction of PCA in glial cells.*

It is well known that the brain is a rich source of thromboplastin. Indeed, brain homogenates are commonly used in commercial thromboplastin preparations. In addition, the CSF of patients with CNS trauma contains significant levels of thromboplastin and as such is a sensitive indicator of damage (Graebar and Stuart, 1978).

Although the fibrin deposition in EAE has been attributed to vascular leakage, the possibility exists that neural cells themselves are a source of inducible procoagulant activity, and may contribute to the fibrin deposition in EAE lesions.

Astrocyte- and oligodendrocyte-enriched cell populations were obtained from newborn rats by the method of McCarthy and de Vellis (1980). Cells were incubated at $2x10^6$/ml with LPS (0.1, 1, 10, 100 ug/ml) or with twofold dilutions of TCGF in a volume of 0.5ml. Cells were harvested at 12 and 24 hr, and tested for procoagulant activity. In addition, aliquots of cells were washed, frozen and thawed 3 times to test for intracellular thromboplastin. Procoagulant activity was not induced in any of the cultures by either TCGF or by LPS, nor was intracellular procoagulant activity increased. The thromboplastin was found to reside mainly in the astrocyte fraction, oligodendrocytes having little procoagulant activity (Table 3.5).

Table 3.5 Procoagulant activity of glial cells.

Treatment	Clotting time of rat plasma (secs.)*	
	Astrocytes	Oligodendrocytes
TCGF (1/4)	149.4	145.6
LPS (10ug/ml)	147.3	154.1
-	146.8	141.7
Frozen/thawed 3X (diluted 1/4)	41.4	106.2

* Cell cultures were established in triplicate, 10 cells/0.5ml, and harvested at 12 hr.
Standard deviations were all less than 10%.

3.11 Discussion.

EAE in the Lewis rat is an acute inflammatory disease, accompanied by strong DTH responses. The macrophage procoagulant assay (MPCA) is a reliable indicator of DTH in rats with EAE.

MPCA activity of lymphoid cells was optimum at an antigen concentration of 10 ug/ml, and little activity was detected below 5 ug/ml, suggesting that the sensitivity of the MPCA for EAE is similar to other in vitro tests for DTH, such as macrophage migration inhibition and lymphoproliferation. By contrast, reactivity to PPD in man has been reported to be highly sensitive, results being obtained with as little as 1 ng antigen (Geczy and Meyer, 1982; Geczy, personal communication).

Tests of LNC and PEC from rats with EAE showed that reactivity to MBP as measured by the MPCA could be detected as early as 6 days after sensitization with whole spinal cord homogenate; peak levels were reached by days 8-10, and declined thereafter, although LNC still responded 60 days after initial sensitization. Procoagulant activity correlated well with lymphoproliferative responses to GPMBP, and with cellular infiltration into the CNS. However, neither histology nor MPCA status correlated well with clinical signs, or duration of disease; nor were MPCA responses on day 10 a good indication of future severity of disease. Poor correlation of DTH with clinical signs does not necessarily negate a role for DTH in the pathology of EAE; drugs which suppress cell-mediated responses have been used successfully to treat affected animals

and it may be that other factors, associated with the DTH response, are involved in the generation of clinical symptoms.

Fibrin deposition, as occurs in sites of DTH inflammation has been suggested as a cause of paralysis in EAE (Paterson, 1976). The main source of fibrin is thought to be via extravasation of plasma proteins; whether macrophages producing PCF within lesions of EAE contribute to fibrin deposition is not known. Indirect evidence for such a role is suggested by Geczy et al (1984) who found that peritoneal macrophages, taken from guinea pigs with EAE, produced procoagulant factor on stimulation with MBP, but that during recovery such PEC produced an anti-coagulant factor, the nature of which is unknown.

Astrocytes were shown to contain high levels of thromboplastin, and damage to astrocytes is presumably a source of procoagulant factor found in the CSF as a result of trauma (Graebar and Stuart, 1978). However, procoagulant could not be induced on the surface of intact astrocytes by stimulation with LPS or TCGF *in vitro*. CSF taken from rabbits with severe chronic EAE (section 4.19) contained significant amounts of PCA. Whether this is blood-derived, or resulted from cellular damage within the CNS, is not known.

CHAPTER 4. SUPPRESSION OF EAE BY DESFERRIOXAMINE.

4.1 Introduction.

Although strong DTH responses can be demonstrated in EAE, the contribution of the hypersensitivity response to the pathology of the lesion, and to the clinical expression of disease, is not clear. Iron-deficiency anaemia is associated with poor cell-mediated, but normal antibody responses (Joynson et al, 1972; Fletcher et al, 1975; Kuvibilida, Baliga and Suskind, 1981; Baliga, Kuvibilida and Suskind, 1982; Kuvibilida et al, 1983); conversely, incorporation of iron in the adjuvant used to induce EAE results in severe symptoms and prolongation of disease (Levine and Sowinski, 1970). In addition, iron has been shown to participate in free radical-generated lipid peroxidation, as outlined in section 1.12. If such a process is involved in the pathology of the EAE lesion, depletion of catalytic iron might be expected to reduce oxidative damage within the lesion, resulting in amelioration of disease.

The choice of Desferrioxamine (DFO), therefore, to deplete animals of iron was based on two properties. One was its high specificity for non-haem, ferric iron, which effectively depletes tissues of traces of iron which would normally be available to generate the delayed hypersensitivity response, and participate in iron-catalysed free-radical formation. The second property is the ability of DFO to act as a radical scavenger in its own right, (Hoe, Rowley and Halliwell, 1984). DFO was administered to rats

with EAE, to examine the effect, if any, on the disease process, and the pathology of CNS lesions.

4.2 Properties of desferrioxamine.

Desferrioxamine is a fungal metabolite obtained from *Streptomyces pilosis*. It was first isolated by Bickel (1960), and has proved a valuable drug in the treatment of iron overload caused by multiple transfusions (e.g. in thalassemia and haemodialysis) and in iron poisoning (Modell and Beck, 1974). Its high affinity for ferric iron (Table 4.1) means that it can be used to great advantage both *in vitro* and *in vivo*, without significantly affecting levels of other essential ions, such as calcium and magnesium. As it is not absorbed from the gastrointestinal tract, it is usually given parenterally. DFO binds iron in a 1:1 ratio, forming a tight complex (Figure 4.1), which is then excreted via the kidneys, turning the urine a reddish-brown colour. The colour of the iron-bound ferrioxamine complex forms the basis of its assay in plasma (Section 2.10), since it can be measured spectrophotometrically.

The half-life of DFO in blood is approximately 30 min, with complete excretion by 4 hr. Although some metabolism occurs in vivo by an α globulin enzyme found in plasma, degradation accounts for less than 30% of available DFO in 3 hr (Keberle, 1964), and is therefore probably insignificant in the overall in vivo metabolism of DFO.

Table 4.1 Binding constants for various metal ions of desferrioxamine and transferrin.

Metallic ion	Desferrioxamine	Transferrin
Fe^{3+}	10^{31}	$10^{28,29}$
Ca^{2+}	10^{2}	
Mg^{2+}	10^{4}	
Zn^{2+}	10^{11}	
Cu^{2+}	10^{14}	
Fe^{2+}	10^{10}	

From Keberle (1964).

Desferrioxamine B

Ferrioxamine B

Figure 4.1 The structure of desferrioxamine B and ferrioxamine B.

(from: Keller-Schierlein, 1975; Pitt and Gupta, 1975).

DFO removes iron from the iron-storage proteins ferritin and hemosiderin very effectively without affecting haem iron (Propper, Shurin and Nathan 1975). In vitro studies have shown that DFO will bind up to 14% of transferrin-bound iron (Keberle, 1964), but this could not be demonstrated in vivo

(Hallberg and Hedenberg, 1965). In general, the amount of iron chelatable by DFO *in vivo* is related to the amount of storage iron, particularly to the concentration of ferritin (Jacobs, Kaye and Trevett, 1969; Balcerzak, Jensen and Pollack, 1966). Propper, Shurin and Nathan (1975) have concluded that DFO appears to act upon an intermediate compound in the pathway of iron transfer from storage (haemosiderin, ferritin) to plasma (transferrin) pools (See Fig. 4.2). Since DFO binds trivalent iron only, and storage iron is in the 2+ state, an oxidation step is necessary. Ceruloplasmin is the most likely source of ferroxidase *in vivo*, although ascorbate can also act in this way. Frieden (1971) has shown that the rate of Fe^{++}oxidation in the presence of 2 uM ceruloplasmin is 10-100 times faster than non-enzymatic oxidation under physiological conditions.

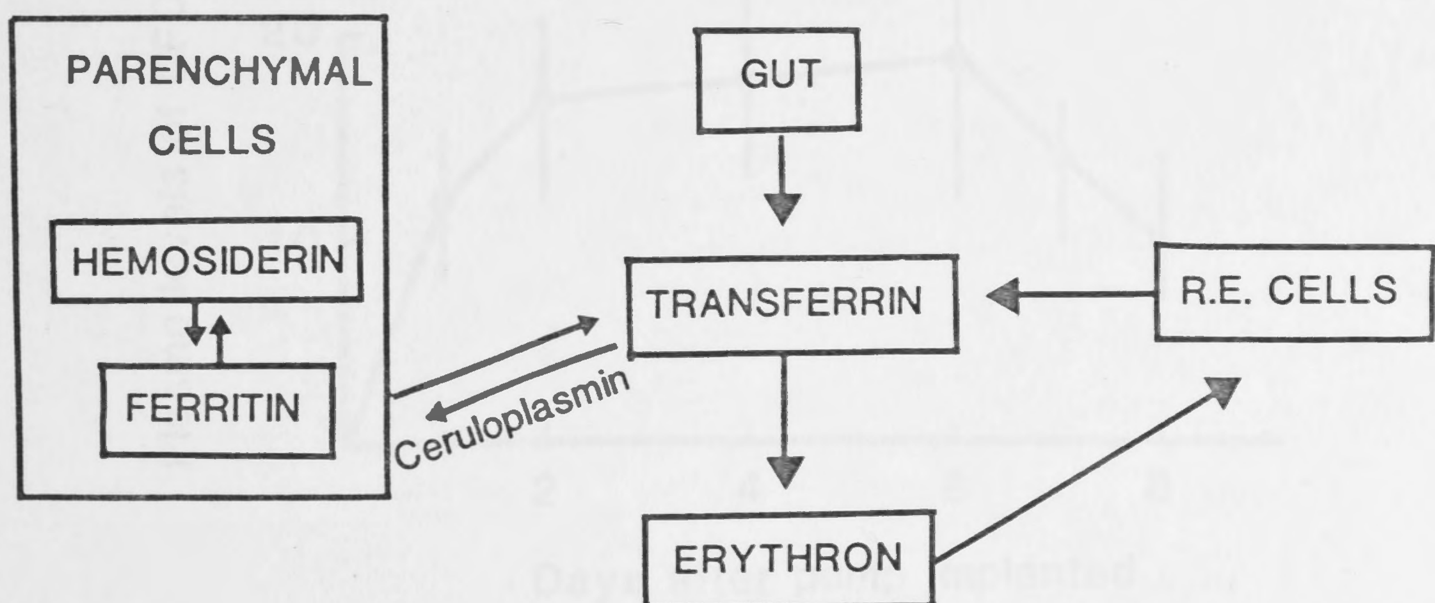

Figure 4.2 Major iron pools of the body.

(from: Propper, Shurin and Nathan, 1975)

4.3 Estimation of plasma and CSF levels of Desferrioxamine.

Initial experiments showed that intramuscular injections of DFO into rats with EAE did not alter the disease process. Since DFO is excreted rapidly, with a half-life in plasma of about 30 minutes, a continuous infusion was effected by means of an Alzet osmotic pump which could be implanted subcutaneously, delivering 3 mg DFO/hr for 7 days. Five normal rats were bled at various times after implantation of Alzet osmotic pumps containing 500mg DFO. Maximum levels of DFO in plasma were reached within 24hr of starting infusion, and were maintained at a steady rate of approximately 10-20ug/ml throughout (Fig 4.3).

Fig 4.3 Plasma levels obtained after implantation of osmotic pumps containing 500mg Desferrioxamine.

The maximum amount of DFO that can be loaded into an osmotic pump is 500 mg and, since levels of 10 ug/ml were subsequently shown to be necessary for suppression of lymphoproliferation *in vitro*, the amounts used here would be marginal for effective immunosuppression. Animals which are much larger than those used in these experiments (more than 150 g) would theoretically need larger doses of DFO. In addition, storage iron levels may vary between individual animals, and certainly does vary according to age, since the iron concentration in spleen is about 10 times lower in young (20 day) animals than in adult rats (Kochanowski and Sherman, 1983). Investigations are currently in progress to evaluate other continuous drug delivery systems, so that dose responses can be assessed.

Because EAE is a disease that affects the CNS, it was important to determine whether DFO was able to cross the blood-brain barrier. Levels were determined in rabbits, since it is difficult to obtain CSF from rats without blood contamination. Blood and CSF were taken at various times after a single injection of 1g of DFO. Maximum levels of 150ug/ml were reached in the plasma 1 hour after injection, and in the CSF 3 hours later (Figure 4.4). This showed clearly that DFO was able to cross the blood-brain barrier, and the delay of two hours indicates that it has probably diffused through the CNS into the CSF. It is therefore likely that the drug would be available to act within lesions of EAE.

Fig. 4.4 Levels of DFO in plasma and CSF of rabbits given a single injection of 500mg DFO.

4.4 Effect of desferrioxamine on clinical EAE.

Treatment of GPSCH-FCA-sensitized rats with DFO prior to the onset of symptoms either completely prevented the weight loss that is associated with clinical EAE (days 0-7, Fig 4.5a), or greatly reduced the magnitude of such weight loss (day 8-15, Fig 4.5b). The same general effect is seen in rats given the drug from day 11-18 (Fig 4.5c). Also, clinically affected rats that were treated from day 14-21 regained weight more rapidly than untreated controls (Fig 4.5d). Administration of DFO to normal rats caused no change in normal weight gain.

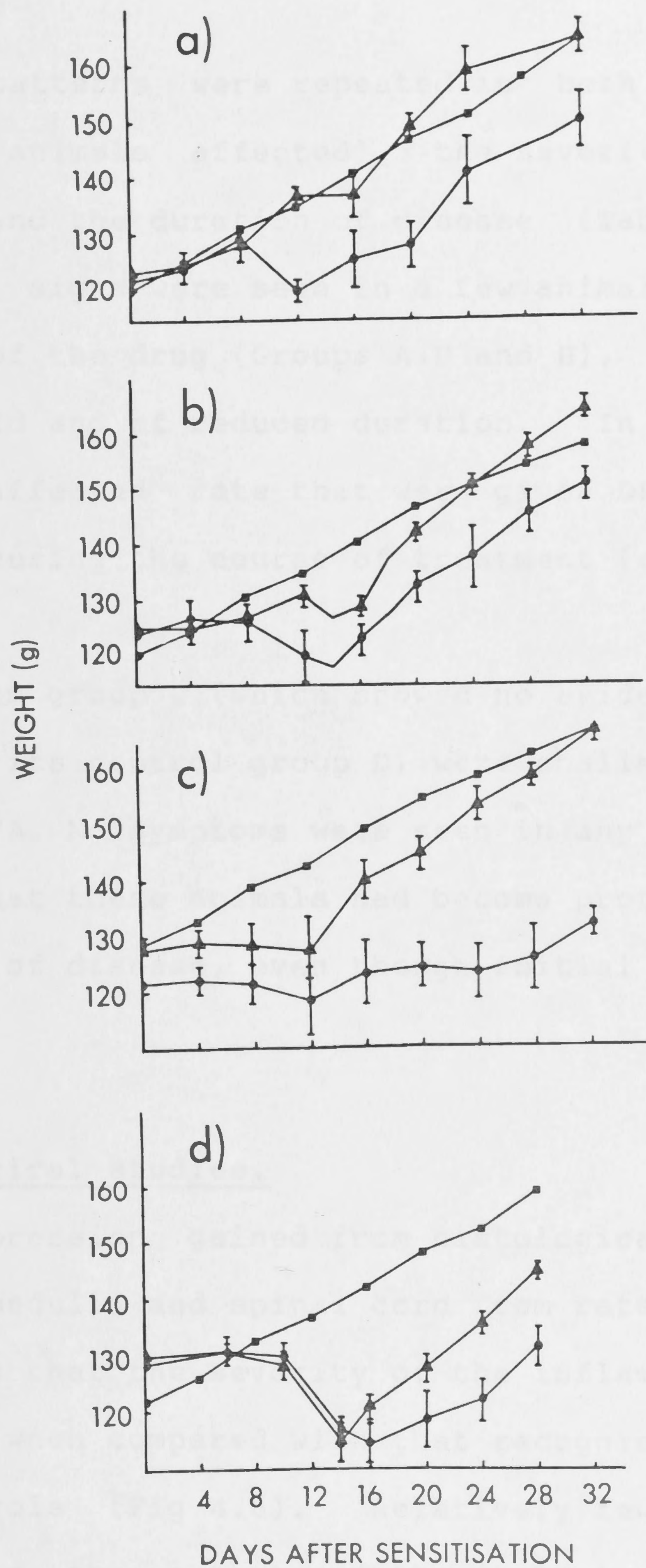

Figure 4.5 Effect of DFO on clinical course of EAE.

Rats were given DFO on: days 0-7 (a), days 8-15 (b), days 11-18 (c) and days 14-21 (d). The groups were:

▲—▲ GPSCG-CFA-primed and treated with DFO.

●—● GPSCH-CFA-primed and untreated.

■—■ Unprimed and given DFO.

5 rats per group.

These patterns were repeated in both the incidence (number of animals affected), the severity of clinical impairment and the duration of disease (Table 4.2). Some neurological signs were seen in a few animals subsequent to withdrawal of the drug (Groups A,D and H), but these were generally mild and of reduced duration. In addition, the clinically affected rats that were given DFO showed rapid improvement during the course of treatment (compare G and H, Table 4.2).

Rats from group C (which showed no evidence of primary disease) and its control group D, were challenged at day 35 with GPSCH-CFA. No symptoms were seen in any animals, indicating that these animals had become protected against re-induction of disease, even though initial symptoms were absent.

4.5 Histological studies.

The impression gained from histological analysis of sections of medulla and spinal cord from rats treated early with DFO was that the severity of the inflammatory process was minimal when compared with that recognised in the EAE-primed controls (Fig 4.6). Relatively few lesions were observed, and the extent of the cellular invasion was slight. Also, it was noted that sections from those animals that had already developed symptoms at the commencement of treatment showed evidence of numerous old lesions, with fibrin depostion but little cellular infiltration. The few cells that were present had the general appearance of macrophages.

Table 4.2 Effect of DFO on the duration and severity of symptoms of EAE.

Group	Number in group	Treatment DFO	Treatment Days	Days after sensitization 12	14	16	18	20	22	24	28
A	5	+	0-7	0	0	1.0(5)*	0	0	0	0	0
B	5	-		0	3.0(5)	3.5(5)	2.0(5)	0.5(2)	0	0	0
C	5	+	8-15	0	0	0	0	0	1.5(1)	0	0
D	5	-		3.3(3)	4.0(3)	3.0(4)	1.0(2)	2.0(2)	3.0(1)	0	1.0(1)
E	5	+	11-18	0	0	0	0	0	0	0.5(1)	0
F	5	-		1.0(3)	2.0(4)	1.7(3)	1.0(3)	1.0(2)	2.0(2)	0	0
G	10	+	14-21	0	1.5(2)	1.4(8)	1.0(3)	0	0	1.0(1)	0
H	10	-		0	1.5(2)	2.0(7)	2.7(6)	1.5(2)	2.3(3)	0.8(2)	0

Osmotic pumps delivering 70 mg of DFO per day were implanted into rats (5 per group). Controls were sham-operated. The results for days 14-21 are cumulated data for 2 groups of 5 animals: the two animals that relapsed on day 24 were from the same group.

* The results are given as: mean clinical score for those with neurological signs (number of clinically affected animals).

X150

Figure 4.6 Histological sections from desferrioxamine (DFO)-treated rats.

Haematoxylin and eosin-stained sections from medulla of rats, 10 days (a,b) and 16 days (c,d) after injection of GPSCH-CFA.

a) and c): from untreated rats, showing typical lesions.

b) and d): from rats given DFO on days 0-7 (b) and 7-14 (d) showing absence of small mononuclear cells in lesions.

Examination of histological sections from rats given DFO from day 14 onwards (i.e. after the appearance of symptoms) indicated that fewer cells could be found in lesions by 48 hr after DFO treatment was started. This was confirmed by ^{125}IUdR labelling (see section 4.13).

4.6 Patterns of lymphocyte responsiveness.

The initial hypothesis was that DFO may limit the severity of EAE by removing free iron necessary for generating hydroxyl radicals. However the apparent loss of mononuclear cells from lesions (Section 4.5) raised the possibility that the drug might be either directly affecting lymphocyte function, or affecting lymphocyte migratory patterns. The first possibility was examined by assaying PEC for their procoagulant activity to MBP.

Peritoneal exudate cells were taken at intervals from the rats that had been examined clinically (Fig 4.5, Table 4.2) and were tested for procoagulant activity. The level of response was depressed for PEC sampled from 1-3 days after the cessation of DFO treatment (Groups A and B d10, C and D d16, E and F d21, Table 4.3). This diminution in activity was least apparent for the rats that were clinically affected when drug treatment was started on day 14, though evidence of suppressed lymphocyte function was still apparent in these animals (Groups G and H d21, Table 4.3). However, by day 32 (Table 4.3), PEC from all groups of rats given DFO showed levels of PCA that were comparable to

Table 4.3 Levels of T cell responsiveness in peritoneal exudates.

Group[a]	Treatment		Procoagulant activity on days[b]					
	DFO	Days	7	10	14	16	21	32
A	-	0-7	-	12.0±1.7	-	-	23.4±1.0	23.4±2.0
B	+	0-7	-	3.3±1.4[c]	-	-	23.0±1.8	18.6±2.4
C	-	8-15	5.5±1.3	-	-	21.6±1.9	-	52.2±2.1
D	+	8-15	5.5±1.1	-	-	3.1±1.1	-	54.4±6.2
E	-	11-18	-	9.5±2.6	-	-	23.7±2.3	45.5±2.9
F	+	11-18	-	9.5±2.8	-	-	1.0	49.8±2.7
G	-	14-21	-	-	18.7±1.5	-	24.1±1.8	24.4±2.7
H	+	14-21	-	-	19.2±1.7	-	16.7±3.5	20.8±3.3

[a] These are the same groups as those shown in Table 4.2 and Figure 4.5.
[b] Mean ± SD for 5 animals; rats were injected i.p. with thioglycollate 3 days before sampling.
[c] Statistically significant $P<0.05$.

those seen in the EAE controls. Thus, the capacity of lymphocytes to respond to MBP was completely restored within 11 days of stopping the drug treatment (Table 4.3). This may be due to persistance of the antigen in the injection site (Tabira, Itoyama and Kuroiwa, 1984). Even so, recovery of lymphocyte responsiveness did not usually lead to the development of symptoms, and 12 of the 15 animals that were given DFO from day 8 onwards remained completely free of the disease subsequent to treatment (Groups D, F and H, Table 4.3)

Popliteal lymph node cells were also tested from other rats that were subjected to the same treatment schedules used for the clinical evaluation (Groups A to F, Table 4.3). These animals were killed at various times after removal of the osmotic pumps. The patterns of response seen (Table 4.4) were generally similar to those observed for the PEC populations (Table 4.3). Lymph node cells taken up to 6 days after cessation of DFO-treatment showed reduced MPCA activity, however they had returned to normal levels by 16 days after treatment was stopped. These results suggested that the reduced severity of disease in DFO-treated animals might be due to a reduction in the cellular immune response.

The "rebound effect" (Table 4.4) could be due to 1) a delay in the normal immune response, so that the peak occurs later in treated animals; 2) rapid mobilization of storage iron after depletion by desferrioxamine, or 3) may reflect the fact that lymphocytes are not killed, but remain synchronised in G1, resulting in an accelerated response once DFO is withdrawn.

Table 4.4. MPCA-promoting activity of lymph node cells.

Days DFOM given	Day of sampling	% reduction in clotting time*
0-7	13	9.7±0.9
Nil	13	39.0±3.3
8-15	31	51.1±2.5
Nil	31	33.7±2.0
11-18	18	14.0±1.1
Nil	18	28.7±2.3
11-18	35	49.8±2.4
Nil	35	35.8±1.9

* Supernates from cultures with MBP were assayed using the PU5-1.8 cell line.

4.7 Suppression of T cell function.

In order to determine whether DFOM was inhibiting macrophage or T cell function, PEC from DFOM-treated rats were incubated with lipopylysaccharide B (LPS) which is known to directly stimulate procoagulant activity in macrophages (Farram et al, 1983). The PEC from DFO-treated rats responded to LPS at least as well as normal PEC (Table 4.5). Also, the addition of lymph node cells from rats primed with GPSCH-CFA 18 days previously, restored the capacity of PEC from the DFO-treated animals to produce procoagulant factor when stimulated with MBP. In contrast, lymph node cells from EAE/DFO rats were unable to stimulate normal macrophages in the presence of MBP. This suggested that the defect resulting from DFO treatment was in the lymphocyte rather than the macrophage population.

Table 4.5 Restoration of PEC reactivity by addition of lymph node cells from GPSCH-sensitized rats.

PEC*	LNC	%reduction in clotting time MBP	LPS
Normal	None	4.1	39.3
EAE/DFOM (dl1-18)	None	1.0	42.3
EAE/DFOM (dl1-18)	EAE LNC	36.5	
EAE/DFOM (dl1-18)	Normal LNC	4.5	
EAE (d18)	None	36.0	
Normal	EAE LNC	38.0	
None	EAE LNC	3.9	
Normal	DFOM/EAE LNC	2.4	
None	DFOM/EAE LNC	1.5	

* PEC were tested for procoagulant activity by incubation with antigen (MBP, 5 ug/ml or LPS, lug/ml) for 16 hr. LNC from EAE (day 18 PI), DFOM/EAE (DFOM d 11-18) or normal rats were added at $3x10^5/10^6$ PEC, the optimum ratio for stimulation.

Plasma levels of DFO achieved *in vivo* were approximately 15 uM (10 ug/ml) (see page 86). The concentration routinely used *in vitro* to suppress proliferation was the same, 15 uM.

4.8 Effect of DFO on production of procoagulant factor by PU5-1.8 cell stimulated by lymphokine.

DFO appeared to inhibit lymphocyte reactivity, but had little effect on macrophages when sensitized lymphocytes were present (and therefore, a source of lymphokine). In order to determine whether production of procoagulant factor could be inhibited by DFO, PU5-1.8 cells were incubated in the presence of T cell growth factor (TCGF) and various dilutions of DFO, as shown in Table 4.6. DFO did not inhibit the production of PCF, at concentrations routinely used in vitro. This suggested that DFO had no effect on the ability of lymphokine to stimulate the production of procoagulant factor on PU5-1.8 cells.

Table 4.6 Effect of DFO on procoagulant activity of PU5-1.8 cells stimulated with lymphokine.

DFO (uM)	clotting time (secs) +TCGF	-TCGF	%RCT
-	79.9	126.9	37.0
2.4	68.9	125.9	46.8
12	63.6	110.4	47.4
60	63.9	120.9	43.4
300	56.9	112.9	47.3
1500	40.2	66.4	39.4

4.9 Suppression of lymphocyte proliferation in vitro.

A further experiment was done in vitro to establish whether the effect of DFO was indeed mediated via the removal of loosely-bound iron. Lymph node cells from EAE-primed rats were incubated in vitro with MBP. The resultant proliferative response was completely inhibited by the addition of DFO to the medium (Table 4.7). However the inhibitory effect of DFO was substantially reversed when the drug was saturated with Fe^{+++} before adding it to the culture, indicating that DFO suppresses lymphocyte responses by chelating loosely-bound iron.

Table 4.7 Effect of DFO and $FeCl_3$ on T cell proliferation.

	Stimulation index*				
LNC	Con A	MBP	MBP+DFO	MBP+SDFO	$MBP+Fe^{+++}$
EAE d10	4.0	2.9	1.0	2.3	2.3
Normal	6.4	1.0	1.0	1.0	1.1

* Results are expressed as stimulation index relative to counts for unstimulated control cultures.

4.10 Phagocytosis of carbon particles by macrophages.

An additional experiment was carried out to rule out any effect of DFO on macrophage function. Macrophages were tested for their ability to phagocytose carbon particles in the presence of DFO.

PEC were obtained from the peritoneal cavities of rats injected with thioglycollate broth 3 days previously. The cells were allowed to adhere to plastic petri dishes overnight; non-adherent cells were removed and the medium was replaced with RPMI + 10% HIFCS and 1:10,000 "Pelikan" brand Indian ink, which contains a suspension of carbon particles in a non-toxic base. Dilutions of DFO ranging from 1 uM to 100 uM were added to the cultures simultaneously, and the cells were incubated for 1 hr at 37°C. Electron microscopy revealed that phagocytosis of carbon particles was undiminished at dilutions of DFO up to 50 uM (Figure 4.7). Some inhibition occurred at 100 uM DFO, although occasional cells could still be seen containing carbon particles within lysosomes.

4.11 Production of antibody to Myelin Basic Protein.

Production of antibody to MBP is generally considered to be dependent on T help (Bernard, Leydon and Mackay, 1976; Ortiz-Ortiz and Weigle, 1976). Since DFO appeared to inhibit sensitization of T lymphocytes to MBP, and symptoms of EAE, it was important to determine whether the drug had any effect on antibody production.

Figure 4.7 Phagocytosis of carbon particles by macrophages.

Rat PEC were incubated for 1 hr in the presence of various concentrations of DFO.

1) Carbon uptake by PEC in the absence of DFO.
2) In the presence of 50 uM DFO, carbon uptake was undiminished.
3) At a concentration of 100 uM DFO, occasional sparse lysosomes containing carbon could be found.
4) PEC incubated without addition of carbon or DFO.

1

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Groups of 5 rats were bled at various times after DFO treatment, as shown in Table 4.8, and the sera were tested for antibody to MBP. In addition, 2 groups of animals were tested which had been treated with the antioxidants α-tocopherol and BHA (see section 4.12).

Antibody production in the DFO treated groups followed the same pattern as in EAE controls (Table 4.8). This means that antibody production proceeds normally in the face of a marked reduction in T cell responses, as measured by the MPCA assay and severity of clinical disease.

Table 4.8 Anti-MBP antibody titres of rats with EAE.

Treatment	Days after sensitization			
	7	14	21	28
None	ND*	128	2048	8192
DFO day 0-7	ND	256	4096	8192
DFO day 7-14	ND	256	8192	2048
DFO day 11-18	ND	128	1024	8192
DFO+$FeCl_3$ day 7-14	ND	512	1024	8192
α-tocopherol	ND	64	4096	2048
BHA	ND	16	1024	4096

Antibody titres are expressed as the reciprocal of the serum dilution. ND = not detected.

4.12 Other iron-chelators and antioxidants in vivo.

It was still unclear whether DFO was acting in the suppression of EAE by chelating iron, thereby inhibiting lymphocyte proliferation, or by scavenging free radicals, and preventing lipid peroxidation in the CNS. Several anti-oxidants, iron-chelators and inhibitors of the enzyme ribonucleotide reductase (RR) were tested for their ability to suppress EAE. Butylated hydroxyanisole (BHA), a-tocopherol, dimethyl sulphoxide (DMSO), and hydergine have been shown to inhibit lipid peroxidation in vitro (Gutteridge, 1978; Koreh Seligman and Demopoulos, 1982). The iron chelators HBED and PIH (described in 2.2) can be given orally, and therefore have the advantage of being easier to administer. Hydroxyurea is an antineoplastic drug which acts by inactivating RR, thereby preventing DNA synthesis. Another compound which inhibits RR activity, PATS (described in Section 2.2), and its iron-containing counterpart Fe-PATS, were also tested to determine whether inactivation of RR could be reversed by the addition of iron.

Of all the compounds tested, the most effective were DFO and hydroxyurea (Table 4.9). PIH, PATS and FePATS appeared to be toxic, in that soon after starting treatment the animals exhibited ruffled fur and loss of activity and eating, not associated with EAE. In addition, 8 of the 10 animals receiving PATS died on days 12-13 from causes other than EAE. Some reduction in both the length and severity of disease was seen in all groups of rats which received

Table 4.9 Effects of various iron-chelators and antioxidants on the clinical course of EAE.

Expt. No.	Treatment (d7-14)	Dose (mg/kg/day)	Route	Days after sensitization 12	14	16	18	20	22	24	28
1.	BHA	150	food	1.5(2)*	1.6(5)	1.8(8)	1.9(6)	2.0(6)	1.5(5)	1.4(5)	0
	Vitamin E	150	i.p.	4.0(2)	3.0(6)	2.2(10)	1.6(6)	1.0(4)	1.0(2)	0	0
	DMSO	1300	water	1.8(6)	1.5(7)	1.2(8)	1.2(6)	1.0(6)	0.7(3)	0	0
	Hydergine	80ug	water	2.3(3)	2.3(4)	2.2(5)	1.7(4)	1.3(3)	1.0(2)	0	0
	Nil	-		2.2(6)	2.6(6)	2.9(7)	2.7(7)	2.5(7)	2.5(7)	2.5(6)	2.2(4)
2.	DFO	400	pump	0	2.0(1)	2.0(2)	1.0(2)	0	0	0	0
	Hydroxyurea	25	pump	0	1.0(2)	2.2(4)	1.7(3)	1.5(2)	0	0	0
	FePATS	10	pump	1.8(10)	1.8(9)	2.0(8)	1.8(8)	1.6(6)	1.3(6)	1.0(5)	1.0(1)
	PATS**	5	pump								
	Nil	-		1.5(3)	1.7(7)	2.0(8)	1.8(7)	1.5(6)	1.5(5)	1.0(4)	0
3.	SDFO	400	pump	2.3(6)	2.8(8)	3.2(10)	3.5(10)	3.0(10)	3.0(10)	2.9(10)	3.0(2)
	FeCl	30	pump	1.0(6)	1.5(6)	1.7(6)	1.8(10)	2.0(10)	3.0(7)	3.0(3)	3.0(2)
	PIH	50	pump	2.0(6)	2.0(8)	2.1(9)	1.8(8)	1.4(7)	1.8(5)	2.0(2)	0
	HBED	50	pump	2.3(4)	2.1(7)	2.2(8)	1.8(6)	1.6(5)	1.5(5)	1.0(3)	0
	Nil	-		2.2(6)	2.2(7)	2.1(8)	1.9(7)	1.5(6)	1.3(6)	1.0(2)	1.0(1)

* The results are given as: mean clinical score for those with neurological signs (no. of animals sick). for groups of 10 rats.

** All but 2 of this group died from days 12-13. The remaining 2 showed no signs of disease.

antioxidants (Experiment 1, Table 4.9), however, the effect was not as great as in those receiving DFO or Hydroxyurea. Supplementation with saturated DFO (DFO:$FeCl_3$ in a 1:1 molar ratio) greatly enhanced the severity of disease; this group did not recover normal weight until 3-4 weeks after other symptoms had resolved (days 51-58), compared with the EAE controls which recovered normal weight by day 36.

Therefore, it appears that other iron-chelators do not significantly alter the clinical couse of EAE. Antioxidants are partially effective in reducing the severity of disease. However the greatest effect is seen with DFO and hydroxyurea, both of which may act by reducing lymphocyte proliferation.

4.13 ^{125}IUdR labelling of infiltrating cells.

Histological studies (Section 4.5) suggested that small mononuclear cells which accumulate in lesions of EAE were absent from animals which had been treated with DFO. Scavengers of free-radicals have been shown to inhibit the increase in vascular permeability caused by free-radical generating systems (del Maestro, Bjork and Arfors, 1981). It was possible that DFO was acting by decreasing blood-brain barrier permeability, and preventing entry of sensitized lymphocytes into lesions of EAE. To test this possibility, BBB permeability to specifically sensitized lymphocytes, and to a non-specific protein, bovine serum albumin (BSA) was measured in rats with EAE. 5-iodo-2'-deoxyuridine (^{125}IUdR) is incorporated into rapidly dividing cells, and is a

sensitive measure of the accumulation of such cells in inflammatory lesions. (Stohl, Kaplan and Gonatas, 1979; Linthicum, Horvath and Carnegie, 1979). Groups were matched according to day of onset and clinical score on day tested. On days 14, 17 and 20 after injection of GPSCH-CFA, levels in EAE controls were similar, at about 4,000 counts per minute (Fig 4.8). Rats given DFO from day 14 onwards showed a marked drop in ^{125}IUdR label on day 17, which reached almost normal levels by day 20. There was no significant difference in BBB permeability to ^{125}I-BSA between DFO-treated groups and EAE controls on days 14 and 17; however counts on day 20 in the DFO-treated group were almost half that of EAE controls. This may reflect the repair of the blood-brain barrier as lesions resolved in the treated groups. However the finding for day 14 and day 17 indicates that there is direct inhibition of the inflammatory process that is not a consequence of repair to the blood-brain barrier.

counts per minute x 10^3

7
6
5
4
3
2
1

day 14 day 17 day 20

^{125}I-BSA

day 14 day 17 day 20

^{125}I-UdR

Fig 4.8 Permeability of the CNS to I-BSA and to IUdR-labelled cells.

Permeability to a) ^{125}I-BSA and b) ^{125}IUdR in rats with EAE (☐), rats with EAE given DFO from days 14-21 (▨), and normal controls (▤).

4.16 Alteration of normal migration patterns by DFO.

From the previous results it appeared that the migration of specifically sensitized lymphocytes into the CNS may have been reduced by DFO treatment. This could be due to suppression of proliferation of these cells, as was indicated by in vitro results (Sections 4.7, 5.3). Another possibility is that DFO may alter, in some way, the migration of normal lymphocytes, since it has been suggested that iron may influence lymphocyte migratory patterns (de Sousa, 1978). This was examined by labelling normal spleen cells with the irreversible DNA stain, Hoechst 33342 (H33342) dye, and following their migration in normal or DFO-treated recipients. DFO-treated CBA mice received osmotic pumps containing 50mg DFO, which delivered 7mg over a 24 hr period. Pumps were implanted subcutaneously 16 hr before injection of cells. Normal CBA mouse spleen cells were treated by rosetting to remove Ig+ cells ("T" cell fraction), or by anti-Thy 1 serum to remove Thy 1+ cells ("B" cell fraction). Each mouse received 2x 10^7 cells i.v. After 1 hr, spleens were removed and examined by UV microscopy. There was no difference in the pattern of B cell migration between the DFO-treated and untreated animals (Figure 4.9a,b). In both groups B cells migrated to outer follicular areas of white pulp. In contrast, T cell migration in DFO-treated animals was markedly abnormal;the cells were found in the marginal zone between red and white pulp, and in a "doughnut-shaped" area around the peri-

Fig. 4.9 Migration of normal spleen cells to spleen.

a) B cells into normal mice.

b) B cells into DFO-treated mice.

c) T cells into normal mice.

d) T cells into DFO-treated mice.

Magnification X60.

arteriolar sheath (Fig 4.9d). T cells in untreated mice were found in close proximity to the periarteriolar sheath, as is normally seen.

Thus, DFO was found to alter the migration of normal T cells to T-dependent areas of the spleen. The migration of B cells was not affected, however.

4.15 Suppression of DTH to sheep red blood cells.

In the previous section it was shown that DFO altered the homing of normal T cells to T-dependent areas of the spleen. Another way of examining this question is by placing both antigen and sensitized cells into a suitable site, such as the footpad. This procedure normally results in swelling of the footpad to approximately twice the normal thickness, and overcomes the necessity for specifically sensitised effector cells to migrate into sites of inflammation.

The footpad swelling response to sheep red blood cells (SRBC) is a standard procedure which is widely accepted as a classical model for delayed hypersensitivity, which can be readily measured. An outline of the experimental protocol is given in Fig 4.10. CBA/H mice were sensitized to SRBC by an intravenous injection of 10^8 cells. Five days later, 10^8 spleen cells from these mice were transferred into naive recipients by intravenous injection. At the same time, an eliciting dose of 10^8 SRBC in 50 ul PBS was injected in the dorsum of the hind footpad. 50 ul saline was injected in the opposite foot to serve as a control. The swelling was

*

Direct injection of sensitized lymphocytes together with antigen into the footpad, failed to induce an inflammatory response in mice treated with DFO. This suggests that the alteration of migration of sensitized effectors is not the only means by which expression of DTH is inhibited. DFO may also inhibit cell proliferation or the migration of recipient cells (which form the majority of cells in DTH inflammatory sites).

measured 4, 24 and 48 hr later, as described in Section 2.13. A variation of this method was to combine the spleen cells with the SRBC in 50 ul, which was then injected into the footpad. As mentioned previously, the injection of both antigen and sensitized lymphocytes into a local site obviates the need for specific effector cell migration.

Mice which were treated with DFO by implantation of osmotic pumps 3 days before challenge with SRBC did not express DTH to SRBC, either when sensitized spleen cells were given intravenously or with SRBC into the footpad (Figure 4.10). However, the same mice developed normal antibody responses to SRBC (results not shown).

Haematoxylin and eosin-stained sections of the footpads of these mice showed that in place of the massive infiltrate of mononuclear cells characteristic of DTH responses (Fig. 4.11b), the footpads appeared essentially normal, with a thin layer of cells beneath the dermis consisting of the injected erythrocytes and mononuclear cells (Fig. 4.11c).

These results suggest that the lack of oedema and failure to recruit non-specific inflammatory cells such as macrophages into the inflammatory site which was seen in the DFO-treated animals is not due solely to the absence of sensitized lymphocytes.*

10^8 srbc i.v.
spleen 5 days
spleen cells i.v.
TEST FOR DTH: 10^8 srbc fp.
spleen cells+srbc fp.

24 hr footpad swelling x0.1mm

15
10
5
a
b
Imm DFO Nor Imm DFO Nor

Figure 4.10 Footpad swelling response to SRBC.

a) Spleen cells i.v., SRBC in footpad.
b) spleen cells + SRBC in footpad.

Imm: sensitized spleen cells into normal mice.
DFO: sensitized spleen cells into DFO-treated mice.
Nor: normal spleen cells into normal mice.

Figure 4.11 (next page) Histological sections of mouse footpad, showing

a) normal spleen cells and SRBC injected together into a normal mouse footpad.

b) Sensitized spleen cells and SRBC injected together into a normal mouse footpad, showing the massive mononuclear infiltrate.

c) sensitized spleen cells and SRBC injected together into footpad of a mouse given DFO from -2 days.

a

b

c

4.16 Suppression of generation of T suppressor cells.

Animals which were injected with GPSCH-CFA, treated with DFO, and did not develop symptoms, were nevertheless resistant to reinduction when challenged on day 35 (Section 4.4). It is thought that resistance to re-induction of EAE is primarily due to T suppressor cells (reviewed in Section 1.9). It was important, therefore, to determine whether T suppressor cells were generated during DFO treatment.

Spleen cells from mice given SRBC 4 days previously were injected into recipients at the time of sensitization, a procedure which has been shown to suppress the DTH response in the recipients (Ramshaw, Bretscher and Parish, 1976). In this experiment, the footpad swelling was reduced by approximately 60% (Figure 4.12). However, treatment of donors by implantation of osmotic pumps containing DFO at the time of sensitization abrogated the suppression, suggesting that these animals did not generate T suppressor cells (Figure 4.12).

Figure 4.12 Effect of DFO on the generation of suppressor cells.

Mice were sensitized with SRBC, 4 days later 1 spleen equivalent (containing the putative T suppressor cell population) was transferred to naive recipients, and mice were challenged with SRBC in the footpad.

■ sensitised, untreated donors.
□ sensitised, donors treated with DFO.
⊡ unsensitised donors.

4.17 Effect of DFO on non-specific inflammation.

Desferrioxamine appeared to be a potent suppressor of the expression of delayed hypersensitivity, whether or not the sensitized effector cells were present in the inflammatory site, since it inhibited the footpad swelling induced by injection of sensitized spleen cells both intravenously and directly into the footpad (Section 4.15). Much of the expression of DTH, i.e. oedema and erythema, is mediated by macrophages. It has been suggested that superoxide, produced by macrophages, is one of the principal mediators in sites of inflammation. McCord and Wong (1979) have shown that superoxide dismutase (SOD), which scavenges superoxide, prevents the inflammatory oedema generated by macrophages. If DFO were able to scavenge free radicals *in vivo*, then some inhibition of inflammation could be expected. Carrageenan-induced footpad swelling is ideally suited for this purpose, since it is mediated principally by macrophages (di Rosa et al, 1971; Oyanagui, 1976), and is inhibited by SOD but not by catalase (McCord and Wong, 1979), indicating the operation of an O_2^--dependent process. The injection of 25 mg Carrageenan into the footpad resulted in an acute swelling of 50-60%, reaching peak levels 4 hr after injection (Fig 4.13). Five rats received osmotic pumps (delivering 70 mg DFO per day) 24 hr before injection with carrageenan. In this group there was a small but significant reduction in footpad swelling of about 8% at each hourly timepoint tested. However, the reduction was not sufficient

to invoke superoxide inhibition as a major mechanism for the inhibition of inflammatory processes in EAE.

Figure 4.13 Effect of DFO on carrageenan-induced footpad swelling.

Carrageenan (25mg) was injected into the footpads of (●——●) normal rats, and (o·······o) rats which had received DFO in osmotic pumps 24 hr previously. ▒ shows footpad thickness of normal controls.

The injection of Con A into the footpad induces an infiltrate of small mononuclear cells 22 hr later. This type of inflammation has not been well documented, but is useful here in that the mononuclear infiltrate is similar to that found in DTH reactions (Oyanagui, 1982), appearing 22-24 hr after injection of 50ug Con A, and being composed of small lymphocytes, with few macrophages at 22 hr (Fig. 4.14a). This response was totally inhibited by prior treatment with DFO (Fig. 4.14b). Since Con A is a potent lymphocyte mitogen, this again indicates an effect of DFO on rapidly dividing lymphocytes.

Thus, DFO effectively suppressed the non-specific inflammation induced by Con A, which is mediated by small lymphocytes, but had little effect on the macrophage-mediated footpad swelling produced by carrageenan.

4.18 Products of lipid peroxidation in rabbit CSF.

Rabbits were used in this experiment, since it is difficult to obtain cerebrospinal fluid from rats without some blood contamination. A total of 7 rabbits were injected with GPSCH-CFA; all developed paralysis on the days shown in Table 4.10. Rabbits #3 and #4 developed severe chronic paralysis, and were killed on day 80. At various times after injection, rabbits were anaesthetized with nembutal, and 0.5-10 ml of CSF was withdrawn from the cisterna magna, cells were removed by centrifugation, and nitrogen was bubbled through the sample to prevent autoxidation. Samples were frozen at $-70^{0}C$

Figure 4.14 Suppression of Con A response in mouse footpad.

a) Inflammatory response, accompanied by massive cellular infiltration, 22 hr after injection of 50 ug Con A.

b) Mouse footpad, 22 hr after injection of Con A and 46 hr after implantation of osmotic pumps containing DFO.

c) Normal mouse footpad, after injection of 50 ul saline.

a

b

c

until tests could be carried out. CSF samples were tested for products of lipid peroxidation by a sensitive test for aliphatic and aromatic aldehydes (Jacobsen, 1974), which is sensitive to 0.2 ppm. None was detected in any of the samples.

A second test to measure small amounts of catalytic iron, the bleomycin assay, was also negative for all samples. This indicates that the amount of "free" iron which could derive from damage to erythrocytes in lesions of EAE, is not sufficient to be detected in CSF, although it could theoretically act within the local environment of the lesion.

Thirdly, samples were tested for procoagulant activity. Brain cells contain large amounts of thromboplastin, and damage within the CNS is easily measured by the ability of CSF to reduce the clotting time of normal plasma. This method has been used as a sensitive indicator of trauma due, for example, to stroke and spinal injury in humans (Graebar and Stuart, 1978). CSF (0.1ml) was added to normal rabbit plasma (0.1ml), CaCl (0.1ml of 0.3M) was added, and the clotting time estimated as for the MPCA (section 2.14). All of the rabbits tested had high levels of procoagulant activity compared with uninjected controls (Table 4.10), indicating that damage within the CNS had occurred.

Table 4.10 Procoagulant activity of CSF from rabbits with EAE.

Rabbit No.	Day symptoms first appeared	day tested	% RCT*
1	19	28	60.3
2	20	29	57.6
3	17	80	78.7
4	25	80	76.3
5	20	29	76.4
6	23	29	83.1
7	18	29	75.5

* % reduction in clotting time is $\frac{\text{CT normal CSF} - \text{CT test CSF}}{\text{CT normal CSF}} \times 100$

4.19 Discussion.

The severity of EAE was greatly diminished in rats given the iron-chelating agent desferrioxamine (section 4.3). The effects ranged from complete prevention of the development of neurological symptoms, to more rapid recovery in rats that were already clinically affected. The clinical pattern was broadly reflected in the extent of histopathological changes (section 4.4). Recovery of lymphocyte responsiveness after the cessation of therapy, however, did not lead to the development of severe disease.

The protective effect of DFO in EAE was shown to be directly related to the level of responsiveness for lymphocytes of the DTH/helper subset, taken from both the lymph nodes and the peritoneal cavity (section 4.5). ^{125}IUdR-

labelling of rapidly dividing mononuclear cells in lesions also suggested that effector cells were diminished in number (section 4.12), however, antibody production was not reduced. Thus, the most likely reason for the reduction in severity of disease is that DFO is a potent immunosuppressive agent for DTH effector T cells. The finding that DFO suppresses lymphocyte proliferation in vitro and the fact that this is reversed by the addition of $FeCl_3$ (Section 4.9), indicates that the depressive effect may operate via the removal of iron, most of which is bound to transferrin in the serum. The increase in severity of symptoms of rats given an iron supplement (Section 4.12,) further supports the concept that serum iron levels influence the magnitude of DTH responses. Dietary iron deficiency is associated (in various antigen systems) with reduced delayed hypersensitivity (Joynson et al, 1972), failure of lymphocytes to bind to target cells (Baliga, Kuvibidila and Suskind, 1981), and decreased DNA synthesis (Robbins and Pederson, 1970). Iron is also thought to be necessary for other aspects of lymphocyte function, including recirculation and homing (de Sousa, 1978). The alteration of normal T cell migration patterns by DFO (Section 4.14) supports this idea.

Incubation with DFO appeared to have little effect on macrophages in vitro, in that it did not inhibit the production of procoagulant in response to endotoxin stimulation or lymphokine (section 4.9), nor did it inhibit the phagocytosis of carbon particles at concentrations which depress lymphocyte proliferation (4.10). The macrophage-

mediated inflammation produced by carrageenan was not as greatly modified by DFO, compared with the marked effects of the drug on both the footpad swelling induced by Con A and the DTH response to SRBC (Sections 4.15 and 4.17). Since there is an absolute requirement for clonal expansion on contact with antigen (or mitogen) in DTH reactions (Oppenheim, 1968), these results favour the interpretation that DFO acts directly on T lymphocytes, preventing their proliferation and homing.

Two observations indicate that the reduction of lymphocyte proliferation may lead to a decrease in inflammation within EAE lesions : firstly, a more rapid resolution of the disease was seen in rats that were treated after the development of symptoms. Secondly, the number of rapidly dividing mononuclear cells which were labelled with ^{125}IUdR was greatly reduced within 2 days after beginning DFO treatment. Decreased production of procoagulant in inflammatory lesions might be expected to reduce the deposition of fibrin in white matter in EAE (Paterson, 1976). Chelation and removal of "free" iron from sites of inflammation may also prevent free radical-induced lipid peroxidation and production of chemotactic factors resulting from tissue damage. An excess of free iron (produced by direct injection of $FeCl_3$) in the CNS is known to contribute directly to such processes, and to the development of oedema and gliosis (Willmore and Rubin, 1978 and 1982; Hiramatsu, Mori and Kono, 1984). So far, efforts to detect products of lipid peroxidation in EAE have failed. No malonaldehyde could be

detected in the CSF of rabbits with severe chronic EAE (4.19), using a sensitive method which is able to detect nanomolar amounts in serum. Nevertheless, future experiments are planned which aim to detect H_2O_2 and long-lived products of lipid peroxidation in brain slices containing EAE lesions.

It is of interest that iron deposits in the form of hemosiderin, which could be readily mobilised by DFO (Shoden and Sturgeon, 1962) have been found in multiple sclerosis plaques (Craelius et al, 1982). In fact, in one study, iron levels in such plaques were found to be approximately 5 times the level found in normal nervous tissue (Craelius, Jacobs and Lee-Jones, 1980). Therefore, DFO could possibly be of value in multiple sclerosis by removing iron from plaques (thus reducing the potential for iron-catalysed lipid peroxidation), and by acting as a mild, but reversible T cell immunosuppressant.

CHAPTER 5. SUPPRESSION OF T CELL RESPONSES BY DESFERRIOXAMINE.

5.1 Introduction.

The principal effects of DFO *in vivo* appeared to be the inhibition of sensitization to MBP, and alteration of T cell migration. Since both of these functions may be dependent upon proliferation, (Oppenheim, 1968; van Loveren and Askenase, 1984b), and it appeared that T cells were selectively affected, there was a possibility that DFO was acting to suppress EAE solely by preventing T cell proliferation.

The effects appeared to be reversible in that LNC taken from rats treated with DFO responded poorly to MBP (indicating a lack of sensitization *in vivo*), although the same cells were stimulated normally by Con A *in vitro*. Mainou-Fowler and Brock (1985) showed that T cell mitogenic responses were inhibited if carried out in serum from iron-deficient rats, but T cells taken from iron-deficient animals responded normally in normal serum. It has frequently been shown that people suffering dietary iron deficiency show poor responses to T cell mitogens and delayed skin reactivity (Joynson et al, 1972), although antibody production and B cell numbers appear to be normal. Similarly, DFO suppressed the *in vivo* expression of DTH, although antibody responses were normal. Iron may be needed for DNA synthesis, since tumour cells cultured in the presence of DFO showed selective inhibition of DNA but not RNA or protein synthesis (Robbins and Pedersen, 1970).

In order to analyse further the mechanism of action, the effect of DFO on a variety of in vitro T cell proliferative responses was assessed.

5.2 Toxicity of DFO in vitro.

Desferrioxamine was not directly toxic to cells in vitro, as shown by trypan blue exclusion (Table 5.1). Primary cultures of rat LNC and the continuous macrophage cell line PU5-1.8, were grown in RPMI-1640 medium containing DFO in concentrations ranging from 1 uM to 1 mM. Although no loss of viability occurred during the 3 days incubation at 10 uM DFO, PU5-18 cells gradually lost adherence to the culture flask (Table 5.1). Desferrioxamine was toxic at concentrations greater than 100 uM.

Table 5.1 Viability and adherence of cells cultured with desferrioxamine.

days in culture	DFO (uM)	% live cells*		% cells adherent
		LNC	PU5	PU5
1	10	95	99	98
2	10	92	94	56
3	10	87	91	22
3	100	38	25	11.5

* Figures given are no. of live cells/100 cells counted
Absolute cell numbers in cultures did not vary significantly over 3 days.
Standard deviations are less than 10%.

5.3 Effect of desferrioxamine on the proliferative response of lymphocytes to Con A and LPS.

The proliferative response of mouse spleen cells to Con A and LPS in the presence of various concentrations of DFO is shown in Table 5.2. Spleen cells were stimulated with Con A (1 μg/ml) or LPS (50 μg/ml) in the presence of various concentrations of DFO for 48 hr. ^{3}H-thymidine was added during the final 6 hr of incubation. DFO at concentrations of 15uM completely suppressed the Con A-induced T cell proliferation. Higher concentrations of the drug (60μM) were needed to obtain the same degree of suppression in B cells responding to LPS (Table 5.2).

Table 5.2 The effect of desferrioxamine on the proliferative response of lymphocytes to Con A and LPS.

	^{3}H-thymidine uptake (cpm x10^3 ± SE)	
DFO (μM)	Con A	LPS
0	258.0 ± 8.4	169.6 ± 6.5
1	239.0 ± 7.3	177.1 ± 4.1
15	5.5 ± 2.3	52.6 ± 2.3
30	3.2 ± 1.4	21.4 ± 3.0
60	1.4 ± 0.2	2.0 ± 0.1

5.4 Effect of desferrioxamine on the proliferative response of human peripheral blood lymphocytes to mitogens.

The possibility that DFO may be of value in suppressing lymphocyte proliferation in humans, and the fact that DFO is beginning to be used to treat conditions other than iron overload (where iron depletion does not exceed normal levels) e.g. rheumatoid disease (Blake et al, 1981) raised the question of whether human lymphocyte responses are similarly suppressed in the presence of DFO.

Peripheral blood lymphocytes from normal subjects were stimulated with PHA, Con A, PWM or LPS in the presence or absence of various concentrations of DFO. Proliferation was assessed after 3 days, ^{3}H-thymidine being added during the final 24 hr incubation.

DFO inhibited the proliferation of PHA-, Con A- and PWM-stimulated lymphocytes (Fig 5.1), and this was prevented by saturating levels of iron. DFO was less effective at suppressing LPS-induced proliferation in human PBL (Fig. 5.1) and mouse spleen cells (Table 5.2). PHA and Con A activate T cells almost exclusively (Greaves, Owen and Raff, 1974). Human T cells, in contrast to those from mice, are not only activated by PWM but are also required for B-cell differentiation induced by that mitogen (Keightly, Cooper and Lawton, 1976; Dimitriu and Fauer, 1978). LPS, in the mouse at least, is a known B cell mitogen (Andersson et al, 1972). Thus, it appears that T cells are more susceptible to suppression by DFO than B cells.

Figure 5.1 Effect of DFO on the proliferative response of human peripheral blood cells to a variety of mitogens.

a) mitogen alone, b) mitogen + DFO (15μM),

c) mitogen + DFO (15μM) + $FeCl_3$(100μM) d) unstimulated cells.

5.5 Binding of Con A to lymphocytes.

The suppression of mitogen responsiveness was not due to failure of Con A to bind to the cell membrane, since incubation of mouse spleen cells with fluorescein-conjugated Con A (FITC-Con A) in the presence of DFO in concentrations ranging from 1 μM to 1 mM, did not result in any reduction of fluorescent staining, as assessed visually by fluorescence microscopy (Fig 5.2). In addition, DFO did not prevent Con A-agglutination of sheep red blood cells, at concentrations of Con A (1-5 μg/ml) and DFO (1-50 μM) used in the lymphoproliferative assay (results not shown). These results indicate that DFO does not interfere with the binding of mitogen to the cell surface.

Figure 5.2 Binding of FITC-Con A to spleen cells.

DFO was added to 10^6 LNC in 1 ml PBS, 50 μl FITC-Con A was added immediately, and the cells were incubated at 4^0C for 30 min. After 3 washes in PBS, cells were resuspended in buffered glycerol saline and examined under the UV microscope.

a) Mouse spleen cells stained with FITC-Con A.

b) As for a), in the presence of 50 μM DFO.

5.6 Reversal of DFO-induced inhibition with free iron.

To determine whether the iron-chelating capacity of DFO was responsible for the observed inhibition of lymphocyte proliferation, Con A-stimulated CBA mouse spleen cells were cultured with DFO (15μM) and varying concentrations of ferric chloride ($FeCl_3$) for 48 hr, and pulsed with ^{3}H-thymidine for a further 6 hr. Table 5.3 shows that the inhibitory effect of 15uM DFO could be completely reversed by adding 100uM ferric chloride. Higher concentrations (1mM) of ferric chloride were toxic to lymphocytes in that control cultures without desferrioxamine failed to respond to Con A.

Table 5.3 Ability of ferric chloride to reverse the inhibitory activity of desferrioxamine.

Treatment			^{3}H-thymidine uptake
Con A	DFO (15μM)	$FeCl_3$	(cpm x10^3 ±SE)
+	-	-	230.2 ± 18.5
+	+	-	5.5 ± 0.2
+	+	1uM	3.9 ± 1.2
+	+	10uM	9.8 ± 2.5
+	+	100uM	286.5 ± 10.4
+	+	1mM	8.3 ± 2.4
+	-	1uM	224.6 ± 7.8
+	-	10uM	223.8 ± 9.6
+	-	100uM	260.4 ± 14.6
+	-	1mM	3.6 ± 0.2

The ability of iron to reverse the DFO-induced inhibition of cell proliferation is also demonstrated in Figure 5.3, in which Con A-stimulated spleen cells were cultured with 15μM DFO and a saturating dose of $FeCl_3$ (100μM) was added at 24 hr intervals. The cultures were pulsed with ^{3}H-thymidine 24 and 48 hours later. $FeCl_3$ completely reversed the DFO-induced inhibition when added 24 or 48 hours later. There was some loss in proliferative capacity when iron was added 72 hours after the establishment of the cultures.

Fig 5.3 Ability of $FeCl_3$ to reverse the activity of DFO

Spleen cells were cultured with Con A (1μg/ml) and DFO (15μM). At 24 hr intervals $FeCl_3$ (100μM) was added and cell proliferation measured 24 and 48 hr later.

These experiments indicate that DFO is not directly toxic to lymphocytes, since iron could reverse the observed inhibition of cell proliferation even after prolonged exposure to the drug. To test for the toxic effects of higher concentrations of the drug, spleen cells were cultured with DFO for 48 hours, washed and stimulated with various concentrations of Con A. Table 5.4 shows that DFO at 10 times (150μM) the inhibitory concentration had only marginal effects upon the subsequent proliferative capacity of spleen cells. DFO at 1.5mM was regarded as toxic in that the subsequent proliferative capacity of spleen cells was impaired at some concentrations of Con A tested. These results suggest, therefore, that there were no irreversible direct toxic effects of DFO on lymphocytes at concentrations that inhibited cell proliferation.

5.7 Effect of other metal salts on the inhibitory activity of desferrioxamine.

Although desferrioxamine has a particularly high affinity for Fe^{3+} ions the drug does bind other metal salts at a much lower affinity (Table 5.5). Con A-stimulated spleen cultures were established with DFO (15μM) and different molar ratios of zinc, copper and calcium metal salts, to determine whether such salts would also reverse the DFO-induced inhibition. Various concentrations of metal salts were added as shown in Table 5.5, and the effect on cell proliferation was measured 48 hr later by pulsing with ^{3}H-

Table 5.4 The effect of culturing lymphocytes with desferrioxamine on subsequent proliferative responses to Con A.

DFO concentration	^{3}H-thymidine uptake (cpm ± SE) Con A concentration (μg/ml)						
	0	0.5	1.0	2.0	4.0	8.0	16.0
0	5440 ±1548	72482 ±13824	169594 ±18781	276018 ±26026	230414 ±68983	253923 ±51553	28710 ±4361
15uM	16360 ±1978	79898 ±9646	93369 ±16759	288973 ±23203	178570 ±16774	116471 ±17954	46075 ±3877
150uM	18212 ±4265	66593 ±13157	78165 ±28545	190438 ±4965	75400 ±7236	47550 ±8052	20005 ±9642
1.5mM	12771 ±727	36228 ±8204	72978 ±25408	10898 ±2887	4685 ±1490	8927 ±1851	6880 ±1253

Spleen cells were cultured with various amounts of desferrioxamine for 48 hr, washed three times in medium and stimulated with Con A. The cells were then pulsed 48 hr later with ^{3}H-thymidine.

thymidine. Calcium, zinc and copper ions had little or no effect on the inhibitory activity of DFO (Table 5.5) at concentrations that were not directly toxic to lymphocytes.

Table 5.5 The ability of various metal salts to reverse the inhibition of lymphocyte proliferation.

Metal salt	Concentration (uM)	% inhibition of H-thymidine incorporation	
		Con A	Con A + DFO
Ferric chloride	0	-	98
	10	0	97
	100	0	0
Zinc sulphate	0	-	97
	25	0	88
	50	16	87
	100	98	99
Copper chloride	0	-	97
	30	53	96
	60	78	98
	120	85	97
	250	83	93
	500	85	87
Calcium chloride	0	-	98
	8.5	0	98
	17.0	0	98

Spleen cells were cultured with Con A (1 ug/ml) with or without the addition of DFO (15 uM). Various concentrations of metal salts were added and the effect on cell proliferation measured 48 hr later by pulsing with ^{3}H-thymidine.

5.8 The effect of desferrioxamine on mixed lymphocyte culture responses.

To test whether DFO could also prevent other proliferative responses, mixed lymphocyte cultures were established in which F1 (BALB/c xSJL/J) lymph node cells were stimulated with irradiated CBA/H spleen cells. Cell proliferation was measured at 72 hours by pulsing with ^{3}H-thymidine. The cytotoxic T cell response generated was measured after 5 days by incubating effector cells with L929 target cells at various ratios. The level of cytotoxicity was measured after 4 hr by determining the amount of neutral red taken up by residual target cells. Table 5.6 shows that 15uM of DFO could completely suppress both cell proliferation and the generation of cytotoxic T cell effectors. This inhibition could be reversed if saturating (equimolar) levels of $FeCl_3$ were added to the cultures. However, DFO had no effect on the ability of cytotoxic T lymphocytes to kill target cells (Table 5.6).

5.9 Effect of adding desferrioxamine at different times after initiation of proliferation.

Spleen cell cultures were stimulated with Con A and DFO was subsequently added at various times between 0 and 48 hr. Forty-eight hours after the initiation of the cultures, the lymphocytes were pulsed with ^{3}H-thymidine and the level of uptake measured. Table 5.7 shows that DFO added even as late as 22 hr after the initiation of the culture still suppressed the proliferative response of the lymphocytes. There was no effect of the drug when added at 48 hrs, that is just prior to

Table 5.6 The effect of desferrioxamine on cell proliferation and the generation of cytotoxic T cells in a mixed lymphocyte culture.

Treatment	^{3}H-thymidine incorporation (cpm ± SE)	% cytotoxicity ± SE effector/target cell ratio			
		36:1	18:1	9:1	4:1
Nil	67484±3824	94±8.1	88±7.0	88±7.5	55±12.5
DFO 1.5 uM	39275±2497	81±10	53±10	53±7.2	ND
DFO 15 uM	719±206	0	0	0	ND
DFO (15uM)+$FeCl_3$(100uM)	41245±1838	87±5	69+5.2	40±1.7	ND
DFO 15 uM*	-	95±14	91+6.1	81±10	ND

F1 (BALB/c x SJL) lymph node cells were stimulated with irradiated CBA/H spleen cells and 72 hr later cell proliferation was measured by a 6 hr pulse with ^{3}H-thymidine. The activity of cytotoxic T cells was measured at 120 hrs against L929 target cells.

* Desferrioxamine was added at the cytotoxic assay stage.

ND Not determined.

pulsing with ^{3}H-thymidine. These results indicate that DFO can inhibit an on-going proliferative response, but if added after 22 hr, has no suppressive effect. This is of interest since Tormey and Mueller (1972) found that, in the proliferative response to Con A, there is a transferrin-sensitive period about 28 hr after stimulation.

Table 5.7 The effect of adding DFO, at various times after stimulation with Con A, on cell proliferation.

Time between addition of Con A and DFO	^{3}H-thymidine uptake (cpmx10^3±SE)*			
	Desferrioxamine concentration (μM)			
	0	15	30	60
0	278.2 ±4.7	3.0 ±0.7	1.5 ±0.7	0.5 ±0.1
6		2.7 ±0.1	2.0 ±0.7	1.2 ±0.3
22		27.5 ±4.1	8.7 ±0.5	2.4 ±0.3
48		310.3 ±16.7	298.1 ±6.8	254.6 ±6.5

Unstimulated 12.9±0.5

* Cells were incubated for 48 hr and pulsed with ^{3}H-thymidine for a further 4 hr before harvesting.

5.10 Effect of desferrioxamine on the triggering signal for cell proliferation.

Within 24 hours Con A stimulated lymphocytes become fully committed to DNA replication (Stenzell, Rubin and Novogrodsky, 1978). Removal of Con A at this time has no effect on subsequent blastogenesis. This can be demonstrated by adding α-methyl-mannoside (α-MM), a saccharide that competes with sites on the lymphocyte cell surface which bind the lectin: α-MM (0.1M) can competitively inhibit blastogenesis when added up to 5 hr after Con A (Stenzell, Rubin and Novogrodsky, 1978). To test whether DFO interfered with a proliferative signal to the lymphocytes, spleen cells were cultured for 24 hr with Con A (1 μg/ml) and DFO (15 μM). The activity of both Con A and DFO was then reversed by adding $FeCl_3$ (100uM) and α-MM (0.1M) and the subsequent proliferative capacity of the cells was measured 24 hr later. If DFO interferes with a cellular signal for proliferation, then the addition of ferric chloride and α-MM should prevent subsequent blastogenesis. Table 5.8 shows that α-MM completely inhibited cell proliferation when added at 0 hr, but not at 24 hr after the establishment of Con A-stimulated spleen cell cultures. DFO also inhibited lymphocyte proliferation, but this was reversed when $FeCl_3$ was subsequently added. Adding both α-MM and $FeCl_3$ at 24 hr had little effect on the proliferative response of the cells, suggesting that DFO did not interfere with the proliferative signal to the cells.

Table 5.8 Effect of DFO on the triggering signal for cell proliferation.

Treatment		^{3}H-thymidine uptake
0 hr	24 hr	cpm x10^3 ±SE
Con A	-	355.3 ± 14.6
Con A + α-MM	-	7.8 ± 0.4
Con A	α-MM	376.3 ± 7.9
Con A + DFO	-	3.2 ± 0.1
Con A + DFO	$FeCl_3$	187.0 ± 4.9
Con A + DFO	α-MM+$FeCl_3$	171.4 ± 2.3

Spleen cells were cultured with Con A (1µg/ml) with or without the addition of α-MM (0.1M), desferrioxamine (15µM) or $FeCl_3$ (100µM) for 48 hr and pulsed with ^{3}H-thymidine for 6 hr before harvesting.

5.11 Flow cytometric analysis of desferrioxamine-treated lymphocytes.

Desferrioxamine seems to inhibit cell division as measured by thymidine uptake. Flow cytometric analysis was used to determine whether cells are blocked at a particular phase of the cell cycle. Figure 5.4 shows that lymphocytes stimulated for 48 hr with Con A developed the characteristic G2M phase peak associated with cell division. However, this peak did not occur in lymphocytes exposed to DFO and the DNA

distribution was similar to that of an unstimulated lymphocyte population. This result suggests that DFO blocks lymphocyte cell division in the Gl phase.

A

G_1 G_2M

B

C

D

Figure 5.4 FACS analysis of DNA content of mouse spleen cells. Cells were stained with mithramycin, after 72 hr in culture.

a) Con A stimlated spleen cells showing peak in G2M phase.
b) Unstimulated cells.
c) Spleen cells incubated with Con A in the presence of 15uM DFO.
d) Spleen cells incubated with Con A, DFO (15uM) and FeCl (100uM)

5.12 Interleukin production.

Supernatants from cultures of Con A-stimulated spleen cells incubated with or without DFO were assayed for interleukin production by 1) the ability to promote growth of Con A blasts (IL-2), 2) the induction of procoagulant factor on the surface of macrophages, and 3) production of IL-1 by PU5-1.8 cells.

CBA mouse spleen cells were incubated with Con A (2 ug/ml) in the presence of varying concentrations of DFO, as shown below. After 48 hr incubation, cells were removed by centrifugation, and equimolar concentrations of $FeCl_3$ were added to the supernatant to saturate the DFO. Twofold dilutions of the supernatants were added to Con A blasts to measure IL-2, or to PU5-1.8 cells to measure PCIF, and assayed as in sections 2.14 and 2.20. DFO + $FeCl_3$ was not stimulatory for Con A blasts.

Neither IL-2 nor PCF induction were suppressed by concentrations of DFO up to 100 uM, levels which completely suppresses proliferation in <u>vitro</u> (Table 5.9).

Table 5.9 Failure of DFO to suppress lymphokine production.

	IL-2	PCIF
DFO (μM)	^{3}H-thymidine uptake (cpm x 10^3 ±SE)	% reduction in clotting time
1	25.8 ± 2.1	45.7 ± 2.2
10	34.6 ± 1.7	49.6 ± 2.1
50	29.4 ± 1.3	49.6 ± 1.7
100	33.3 ± 1.5	55.1 ± 2.4
-	29.1 ± 1.4	37.0 ± 1.3
Unstimulated	1.6 ± 0.1	0.0

LNC were incubated with Con A (2μg/ml), and concentrations of DFO as shown. The supernatants were tested for their ability to support proliferation of Con A blasts (IL-2) and for induction of PCF on PU5-1.8 cells (PCIF)

Interleukin 1 (IL-1) is produced by PU5-1.8 cells upon stimulation by LPS, and assayed by augmentation of proliferation of C3H/HeJ thymocytes incubated with sub-optimal concentrations of PHA. C3H thymocytes do not react to LPS, so the assay is specific for IL-1. Thymidine uptake by C3H thymocytes in response to IL-1 is less than that obtained with other mitogens, nevertheless, the stimulation index is usually about tenfold higher than unstimulated cells. PU5-1.8 cells were incubated with various concentrations of DFO, and 20 μg/ml LPS. Both cells and supernatant were assayed, since up to 90% of the IL-1 produced by LPS stimulation remains intracellular (Gery and Lepe-Zuniga, 1984). There was some reduction in IL-1 at

concentrations of DFO greater than 50 ug/ml, but no significant difference at 10 μg/ml (Table 5.10).

Table 5.10 Production of IL-1 by PU5-1.8 cells.

DFO (uM)	$FeCl_3$ (uM)*	^{3}H-Thymidine uptake (cpm x10^3)		
		Cells	Supernatant	Total
1	-	2235	2393	4628 ± 254
10	-	1873	1191	3264 ± 221
50	-	1605	924	2529 ± 142
100	-	400	658	1058 ± 54
1	1	1838	1695	3533 ± 239
10	10	1377	1882	3259 ± 232
50	50	1792	1654	3446 ± 268
100	100	2230	1292	3522 ± 125
-	-	1538	2020	3558 ± 245
Unstimulated		256	172	428 ± 39

* $FeCl_3$ added at the same time as DFO.

5.13 FACS analysis of transferrin receptors.

The appearance of transferrin receptors on the surface of stimulated lymphocytes occurs soon after the induction of IL-2 receptors, and appears to be dependent upon the latter. Neckers and Cossman (1983) have shown that blocking of IL-2 receptors by the monoclonal antibody anti-TAC prevented both

the appearance of transferrin receptors and subsequent DNA synthesis. Inhibition of DNA synthesis occurred only if anti-TAC was administered before the appearance of transferrin receptors. Conversely, Neckers and Cossman (1983) found that blockade of transferrin receptors abrogated the mitogenic capacity of IL-2. Although DFO was found not to inhibit IL-2 production (section 5.12) there was a possibility that the appearance of transferrin receptors might be altered, either by inhibition of induction or by blocking of receptors. This was determined by FACS analysis on PHA-stimulated human lymphocytes, using the monoclonal antibody to human transferrin receptors, 5E9 (Haynes et al, 1981).

DFO was added to $2x10^6$/ml normal human lymphocytes to give a final concentration of 15 μM. Immediately, 50 ug PHA was added, and the cells were incubated at 37 ^oC for up to 3 days. At various times, aliquots were removed and stained with 5E9 antibody. FACS analysis showed that cells incubated with PHA developed transferrin receptors after 8 hr, reaching peak levels at 48 hr, and diminishing by 72 hr. In the presence of DFO, receptors appeared at the same rate, but accumulated on the surface, and were still evident after 3 days incubation (Fig. 5.5). This result is in accord with Mattia et al (1984) who demonstrated a threefold increase in the number of transferrin receptors when K562 (human leukaemic) cells were incubated in 50 μM DFO. The increased number of receptors was due to a specific increase in the rate of receptor synthesis, which reached 3-4 times that of untreated cells within 6 hr after addition of DFO (Mattia et al, 1984).

Thus DFO not only fails to inhibit, but appears to enhance the synthesis of transferrin receptors.

a

b

c

Figure 5.5 FACS analysis of transferrin receptors.

Spleen cells were incubated with mitogens in the presence or absence of DFO for 72 hr, then stained with the MAb 5E9, followed by FITC-conjugated goat anti-mouse antiserum.
a) PHA-stimulated cells, b) PHA-stimulated + DFO, 10 ug/ml, c) unstimulated cells.
Arrows indicate transferrin-positive peaks.

5.14 Depletion of transferrin-bound iron by desferrioxamine.

It is generally thought that iron chelators are unable to remove iron from transferrin (Rubin, Houlihan and Princiotto, 1960; Cleton, Turnbull and Finch, 1963; Brown, Hwang and Allgood, 1967). However, there is ample evidence that iron can be removed by dialysis of transferrin against EDTA, nitriloacetic acid (NTA) or citrate (Charley et al, 1960; Bates, Billups and Saltman, 1967) and this can be reversed by adding back iron, but not other metal ions (Aasa et al, 1963; Feeney and Komatsu, 1966). Indeed, dialysis of iron was one of the methods used to determine metal binding constants for transferrin (Aasa et al, 1963).

Removal of iron from transferrin depends upon both the association constant (see Table 4.1), and a kinetic factor, for example, NTA and citrate are able to chelate 50% of the iron in transferrin in 3 hours, while EDTA takes more than 4 days. The amount of transferrin in serum varies, the average for bovine serum being 2.6 mg/ml (Brock and Mainou-Fowler, 1983), and is 30% saturated with ferric iron. Saturation of transferrin below 24% or above 70% has been shown to inhibit lymphocyte proliferation in vitro, (Brock and Mainou-Fowler, 1983). Thus, it is possible that DFO could remove sufficient iron from transferrin to render it inhibitory.

The following experiment was done to determine whether DFO was capable of removing iron from transferrin, (see Fig. 5.6). DFO was added to 10 ml of HIFCS to give a final concentration of 100 µg/ml. This mixture was left at $4^{o}C$ for

16 hr, then spun through a Centrilfo ultrafiltration cone to remove molecules less than 10,000 daltons. Iron bound to DFO has a molecular weight of 657 and should pass through the filter, while transferrin (MW 76,500) should be retained. A solution of 2.6 mg/ml transferrin in RPMI (30 % saturated with iron), and an aliquot of FCS without DFO was treated in the same way. After centrifugation at 3,000 rpm for 20 min, the volume remaining in the cone was 1/10th of the original 10 ml. This was then made up to the original volume with RPMI medium, and used as the source of transferrin in Con A-stimulated cultures of rat lymph node cells. The assay medium contained a final concentration of 1 ug/ml DFO, well below the level which inhibits proliferation _in vitro_ (see section 5.3). When used at a concentration of 5%, FCS treated in this way did not support Con A stimulation of spleen cells (Table 5.11). Filtered FCS without DFO was not altered by the treatment. Addition of 10 uM DFO to FCS also inhibited proliferation, as expected (Table 5.11). Approximately 90% of the DFO was recovered in the serum filtrate, and 97% in the transferrin filtrate (not shown). DFO may bind to another large protein in serum, or may have been catabolised by a serum enzyme; however the treated transferrin failed to support proliferation to a similar extent, indicating that retention of DFO, if it occurred, was not sufficient to account for the suppressive effect.

It was more likely that DFO had removed iron from transferrin, rendering it "undersaturated", and unable to support lymphocyte proliferation.

Fig. 5.6 Removal of transferrin-bound iron by DFO.

DFO was added to FCS or transferrin (30% saturated) to give 100 ug/ml. The mixture was then spun through an ultrafiltration cone to remove ferrioxamine. The serum and transferrin remaining in the cones were tested for their ability to support Con A-stimulation of rat lymph node cells, shown by the uptake of ^{3}H-thymidine.

Table 5.11 Inhibition of lymphoproliferation by removal of transferrin-bound iron.

Treatment	^{3}H-Thymidine uptake (cpm x 10^3)		
	Final concentration of serum used in assay		
	10%	5%	2%
DFO-FCS	36.7±4.7	7.6±0.7	0.6±0.03
FCS	74.5±2.5	44.0±1.4	5.4±0.1
FCS+DFO added	3.4±0.1	7.2±0.9	4.8±0.3
FCS untreated	78.5±4.7	44.5±0.3	6.1±0.3
Unstimulated	4.9±0.4	3.8±0.3	1.5±0.03

The addition of transferrin (30% saturated with iron) or $FeCl_3$ to cultures containing 10% DFO-treated FCS, fully restored the lymphoproliferative response at a concentration of 500 µg/ml transferrin, or 5 µg/ml $FeCl_3$ (Fig. 5.7). Apo-transferrin, which does not contain iron, was unable to reverse the inhibition.

The normal concentration of transferrin in serum varies, but is usually about 2.5 mg/ml (Brock and Mainou-Fowler, 1983). Levels below this are inhibitory. Using the known 1:1 binding ratio for DFO and Fe, and known amounts of transferrin in this experiment, it would seem reasonable to postulate that DFO was reducing the iron saturation of TF to about 20%, and that addition of 5µg/ml Fe could restore saturation to 30%, thus reversing the inhibition.

Figure 5.7 Restoration of the lymphoproliferative response by the addition of transferrin or $FeCl_3$.

Saturated transferrin (a) or $FeCl_3$ (b) were added to DFO-treated FCS at the concentrations shown, and used as a source of FCS in the response of rat LNC to Con A (2μg/ml).

■ Transferrin, □ apotransferrin, U untreated FCS.

Restoration of the proliferative response of LNC by 500ug/ml transferrin (which by itself does not support proliferation) and by 5ug/ml $FeCl_3$, indicates that DFO probably acts by removing iron from transferrin, however further experiments using ^{59}Fe are needed before any firm conclusions can be drawn.

5.15 Intracellular iron levels in lymphoid cells.

Previous experiments showed that T cell proliferation was suppressed in the presence of 15uM concentrations of DFO (sections 5.3, 5.4); however B cell stimulation *in vitro* (sections 5.3, 5.4) and antibody production *in vivo* (section 4.11), was not adversely affected. Macrophage function also appeared to be normal (sections 4.7, 4.8, 4.10). In contrast to B cells and macrophages, T cells do not synthesize iron-storage proteins in response to increased iron levels. Intracellular iron was determined by carbon rod analysis in order to determine whether intracellular iron stores were greater in B cells and macrophages than in T cells, and could thus overcome the depletion of extracellular iron by DFO. Two sources of T cells were used; CBA thymocytes, and CBA spleen depleted of Ig+ cells by rosetting (section 2.16). Both T cell suspensions contained the same amount of iron, i.e. 0.38 ug/10^8 cells. A B cell-enriched fraction was obtained by anti-thy 1.2 treatment of spleen cells. Macrophages were obtained from the peritoneal cavity; after incubation at 37^0C for 5 hr in RPMI-1640 + 10% HIFCS, non-adherent cells were washed off. All glassware was washed in Chelex-treated water. Cells were resuspended in Chelex-treated distilled water containing 0.1% SDS, homogenized,

and kept at $-20^{o}C$ until iron determinations were made. Iron levels were measured at the Research School of Chemistry, ANU, using a Varian carbon rod Atomiser CRA-90.

Cell size was measured using a calibrated slide and a microscope fitted with an eyepiece graticule. The cells were placed in medium in a petri dish so that their spherical shape was maintained.

Total iron determinations showed wide differences between cell types. However when the cell volume was taken into account, the iron content was similar, T cells and adherent peritoneal macrophages having the same amount (Table 5.12).

Table 5.12 Total iron content of lymphoid cells.

	Total iron (μg)/10^8 cells	Cell diameter (mμ)	Cell volume*	Total Fe content (μg/cc^3 cell volume)
T	0.38	6.5+0.3	143	2.6
B	2.94	11.5+0.7	796	3.69
Mϕ	4.60	15.0+1.1	1768	2.6

* Cell volume was calculated by the formula $4/3\pi r^3$. The cells were placed in a petri dish and counted immediately to maintain their spherical shape. Ten cells of each type were measured.

5.16 Other antioxidant and iron chelators in vitro.

Several compounds which are known to have efficient iron-binding or radical-scavenging properties were tested for their ability to inhibit lymphocyte proliferation *in vitro*. All of these had previously been tried *in vivo* for possible therapeutic effects in EAE (section 4.12); although none of the iron-chelating compounds appeared to be effective *in vivo*, the antioxidant BHA, Hydergine, and the antineoplastic drug Hydroxyurea all reduced the severity of EAE (section 4.12). HBED, BHA and Hydroxyurea were also effective at inhibiting proliferation *in vitro*, however they were toxic to cells at the concentrations which suppressed proliferation, as indicated by the inhibition of H-thymidine uptake of unstimulated cells (Table 5.13). BHA, being relatively insoluble in water, formed an oily suspension which was detrimental to cell cultures. Hydergine, which has been shown to inhibit lipid peroxidation *in vitro* (Koreh, Seligman and Demopoulos, 1982), did not inhibit lymphoproliferation. The ribonucleotide reductase-inhibitor PATS, effectively inhibited proliferation; this could be reversed by incorporation of iron in the compound Fe-PATS, indicating that the suppression is not due to irreversible inactivation of the tyrosyl radical.

In summary, inhibitors of lipid peroxidation, although effective at suppressing EAE *in vivo*, did not suppress lymphoproliferation *in vitro*. In contrast, the iron chelator HBED, while ineffective *in vivo*, suppressed proliferative responses to Con A *in vitro*. It may be that DFO acts to suppress

EAE by inhibiting both iron-catalysed lipid peroxidation and lymphoproliferation.

Table 5.13 Effects of various iron-chelators and anti-oxidants on lymphoproliferation.

Compound	Con A	^{3}H-Thymidine uptake (cpm x 10^3) concentration (uM) 1	5	10	50	100
DFO	+	128.5	77.2	13.3	10.0	10.3
	-	8.0	7.1	6.1	6.5	6.0
HBED	+	79.2	38.8	14.5	4.5	5.5
	-	9.9	11.5	7.4	3.3	4.8
PATS	+	152.1	150.6	3.5	4.5	4.5
	-	5.5	6.2	7.3	0.8	0.2
Fe-PATS	+	171.5	192.1	220.5	243.7	16.3
	-	5.7	8.1	4.6	0.15	0.13
Hydroxyurea	+	130.3	34.4	6.0	2.2	1.0
	-	8.4	3.1	2.9	3.3	2.4
Hydergine	+	121.6	118.4	116.0	113.9	112.2
	-	10.0	10.7	10.3	9.9	9.8
BHA	+	154.7	145.3	139.2	2.5	0.6
	-	8.0	8.3	3.8	0.5	0.4
DMSO	+	171.8	137.9	121.6	1.8	0.4
	-	8.3	8.0	0.4	0.4	0.35
Nil	+	176.4				
	-	8.4				

5.17 Suppression of mitogenic effect of the Calcium ionophore A23187.

The calcium ionophore A23187 is a fungal metabolite obtained from *Streptomyces chartreusensis*. It induces proliferation in lymphocytes by increasing cell membrane permeability to Ca++ ions. Mitogens such as Con A bind to the cell surface, and subsequently Ca channels are opened, resulting in an increase in cytoplasmic Ca. A23187 acts differently in that is a mobile ion carrier, diffusing into the lipid bilayer, thereby creating a steep electrochemical gradient. As a result, Ca++ ions rush into the cytosol. The effect is transient at low concentrations. At mitogenic concentrations, however, the Ca++ uptake is sustained (Alberts et al, 1983) and inability to maintain cytoplasmic Ca levels within the normal nanomolar range results in death of the cell. For this reason, A23187 is eventually toxic to cells, even at mitogenic doses. A23187 has been shown to be a specific mitogen for T helper cells (Akerman and Andersson, 1984). It appears to act independently of lymphokines, since it is a poor IL-2 inducer; blockade of IL-2 receptors by the monoclonal antibody anti-TAC does not inhibit proliferation; and addition of IL-2 to the medium does not augment the response at sub-mitogenic responses (Koretzky et al, 1983). In addition, macrophage-depleted cultures are able to respond, indicating that IL-1 is not necessary for its action. Finally, A23187 can induce proliferation in the absence of serum (Brock, 1981). Thus, A23187 is able to act by increasing transport of Ca across the cell membrane, and independently of transferrin (and

Figure 5.9 Suppression of ionophore-induced proliferation by DFO.

Rat lymph node cells were incubated with mitogens and various concentrations of DFO as shown, for 48 hr.

▲—▲ Con A (1ug/ml) + DFO

△- -△ A23187 (1uM)

●—● A23187 (1uM) + DFO

o- -o A23187 (1uM) + DFO + equimolar $FeCl_3$

..... unstimulated

hence iron uptake). Therefore, one might expect an iron chelating agent such as desferrioxamine, which has a high binding affinity for iron (10^{31}) and low affinity for calcium (10^{2}), to be ineffective in blocking ionophore-induced proliferation. Figure 5.9 shows that at concentrations of 0.5 µM, DFO completely inhibited the proliferative response induced by A23187. This is 1/30 of that used to suppress all other mitogens tested. Suppression of proliferation was reversed by saturating DFO with equimolar amounts of $FeCl_3$; at concentrations of $FeCl_3$-saturated DFO above 10µM, ^{3}H-thymidine uptake was markedly reduced. This was not due to loss of cell viability (checked by trypan blue exclusion). The reason for this apparent suppression is not clear.

5.18 Ultrastructure of A23187-stimulated lymphocytes.

Incubation of lymphocytes with A23187 at concentrations that are mitogenic, or higher concentrations which are toxic to cells, results in increased uptake of Ca++ into the cytosol. Most of the calcium is concentrated in the mitochondria, and as the rate of uptake exceeds that at which it can be pumped out into the cytoplasm, the mitochondria become swollen and distorted, and eventually disrupt (Lichtman, Segal and Lichtman, 1983). Incorporation of 1 uM DFO in the culture medium protected mitochondria from such damage, as shown in Fig 5.10. It was also interesting to note that cells incubated with A23187 and DFO showed dense chromatin and prominent Golgi apparatus, indicating that RNA and protein synthesis were not inhibited.

Figure 5.10 (following page) Ultrastructure of A23187-stimulated lymphocytes.

Transmission electron micrographs of mouse lymph node cells incubated for 1 hr with 1 μM A23187.

a) Untreated cell, showing intact mitochondria
b) and c) Cells exposed to A23187, showing swollen, distorted mitochondria.
d) Lysed cell. Even at mitogenic concentrations, A23187 was toxic to lymph node cells.

a

b

c

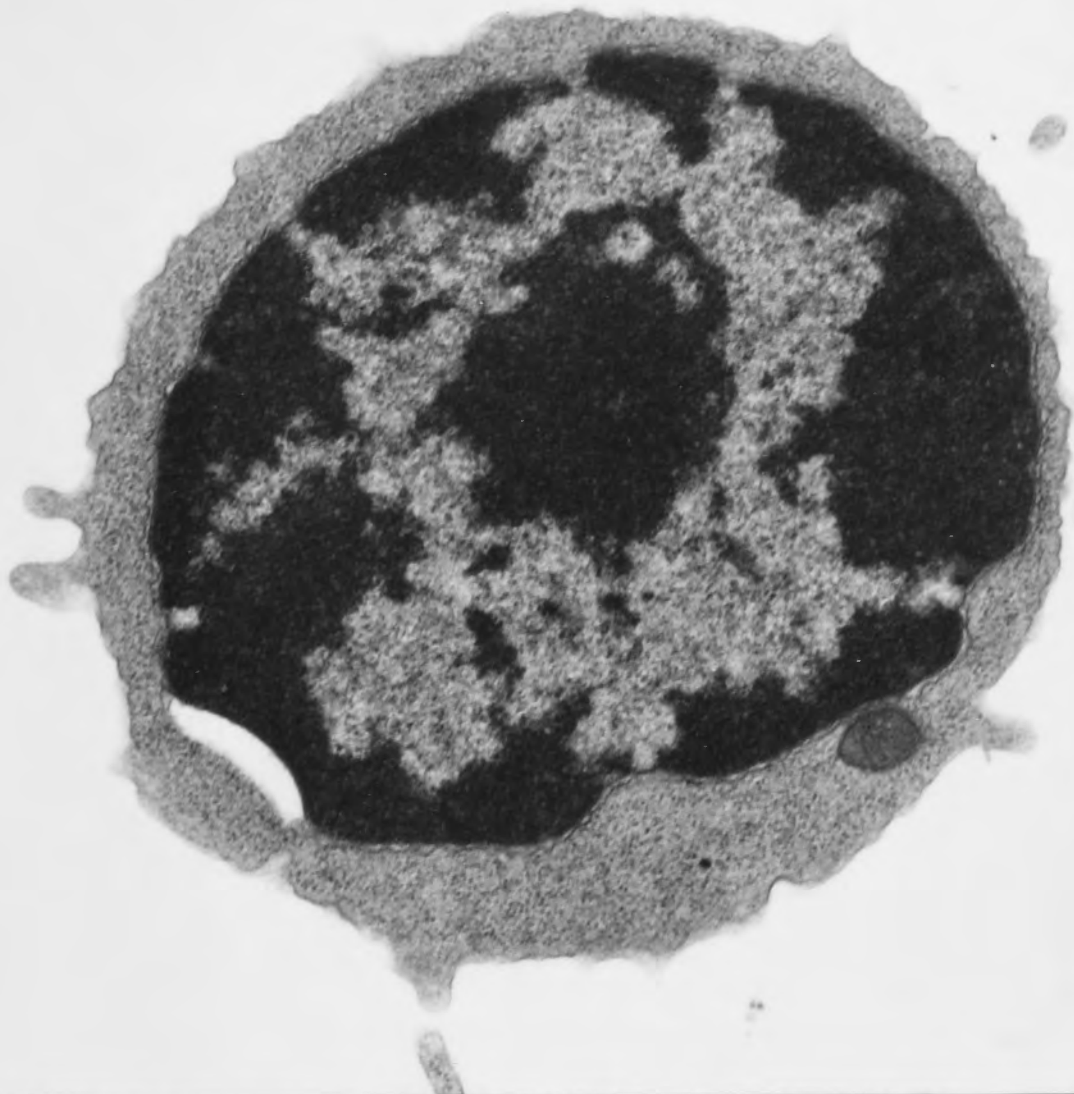
d

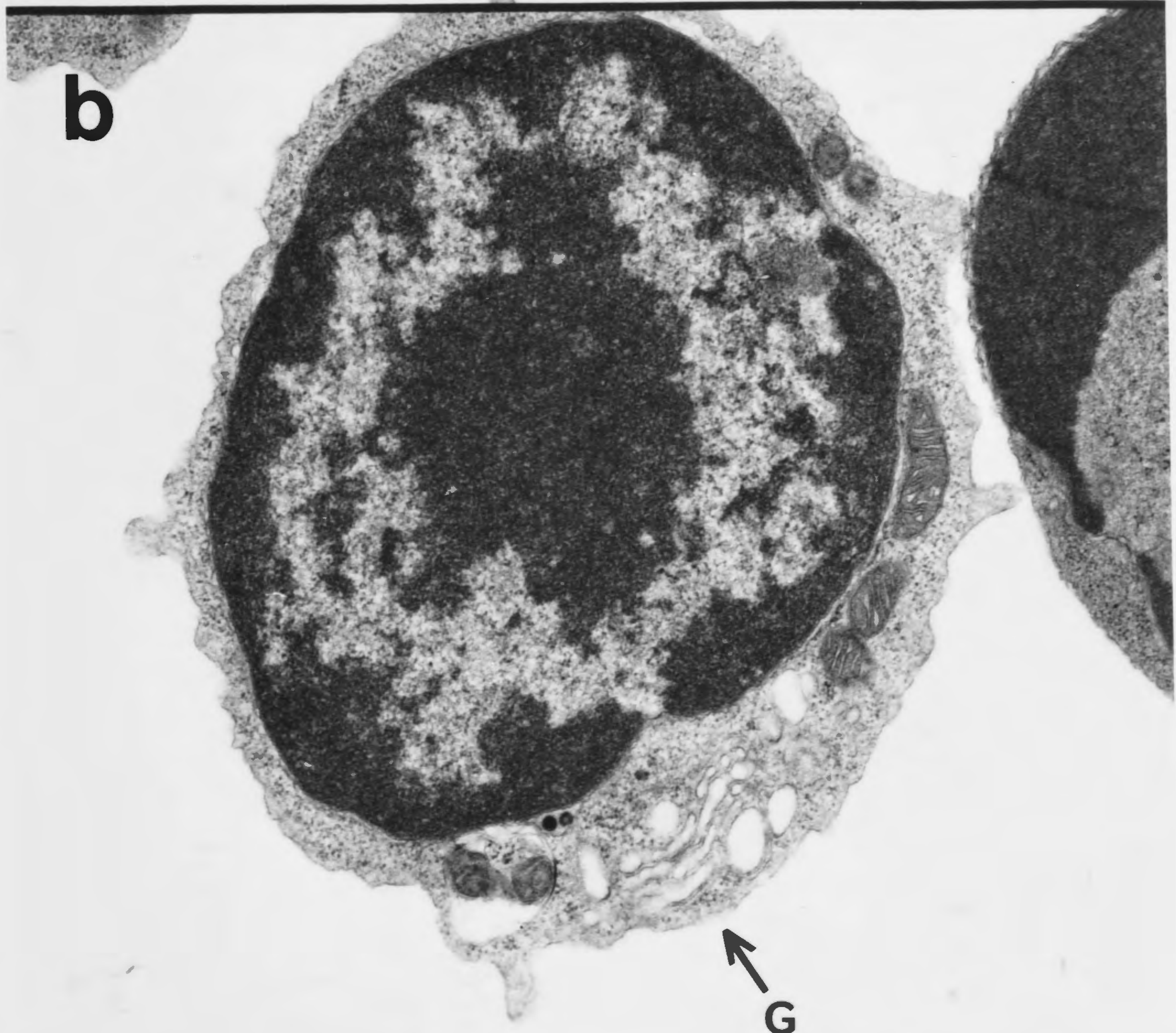
Ultrastructure of A23187-stimulated lymphocytes.

a) Normal lymph node cell, showing intact mitochondria.

b) Cells were incubated with A23187 for 16 hr, in the presence of 10 μM DFO. Note intact mitochondria and prominent Golgi apparatus (G).

a





5.19 Measurement of cytoplasmic ca++ changes by quin2.

Since DFO appeared to prevent the damage to mitochondria caused by increased calcium uptake it seemed relevant to measure Ca++-uptake into the cytosol, to determine whether DFO was inhibiting this early mitogenic change.

One of the earliest, measurable events after binding of mitogen to the cell surface is the increased uptake of calcium. This can be measured by loading cells with quin2, which fluoresces upon binding to calcium. Figure 5.11 shows the traces obtained when CBA/H thymocytes were incubated with Con A (Fig. 5.11a) or with A23187 (Fig. 5.11c). The intensity of fluorescence increased markedly within seconds. Addition of 10 uM DFO did not significantly affect the increase in cytoplasmic Ca++ (Fig. 5.11b). There was some reduction in A23187-stimulated cells (Fig. 5.11d). This was reproducible and is probably a real effect. However, the reduction did not seem to be large enough to account for the suppression of proliferation, particularly at 10 µM DFO. These results are of relevance in the light of a recent report by Halliwell (1984). Two patients undergoing DFO therapy for rheumatoid arthritis, who received the phenothiazine prochlorperazine concurrently, lost consciousness for 48-72 hr. As phenothiazine is a calmodulin antagonist (Cheung, Grinstein and Gelfrand, 1983), the combined effects of these two drugs may have unfortunate effects, particularly in excitable cells which require Ca++ for release of neurotransmitters.

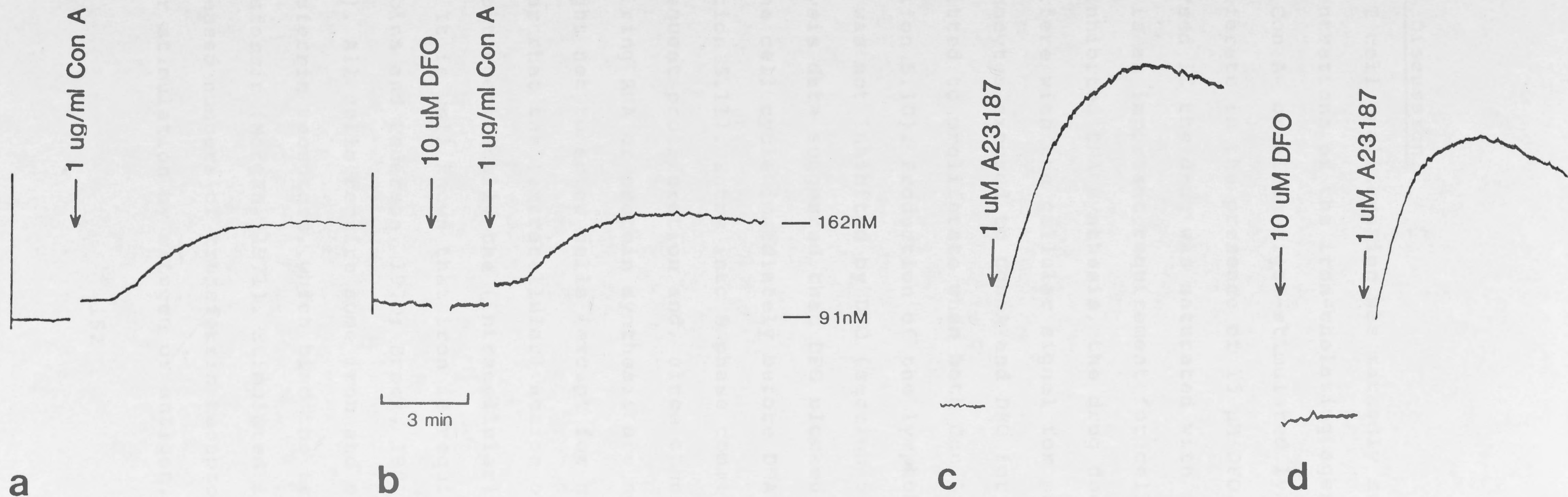


Figure 5.11 Measurement of cytoplasmic Ca++ changes by quin2 fluorescence.

CBA/H thmocytes were incubated with Con A (a,b) or A23187 (c,d), and the increase in cytoplasmic Ca++ was measured by quin2 fluorescence in the absence (a,c) or presence (b,d) of 10 μM DFO.

5.20 Discussion.

T cell proliferation was markedly suppressed by non-toxic concentrations of the iron-chelating agent desferrioxamine. Thus Con A- or alloantigen-stimulated lymphocytes failed to proliferate in the presence of 15 μM DFO. This effect could be reversed if the drug was saturated with iron, suggesting that iron is an important requirement for cell division. Although DFO inhibited DNA synthesis, the drug does not seem to interfere with the cellular signal for proliferation. Thus, lymphocytes exposed to Con A and DFO for 24 hours were still committed to proliferate when both Con A and DFO were removed (section 5.10). Production of the lymphokines IL-1, IL-2 and PCIF was not inhibited by DFO (section 5.12). Flow cytometric analysis data suggested that DFO blocked cells in G1, the stage in the cell cycle immediately before DNA synthesis begins (section 5.11). Entry into S phase commits the cell to subsequent proliferation and, since other cell functions requiring RNA or protein synthesis are not inhibited and DFO is thought not to enter cells (except for hepatocytes), it would appear that the (extracellular) action of the drug is able to inhibit specifically the (intracellular) synthesis of DNA.

It is well known that iron is required for DNA synthesis (Robbins and Pederson, 1970; Brock, 1981; Novogrodsky et al, 1982). All cells require some iron and express low levels of transferrin receptors, which bind the iron-transporting protein transferrin (Morgan, 1971). Stimulated lymphocytes express increased numbers of transferrin receptors approximately 6 hr after stimulation by mitogen or antigen. The iron-transferrin

complex is internalised and the iron released from transferrin at an intracellular site by a mechanism which is still poorly understood. The iron-depleted transferrin is returned to the plasma undegraded (Bomford et al, 1983). Uptake of transferrin-bound iron in all tissues is regulated by the number of transferrin receptors, rather than the amount of iron available in serum (Huebers and Finch, 1982). The accumulation of transferrin receptors on the surface of cells incubated with DFO may represent a fruitless attempt to acquire iron. Experiments in which iron was removed from transferrin by ultrafiltration of ferrioxamine (section 5.14), may have rendered it "under-saturated", a process which results in poor binding capacity, and, consequently, poor stimulation. (Brock,1981).

The sensitivity of cells to iron depletion is illustrated by an interesting report by Hibbs, Taintor and Vavrin (1984). The cytotoxic effect of activated macrophages on leukaemia cells was shown to be associated with release of iron from the targets and uptake of iron by the macrophages, occurring at the same time as DNA synthesis in the leukaemia cells ceased, i.e. at 4-6 hr.

It is known that the continual presence of oxygen and iron are required for the activity of ribonucleotide reductase (Thelander, Graslund and Thelander, 1983), a cytoplasmic enzyme which catalyses the first step in DNA synthesis: that of the reduction of ribonucleotides to deoxyribonucleotides. Mammalian RR is made up of two subunits, Ml (MW 84,000), which has

receptors for nucleoside triphosphates, and M2 (MW 58,000) containing an iron-tyrosine-free radical structure. The enzyme has a short half-life of 10 minutes, and it is thought that reconstitution of the tyrosine-radical centre is the primary mode of regulation of DNA synthesis (Thelander, Graslund and Thelander, 1983). RR is dependent upon iron for regeneration of the tyrosine-radical, and inhibition of RR by hydroxyurea and thiosemicarbazones, compounds which complex with the iron-tyrosil radical, can be partially reversed by the addition of ferrous ions (Moore, 1969). Mammalian RR differs in two respects from <u>E. coli</u> RR which, until recently, was the primary source for such studies. Firstly, mammalian RR is inhibited by hydroxyurea, but the effect is fully reversible if the drug is washed out (Thelander and Reichard, 1979). This is in contrast to RR from <u>E. coli</u>, in which the tyrosyl radical is irreversibly inactivated. The reversibility of mammalian RR indicates a different catalytic process, with the radical possibly being formed during catalysis (Thelander and Reichard, 1979). Secondly, mammalian RR (in calf thymocytes) appears to be more sensitive to withdrawal of iron from the medium than <u>E. coli</u> RR (Thelander and Reichard, 1979).

Incubation of PHA-stimulated lymphocytes with DFO (1mM) results in a decrease in total ribonucleotide reductase to 11% of normal levels within 45 min, and a concomitant fall in dATP levels, with a rise in dCTP, dGTP and dTTP (Hoffbrand et al, 1976; Ganeshaguru et al, 1980; Lederman et al, 1984). This is consistent with the inhibition of RR by hydroxyurea.

Lymphocytes from patients with iron-deficiency anaemia also

show reduction in all four ribonucleotides (Hoffbrand et al, 1976.

The ability of desferrioxamine to inhibit a mixed lymphocyte culture suggests that the drug may be useful in preventing allograft rejection. Experiments are in progress to investigate whether DFO prolongs the survival of allogeneic pancreatic islet transplants (Lafferty, personal communication). The in vitro proliferation of rat, mouse and human lymphocytes is suppressed to the same extent by comparable doses of desferrioxamine. These findings, together with results presented in the previous chapter, suggest that one way in which DFO probably exerts its effects is by selectively inhibiting T cell proliferation in vivo; it seems likely that this occurs as a result of depletion of iron saturation of transferrin, with diminished uptake by transferrin receptors, and consequently, inhibition of the regeneration of the iron-tyrosyl radical centre of ribonucleotide reductase.

Desferrioxamine seems to be more inhibitory for T cell than for B cell responses. Indeed, human B cell proliferation in response to LPS in vitro is largely unaffected by concentrations of desferrioxamine that totally inhibit T cell responses. This may be due to particular properties of LPS, since Lederman et al, (1984) obtained complete suppression of B cell proliferation by DFO, using the cloned B cell line B8 from human tonsil and the B cell mitogen STA, which is derived from Staph. aureus.

Another reason for the apparent lack of effect on B cells and macrophages may be that DNA synthesis is not required for the secretion of lymphokines, which activate B cells (BAF) (Bloom, Gaffney and Jiminez, 1972) and macrophages (MIF, PCF, etc.). The same is true for both the production of antibody and for phagocytosis. Similarly, the cytotoxic action of lymphocytes occurs without DNA synthesis (Mauel et al, 1970, Perlmann and Holm, 1969). In addition, Neckers, Yenokida and James (1984) have shown that transferrin receptors are not required for B cell activity, since antibody secretion occurs in PHA-stimulated cells whose proliferation has been blocked by the monoclonal antibody 42/6, which binds human transferrin receptors.

CHAPTER 6. ROLE OF ACUTE PHASE PROTEINS IN EAE.

6.1 Introduction.

The injection of CFA into rats produces a chronic, multi-organ, adjuvant disease which is accompanied by an acute phase protein response (Waksman et al, 1960). In view of the report by Brosnan et al (1980) describing the inhibition of the clinical symptoms of EAE by the injection of a variety of proteinase inhibitors, it was relevant to examine the role of the endogenous proteinase inhibitor α_2-macroglobulin (α_2M) in EAE. α_2M irreversibly binds neutral endopeptides of all four classes, serine, metallo, thiol and carboxy peptidases (Starkey and Barrett, 1977). The Lewis rat is a high responder with respect to α_2M. In response to an intense inflammatory challenge, α_2M levels in plasma rise from microgram levels to as high as 15 mg/ml (Baldo et al, 1981)

In addition to its role in protein capture, α_2M has additional properties which are relevant to a role in EAE. α_2M in either free or proteinase-bound form is reported to modulate the secretion of proteinases by macrophages (Johnson et al, 1982). At high concentrations, α_2M inhibits the mixed lymphocyte response (Hubbard et al, 1981). Also the enzyme-bound form of α_2M is reported to inhibit the release of superoxide anion from stimulated macrophages (Hoffman et al, 1983).

The major role of Ceruloplasmin (Cp) is the binding and transport of copper, however it also acts as a major tissue antioxidant, and may be particularly important in the CNS,

where levels of catalase are low (Hartz et al, 1983). Cp also plays a significant role in the mobilization of iron from tissues, by oxidizing Fe++ to the Fe+++ state, making it available for uptake and removal by transferrin. Apart from the hereditary deficiency of Cp (Wilson's disease), lower than normal Cp levels do not occur. Cp levels are raised in acute and chronic infections (Markowitz et al, 1955), pregnancy, chronic and autoimmune inflammatory disease, such as systemic lupus erythematosis (Denko and Gabriel, rheumatoid arthritis (Scudder et al, 1978) and sarcoidosis (Koskelo et al, 1966).

The acute phase proteins are synthesized by hepatocytes in response to the release of IL-1 from macrophages (Kampschmidt, Upchurch and Pulliam, 1982). There is, however, additional regulation, particularly in the case of α_2M. Steroids play an important, but as yet undefined, role in the response of this protein to inflammatory stimuli (Thomas and Schreiber 1985).

The purpose of the following experiments was to document the acute phase response in EAE, and in particular to relate plasma levels of α_2M, fibrinogen and Cp to the onset, severity of disease, and recovery phases. In addition, the effect, if any, of DFO on the acute phase response, was examined. Finally, in view of the protective antioxidant role of Cp, and previous conflicting reports, levels of Cp in serum of MS patients were determined.

6.2 The acute phase response in EAE.

Serum levels of Cp were consistently elevated over a 60-day period in a group of 5 rats injected with GPSCH-CFA (Table 6.1). In contrast, α_2M levels had fallen to normal values by day 10, just before the onset of clinical signs on day 12. There was a small but significant rise on day 18, when the symptoms were subsiding, which again fell to normal levels by day 27, and remained at this level.

Table 6.1 Levels of acute phase proteins in rats during EAE.

Rats injected with	Day tested	Clinical score*	α_2M (mg%)	Cp (mg%)	Fibrinogen (mg%)
GPSCH -CFA	6	-	82.5±51.2	74.5±27.0	38.8±4.5
	10	-	4.5±1.5	72.0±4.0	32.0±1.5
	14	2.0(4)			
	18	1.2(4)	15.7±5.0	85.2±25.2	26.8±8.5
	22	-			
	27	-	8.2±2.5	121.5±19.9	19.2±3.0
	32	-			25.7±1.6
	46	-	2.5±0.2	74.7±4.0	
	60	-	3.6±1.6	199.0±24.5	
CFA	10	-	2.5±1.5	61.0±4.5	
	30	-			
	60	-			
Nil	-	-	6.0±2.0	50.5±6.5	20.5±1.4

* clinical score is given as mean for group of 5 rats (no. sick).

6.3 Infusion of alpha globulin into rats with EAE.

The abrupt decline in α_2M levels before disease onset (table 6.1), and the increase coinciding with recovery, suggested the possibility that the decrease in α_2M levels might be associated with the development of symptoms.

In a preliminary study an alpha globulin preparation, prepared as described in Hunter et al (1985), containing 1mg/ml α_2M, was given to rats via osmotic pumps at the rate of 1mg/rat/day for a period of 7 days beginning on day 8 after injection of GPSCH-CFA. Rats receiving α_2M were completely protected from clinical signs of EAE. Rats receiving buffer alone showed symptoms from days 13-23, of similar severity to untreated controls (Table 6.2). The plasma levels of fibrinogen and α_2M in the α_2M-infused group are shown in Table 6.2. Fibrinogen levels in rats injected with GPSCH-CFA were variably elevated over a 32 day period. Infusion of α_2M from day 8-15 resulted in a further elevation of fibrinogen levels. Similarly, there was a marked increase in α_2M levels following infusion of alpha globulins.

Table 6.2 Infusion of alpha globulin into rats with EAE.

Rats injected with	alpha globulin infused*	day tested	clinical score **	α_2 M	fibrinogen
GPSCH-CFA	-	4	-	15.4±2.1	38.5±3.6
		8	-	3.5±0.6	33.8±5.5
		12	-	2.5±0.5	29.5±1.3
		16	1.8(4)	2.8±0.8	31.0±8.4
		20	1.4(3)	2.7±0.3	22.8±2.6
		25	-	0.6±0.1	24.2±2.5
		28	-	2.3±0.5	19.2±2.2
		32	-	2.5±0.3	25.8±1.3
GPSCH-CFA	+	8	***	2.8±0.2	29.7±0.9
		21	-	13.7±3.6	39.8±2.5
		25	-	7.4±1.9	50.5±2.3
CFA	-	10	-	4.8±1.6	31.3±3.1
		17	-	0.6±0.1	26.1±2.2
		23	-	0.6±0.1	24.6±2.5
Nil	-	-	-	4.2±0.8	21.0±1.3

* given α_2 M days 8-15 after injection of GPSCH-CFA, lmg/rat/day
** mean clinical score for group of 5 rats (no. sick)
*** 3 rats showed clinical scores of 1,1,1 on day 16 only.

6.4 Infusion of purified α M into rats with EAE.

Following the results with infusion of an alpha globulin fraction it was decided to infuse purified α_2M to determine whether this protein protected against EAE.

Purified α_2M was given via osmotic pumps to rats which had been injected with GPSCH-CFA 8 days previously, infusing 1 mg/rat/day for a period of 7 days. Control groups received either PBS or BSA (1mg/rat/day) in osmotic pumps. Rats which received α_2M were effectively protected from clinical signs, as shown in Table 6.3. Plasma α_2M levels rose rapidly in all groups, declining on day 8 (Figure 6.1). However, the α_2M-infused animals showed a marked increase in α_2M on days 16 and 20, and overall increases in fibrinogen and ceruloplasmin levels suggesting that the infusion of α_2M had re-stimulated the acute phase response. The infusion of 7 mg of α_2M over a 7-day period from day 8-15 could not account for the level of 6 mg/ml of plasma α_2M on day 20.

H and E sections of brain and spinal cord taken from rats on day 16 after injection of GPSCH-CFA, showed that protected animals had extensive cellular infiltration, similar to EAE controls. In addition, the response of sensitized lymph node cells, taken on day 20 after injection of GPSCH-CFA, to MBP in the MPCA were similar for both diseased and protected animals (results not shown).

Table 6.3 Clinical scores for rats infused with purified alpha$_2$ macroglobulin.

Days after sensitisation	BSA-CFA	GPSCH-CFA	GPSCH-CFA α_2M days 8-15
No. in group	8	10	5
0-12	0	0	0
13	0	0	1.0(1)*
14	0	1.0(6)	0
15	0	1.5(6)	0
16	0	1.6(5)	1.0(3)
17	0	2.0(4)	0
18	0	2.0(4)	0
20	0	1.6(3)	0
20+	0	0	0

* scores are given as the mean for the group (no. sick).

NEXT PAGE:

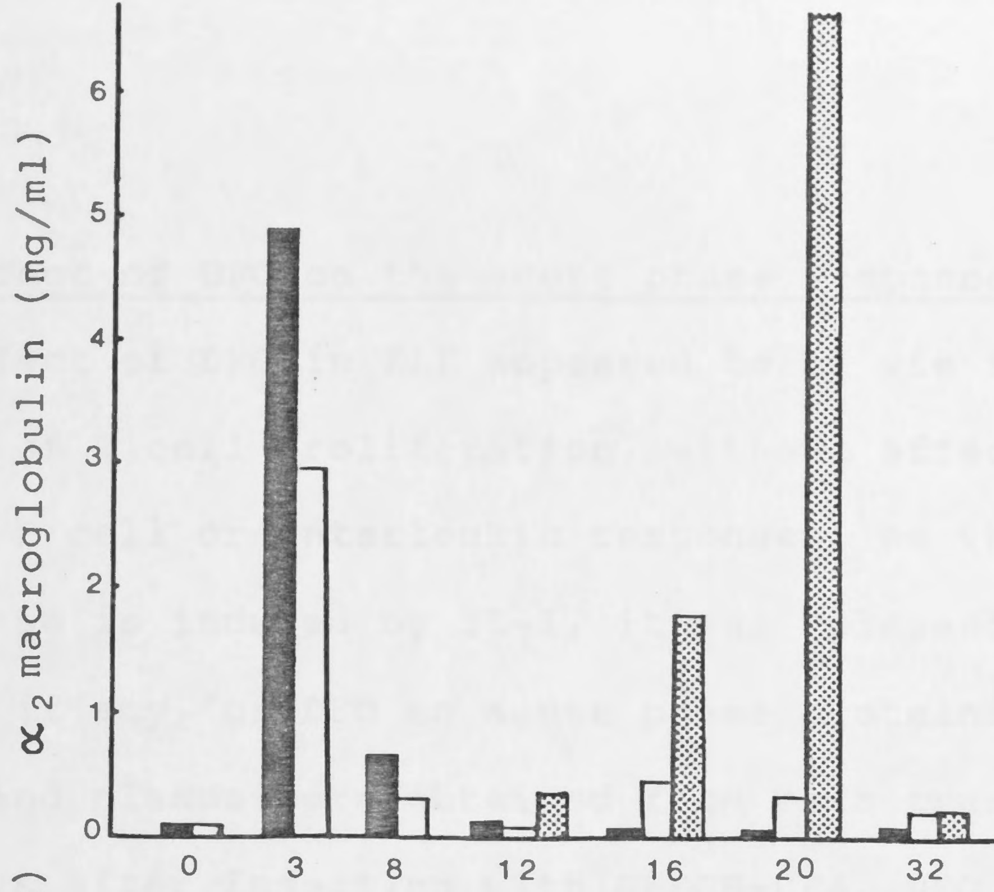
Figure 6.1 The acute phase response in rats with EAE.

Plasma levels of the acute phase proteins a) α_2 macroglobulin, b) ceruloplasmin and c) fibrinogen were measured in rats given

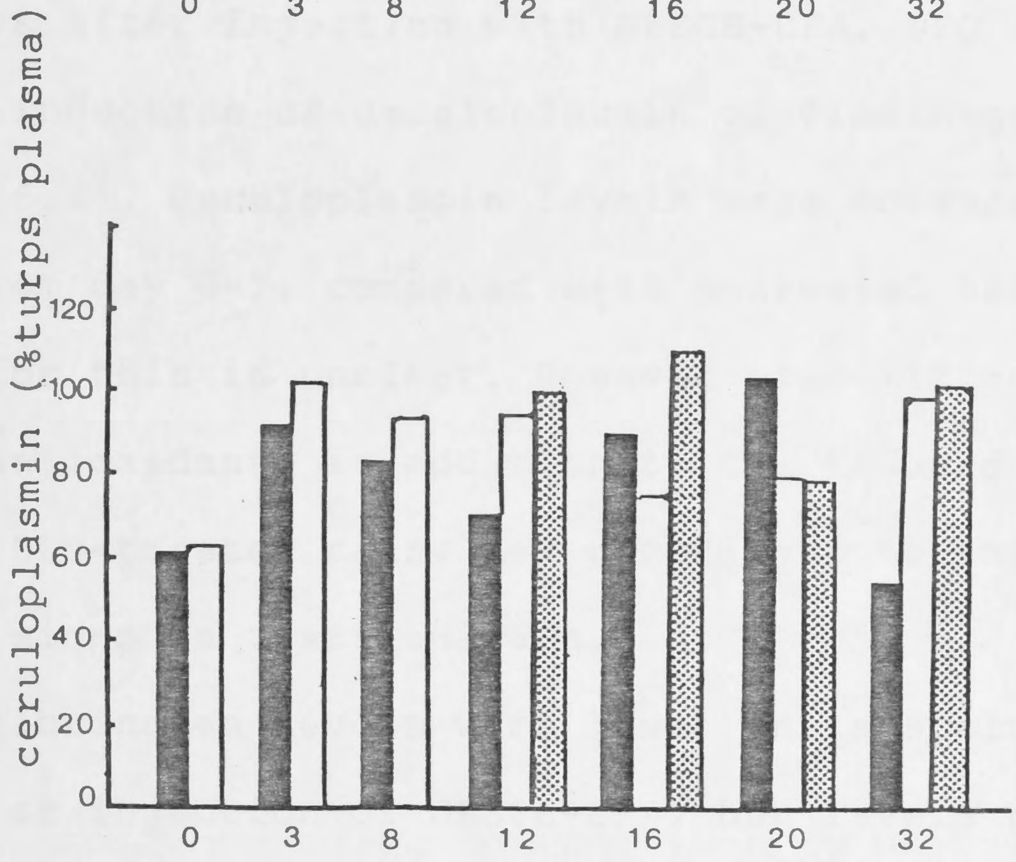
■ BSA-CFA on day 0

□ GPSCH-CFA on day 0

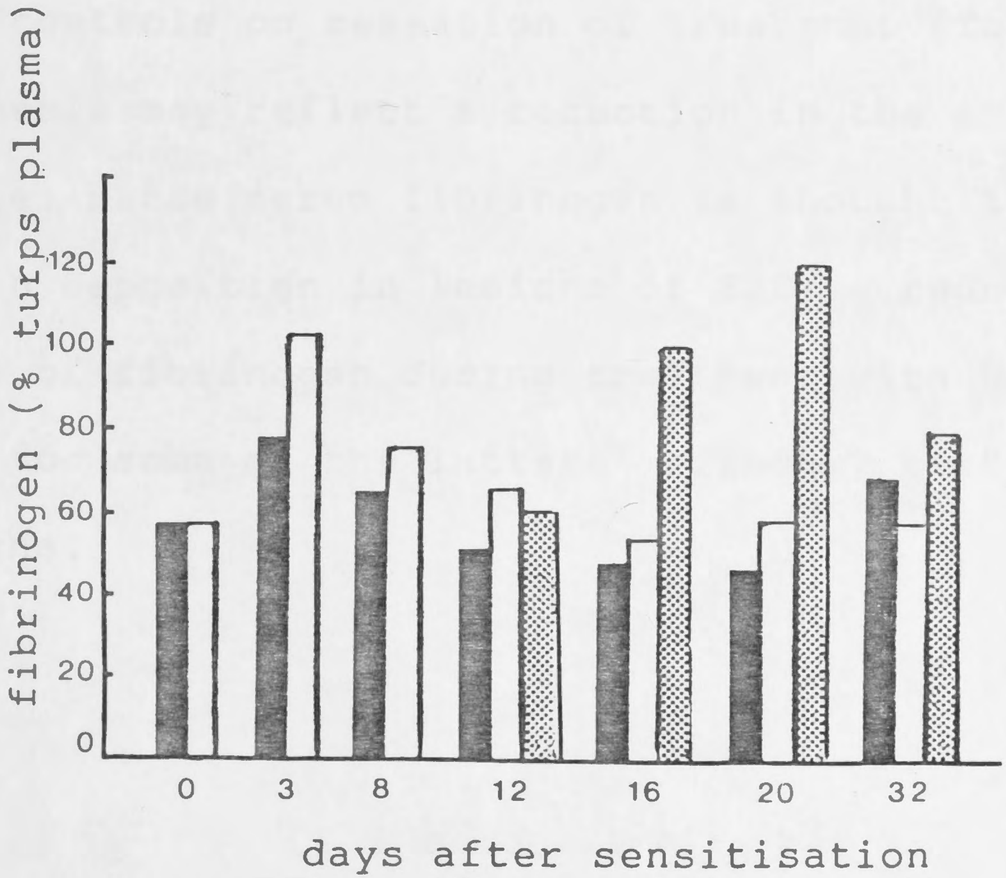
▩ GPSCH-CFA on day 0 and infused with α_2 M from days 8 to 15.



α2 macroglobulin (mg/ml)
0
1
2
3
4
5
6
0
3
8
12
16
20
32



ceruloplasmin (%turps plasma)
0
20
40
60
80
100
120
0
3
8
12
16
20
32



fibrinogen (% turps plasma)
0
20
40
60
80
100
120
0
3
8
12
16
20
32
days after sensitisation

6.5 The effect of DFO on the acute phase response.

The effect of DFO in EAE appeared to be via the suppression of T cell proliferation, without affecting macrophage, B cell or interleukin responses. As the acute phase response is induced by IL-1, it was relevant to examine the effect, if any, of DFO on acute phase proteins.

Serum and plasma were obtained from rats treated with DFO 0-7 days after injection with GPSCH-CFA. DFO did not prevent the induction of ceruloplasmin or fibrinogen in EAE rats (Table 6.4). Ceruloplasmin levels were enhanced in rats given DFO from day 0-7, compared with untreated EAE controls. The reason for this is unclear. However stimulation of this protective antioxidant, in addition to the reduced T cell function in DFO-treated rats, may contribute to the reduction of clinical signs in these animals.

Serum fibrinogen levels were lower in rats given DFO on days 0-7 after injection of GPSCH-CFA, but levels returned to that of EAE controls on cessation of treatemnt (Table 6.4). The lower levels may reflect a reduction in the amount of tissue damage. Since serum fibrinogen is thought to contribute to the fibrin deposition in lesions of EAE, a reduction in serum levels of fibrinogen during treatment with DFO may be responsible for some of the latters' effects, particularly on clinical signs.

Table 6.4 Ceruloplasmin and fibrinogen levels in rats treated with DFO.

Rats injected with	DFO day 0-7	day tested	ceruloplasmin (mg/100ml)	fibrinogen (mg/ml)
GPSCH-CFA	-	3	NT	91.2±12.5
		7	76.5±9.0	47.0±7.1
		10	93.0±11.0	41.5±3.8
		14	162.0±10.0	57.5±10.2
GPSCH-CFA	+	3	NT	31.1±5.5
		7	101.0±11.3	55.2±8.6
		10	175.2±23.3	43.5±9.5
		14	148.4±13.2	85.0±7.5
Nil	-	-	45.5±9.9	49.7±8.3

6.6 Ceruloplasmin levels in multiple sclerosis patients.

A study by Plum and Hansen (1960) showed that in 50 MS patients studied, all had low levels of ceruloplasmin. This rather unusual finding has not been followed up until recently. Three small studies have shown that patients with recently diagnosed MS (onset less than two years) tend to have low levels of ceruloplasmin while older patients, or those with chronic disease (onset over five years) have increased levels, up to 40% above normal controls. Most, however, fall

within the normal range, and the means of such groups are similar to controls (Voiculescu et al, 1983; Cendrowski and Szajbel, 1966; Becus, Popoviciu and Palade, 1971). Ceruloplasmin increases with age, with concomitant infection (which is not uncommon in MS) and with ACTH therapy. However, the low levels found in recently diagnosed patients are interesting.

Sera from 24 MS patients were kindly provided by Prof. A. Basten, Dept. of Immunology, University of Sydney. Control sera were obtained from the Blood Bank at Woden Valley Hospital, Canberra. Ceruloplasmin levels are shown in Figure 6.2. Control sera were within the normal range of 30 $\pm$ 6 mg/100ml (Scheinberg and Sternlieb, 1963). The MS sera showed greater variation, however. In particular, 3 patients had higher than normal values (42.3, 52.7, 75.3 mg%), and two were lower than normal (12.3,17.5). These patients all had mild relapsing MS with a disability of 0-3 on the Kurtzke scale, and were not receiving therapy at the time of sampling.

No conclusion could be reached from this small study, although the variation in ceruloplasmin levels found in MS patients in this and the reports cited above is worthy of further investigation.

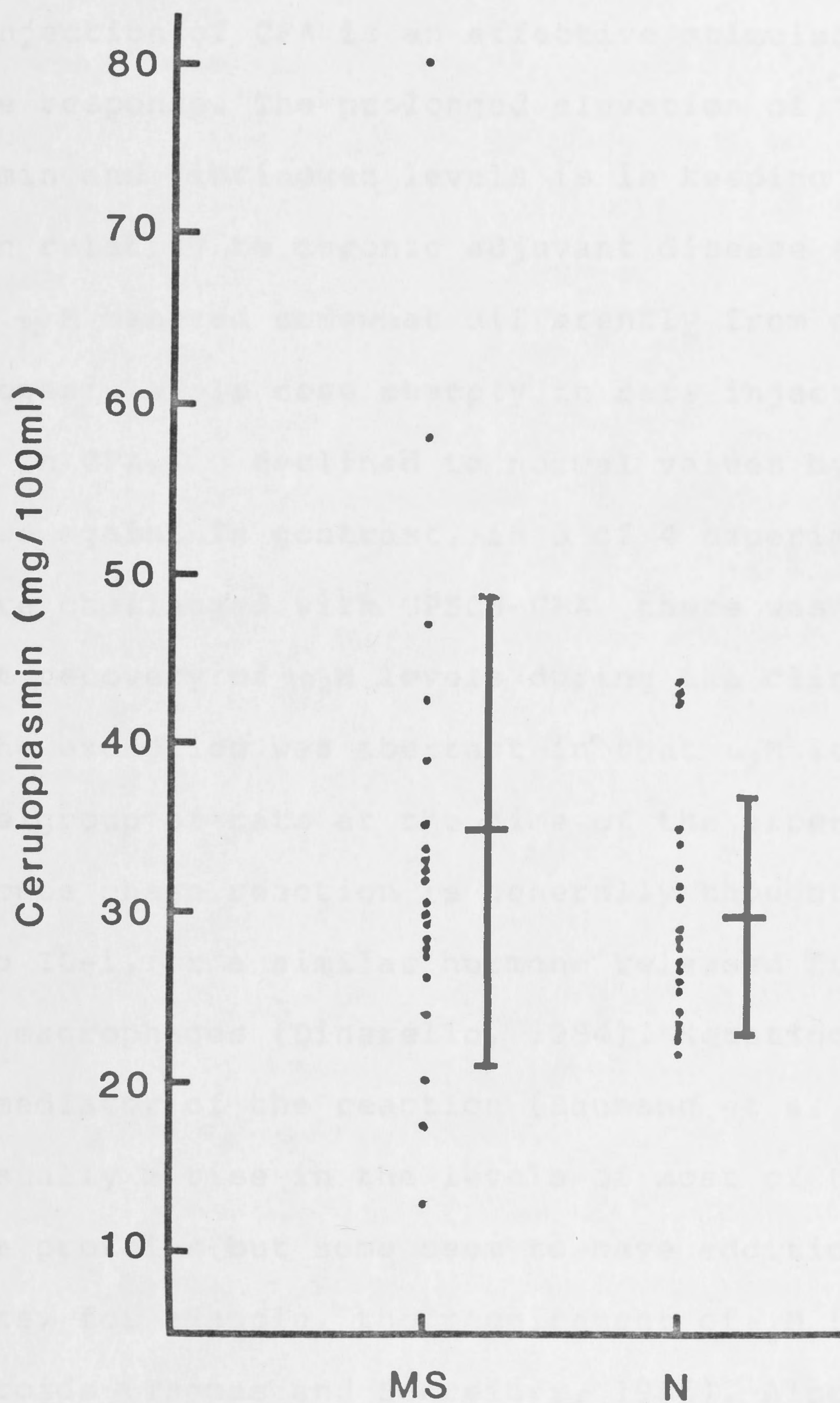


Figure 6.2 Serum ceruloplasmin levels in MS patients.

Serum ceruloplasmin levels were detrmined from 24 MS patients (MS) and 24 normal controls (N).

6.7 Discussion.

The injection of CFA is an effective stimulus of the acute phase response. The prolonged elevation of ceruloplasmin and fibrinogen levels is in keeping with the information relating to chronic adjuvant disease (Pearson et al, 1961). α_2 M behaved somewhat differently from ceruloplasmin and fibrinogen; levels rose sharply in rats injected with BSA emulsified in CFA, declined to normal values by day 12, and did not rise again. In contrast, in 3 of 4 experiments where animals were challenged with GPSCH-CFA, there was a small but significant recovery of α_2M levels during the clinical disease. The exception was aberrant in that α_2M levels were high in the group of rats at the time of the experiment.

The acute phase reaction is generally thought to be a response to IL-1, or a similar hormone released from stimulated macrophages (Dinarello, 1984). Keratinocytes also produce a mediator of the reaction (Baumann et al, 1984). There is usually a rise in the levels of most of the group of acute phase proteins but some seem to have additional requirements, for example, the requirement of α_2M for corticosteroids (Thomas and Schreiber, 1985). Also, an increase in the plasma concentration of ceruloplasmin in the absence of a general acute phase reaction, has been reported in rats treated with hyperbaric oxygen (Moak and Greenwald, 1984)

Interelukin 1 released from stimulated macrophages is the probable inducer of the acute phase response in CFA-challenged animals. The functions of the acute phase proteins, although

incompletely understood, seem to be essentially anti-inflammatory and tissue protective (Kushner, 1982). In contrast, IL-1 also promotes the immune response directly by acting as a co-stimulant for T lymphocytes and a maturation factor for B lymphocytes (Dinarello, 1984), and indirectly by promoting fever which is reported to augment lymphocyte function (Duff and Durum, 1983). By stimulating fibroblast-like cells to secrete collagenase, IL-1 could also have tissue degrading effects.

One explanation for the data presented here is that clinical disease represents a temporary imbalance between pro-inflammatory events promoted in part by IL-1 and anti-inflammatory activities of the acute phase proteins, also promoted by IL-1.

On a molar basis, α_2M was one hundred thousand to one million times more effective in protecting rats from EAE than the low molecular weight proteinase inhibitors used by Brosnan et al (1980). This suggests that the proteinase binding properties of α_2M are not the only basis for protection. This should be considered, however, in the context of the broad spectrum of inhibitory activities of α_2M (Starkey and Barrett, 1977) compared with other proteinase inhibitors; α_2M may bind a variety of proteinases which could contribute to clinical disease.

The infusion of α_2M contributed in only a minor way to the increased plasma levels of this protein. The infusion of an albumin preparation also restimulated the acute phase

response. The injection of α_2M in the free or proteinase-bound form into normal animals did not result in an increased acute phase response above that produced by injection of buffer alone. The response of animals with an ongoing inflammatory process could, however, differ from normal animals.

The major difference between the group of rats infused with a low dose of α_2M and albumin was the higher level of α_2M on days 14 and 17 in the α_2M infused animals. It could be that a minimum threshold of α_2M is necessary for protection. This is supported by the drop in level of protection in this group. Another possibility is that there is a special characteristic of the α_2M preparation. In this regard Nieuwheizen et al (1979) reported that an α_2M preparation with high levels of esterolytic activity, which may thus be in the proteinase-bound form, effectively reduced edema induced by carrageenan injection while a preparation shown to be predominantly in the "free" form was ineffective. Proteinase bound α_2M was not detected in the preparation used in the present study, however. An alternative explanation is the possibility of isomeric forms of α_2M with differing biological properties. Isomers have been reported for human α_2M (Frenoy and Bourrillon, 1974) and there was an apparent heterogeneity in the elution of α_2M from the Coomassie blue affinity gel used in these studies (Hunter et al, 1985).

There are a number of possible steps where α_2M could intercede in the inflammatory reaction, including suppression of the immune response, inhibition of proteinase secretion,

inhibition of oxygen radical formation and binding of proteinases (Goldstein and Charo, 1983). The data suggest that α_2M does not prevent lymphocyte sensitization or migration into the central nervous system. If α_2M is the protective agent then it seems that it works by blocking a late stage in the effector pathway, perhaps by interfering directly with the DTH reaction.

The rat, like other rodents (but unlike man), has two major macroglobulin inhibitors. In this respect the rat provides a good model for studying the biological properties of α_2M, because the protein is a major acute phase protein in this species. Further studies are in progress to determine if α_2M is a key protein in the regulation of the disease process.

CHAPTER 7 GENERAL DISCUSSION

Rats which were treated during the course of EAE with the iron-chelating agent desferrioxamine, showed a marked reduction in the severity and duration of symptoms. Some groups showed mild symptoms about 1 week after withdrawal of the drug, indicating that regulatory mechanisms which prevent re-induction of EAE (possibly T suppressor cells) were not fully functional. That suppressor mechanisms did eventually develop was indicated by the results in sections 4.4 and 4.6. Firstly, recovery of lymphocyte responsiveness after the cessation of therapy did not lead to the development of severe symptoms, and secondly, in a group of rats which had been treated on days 8-15 with DFO, and did not show any symptoms, the disease could not be induced by challenge on day 35 with GPSCH-CFA. Since DFO inhibited the generation of T suppressor cells in response to SRBC in mice (section 4.16), it may be that other protective mechanisms such as antibody or acute phase proteins are able to confer resistance to (re)induction in EAE. Alternatively, the generation of T suppressor cells may be delayed, a possibility which could easily be tested in the future. The T suppressor cell of DTH to SRBC in mice has been shown to be Lyt $1{+}23^{-}$ (Ramshaw, Bretscher and Parish, 1976); T suppressor cells in EAE may be Lyt $1^{-}23{+}$, and it is possible that DFO may preferentially suppress the proliferation of T cells of the DTH/helper type, allowing the development of T suppressor cells.

The protective effect of DFO in EAE was directly related to the level of responsiveness for lymphocytes of the DTH/helper subset. The finding that DFO suppressed lymphocyte proliferation *in vitro*, and the fact that this was reversed by the addition of $FeCl_3$, indicates that the inhibition may operate via the removal of iron. There is abundant evidence that iron- deficiency anaemia is associated with diminished cell-mediated responses, while antibody production is not affected (Joynson et al, 1972). Furthermore, Mainou-Fowler and Brock (1985) have shown that lymphocytes, incubated in serum from iron-deficient animals, respond poorly to mitogens, while incubation in normal serum restores the lymphoproliferative capacity of lymphocytes from iron-deficient animals. The inhibitory effect of iron-deficient serum can be reversed by addition of iron, and Mainou-Fowler and Brock (1985) suggested that the inhibition is related to the degree of transferrin saturation.

Histological sections taken from DFO-treated rats showed an absence of small mononuclear cells from CNS lesions, although macrophages could be found. The effect of treatment on small lymphocytes was confirmed by ^{125}IUdR labelling (section 4.13), in which a decrease was found in the numbers of rapidly dividing cells in lesions within 48 hr of starting DFO treatment. This could be due to the suppression of proliferation. Alternatively, DFO may, by its iron-chelating capacity, alter the migration of T lymphocytes. The "homing" of lymphocytes to iron deposits in the synovium of rheumatoid arthritis patients (Blake et al, 1983), and in the gut and

spleen (de Sousa, 1978 and 1981), has been suggested as a mechanism of attracting lymphocytes to sites where they obtain iron for proliferation. Conversely, iron depletion may result in a failure of lymphocytes to migrate to appropriate areas of lymphoid tissue, leading to a decrease in the numbers of immunologically competent cells (de Sousa, 1981). The migration of T cells to T-dependent areas of the spleen was altered in mice treated with DFO (section 4.14). Perhaps the failure of lymphocytes to migrate to the CNS in EAE rats treated with DFO (section 4.5) could similarly be a result of a decrease in antigen stimulation within lymphoid tissue. This is consistent with the failure of lymph node cells from DFO treated rats to respond to MBP (section 4.9), although such cells were capable of responding to Con A *in vitro*.

It is well known that primary sensitization to antigen *in vivo* requires the clonal expansion of T effector cells (Oppenheim, 1968). Subsequent expression of DTH is mediated by two T lymphocyte populations acting in sequence (van Loveren and Askenase, 1984; van Loveren et al, 1984). The first, appearing early in the response (12-24 hr), is Ly-1+ and radiation-insensitive; the second, appearing 3-4 days later, is also Ly-1+, but radiation-sensitive. In these studies, and those of Kettman and Matthews (1975), irradiation of cells that transfer the late component of DTH resulted in defective recirculation of these cells into sites of inflammation. Injection of the cells with antigen, bypassing the need for recirculation, did not affect their ability to mediate a local

DTH reaction.

Proliferation of recipient cells is obligatory for expression of DTH, since transfer of sensitized lymphocytes into irradiated recipients inhibits the inflammatory response (Kettman and Matthews, 1975).

Thus, treatment of rats with EAE by infusion of DFO, by suppressing proliferation, could inhibit the expression of DTH at three separate stages: 1) sensitization to MBP in the draining lymph node, 2) the homing of specifically sensitized T lymphocytes into the CNS, and 3) Infiltration of non-specific inflammatory cells into CNS lesions.

The proliferative response of mouse, rat and human lymphocytes to T cell mitogens was markedly suppressed by non-toxic (15μM) concentrations of DFO *in vitro*. DFO did not seem to interfere with the cellular signal for proliferation, since lymphocytes exposed to Con A and DFO for 24 hr were still committed to proliferate when both Con A and DFO were removed.

Analysis of the DNA content of lymphocytes incubated with Con A and DFO showed that cell proliferation was halted in G , that is, the stage immediately before DNA synthesis begins. DFO was ineffective at suppressing antibody production, phagocytosis by macrophages, secretion of IL-1, IL-2 and PCIF, and cytotoxicity of T lymphocytes, none of which require DNA synthesis for their action (Perlmann and Holm, 1969; Mauel et al, 1970; Bloom, Gaffney and Jiminez, 1972). These results indicate that DFO may selectively inhibit DNA synthesis, a view supported by Robbins and Pederson (1970), who also found

that tumour cells cultured in the presence of DFO showed a selective inhibition of DNA but not of RNA or protein synthesis.

Thus, it appears that DFO selectively suppresses T cell proliferation via the inhibition of DNA synthesis. That this effect can be reversed with iron, but not with other metal salts, suggests that the inhibition is related to the iron-binding capacity of the drug. Since DFO is thought not to enter cells, except for hepatocytes (Morgan 1971; White, Bailey-Wood and Jacobs, 1976), and DFO cannot remove intracellular iron from lymphocytes (Brock and Rankin, 1981), it must exert its effects in the extracellular medium. Nearly all of the iron in plasma (and in serum-containing culture medium) is bound to transferrin (Hahn and Ganzoni, 1980). Stimulated lymphocytes express transferrin receptors, which take up transferrin-bound iron, internalise the complex, and release the apotransferrin. DFO does not interfere with binding of transferrin to its receptors (section 5.13).

There are at least two possible ways DFO could be exerting its effects *in vitro*. Firstly, DFO binds iron present in the culture medium, which then cannot be bound by transferrin. Transferrin receptors appear about 6 hr after stimulation, reaching a peak at 20-24 hr, with a half-life of approximately 8 hours (Mattia et al, 1984). Subsequent uptake of (30% saturated) transferrin and release of apotransferrin results in necessity of further supply of iron for a second round of transferrin saturation and uptake. Brock (1981) has

shown that 8 ng DFO is sufficient to bind the iron present in serum-free medium, preventing its subsequent uptake by apotransferrin, and proliferation. DFO may act in this way *in vitro*, however *in vivo* there would be sufficient saturated transferrin in serum to overcome such a deficit.

Secondly, DFO may compete with transferrin for iron, rendering it "undersaturated". Normal serum transferrin is approximately 30% saturated with iron; it has been shown that saturation of less than 24% results in poor binding of transferrin to its receptors, and hence, poor proliferative responses (Brock, 1981; Brock and Mainou-Fowler, 1983). Keberle (1964) showed that DFO could remove about 14% of transferrin-bound iron. It is possible that DFO, at concentrations of 15μM, could remove sufficient transferrin-bound iron to inhibit uptake by lymphocytes. The results in section 5.14 suggest that this might be the case. However more definitive experiments using radiolabelled iron need to be done before any firm conclusion can be drawn.

Carotenuto et al (1985) have suggested that DFO may inhibit the expression of IL-2 receptors on PHA-stimulated lymphocytes. However, the appearance of transferrin receptors was not diminished by DFO and, since the appearance of the latter is dependent upon the prior appearance of Il-2 receptors (Neckers and Cossman, 1983) this is unlikely. However, DFO may interfere with binding of IL-2 to its receptors.

The apparent lack of effect of DFO on B cells could be explained by the fact that neither B cell proliferation nor

uptake of transferrin-bound iron is essential for antibody production (Neckers, Yenokida and James, 1984). In addition, irradiated T cells can provide help for antibody production, and secretion of B cell-activating factor (BAF) occurs in non-proliferating T cells (Bloom, Gaffney and Jiminez, 1972)

The importance of iron in maintaining normal DNA synthesis is well recognized (Robbins, Faut and Norton, 1972; Hoffbrand et al, 1976). The decrease in DNA synthesis resulting from iron-deficiency probably occurs in all cells, although the effect is most noticeable in rapidly dividing cells such as stimulated lymphocytes. There are several iron-dependent enzymes which may be affected by iron-deficiency. These appear to vary according to the tissue studied. In the liver, for example, only succinate-cytochrome c reductase activity is affected (Bailey-Wood et al, 1975). The suppression of DNA synthesis in lymphocytes from patients with iron-deficiency anaemia has been linked to a reduction in the enzyme ribonucleotide reductase, leading to a reduction in deoxyribonucleotide levels, particularly deoxyadeno-triphosphate (dATP). A similar reduction in dATP has been found <u>in vitro</u> by incubation of PHA-stimulated lymphocytes with DFO (Hoffbrand et al, 1976).

The effect of DFO on iron-catalysed lipid peroxidation in EAE is less clear. While other antioxidants were able to inhibit clinical signs of EAE, none was as effective as DFO or hydroxyurea, both of which effectively inhibit lymphoproliferation. Attempts to detect products of lipid

peroxidation in the CSF of rabbits with severe, chronic EAE were unsuccessful (section 4.18). Further experiments are intended to establish whether lipid peroxidation occurs within CNS lesions of EAE.

There is evidence to suggest that DFO may prevent oxidative damage in vivo. Clark and Hunt (1983) have shown that DFO blocks the haemolytic action of several radical-generating drugs in mice. In humans, DFO has been used successfully in the treatment of encephalopathy and osteomalacia due to chronic haemodialysis (Ackrill et al, 1980; Brown et al, 1982), neuronal ceroid lipofuscinosis (Westermarck and Santavuori, 1984) and rheumatoid arthritis (Giordano et al, 1984). In all three studies, DFO was thought to act via the removal of excess iron, preventing the oxidative damage which occurs as a result of iron deposition. Some caution needs to be exercised in the use of long-term, high-dose DFO therapy in conditions other than iron overload; in a preliminary trial of seven rheumatoid arthritis patients given DFO (3g daily 5 days/week for 1-3 weeks) three developed ocular abnormalities that were reversed on withdrawal of the drug (Blake et al, in press). In the same study two patients, who also received the anti-emetic drug prochlorperazine, lost consciousness for 48-72 hours and then fully recovered.

DFO may be beneficial in the treatment of MS for two reasons: firstly, lesions of MS contain cellular infiltrates of T lymphocytes in similar proportions to those found in EAE (Traugott, Reinherz and Raine, 1983a, 1983b). The effects of DFO on migration of T cells into the CNS and in reducing

proliferation of sensitized T cells indicates that it may be useful in reducing cellular infiltration into the CNS.

Secondly, since DFO is able to cross the blood-brain barrier, it could act within lesions to remove catalytic iron, thus preventing lipid peroxidation. Evidence for oxidative processes occurring in MS is conflicting. The occurrence of lipid peroxidation is usually accompanied by a rise in protective antioxidant mechanisms such as catalase, glutathione peroxidase, vitamin E and ceruloplasmin. Studies of MS patients have yielded variable results, ranging from decreased to increased levels of antioxidant activity (Plum and Hansen, 1960; Wikstrom et al, 1976; Szeinberg et al, 1981; Mehlert et al, 1982; Voiculescu et al, 1983). Recently, Hunter et al (1984) have shown that MS patients undergoing hyperbaric oxygen therapy exhibit up to six-fold increases in catalase levels. These authors have suggested that the beneficial effect of hyperbaric oxygen experienced by some MS patients (Neubauer, 1985) may be due to the increase of antioxidant enzymes, particularly catalase, induced by oxidative stress.

In a group of MS patients used as controls in study by Gutteridge et al, (1982) no increase in CSF non-protein-bound iron was found, nor was CSF antioxidant activity different from normal controls. However, Craelius, Jacobs and Lee-Jones (1980) found that iron levels in plaque material from MS patients were approximately five times the level found in normal white matter. The source of such iron was presumed to be red blood cell extravasation and breakdown. In addition,

iron deposits in the form of haemosiderin, which can be mobilized by DFO, (Shoden and Sturgeon, 1962) have been found in multiple sclerosis plaques (Craelius et al, 1982), although this finding was not confirmed by Walton and Kaufmann (1984). The presence of iron in plaques, apart from contributing directly to iron-catalysed lipid peroxidation in myelin-rich areas, may attract lymphocytes to sites of inflammation, as has been suggested for rheumatoid arthritis (Blake et al, 1981). Therefore, removal of iron from plaque areas might be expected to have a beneficial effect.

Since DFO does not prevent the production and release of lymphokines, nor their action on macrophages and B cells, immune surveillance to other antigens, and to cancer cells, should be intact. Thus, the prevention of proliferation of clones of effector cells, together with an inhibition of migration of these cells into the CNS, suggests that DFO may be of value as a reversible, non-toxic immunosuppressive agent to prevent both autoreactive and rejection processes involving T lymphocytes.

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