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MODULATIONOF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN THE SJL/J MICE BY CYTOKINES AND ANTI-CYTOKINE ANTIBODIES

by

TRANG THI DUONG

Department of Microbiology and Immunology

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
June 1995

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ABSTRACT

Immunization with myelin basic protein in complete Freund's adjuvant resulted in only mild chronic-relapsing experimental allergic encephalomyelitis (EAE) with very low incidence in SJL/J mice, and no disease at all in A/J, BALB/c, C3H/HeJ, DBA/2, AKR and NZW mice. However, treatment of mice with anti-IFNγ (aIFN) monoclonal antibody (mAb) at the time of immunization determined severe disease in all those strains except AKR. Similarly, the passive induction of EAE in the SJL/J mice and in two of three resistant strains examined was significantly enhanced by aIFN mAb administration. In the SJL/J mice, administration of anti-interleukin 2 (aIL2) mAb had only a marginal effect in the active induction, but drastically reduced the manifestation of passive EAE. This was also observed even when aIL2 mAb was mixed with a disease-enhancing dose of aIFN mAb.

Kinetic studies revealed that aIFN mAb exerted its effect within 24 hr following the challenge or cell transfer. In several experiments, biotinylated aIFN (bt-aIFN) mAb was used to allow its deliberate removal by avidin. The results of these studies demonstrated that (i) the aIFN mAb exerted its effect within 24 hr after its administration, even prior to cell transfer, and (ii) given a period of 24 hr after the removal of bt-aIFN, the animals appeared to revert to the pretreatment disease susceptibility status.

MBP-challenged, aIFN mAb-treated animals exhibited higher percentages of CD4 and CD8 cells expressing the very late antigen-4 (VLA-4) in the lymph nodes in comparison to challenged but nontreated animals. Furthermore, immunostaining of brain sections of challenged and aIFN mAb-treated mice

revealed higher expression of VLA4, intercellular adhesion molecule-1 (ICAM-1) and of vascular cell adhesion molecule-1 (VCAM-1). Lymph node cells isolated from challenged and aIFN mAb- treated animals also produced less interferon-gamma (IFNγ) and more tumor necrosis factor α/β in comparison to those from untreated mice.

The function of IFNγ and aIFN mAb in the early events of antigen-induced T cell activation was investigated *in vitro*. Pretreatment of murine peritoneal exudate cells (PEC) with IFNγ led to a significant increase in their ability to activate antigen (Ag) specific, short-term T cell lines. When exogenous IFNγ was added to cocultures of T cells and Ag-pulsed PEC or Ag-pulsed thymocytes or splenocytes, the T cell proliferation and interleukin 3 (IL3) production were considerably reduced. Anti-IFNγ mAb added to these cultures neutralized the inhibitory effect of the exogenous IFNγ but had no detectable effect on class II major histocompatibility gene expression by the Ag-pulsed PEC present in the same cultures. A reduction in T cell proliferation and IL3 production was also observed when the T cells were treated with IFNγ prior to coculture with the Ag-pulsed PEC. Furthermore, both the expression of IL2 receptor and calcium flux were reduced in the IFNγ treated T cell population. Determination of cell viability revealed that IFNγ rendered T cells less responsive to the Ag-pulsed PEC but did not kill them.

Inasmuch as IFNγ and interleukin 4 (IL4) antagonized each other in many aspects, the role of IL4 in EAE was also examined. The disease-enhancing effect of aIFN mAb in the active induction of EAE was clearly outweighed by anti-IL4 receptor (aIL4R) mAb administration. Both disease severity and incidence were

reduced by the inclusion of aIL4R to the aIFN mAb treatment. When mouse recombinant IL4 was given under conditions that would increase its retention time and biological activities, severity and incidence of actively induced EAE were enhanced.

Taken collectively, the work in this thesis demonstrated that whereas IL2 and/or IL4 is involved in EAE induction, IFNγ plays a disease-limiting role at least during the early stages of disease induction. Our data support the notion that IFNγ may down-regulate the disease through multiple mechanisms. A model of immunoregulation early in the development of EAE by IL2, IFNγ and IL4 will be discussed.

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ABBREVIATIONS

aa	amino acid(s)
Ab	antibody
aIFN	anti-IFNγ
aIL2	anti-IL2
aIL4	anti-IL4
aIL2R	anti-IL2 receptor
aIL4R	anti-IL4 receptor
Ag	antigen(s)
APC	antigen presenting cells
ATCC	American type culture collection
Av.	avidin
BBB	blood brain barrier
BCDF	B cell differentiation factor
Bp	*Bordetella pertussis*
BSA	bovine serum albumin
BSF-1	B cell stimulating factor-1
BSS	Hanks' balanced salt solution
bt-	biotinylated-
Ca^{++}	calcium
CFA	complete Freund's adjuvant
clin. score	clinical score
CNS	central nervous system
cr-EAE	chronic-relapsing EAE
CSF	cerebrospinal fluid
CSIF	cytokine synthesis inhibitory factor
dpm	disintegrations per minute
Δdpm	delta disintegrations per minute
DTH	delayed-type hypersensitivity
EAE	experimental allergic encephalomyelitis
EAU	experimental autoimmune uveoretinitis
ELISA	enzyme-linked immunosorbent assay
Exp.	experiment
H & E	hematoxylin and eosin
hsp65	heat shock protein 65
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
GC	galactocerebroside
hr	hour(s)
^{3}H-TdR	(methyl-^{3}H) thymidine
ICAM-1	intercellular adhesion molecule-1
(r)IFN	(recombinant) interferon

Ig	immunoglobulin
IL	interleukin
IL2R	IL2 receptor
IL4R	IL4 receptor
ip	intraperitoneally
iv	intravenously
KLH	keyhole limpet hemocyanin
LFA	lymphocyte function-associated antigen
LNC	lymph node cells
LT	lymphotoxin
mAb	monoclonal antibody
max.	maximal
MBP	myelin basic protein
MBP_{87-103}	MBP peptide spanning amino acids 87-103
MBP-CFA	MBP in CFA
MCS	mean clinical score
MHC	major histocompatibility
min	minutes
MS	multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
MW	molecular weight
NK	natural killer cells
NP	nitroiodophenyl acetate
PBL	peripheral blood lymphocytes
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEC	peritoneal exudate cells
PFA	4 % paraformaldehyde in PBS
PLP	proteolipid protein
RCAS	concanavalin A-activated rat spleen cell supernatant
SAAP	streptavidin conjugated alkaline phosphatase
sal	saline
Sc	spinal cord
sc	subcutaneous(ly)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SI	stimulation index
SPL	splenocytes
TcR	T cell receptor
TGF	transforming growth factor
Th	T helper
THYM	thymocytes
(r)TNF	(recombinant) tumor necrosis factor

V	variable
VCAM-1	vascular cell adhesion molecule-1
VEC	vascular endothelial cells
VLA-4	very late activated antigen-4

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Chapter 1: INTRODUCTION

Multiple sclerosis (MS), which involves the human central nervous system (CNS), is the most common neurological disease, and is the paradigm of human demyelinating diseases (Paterson, 1976; Raine, 1984). It can follow a progressive course or a chronic-relapsing course with the most common symptoms such as fatigue, double vision, tremors, etc. MS is most prevalent in young adults, with the majority of cases occurring between the ages of 20-40. As an accepted, time-honored animal model for immune-mediated demyelination, experimental allergic encephalomyelitis (EAE) has offered a variety of useful immunopathogenetic and therapeutic approaches pertinent to MS. To understand better the mechanisms that lead to exacerbation or suppression of MS, the aim of this study is to examine the aspect of immunoregulation in EAE by lymphokines such as interleukin-2 (IL2), IL4 and in particular, interferon gamma (IFNγ).

1.1 The central nervous system (CNS)

1.1.1 Blood brain barrier

The CNS is a specialized organ well sheltered from the blood. For this reason, it is also an immunological privileged site. To isolate and protect this environment, the system is enclosed behind a blood brain barrier (BBB), which itself is special in its structure and functions.

The interface between the vascular and extracellular compartments including the CNS, and the surface across which blood-tissue exchanges take place is the capillary wall which is made up of endothelial cells arranged as a single layer set upon a basement membrane. Several major differences exist between nonneural

and neural capillary wall that makes the BBB unique. In a general capillary, passage of fluid and/or its constituents can occur in two ways: a) through pinocytosis, and b) through the spaces between adjacent endothelial cells. On the other hand, in the CNS capillary, the passage is limited due to the absence of intercellular spaces, and to reduced pinocytosis (Oldendorf, 1975; Milhorat, 1987). The epithelial cells of the BBB are also unique in that they contain more mitochondria believed to serve the barrier both as a means of trapping certain substances, and for facilitating the transport of others (Cutler, 1980).

Overall, the BBB is a selective barrier that regulates the entry of extracellular fluid and its constituents into the CNS, and maintains the internal milieu as a stable environment. Any injury to the BBB or changes in its permeability will have impacts on the function of the CNS.

1.1.2 Cells of the CNS

There are two mains classes of cells in the central nervous system. Nerve cells or neurons are specialized for receiving, conducting and transmitting signals.The neurons are outnumbered by about 1 to 10 in the mammalian brain by the second class of cells known as neuroglia or simply as glia which play important ancillary functions.

The gray matter in the CNS consists of the cell bodies of the neurons embedded in a neuropil made up predominantly of neuronal and glial processes. The white matter, on the other hand, consists mainly of processes of neurons covered by a myelin sheath. Both the gray and white matter contain large numbers of neuroglial cells, and a network of blood capillaries (Barr and Kiernan, 1993).

The neurons: Every neuron consists of a cell body containing the nucleus with a number of branching dendrites and one axon. The dendrites extend from the cell body like antennae to receive signals from other neurons while the axon conducts signals away from the cell body, towards the distal targets. Some neurons have no axons, and their drendrites conduct signals in both directions, to and away from the cell body. Both dendrites and axons divide at its far ends into many branches, and therefore, can receive or pass on signals from and to many cells simultaneously.

Neuroglial cells: The glial cells surround both the cell bodies and processes of neurons, and occupy the spaces between them. The 4 types of glial cells in the CNS are the astrocytes, oligodendrocytes, ependymocytes and microglial cells (Alberts et al., 1989).

Astrocytes are the most plentiful and diverse of the glial cells. Their cytoplasm contains gliofilaments which are made of a protein known as glial fibrillary acidic protein. Astrocytes are thought to provide physical support for other cellular elements of the CNS. Many astrocytic processes are closely associated with capillary blood vessels suggesting that these cells may also be involved in the exchange of metabolites between neurons and the blood.

Oligodendrocytes are responsible for producing and maintaining the myelin sheath wrapping around the axons. Their cytoplasmic processes provide the myelin sheath which enlarges because lipoprotein added to the surface of the central part of an oligodendrocyte diffuses laterally within the plasmalemma, and is quickly incorporated into the whole surface of the cell.

The third type of glial cells, the ependymocytes, form the ciliated epithelial lining of the central cavity of the CNS. Like the astrocytes, these cells often have precesses ending on blood vessels.

About 5% of the total glia are microglial cells which are the equivalent of macrophages of nonneural tissues. They can acquire the expression of major histocompatibility (MHC) class II molecules, and phagocytic properties, and are involved in reactions to tissue damage and infection.

Except for microglial cells which originate in the hematopoietic tissue, all other glial cell types and neurons in the CNS share a common embryonic precursor. Whereas most glial cells remain capable of dividing throughout life, most neurons cannot divide once they have differentiated.

1.1.3 Myelin

The axon of vertebrate neurons are covered by a myelin sheath which functions as an insulator to increase the velocity of conduction of stimuli between a nerve cell body and its target. The myelin sheath is formed by oligodendrocytes in the CNS, and by specialized glial cells, the Schwann cells, in the peripheral nerve system. During myelination, the glial cell extends its cytoplasm and wraps it concentrically around the axon to form a segment of myelin sheath about 1 mm long (Alberts et al., 1989). As it continues to wrap around the axon, all the spaces between the plasma membranes, both cytoplasmic and extracytoplasmic are greatly reduced. The multiple layers of membranes (which can be as many as 50-100 layer thick) become so tightly packed such that the inner leaflets of the membrane bilayer associate to form the major dense line, and the outer leaflets of adjacent layers

associate to form the intraperiodic line. Along the axon, each region of myelin formed by an individual glial cell (internodes) is separated from the next region by a short unmyelinated area called the node of Ranvier (or simply, node). This is the only site where axonal membrane is in direct contact with the extracellular fluid. Each Schwann cell devotes itself to myelinate a segment of a single axon, whereas an oligodendrocyte can myelinate a portion of many separate axons simultaneously.

Myelin, like all membranes, consists of lipids (mainly, phospholipids) and proteins. However, it is specialized because of its considerably higher lipid:protein ratio in the range of 70:30 to 80:20 (Norton and Cammer, 1984). Its protein composition is relatively simple compared to that of other biological membranes. In both the CNS and the peripheral nervous system, the major proteins are found only in myelin and myelin forming cells. Myelin basic proteins (MBP) and proteolipid proteins (PLP) are the most abundant classes in the CNS, and are thought to play a role in the tight stacking of the plasma membranes. Myelin in the peripheral nervous system is constructed of other unique membrane proteins.

1.1.3.1 Proteins of the myelin: Historically, PLP and MBP were first isolated from the brain or spinal cord, the former from brain chloroform-methanol extract, and the latter from acid extracts of delipidated brain (Lees and Brostoff, 1984). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of myelin showed that PLP accounted for around 50% and MBP for 30% of total myelin proteins.

PLP: SDS-PAGE of bovine white matter shows that proteolipids constitute a family of proteins which includes a major band PLP at 25 kDa, and a band of

lower molecular weight (MW), DM-20, at 20 kDa. The primary structure of bovine PLP was first reported by 2 independent laboratories (Lees et al., 1983; Stoffel et al., 1985). PLP is the most conserved protein thus far studied. The amino acid (aa) sequences of rat, mouse and human PLP are identical (reviewed by Lees and Bizzozero, 1992), and only 4 substitutions are found between these sequences and bovine PLP. The protein consists of 276 aa, and since 2/3 of which are nonpolar, PLP is highly hydrophobic. Based on its aa sequence, two similar models of topography of PLP were proposed (Laursen et al., 1984; Stoffel et al., 1985). Both models predict that its amino-terminal segment is at the external face, a carboxyl-terminal segment at the cytoplasmic face, and that a hydrophobic domain enters and exits the same side of the membrane (cis-conformation). These models, thus, suggest an important function of PLP allowing the plasma membrane to stack tightly together during myelination. The close apposition of the cytoplasmic faces of the membranes may be due to the interactions between PLP-PLP or MBP-PLP. Apposition of extracellular faces may be stabilized by the polar regions of PLP acting as struts, whereas cis-domains which embed in the apposing membranes, may be important in strengthening compaction.

MBP: As already mentioned, MBP which is obtained by acid-extraction of defatted CNS tissue, comprises 30 to 40% of the total protein content of CNS myelin. It consists of a family of basic protein of different MW but similar aa sequences. MBP is the most extensively studied CNS protein. The bovine and murine MBP molecules are 169 aa long bearing a sequence which is comparable to that from other species. MBP in different species differ in approximately 10% of

its aa residues. Additions, deletions and substitutions vary from species to species.

Rodent and human myelin contain a family of related MBP isoforms, each produced by alternative splicing of a primary MBP message transcribed from a single MBP gene which is located on the distal end of chromosome 18 in both human and mouse, and which consists of 7 exons in human, or of 6 exons in the mouse and rat (reviewed by Kamholz and Wrabetz, 1992; de-Ferra et al., 1985; Takahashi et al., 1985). In the mouse, exons 1 to 6 encode aa positions 1-56, 57-90, 91-102, 103-113, 114-154 and 155-169, respectively (Takahashi et al., 1985; Kamholz and Wrabetz, 1992). The 4 rodent isoforms share a common aa sequence but can be distinguished from each other. The 18.5 kDa isoform contains all 6 axons, whereas the 14 kDa is missing exon 5. The 21.5 kDa isoform contains all 6 exons plus a stretch of 26 aa inserted between exons 1 and 2. Similarly, the 17 kDa isoform also contains the 26 amino acid insertion between exons 1 and 2, but does not contain exon 5. The 18.5 kDa and 14 kDa isoforms are the most abundant species, and make up of more than 90% of adult mouse MBP (Barbarese et al., 1978). The functional significance of the multiplicity of MBP isoforms is not clear since compact myelin can be produced, even if the smallest isoform is expressed (Kimura et al., 1989).

As the name implies, MBP is a highly basic protein with an pI above 10.5. It contains more than 50% polar aa, has 1 tryptophan residue per molecule, and no cysteine. This is in contrast to PLP, which is rich in cysteine and tryptophan (Norton and Cammer, 1984). In aqueous solution, the molecule has an open and flexible conformation and is highly susceptible to proteolysis (Littlemore, 1978;

Mendz et al., 1982). Its ability to induce EAE and combine with antibodies (Ab) is not impaired when treated with urea or heated to 100°C in aqueous solution. At protein concentrations up to 1 mg/ml, more than 99% of the molecules are monomers. At higher concentrations (6 mg/ml) approximately 15% of the MBP molecules are present in hexamer form (Mendz, 1992). The conformation of MBP varies with intermolecular interactions, and in polymeric form, MBP has been shown to acquire the beta structures (Smith, 1985).

Early studies showed that membrane-impermeant biochemical probes applied to intact myelinated fibers were able to label PLP but not MBP, suggesting that PLP is exposed while MBP is located in the cytoplasmic face of myelin membranes (Poduslo and Braun, 1975; Golds and Braun, 1976). Its location in the cytoplasmic face, and its ability to self-associate have been proposed as a natural occurrence necessary to the compaction of myelin by linking the apposed cytoplasmic surfaces (Braun, 1984). X-ray diffraction studies show that MBP is located in apposition to the bilayer in close contact with lipid headgroups, and with some residue side chains penetrating into the hydrocarbon region (MacNaughtan et al., 1985).

1.2 Multiple sclerosis (MS)

As mentioned earlier, MS is a human inflammatory disease of the central nervous system (reviewed by Weinshenker, 1994). Lesions in patients with MS are characterized by focal T cell and macrophage infiltrations, and demyelination in the white matter. Myelin injury with ultimate destruction of the sheath insulating the axons disrupts transmission of action potentials along the nerves of the CNS. The

lesions are distributed around small venules in the white matter, preferentially in the spinal cord, cerebellum and around ventricles. This pathology is associated with neurological dysfunctions and deficits referable to these areas of inflammation resulting in visual impairment, co-ordination defects, motor paralysis, sensory disturbances, and bowel and bladder dysfunctions (McFarlin and McFarland, 1982; Hafler and Weiner, 1987; Martin et al., 1992).

The etiology of MS remains obscure, and both exogenous and endogenous factors are considered to contribute to disease susceptibility. Currently, environmental, genetic and immunological factors are thought to be involved (reviewed by Waksman, 1985).

1.2.1 Influence of environment on MS

There is considerable evidence to support the involvement of an environmental factor in MS. Worldwide analysis of MS cases has revealed different areas of prevalence with the highest in temperate regions of the world, and that MS is more common in the Northern areas and less common in the South (Kurtzke, 1983; reviewed by Poser, 1994). Evidence supporting the involvement of an infectious environmental factor includes the observations that (i) migration after the age of 15 carries with it the risk associated with the location where one dwelled during the first 15 years, while migration before that age carries the risk associated with the immigrant's destination (Pryse-Phillips, 1990), and (ii) the appearance of numerous MS cases on Faroe Island after, but not before the stationing of British troops (Kurtzke and Hyllestad, 1979). Several DNA and RNA viruses have been implicated in the pathogenesis of MS, but attempts to identify a single infectious

agent have so far been unsuccessful (Booss and Kim, 1990). Recently, human neurotropic corona (MS) and retrovirus (MS) have been added to the list of infectious agents which may trigger the pathological process of MS (Murray et al., 1993; Karpas et al., 1986; Ohta et al., 1986). Coronavirus which is known to cause demyelination (Mendelman et al., 1983), was detected in the brains of 2 MS patients (Burks et al., 1980). Coronarvirus RNA was also detected in active demyelinating plaque of MS autopsy brain (Murray et al., 1992). It is possible that MS results from the slow progression of an as yet unidentified infectious agent. In support of this, a spontaneous disease in sheep with clinico-pathological features very similar to MS has been determined to be caused by a retrovirus, and while viral RNA was easily detected in the CNS, viral particles could not be readily found (Narayan et al., 1985).

1.2.2 Influence of genetic factors on MS

The higher disease incidence in women (roughly 2:1, compared to men), the racial and familial clustering of MS cases all suggest that a genetic component influences susceptibility to MS (reviewed by Oksenberg et al., 1993a, Ragheb and Liszak, 1993, Ebers, 1994, and by Heagert and Marrosu, 1994). Results from family studies have shown that the prevalence of MS within family is significantly higher than that observed in the general population (Ebers, 1983). In addition, studies on twins have shown that monozygotic twins have a 10 fold higher concordance rate for MS (26%) than dizygotic twins (2.3%) while the concordance rate among dizygotic twins is similar to that of siblings (Ebers et al., 1986). Consistent with the notion that MS susceptibility can be influenced by genetic factor(s) are studies that

demonstrated an association of MS with particular MHC molecules such as HLA-DR2 or HLA-DQ6 (Altmann et al., 1991; Oksenberg et al., 1993a; Hillert, 1994; Tienari, 1994). Among certain ethnic groups, MS suceptibility is more strongly associated with DR4 (Jordan and Sardinia), or DR6 (Japanese patients) (Kurdi et al., 1977; Naito et al., 1978; Marrosu et al., 1988). Within the class I MHC genes in Northern American and European populations, MS has been shown to be associated with HLA-A3 and -B7 (Bertram and Kuwert, 1982; Tienari, 1994).

1.2.3 MS as an autoimmune disease

Even though the cause of MS has eluded investigators, over the last decade, a considerable amount of data, especially histological evidence, has been available to suggest that MS is immune-mediated (reviewed by Raine, 1984, 1994ab, and by Utz and McFarland, 1994). Light microscopic examination of the acute or of the active chronic MS plaque reveals extensive perivascular and parenchymal cellular infiltrates consisting of predominantly T cells and macrophages, some B cells, and mast cells are sometimes seen. Characterization of inflammatory infiltrates indicates that both $CD4^+$ and $CD8^+$ T cells are present, with $CD4^+$ population being predominant. There is intense fibrous astrogliosis, and astroglia are often multinucleated and hypertrophic. Progressing demyelination occurs, and at the perimeter of lesions, myelin debris is seen to be taken up into the body of macrophages either as large droplets or small myelin fragments which are sometimes attached to coated pits on the surface of macrophages. Axons are generally spared. Remyelination is frequently seen at the edges of lesions, and in such areas, oligodendroglial hyperplasia is sometimes apparent. It is believed that

coalescence of many small lesions leads to the formation of the grossly visible MS plaque. A minor population of the T cells bearing the γδ T cell receptor (TcR) chains has recently come under scrutiny in MS since these T cells have been found in and around chronic active lesions (Wucherpfennig et al., 1992; Hvas et al., 1993; Shimonkevitz et al., 1993). In addition, γδ T cells were found in MS plaques that also demonstrated heat shock protein 65^{+} (hsp65) oligodendrocytes. While a precise role for γδ TcR^{+} T cells remains to be elucidated, the demonstration of cytolytic activity by human γδ TcR^{+} T cells cloned from cerebrospinal fluid (CSF) on cultured oligodendrocytes supports the postulate that they may perform a similar function *in situ* (Freedman et al., 1991). Oligoclonal Ig bands have also been detected in the CSF, and appear to be the product of B cells (plasma cells) from the inflammatory infiltrates (Prineas and Raine, 1976).

The view that MS is an autoimmune disease is also supported by the observations of increased T cell activation and decreased T suppressor cell function. Several reports demonstrated a significant increase in the proportion of activated peripheral blood lymphocytes (PBL) in MS patients, as measured using the anti-TAC antibody that reacts against IL2 receptor expressed on activated T cells (Bellami et al., 1985; Selmaj et al., 1986). Furthermore, as demonstrated by several laboratories, there was a defect in CD8^{+} suppressor cell function in MS (Paty et al., 1983; Antel et al., 1986).

Acute lesions of MS resemble those seen in post-viral encephalomyelitis which occurred after recovery from measles, rubella and other viral infections, at the time when no virus could be detected. The post-viral inflammatory diseases are

localized to the white matter, with a striking absence of detectable virus in the brain suggesting that the CNS or myelin was the target of attack, and that damage is mediated by CNS directed immune response (reviewed by Wucherpfennig et al., 1991). Similarly, an acute, disseminated encephalomyelitis in which the white matter resembles MS can be observed after rabies vaccination with inactivated virus prepared from infected brains. This implicates myelin components as autoantigens in humans. Autoreactivity to MBP has been observed in these cases, both at the humoral and cellular levels (Hemachudha et al., 1987). Consistent with this notion is the observation that T cell responsiveness to MBP and PLP can be detected in the PBL of MS patients (Allegretta et al., 1990; Olsson et al., 1990). A vast number of studies on the autoimmune basis for MS has concentrated on neuroantigen-specific T cell responses. Because of the well established role of MBP in EAE, these studies primarily focused on demonstrating MBP reactivity. Investigations of MBP reactivity using standard seven day proliferation assays suggest that there is a small but consistent increase in proliferation to human MBP by PBL from MS patients compared to normal individuals or to patients with other neurologic diseases (Liszak and Zweinman, 1977; Johnson et al., 1986; Chou et al., 1992). A number of investigators have compared the selective recognition of particular MBP epitopes in MS patients and control individuals using T cell lines or clones. One study demonstrated multiple T cell epitopes in EAE (Richert et al., 1989), while another showed that MBP-reactive lines derived from the blood of MS patients preferentially recognized human, as opposed to bovine, rat or monkey MBP (Tournier-Lasserve et al., 1988). The human MBP peptide spanning amino

acid positions 83 to 96 has been shown to be the immunodominant epitope in both the MS patients and control non-MS individuals. In addition, the response to this peptide could be blocked by anti-DR antibodies, suggesting that this epitope may be preferentially expressed in the context of MS-related class II MHC (Baxevanis et al., 1989; Chou et al., 1989). In addition, increased frequencies of PLP and PLP-peptide specific-T cells have been demonstrated in MS patients (Olsson et al., 1990; Sun et al., 1991), further supporting the notion that myelin specific-T cells are involved in the pathology of MS.

Several investigations on TcR variable (V) region genes from leukocytes of MS patients by polymerase chain reaction (PCR) and/or Southern blotting techniques have reported marked heterogeneity of Vβ gene usage (reviewed by Utz and McFarland, 1994), in contrast to the limited TcR gene usage observed in EAE (Acha-Orbea et al., 1988; Burns et al., 1989). On the other hand, Oksenberg et al. (1993b) and others have reported that there was a striking proportion of haplotype-matched patients expressing Vβ5.2 genes, and a restricted TcR V α/β repertoire (Schlüsener and Wekerle, 1985).

Currently, two major theories of pathogenesis of MS have been proposed: one, that MS is a viral disease of the CNS, and that the inflammatory response in the brain results from an anti-viral immune response; and two, that MS is an autoimmune disease in which infiltrating T cells recognize self antigens. These two possibilities are not mutually exclusive since molecular mimicry as an inciting event in the generation of auto-reactive T cells is conceivable. Amino acid sequences on both MBP and PLP have been reported to bear some homology to viral proteins

(Fujinami and Oldstone, 1985; Shaw et al., 1986). An example is the hepatitis B virus polymerase, which bears homology to a sequence on MBP. Injection of this hepatitis virus protein in adjuvant into rabbits has been shown to induce inflammatory CNS disease. Furthermore, rats infected with the neurotropic JHM virus develop an inflammatory relapsing disease in which MBP-specific T cells are generated. These cells are capable of passively transferring EAE-like disease to naive recipients (Fujinami and Oldstone, 1985). In addition, three independent computer searches have indicated the presence of homologous peptide sequences in regions of MBP and in antigens of measles, influenza, canine distemper, Epstein-Barr virus and several papova and adenoviruses. Viruses such as measles, rubella and varicella have been also shown to sensitize T lymphocytes to MBP (Fujinami and Oldstone, 1985; Souberbielle et al., 1991). These observations underscore the potential that MS is a virally-induced autoimmune disorder. Moreover, autoreactive T cells expressing certain TcR-β chain may also be activated by superantigens which link MHC class II molecule to specific TcR Vβ chains. Such superantigen activation, which may occur during the course of bacterial infection, may potentially be an initiator for an autoimmune response, thus raising the notion that MS is a multiorigin autoimmune disease.

1.3 Experimental allergic encephalomyelitis (EAE)

1.3.1 History

The first suggestion that injections of nervous tissue may have an injurious effect on the nervous system arose from occurrence of paralytic accidents in a minority of patients who had received the Pasteur rabies vaccine during the 1880's

and 1890's (Paterson, 1976). Occurrence of post-rabies vaccine paralysis after injections of rabbit spinal cord or brain containing killed (phenol-treated) rabies virus raised the possibility that the paralytogenic activity of the vaccine was a property of the nervous tissue, rather than of the virus itself. This led to the investigations on the encephalitogenic activity of nervous tissue in experimental models in 1920's and 1930's by several groups . It was reported in 1933 and 1935 that monkeys injected repeatedly over a period of several months with normal brain tissue developed an acute disseminated encephalomyelitis, a condition similar to that observed in humans after injection of rabies vaccine (Rivers et al., 1933; Rivers and Schwentker, 1935). This work is the first report on EAE.

The development of Freund type adjuvants during the 1940's led to the discovery of EAE as it is known today. Several studies in 1947 reported that accelerated form of disseminated encephalomyelitis was seen in monkeys, guinea pigs and rabbits following injection of CNS homogenates emulsified in complete Freund's adjuvant (CFA) containing paraffin oil and killed mycobacteria (Freund et al., 1947; Kabat et al., 1947; Morgan, 1947; Morrison, 1947). In these species, EAE could be induced within 2 to 3 weeks after the injection of CNS tissue-adjuvant emulsion. These important contributions established EAE as a reproducible model for investigating immunologic forms of inflammation and injury to the CNS in man, such as multiple sclerosis.

1.3.2 Induction of EAE

EAE is inducible in a wide variety of species extending from several birds to many mammalian species including sheep, dogs, monkeys, rabbits, guinea pigs,

rats and mouse (Lee et al., 1954ab; Levine et al.., 1967; Stone et al., 1968; Paterson, 1976).

Active EAE: It can be induced by the intradermal, subcutaneous or footpad injection(s) of whole CNS tissue, or of a single CNS Ag such as MBP or PLP, emulsified in CFA (reviewed by Brostoff, 1984). The encephalitogenic activity of the nervous tissue is due to organ-specific rather than species-specific components. CNS from a member of the same or of another species can be used as a source of antigen. Upon induction, the animals may develop an acute form or a chronic form of EAE with multiple relapses and remissions. Different encephalitogenic epitopes on the MBP and PLP molecules have been identified in species such as guinea pigs, Lewis rats, and in several mouse strains such as SJL, PL, (SJL x PL)F1, and SJW (Kono et al., 1988; reviewed by Tuohy, 1994). The day of onset, severity and duration of disease, and whether the animals developed an acute form or a chronic EAE form are dependent on the antigen used for sensitization, species or strain and/or the age of the animals at the time of injection. In addition, the type and amount of mycobacteria in the adjuvant have been found to be important factors in EAE induction (Lee and Schneider, 1954a; Shaw et al., 1965a).

Clinical manifestations of EAE include incontinence, hindleg paresis progressing to paralysis, quadriplegia and death in some cases. Histological examination of the CNS indicates that like MS, lesions of EAE are most prominent in the white matter and that the spinal cord is the most important target (Brown and McFarlin, 1981; Brown et al., 1982). The lesions are characterized by cellular infiltrates consisting of lymphocytes, monocytes/macrophages and

polymorphonuclear cells, and are often found in the meningeal, perivascular and parenchymal areas. In addition, there is also destruction of myelin and/or necrosis (Brown and McFarlin, 1981; Brown et al., 1982; Raine et al., 1984). Myelin injury often occurs on nerve fibers running through or near to the cellular infiltrates. Demyelination appears to involve an apparent dissociation of myelin. During the process, myelin becomes disassociated from, or loosely attached to nerve fibers, and lies within the extracellular spaces as multilamellar droplets of various sizes. Eventually, naked axons can be seen, and myelin debris is phagocytosed by invading macrophages (Raine et al., 1980 and 1984; Brown et al., 1982). These inflammatory lesions are typically reminiscent of delayed-type hyperreactivity skin reactions (DTH). In addition, using MBP for induction of EAE and skin testing purposes, Shaw et al. (1965b) reported that guinea pigs sensitized to either MBP-adjuvant or guinea pig spinal cord (Sc)-adjuvant exhibit typical DTH skin responses when tested with the encephalitogenic protein.

With the use of strong adjuvants, such as CFA, normally quiescent self-specific T cells can be activated by the injected CNS Ag. The observation that cerebral vascular endothelial cells (VEC) *in vitro* can be induced to express class II MHC and present MBP to MBP-specific T cells supports the hypothesis that upon injection of CNS antigens (Ag) emulsified in CFA, CNS-specific T cells that are normally quiescent become activated, circulate and recognize CNS Ag presented by the VEC that comprise the BBB. This event may stimulate the release of immune mediators that may lead to increased permeability, and disrupt the integrity of BBB, allowing influx of leukocytes into the CNS. However, a causal relationship between

CNS inflammation and clinical symptoms remains controversial. Several studies have shown that clinical symptoms of EAE can be reduced without concurrent reduction in CNS infiltrate (Swierkosz and Swanborg, 1975; Su and Sriram, 1991; Weinberg et al., 1994). Other investigations suggest that disease symptoms may correlate more strongly with the activation of the fibrinolytic system at the BBB and with edema than with the extent of the cellular infiltrate (Simmons et al., 1982; de-Rosbo et al., 1985; Koh et al., 1992 and 1993). One of the earliest immunopathologic findings in EAE in the Lewis rat is the perivascular accumulation of fibrin which can be detected well before cellular infiltrates occur (Oldstone and Dixon, 1968). In addition, in their study on fibrin deposition and BBB permeability, Koh et al. (1992) proposed that fibrinolysis by endothelial cell derived plasminogen activator and production of fibrin-fibrinogen degradation products including vaso-active peptides may be responsible for opening the BBB. This is followed by influx of fluid and other circulating components, by perturbation of nerve conduction, and by neurological deficits. A role for fibrinolysis in EAE is also supported by the finding that the use of a plasminogen activator inhibitor partially suppressed EAE (Koh and Paterson, 1987). Alternatively, lesion location may be of importance rather than the overall quantity.

Since EAE is mediated by T cells, its induction is restricted by the major histocompatibility (MHC) antigens. Furthermore, as in the case of MBP, there are differences in the sequences of MBP of different species. These factors together determine the encephalitogenic epitopes, and may also be responsible for the species and strain differences of EAE activity. The guinea pig is highly susceptible

to EAE. As a result, the fine specificity of the encephalitogenic determinant was first characterized in this species. There are two strain-specific encephalitogenic determinants on the bovine MBP: residues 116 to 122 for the strain 13, and residues 1 to 88 for strain 2. Since these regions are conserved in MBP of the human, chimpanzee, cow, pig, rabbit, guinea pig, and rat, these MBP's are also encephalitogenic for the guinea pig. In the Lewis rat, it is known that guinea pig MBP is ten times more potent at inducing EAE than rat EAE (Happ and Heber-Katz, 1988), and that bovine MBP is least encephalitogenic. Guinea pig and rat MBP peptides spanning amino acid residues 68 to 88 are encephalitogenic in the Lewis rat (reviewed by Hashim, 1978).

It has been well established that T cells that are involved in disease induction are $CD4^+$ and class II MHC restricted. Several studied have demonstrated that the disease can be inhibited by the administration of Ab against CD4 molecules, class II MHC or of peptide which inhibits the binding of encephalitogenic epitope to class II MHC (Swanborg, 1983; Brostoff and Mason, 1984; Sriram and Steinman, 1983; Sriram et al., 1987).

Passive EAE: The 2nd method for induction of EAE is the passive or adoptive transfer. This involves the injection of a suspension of lymphoid cells from animals sensitized against whole CNS tissue, MBP, or PLP in CFA, into either normal or immunologically compromised syngeneic recipients. A clinical syndrome with histopathology similar to actively induced EAE follows 5 to 10 days later. Disease transfer is greatly enhanced if these cells are activated *in vitro* with antigen prior to transfer (Holda et al., 1980). This activation step serves to induce blast

transformation and/or expansion of the encephalitogenic cell population. Further expansion is apparently required within the recipients, as suggested by the inability of *in vitro*-activated, mitomycin-C-treated cells to transfer the disease (Panitch, 1980). Adoptive transfer of EAE, initially reported in 1960 and 1961 (Paterson, 1960; Stone, 1961) represented the first demonstration that EAE had a cell-mediated basis. Since then, a large number of studies have been performed, and showed that the lymphocyte population involved in the adoptive transfer of EAE are T cells (Lennon and Byrd, 1973; Gonatas and Howard, 1974; Bernard and McKay, 1983). The ability of T cell lines or clones to induce full-blown disease in both the rat and mouse models (Ben-Nun and Cohen, 1982; Richert et al., 1985; Vandenbark et al., 1985; Zamvil et al., 1985; Tabira and Sakai, 1987) further confirmed that $CD4^+$ T lymphocytes are involved in the pathogenesis of EAE. However, involvement of other cell types in the induction and/or regulation of the disease cannot be excluded (Gausas et al., 1982; Willenborg and Prowse, 1982; Myers et al., 1992). There are several studies supporting a role for B cells in EAE. Intact B cell activity has been shown to be essential for expression of full-blown EAE (Hashim et al., 1987; Gausas et al., 1982; Myers et al., 1992). In addition, a number of studies have demonstrated that sera from guinea pigs with EAE displayed cytolytic activity to neuroglial cells, and to myelinated nerve fibers in myelinating organotypic brain cultures (Appel and Bornstein, 1964; Brosnan et al., 1983). Ab against oligodendrocyte glycoprotein have also been shown to augment demyelination (Linington, et al., 1988; Meeson et al., 1994). On the other hand , a role for B cells in the down-regulation of EAE has also been suggested (Karpus and

Swanborg, 1991a; Day et al., 1992).

1.3.3 EAE in the mouse

There are obvious advantages to studying EAE in this species. These include the availability of large numbers of inbred and recombinant strains, the meticulously worked out genetics, the availability of reagents to recognize or obliterate sets and subsets of lymphoid cells, and the small size and rapid reproductive rate of the animals, allowing for larger experiments. In addition, in this species, a chronic-relapsing form of EAE (cr-EAE) can be induced, which bears more resemblance than the acute form to chronic MS. Both EAE-susceptible and resistant rodent strains have been identified (William and Moore, 1973; Gasser et al., 1975; Bernard, 1976; Raine et al., 1980; Teitelbaum and Arnon, 1981). The most sensitive strains are SJL, PL and (PLxSJL) F1. Others are considered partially or fully resistant to the disease. Cr-EAE can be induced actively by the injection of Sc-CFA with or without the use of killed *Bordetella pertussis* (Bp) (Raine et al., 1980; Brown and McFarlin, 1981; Brown et al., 1982) as an additional adjuvant. Bp is known to have histaminergic effects, and is believed to render the BBB more permeable (Munoz et al, 1981; Linthicum and Frelinger,1982). Lesions in the CNS of mice with EAE are characterized by lymphocytic and polymorphonuclear meningitis and submenigeal and perivascular infiltrates composed of lymphocytes and polymorphonuclear cells. Macrophages containing myelin debris are also often seen. Histologic examination revealed extensive gliosis, axonal damage and demyelination (Brown et al., 1982; Cross et al., 1987; Moore et al., 1987). Cr-EAE in the mouse can also be adoptively transferred via suspension of CNS- or MBP-

activated lymphocytes or cell lines or clones (Mokhtarian et al., 1984; Zamvil et al., 1985). The chronic disease induced passively follows similar patterns of clinical and histological manifestations as actively induced chronic EAE.

Injection of MBP-CFA along with administration of killed Bp organisms can also induce a chronic-relapsing disease with CNS pathology similar to that of Sc-CFA induced disease (Fritz et al., 1983a). Encephalitogenic epitopes within peptides 1-11 and 87-104 of guinea pig MBP have been identified for the PL and SJL strains, respectively (Fritz et al., 1983b; Kono et al.,1988). The peptide 1-11 has also been shown to be encephalitogenic in the (SJL x PL)F1 animals. An additional encephalitogenic epitope within the peptide 17-27 has been identified for the SJL mice (Fritz et al., 1990). Recently, PLP has been shown as a more powerful encephalitogen than MBP in the mouse. Following PLP-CFA challenge, a high percentage of mice develop severe disease that can be fatal (Tuohy et al., 1988), or cr-EAE with multiple relapses and remissions (Trotter et al., 1987). Encephalitogenic epitopes within the PLP peptides 139-151, 43-64 and 103-116 have been identified for the SJL, PL and SWR, respectively (Sobel et al., 1990a; Whitham et al., 1991a; reviewed by Tuohy, 1994). Lymph node cells, T cell lines as well as clones that are PLP-activated have been shown to passively induce EAE in the SJL mouse (van der Veen et al., 1989 and 1990; Whitham et al., 1991b). Histological examination shows heavy infiltrates consisting of lymphocytes and polymorphonuclear cells and severe demyelination, similar to that seen in chronic EAE induced by MBP-CFA or by whole CNS homogenates.

1.3.4 Adhesion molecules in EAE

As mentioned before, in the intact CNS, endothelial cells form the BBB, a tightly interconnected cellular monolayer that controls the free exchange of solutes and molecules between the blood and the neuropil. Consequently, the CNS has been considered an immunologically privileged site. A number of studies in EAE, however, have demonstrated that activated, but not resting T cells do enter the CNS in a random manner, independent of their Ag-specificity, or phenotype (Hickey at al., 1991; Zeine and Owens, 1992). Their presence in the CNS is transient. CNS-specific lymphocytes, on the other hand, enter the CNS, and can still be found there long after the non-CNS specific cells have been gone (Zeine and Owens, 1992). The exact mechanism by which these cells cross into the CNS remains an open question which has attracted a great deal of research. One notion put forward is that activated cells express certain molecules that allow them to pass through the BBB, but only CNS-specific cells, upon interacting with the CNS antigen, stay in the CNS (Hickey et al., 1991; Zeine and Owens, 1992). Since in EAE, immune cells readily gain access to and can be detected in the CNS even prior to the appearance of clinical signs, the entry of lymphocytes into the CNS is considered a critical early event in disease induction (Cross et al., 1990a). Since studies involving the development of systemic inflammation have shown that interactions between receptors on inflammatory cells such as LFA-1, VLA-4 and their corresponding ligands ICAM-1 and VCAM-1 on vascular endothelial cells are the initial step in leukocyte trafficking, much of the current research has focused on the role of these molecules in leukocyte trafficking into the CNS. Several studies have shown increased expression of adhesion-related molecules and their ligands, and

its correlation with inflammatory cell influx during EAE (Cannella et al., 1990; Cannella et al., 1991; O'Neill et al., 1991). Moreover, in support of a selective involvement of the BBB in EAE are reports on membrane interactions between lymphocytes and CNS endothelium. Among the molecules currently implicated in lymphocyte trafficking, two of the most studied in EAE are LFA-1 on the T cells and its ligand ICAM-1 on the endothelial cells.

LFA-1 (lymphocyte function-associated antigen-1) is a member of the β2 integrin family. It is expressed by T and B lymphocytes, natural killer (NK) cells, granulocytes, dendritic cells and macrophages (Roitt, 1991). There are at least two ligands for LFA-1, ICAM-1 and ICAM-2 (intercellular adhesion molecule-1 and 2) which are members of the immunoglobulin (Ig) gene super family. Both molecules are expressed by endothelial cells. LFA-1 and its ligand ICAM-1 molecules were the first adhesion molecules which were studied in MS. It was reported that both LFA-1 and ICAM-1 are elevated in MS lesions, and also in EAE (Raine et al., 1990a; Sobel et al., 1990b; Raine and Cannella, 1992). However, results from studies on the role of LFA-1 or ICAM-1 in inflammation in the CNS have been inconclusive. On one hand, Welsh and colleagues (1993) reported that passive EAE was augmented by the administration of anti-LFA-1α mAb while studies by Cannella et al. (1993) and by Willenborg et al. (1993) showed that neither anti-ICAM-1 nor anti-LFA-1 was successful in blocking disease development. On the other hand, Archelos et al. (1993) reported that the administration of anti-ICAM-1 Ab was able to prevent EAE.

A more definite role of VLA-4 (very late antigen-4), a member of the β1 integrin family, has been implicated in EAE. It is expressed on monocytes, and its

expression on T lymphocytes is increased upon activation of the T cells (Roitt, 1991). One of its ligand is the vascular cell adhesion molecule-1 (VCAM-1) which is also a member of the Ig gene super family, and is expressed by endothelial cells. The binding of an MBP-specific T cell clone to brain capillary endothelial cells was shown to be inhibited by Ab against VLA-4 or VCAM-1 (Tanaka et al., 1993). In EAE, immunocytochemical analysis of frozen sections of inflamed versus noninflamed brains and spinal cords showed that the vascular endothelium in brains and spinal cords from SJL mice with EAE expressed higher levels of VCAM-1 (Steffen et al., 1994). Furthermore, treatment with mAb against VLA-4 molecules has prevented the development of EAE in rats (Yednock et al., 1992). In addition, VLA-4 molecules have been shown to be expressed in much higher amounts on encephalitogenic, but not on nonencephalitogenic T cell clones (Baron et al., 1993). Moreover, the encephalitogenicity of the T cell clones can be inhibited by incubation of these cells with mAb against VLA-4 prior to transfer (Baron et al., 1993).

1.3.5 Cytokines in EAE

The mechanisms whereby cells induce EAE are not clear. Two types of $CD4^+$ subpopulations of T cells were originally identified by their lymphokine secretion patterns (Mosmann et al., 1986; Cherwinski et al., 1987; Mosmann and Coffman, 1987; Mosmann and Coffman, 1989ab; Street and Mosmann, 1991). Upon activation, T helper (Th) 1 cells secrete IL2, IFNγ and lymphotoxin (LT), can mediate DTH reactions and function as helper for B cells to produce Ab of the IgG2a isotype. On the other hand, activated Th2 cells produce IL4, IL5,IL 6 ,IL10,

and IL13, and help B cells to produce Ab of the IgG1, IgE and IgA isotypes. Both types of cells secrete IL3. In the past few years, the increased availability of recombinant cytokines and/or mAb to cytokines has prompted numerous studies on the role of cytokines in the pathogenesis of EAE. The following cytokines have been implicated in EAE:

Tumor necrosis factor α/β (TNF α/β): TNF α/β are related molecules that appear to have evolved together, probably from the same ancestor. The genes for both molecules are closely linked on the same chromosome, and they act through the same receptor. At the amino acid and nucleotide levels, there is 28% and 46% homology, respectively. TNF α is produced predominantly by activated macrophages and Th1 lymphocytes, while TNF β is produced by activated Th1 lymphocytes but not by macrophages. These 18 kDa molecules are known to have a wide variety of effects on a number of different cell types such as leukocytes, endothelial cells etc., and are involved in inflammatory responses (Burke et al., 1993).

In EAE, it has been demonstrated that treatment with antibodies against TNF α/β protects mice against the disease induced by the transfer of MBP-specific lymph node cells or T cell clones (Ruddle et al., 1990), thus implicating a disease-upregulating role for this cytokine. Furthermore, a recent study by Baker et al. (1994) demonstrated that actively-induced EAE in the Biozzi AB/H mice could be inhibited by the treatment of either Ab against mouse TNF α/β or by bivalent human TNF receptor-immunoglobulin fusion proteins. In addition, Selmaj et al. (1995) reported that passively induced EAE in SJL mice was inhibited by treatment with

soluble TNF receptor. A role of TNF α/β in the development of EAE is further supported by the observation that TNF α increases BBB permeability (Kim et al., 1992), and that *in vitro*, TNF α/β induced apoptosis of oligodendrocytes (Selmaj et al., 1991a). This cytokine has also been shown to mediate myelin and oligodendrocyte damage *in vitro* (Selmaj and Raine, 1988). Furthermore, human TNFα has been reported to augment EAE in rats (Kuroda and Shimamoto, 1991).

Interleukin 1 (IL1): Like TNF molecules, IL1 is known to be involved in inflammatory responses. Two forms of IL1 have been identified, the membrane bound form IL1α and the soluble form IL1β of molecular weight of 17.5 kDa and 17.3 kDa, respectively. IL1 has been reported to be produced primarily by monocytes and macrophages, but also by other cell types such as astrocytes, endothelial cells and microglia. A role for IL1 in EAE has been suggested by the findings that EAE in Lewis rats was suppressed by treating the animals with soluble IL-1 receptor (Jacobs et al., 1991). Furthermore, the same study demonstrated that administration of recombinant IL1α exacerbated EAE induced in Lewis rats.

Interleukin 2 (IL2): A role for IL2 in the development of EAE was first reported by Hayosh and Swanborg (1987). In their study, it was shown that the inclusion of a monoclonal antibody (mAb) against IL2 receptor (IL2R) in cultures of spleen cells during the in vitro MBP-activation stage inhibited the ability of these cells to transfer EAE into naive recipients. Furthermore, in 1991, Rose et al. reported that the elimination of IL2R bearing cells *in vivo* inhibited the development of passively induced EAE. They demonstrated that the administration of human IL2 conjugated to a modified form of *Pseudomonas* exotoxin lacking the cell recognition domain

markedly inhibited the clinical manifestations of EAE, and also reduced both inflammation and demyelination in the SJL mouse.

Interleukin 3 (IL3): Another cytokine that has recently been studied in EAE is IL3, a 20 kDa product of both Th1 and Th2 cells and some other cell types. IL3 has documented activity in both hematopoietic and nonhematopoietic systems. It is involved in a number of activities including induction of B cell proliferation and differentiation (Burke et al., 1993). A recent study has demonstrated that inclusion of IL3 at the *in vitro* activation stage increased encephalitogenicity of the MBP-specific T cell lines upon transfer into naive SJL mice (Zhao et al., 1993).

Interleukin 4 (IL4): IL4 was initially characterized as a B cell stimulatory factor (BSF-1) and as a B cell differentiation factor (BCDF). It was subsequently shown to be pleiotropic with multiple functions on diverse cell types such as monocytes, mast cells, endothelial cells, B and T lymphocytes etc. On macrophages, the lymphokine upregulates the expression of class II MHC antigens and LFA-1 (reviewed by Mosmann and Coffman, 1989b). On the B cells, it increases cell viability and the expression of class II MHC and B7, resulting in the enhancement of antigen-presenting capacity of the B cells (Paul, 1991). IL4 is also an important regulator for isotype switching, inducing the production of IgE and IgG1. On the T cells, IL4 plays a dominant role in the differentiation of naive T cells toward the Th2 cell phenotype (Swain et al., 1991; Seder and Paul, 1994). It also suppresses lymphokine production by Th1 cells (Mosmann and Coffman, 1989b). In addition to Th2 cells (Mosmann et al., 1986), other producers of IL4 include $CD8^+$ T cells (Seder et al., 1992), mast cells (Bradding et al., 1992) and basophils (MacGlashan

et al., 1994). The role of IL4 in EAE has recently received attention. Racke et al. (1994) reported that treatment of SJL mice with IL4 following cell transfer reduced severity of the disease suggesting that IL4 may play a disease-downregulatory role in the development of passively induced EAE. Furthermore, a recent study by Racke et al. (1995) demonstrated that feeding SJL mice with retinoid-supplemented diet reduced severity of EAE, and that IL4 production correlated with improved disease course in the treated animals.

Interleukin 10 (IL10): IL10, originally termed cytokine synthesis inhibitory factor (CSIF) is a product of Th2 cells, B cells and macrophages (Fiorentino et al., 1989). Human and mouse IL10 are made up of 160 aa, and are 81% and 73% identical at the nucleotide and amino acid levels, respectively. This cytokine has also been shown to share homology with the Epstein-Barr virus gene BCRF-1 (Moore et al., 1990). IL10 is a pleiotropic cytokine that can exert either immunosuppressive or immunostimulatory effects on a variety of cell types. It acts on B cells to enhance their viability, cell proliferation, Ig secretion and MHC class II expression (de Waal-Malefyt et al., 1991; Howard et al., 1992). In addition, IL10 is also a growth co-stimulator for thymocytes and mast cells (Suda et al., 1990; Thompson-Snipes et al., 1991). As the name implied, CSIF or IL10 was originally discovered as a cytokine that could inhibit cytokine production by Th1 cells in response to antigens in the presence of monocytes/macrophage antigen presenting cells (APC). This inhibitory effect of IL10 was found to be indirect and due to its effect on the antigen-presenting capacity of monocytes/macrophages (Fiorentino et al., 1991a). Currently, the mechanism by which IL10 inhibits the antigen-presenting and

accessory functions of monocytes/macrophages is not clear, but it has been shown that IL10 down-regulates class II MHC expression on these cells (Howard et al., 1992; de Waal-Malefyt et al., 1991), and that it can inihibit production or function of a macrophage membrane-bound costimulator (B7) required for activation of T cells(Moore et al. 1993). In addition, IL10 can suppress the production of TNF α, IL1, IL6 and IL8 (Fiorentino et al., 1991b), and of reactive nitrogen intermediates by macrophages (Gazzinelli et al., 1992).The lymphokine also induces the production of IL1 receptor antagonist.

Kennedy et al. (1992) showed that in EAE, there was a dramatic rise of IL10 mRNA expression during the recovery phase, and that it remained elevated throughout this phase. More recently, it was reported that treatment of Lewis rats with IL10 prevented the development of MBP-CFA-induced EAE, thus, supporting a disease-dampening role for IL10 in EAE (Rott et al., 1994).

Interleukin 12 (IL12): IL-12 is a 75k Da glycoprotein heterodimer composed of two genetically unrelated subunits. Cells that are known to produce IL12 include monocytes/macrophages, B cells and connective tissue type mast cells (reviewed by Merberg et al., 1992; Trinchieri, 1993). Functionally, IL12 has multiple effects on both NK cells and T cells. It promotes the growth of activated NK cells, enhances NK-mediated cytotoxicity, and induces IFNγ production by both NK and T cells (Wolf et al., 1991; Chan et al., 1991). The lymphokine has also been shown to play a major role in the differentiation of $CD4^+$ cells into TH1 or Th2 cells. Its ability to promote Th1-mediated immune response has been demonstrated both *in vitro* (Manetti et al., 1993) and *in vivo*.

A role of IL12 in the development of EAE was recently reported by Leonard et al. (1995). It was demonstrated that *In vitro* stimulation of antigen-primed lymph node cells with PLP and IL12 enhanced their encephalitogenicity, and that administration of anti-IL12 Ab following the cell transfer inhibited the disease. It was also shown that the effects of IL12 on disease severity was independent of IFNγ production.

Interleukin 13 (IL13): IL13, a product of activated T cells, particularly cells of the Th2 phenotype, is a relatively new member of the interleukin family (Lakkis and Cruet, 1993; Minty et al., 1993; Zurawski and deVries. 1994). The biologically active forms of mouse and human IL13 are made up of 111 and 112 aa, respectively, and share over 60% homology with each other. There are no known actions of IL13 on T cells, but it exhibits pleiotropic activity on monocytes/macrophages, B cells and neutrophils. The lymphokine has been considered a potent inactivator of monocytes/macrophages, in that it inhibits their production of TNF α, IL1, IL6, IL8 and nitric oxides (de Waal-Malefyt et al., 1993; Doherty et al., 1993; McKenzie et al., 1993). IL13 is also known for its ability to stimulate neutrophils to produce IL1 receptor antagonist (Muzio et al., 1994). Recently, it was reported by Cash et al. that treatment of Lewis rats with IL13 suppressed the development of both clinical and histological signs of active EAE (Cash et al., 1994). The exact mechanism is not clear, but it was demonstrated that the IL13 administered appeared to affect cells of the monocyte/macrophage lineage, but not the T or B cells. It was suggested that the ability of IL13 to inhibit EAE development could be accounted for by its inhibitory effects on the

monocyte/macrophage cell population (Cash et al., 1994).

Transforming growth factor β (TGF β): TGF βs belong to a family of at least 2 different homologous disulphate-linked homodimeric polypeptides (TGF β1 and β2) and one heterodimeric form (TGF β1.2) (Assoian et al., 1983). Platelets are the major source of TGF β found in normal serum. Other cell types such as activated lymphocytes can also produce this cytokine. The dimeric 25 kDa form is biologically active while no activity can be detected after dissociation of the chains by reducing agents. Analysis of cDNA clones encoding mammalian TGF βs and appropriate cell culture supernatants indicated that the mature molecule is cleaved from the carboxy terminus of a larger glycosylated precursor, and is secreted in a latent, inactive form (Lawrence et al., 1984; Derynck et al., 1985). It has been reported that TGF β activation by monocytes may require IFNγ-mediated gene expression as well as a cell-associated processing event (Twardzik et al., 1990). Extremes of pH (1.5 or 12) have also been shown to activate TGF β in cell-conditioned media (Lawrence et al., 1985; Lyons et al., 1988). TGF βs have been known to have pleiotropic effects, depending on the target cells. As the name implies, this cytokine exerts a positive effect on certain cell types promoting their growth. On the other hand, TGF β1 and β2 inhibit lymphocyte activation (Kehrl et al., 1986; Wahl et al., 1988 and 1989; Schluesener and Lider, 1989; Schluesener et al., 1990).

In EAE, TGF β1 and β2 have been reported to suppress T cell proliferation in response to MBP, and to protect Lewis rats against EAE (Schluesener and Lider, 1989; Stevens et al., 1994). In addition, Karpus and Swanborg (1991b) reported that $CD4^+$ suppressor cells inhibited the function of effector cells in EAE through a

mechanism involving TGF-β. Similarly, Johns et al. showed that treatment with TGF β inhibited EAE development in SJL mice (Johns et al., 1991). Moreover, several studies have demonstrated that administration of anti-TGF β mAb significantly enhanced both the actively and passively induced EAE in the SJL mice, thus further supporting a disease-downregulatory function for TGF β (Kuruvilla et al., 1991; Racke et al., 1991 and 1993; Johns and Sriram, 1993). A recent study by Santambrogio et al. (1993) on both the suppressive and enhancing effects of anti-TNF α/β and anti-TGF β antibodies, respectively, proposed that TGF β may protect against the induction of active EAE and against relapses in chronic EAE by antagonizing both the production of TNF and its effect.

Interferon γ (IFNγ): The cytokine of particular interest in this thesis is IFNγ which is produced by a limited number of activated cell types, such as Th1 cells and NK cells. This 17-25 kDa molecule was first indentified nearly 30 years ago on the basis of its anti-viral activity (Wheelock, 1965). During the ensuing years, a great deal of information has accumulated which unequivocally establishes that IFNγ is an extremely pleiotropic lymphokine (Ijzermans and Marquet, 1989; Billiau and Dijkmans, 1990; Young and Hardy, 1990; Williams et al., 1993; Farrar and Schreiber, 1993). Its biological activities include antiviral, antitumor, and also strong immunomodulatory functions (Halloran, 1993). On the B cells, IFNγ enhances the production of immunoglobulin of the IgG2a isotype; on NK cells, it significantly enhances their cytolytic activities. IFNγ has also been known to suppress Th2 cells and antagonize their lymphokines (Mond et al., 1986; Mosmann and Coffman, 1989b; Gautam et al., 1992). One of the major physiologic roles of IFNγ is its ability

to regulate MHC class I and II protein expression on a variety of cell types, including endothelial cells, epithelial cells, B cells and macrophages. Whereas it acts to increase MHC class II expression on most cells, it inhibits MHC class II expression on B cells (Mond et al., 1986). In addition, the lymphokine is a potent activator of macrophages. Aside from being an effective inducer of MHC class II expression on macrophages (Beller, 1984), IFNγ also enhances their ability to combat microbial pathogens. It promotes the elaboration of macrophage-derived cytocidal compounds such as reactive oxygen and reactive nitrogen-intermediates and nitric oxides (Ding et al., 1988). IFNγ has also been shown to play a regulatory role in the production of a number of cytokines such as IL1 (Farrar and Schreiber, 1993). Despite intensive studies on IFNγ and its modulatory role in the immune response, its function in inflammatory responses has still been contradictory. Different experimental disease models have yielded conflicting interpretations as to its immunopathologic vs protective roles. One of the models in which IFNγ has been implicated as a causative agent is the development of autoimmune nephritis. Administration of exogenous IFNγ to (NZB x NZW)F1 mice accelerated the progression of spontaneous glomerulonephritis, whereas animals treated with IFNγ specific mAb displayed significant remission and increased survival (Hermans et al., 1978; Jacob et al., 1987). A pro-inflammatory role of IFNγ has been shown in diseases such as experimental autoimmune neuritis in which treatment of rats with antibody against IFNγ protected against the disease (Hartung et al., 1990; Strigard et al., 1989). Interestingly, IFNγ was shown to have opposite effects in adjuvant arthritis, depending on the time of administration (Jacob et al., 1989). An anti-

inflammatory role for IFNγ has been reported in other diseases such as type II collagen-induced arthritis (Nakajima et al., 1990), and in experimental autoimmune thyroiditis (Stull et al., 1992). In those studies, it was shown that treatment with anti-IFNγ antibodies exacerbated the disease. Recently, Caspi et al. demonstrated that treatment of mice with IFNγ ameliorated experimental autoimmune uveoretinitis (EAU), and that administration of anti-IFNγ antibodies was able to upregulate disease expression in some EAU-resistant strains, thus supporting a disease-downregulatory role for IFNγ in EAU (Caspi et al., 1994). In EAE, a role of IFNγ in disease pathogenesis is substantiated by the observation that IFNγ producing cells were detected in CNS lesions (Stoll et al., 1993, Renno et al., 1994a). In addition, it has been shown that during disease development, activated T cells (pgp^{high}) are found in the CNS, and these cells produce relatively high levels of IFNγ (Zeine and Owens, 1992; Reno et al., 1994b). Furthermore, the lymphokine has been known to induce class II MHC expression on the endothelial cells of blood vessels in the CNS, and on astrocytes, which are thought to play an important role in antigen presentation to encephalitogenic T cells (Fontana et al., 1984; McCarron et al., 1985 and 1986; Massa et al., 1987). On the other hand, IFNγ has been shown to alleviate EAE in both the Lewis rats (Voorthuis et al., 1990), and mice (Billiau et al., 1988; Kalman et al., 1992). In addition, an anti-inflammatory role of IFNγ has also been implicated by the observations that treatment with IFNγ ameliorated spinal cord-induced EAE in the susceptible SJL strains while administration of anti-IFNγ mAb enhanced both disease incidence and severity in the partially resistant strain C57/Bl6 (Billiau et al., 1988; Lublin et al., 1991).

1.4 Thesis objectives

T cells produce several lymphokines which in the case of EAE, may be directly or indirectly responsible for the CNS damage. The role of IFNγ in the development of EAE is still controversial (see section 1.3.5). Even though the main focus of the thesis was on IFNγ, we also examined the role of IL2 in EAE since this lymphokine is known to be crucial in T cell proliferation and activation. We examined the effect of anti-IFNγ (aIFN) and anti-IL2 (aIL2) mAb treatment on the clinico-pathological course of actively and passively induced EAE in the SJL and several resistant mouse strains.

With the use of biotinylated aIFN mAb, we determined the temporal requirement of the Ab activity. Since the data demonstrated that aIFN, and by implication, IFNγ, acted early in disease induction, at a time when the cells could not have interacted with the BBB, we investigated whether IFNγ could interfere with the passage of cells into the CNS, through its effect on the expression of cell adhesion molecules and their ligands such as VLA-4 and VCAM-I, and ICAM-1. Bio- and immunoassays were performed to examine the level of IFNγ and TNF α/β production by lymphoid cells from aIFN mAb-treated and nontreated animals.

The effect of IFNγ on T cell activation by antigen was also examined *in vitro*. Calcium flux, IL2R expression, IL3 production and proliferation of MBP-specific T line cells grown with antigen in the absence and presence of exogenously added mouse recombinant IFNγ were compared.

Finally, since IFNγ has been shown to selectively inhibit Th2 cell activation, and thus, reduce IL4 production, an additional objective of the thesis was to

investigate whether IL4 had a role in the development of EAE.

Chapter 2: MATERIALS AND METHODS

2.1 Animals

Female SJL/J (H-2^s), A/J (H-2^a), C3H/HeJ (H-2^k), AKR (H-2^k), DBA/J (H-2^d) and NZW (H-2^z) were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and female BALB/c (H-2^d) from Charles River (St. Constant, Quebec). Unless otherwise stated, all mice were between 6-9 weeks old when used.

Strain 13 guinea pigs were bred in our animal facility from a starting nucleus kindly supplied by Dr. D.W.T. Watson (Veterinary Resources Branch, NIH, Bethesda, MD, USA), and were used as a source of CNS tissue. Brains and spinal cords were removed and stored at -70°C until used.

2.2 CNS antigens

2.2.1 Myelin basic protein (MBP)

Guinea pig CNS tissues from our own colony and those purchased from Pel-Freez Biologicals (Rogers, AK, USA) were pooled. MBP was isolated and purified as described by Deibler et al. (1972), lyophilized and stored at room temperature in a desiccator under vacuum.

2.2.2 MBP_{87-103}

The mouse MBP peptide spanning aa 87 to 103 (MBP_{87-103}) (FKNIVTPRTPPPSQGSG) was synthesized on an Applied Biosytems model 431A automated peptide synthesizer, purified by HPLC as described by Boyer et al. (1990), and supplied to us by Dr. Bhagirath Singh (Department of Microbiology and Immunology, University of Western Ontario, London, Ont.).

2.2.3 Spinal cord (Sc) homogenates

Normal SJL/J mice were sacrificed under deep (ether) anaesthesia. The spine was severed at the neck and at the lumbar region. A 20 cc syringe filled with saline and a 21 gauge needle was then inserted into the spine at the lumbar area. The cord was ejected out by forcing the saline through the spine. Spinal cords from all mice were pooled, and used immediately.

2.3 Other antigens

Keyhole limpet hemocyanin (KLH) was obtained from Pacific Bio-marine Laboratories Inc. (Venice, CA, USA) as a saturated ammonium sulphate slurry. The crude preparation was first dialyzed against 4 changes of 0.01M phosphate buffered saline (PBS) (pH 7.4) for 2 days at 4°C. The dialysate was then centrifuged at 700 x g for 10-15 minutes (min) to remove large debris. The supernatant was collected and centrifuged at 100,000 x g for 2 hr. The pellet was dissolved in saline, and insoluble material was removed by a 15 min spin at 700 x g. All centrifugations were carried out at 4°C. The supernatant containing KLH was then filter-sterilized and kept at 4°C. The protein concentration was determined according to Lowry et al. (1951).

2.4 Monoclonal antibodies (mAb)

Rat-mouse hybridomas secreting neutralizing rat IgG1 anti-mouse IFN-γ (XMG-6) (Cherwinski et al., 1987), rat IgG2a anti-mouse IL-2 (S4B6) (Mosmann et al., 1986), rat IgG2a anti-mouse IL4 receptor (M1) (Beckman et al., 1990), rat IgG1 anti-mouse VCAM-1 (M/k 2.7.2) (Miyake et al., 1991), rat IgG1 anti-nitroiodophenyl acetate (NP) (J4.1) and rat IgG2b anti-NP (J1.2) (Finkelman et al., 1988) were gifts of Drs. T. Mosmann and R. Coffman (DNAX Corporation, Palo Alto, CA, USA). The

rat-mouse hybridomas GL117, and GL113 which secrete rat anti-β-galactosidase of the isotypes IgG2a and IgG1, respectively, and 1D11.2 which secretes rat IgG2b anti-mouse IL4 (Abrams et al., 1992) were gifts of Dr. J. Abrams (DNAX). All mAb were prepared and supplied to us by Dr. F. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD, USA). Briefly, all hybridomas were grown as ascites in Pristane-primed athymic (nude) mice. Antibodies were purified from ascites by a combination of ammonium sulfate precipitation and DE52-cellulose ion exchange chromatography (Finkelman et al., 1986). Gel double-diffusion analysis using isotype-specific antibodies purchased from ICN Immunobiologicals (Lisle, IL, USA), were used to identify fractions rich in rat IgG1 or IgG2a.

For cytofluorometric analysis of VLA-4 expression by $CD4^+$ or by $CD8^+$ lymph node cells and splenocytes (see section 2.12.1), the rat anti-mouse VLA-4 (R1-2) mAb was kindly provided to us by Dr. B. Chan (Department of Microbiology and Immunology, UWO, London, Ont.). Fluorescein isothiocyanate (FITC)-conjugated $F(ab)'_2$ goat anti-rat IgG was purchased from Caltag Laboratories, San Franscisco, CA, USA, and phycoerythrin-conjugated rat anti-mouse CD4 or CD8 from Cedarlane, Hornby, Ont.

For the immunohistochemical staining of brain sections (section 2.12.2), the rat anti-mouse VLA-4, rat anti-mouse CD4 and CD8 Ab were purchased from Cedarlane, rat anti-mouse ICAM-1 Ab from Serotect (Toronto, Ont.), and biotinylated goat anti-rat Ab from Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA.

The rat anti-mouse IFNγ capture antibody (18181D) and biotinylated rat anti-mouse IFNγ (18112D) used in the capture enzyme-linked immunosorbent assay (section 2.13.1) were purchased from Cedarlane. The Ab (Y-3P) used in the cytofluorometric analysis of class II MHC expression (section 2.15.7) was a gift from Dr. C.A. Janeway (Yale University, School of Medicine, New Haven, CT, USA). Y-3P is a mouse Ab reacting against all mouse haplotypes except Ia^{q}.

The rat-mouse hybridoma producing rat IgG1 anti-mouse IL2R (PC 61.5.3) was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were grown in serum free medium (Immuncor Canada Inc., Edmonton, Alberta), and the culture supernatant was used as a source of antibodies without further purification (section 2.15.10).

2.5 Biotinylation of Ab

Anti-IFN-γ and the isotype-matched control antibodies were biotinylated according to the method described by Harlow and Lane (1988). Briefly, antibodies were first diluted in sodium borate buffer (0.1 M, pH 8.8) to a concentration of at least 1-3 mg/ml. To this preparation, an appropriate volume of dimethylsulfoxide (ATCC) containing 10 mg/ml N-hydroxysuccinimide biotin (Sigma Chemical Co., St. Louis, MO, USA) was added to achieve a final biotin/antibody ratio of 75 µg/1 mg. This mixture of Ab and biotin was allowed to stand at room temperature for 4 hr. The biotinylation of Ab was stopped by the addition of 20 µl for every 250 µg of ester used, of 1 M ammonium chloride (Fisher Scietific, Fair Lawn, NJ, USA). After 10 min at room temperature, the mixture was dialysed extensively in PBS for 4-5 days at 4°C with frequent changes of the PBS to remove unreacted biotin. The

dialysate was then collected and stored at 4°C.

The presence of biotinylated Ab (bt-Ab) was detected by enzyme-linked immunosorbent assay (ELISA). Wells of an ELISA plate (Corning glass works, Corning, NY, USA) were first coated overnight at 4°C with 0.1 ml of sodium bicarbonate buffer (pH 8.8) containing 2 µg/ml bt-Ab. The wells were then washed with PBS containing 0.1% bovine serum albumin (BSA) (Sigma) (ELISA buffer), and blocked with PBS containing 3% BSA (blocking buffer) for 2 hr at room temperature. Following several washes, each well received 0.1 ml wash buffer containing 1/1000 dilution of streptavidin conjugated to alkaline phosphatase (SAAP) (Jackson ImmunoResearch Laboratories Inc.), and incubated for 45-60 min at room temperature. At the end of the incubation period, the wells were washed, developed with p-nitrophenyl phosphate (Sigma) and read at 405 nm in a Titertek Multiskan plate reader (Flow Laboratories, Mississauga, Ont.). A positive reaction was an indication of successful biotinylation.

2.6 Active induction of EAE

2.6.1 MBP- or MBP_{87-103}-induced EAE

MBP or MBP_{87-103} was dissolved in saline, and was emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI, USA) supplemented with heat killed *Mycobacterium tuberculosis* (Mtb) H37RA (Difco). Mice were injected with an emulsion consisting of either 400 µg MBP plus 80 µg Mtb or 100 µg MBP_{87-103} plus 200 µg Mtb in a total volume of 0.1 ml, distributed among four subcutaneous (sc) sites draining into the inguinal and axillary lymph nodes. Unless otherwise stated, each animal was injected twice, on days 0 and 7.

2.6.2 Spinal cord (Sc)-induced EAE

In several experiments, homogenates of syngeneic spinal cord were used instead of MBP. Freshly isolated syngeneic spinal cord (100 mg) was homogenized in 1 ml of saline, and the homogenate was then emulsified with an equal volume of CFA supplemented with 3 mg/ml Mtb. Each mouse received 0.2 ml of emulsion equally divided among the 4 subcutaneous sites as described above.

A number of mice were also given 10^{10} paraformaldehyde-fixed Bp organisms (Michigan Department of Public Health, Lansing, MI, USA) intravenously (iv) following the MBP-CFA or Sc-CFA injection on day 0, and again on day 2.

2.7 Passive induction of EAE

Donor mice were immunized with a single dose of MBP-CFA as described above. Ten to twelve days later, inguinal and axillary lymph nodes were removed aseptically, and processed into a single cell suspension. The number of cells was adjusted to 4 x 10^6/ml in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Bocknek, BDH Chemicals, Toronto, Ont.), 25 mM Hepes (BDH), 2 mM L-glutamine (Gibco), 5 x 10^{-5} M 2-mercaptoethanol (Sigma), 1 mM sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 100 µg penicillin, and 100 µg streptomycin (Gibco) per ml (culture medium). MBP was added at either 50 or 100 µg/ml. Cultures were incubated in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) in 30 ml medium at 37°C in 6% CO_2 for 4 days. At the end of the incubation period, live cells were collected by centrifugation over Ficoll-Hypaque made up of 5.8% Ficoll (Pharmacia Biotech, Uppsala, Sweden) and 18.2% Hypaque (Sigma), and washed 3 times in Hanks'

balanced salt solution (BSS) (Gibco). The final cell pellet was resuspended at 40 x 10^6 cells/ml in the same medium. Syngeneic recipients were injected intraperitoneally (ip) with 20 x 10^6 viable cells.

2.8 Clinical assessment

Mice were examined daily for signs of disease, and graded on an arbitrary scale from 0 to 6 (Pettinelli and McFarlin, 1981): 0, no abnormality; 0.5, slow reflexes; 1, floppy tail with mild hindlimb weakness; 2, floppy tail with moderate hindlimb weakness; 3, hindleg paresis with or without mild forelimb weakness; 4, hindleg paralysis with or without moderate forelimb weakness; 5, quadriplegia; 6, dead or moribund requiring sacrifice.

2.9 Histopathology

Immediately after sacrifice, the animals were perfused with isotonic saline. The entire CNS was dissected and immersed in 10% buffered formalin. After fixation, the tissue was serially sectioned into 1-2 mm coronal cuts, and the entire specimen was serially imbedded in paraffin. Sections were cut at 5 µm, and stained with hematoxylin and eosin (H & E), and Solochrome R. The slides were reviewed by one observer independently, and without prior knowledge of the treatment or clinical status of the animals at the time of sacrifice.

The pathological changes were scored as described previously (Strejan et al., 1985) with slight modifications. Briefly, cellular infiltrates and demyelination were scored separately from 0 to 4. For the cellular infiltrates, since certain changes were more likely than others to have clinical relevance, they were weighed as follows: meningeal involvement 1x, perivascular infiltrates 2x, and encephalitis

3x. Thus, to achieve a maximum score of 4, meningeal involvement would represent 0.66, perivascular infiltrates 1.33, and encephalitis 2.0. For demyelination, scoring was done according to the number and severity of demyelinating plaques.

2.10 *In vivo* treatment with mAb and/or avidin

Ab was diluted in BSS, and was injected iv in volumes of between 0.2-0.5 ml. In some experiments as indicated at Results, the Ab was mixed with the MBP-activated cells prior to cell transfer, and this mixture of cells and Ab was injected ip into recipients. Unless otherwise stated, all Ab were given at 1 mg/mouse. In experiments where the animals were also treated with avidin, the avidin was first dissolved in BSS or PBS. Unless otherwise stated, each animal was given an iv dose of 2 mg avidin in 0.2 ml volumes. Ab and/or avidin was given at various time points as indicated at Results. In cases where both the mAb and avidin were given on the same day, the avidin was injected within 60 min after the Ab.

2.11 *In vivo* treatment with IL4 and anti-IL4 mAb (IL4/aIL4 mAb) complexes

Murine recombinant IL4 and anti-IL4 (aIL4) mAb (gifts of Dr. Finkelman, USUHS. Bethesda, MD, USA) were mixed at molar ratios indicated at Results. The mixtures were incubated at room temperature for 20-30 min prior to injection. Mice were injected iv or ip with 0.2 ml volumes containing various amounts of IL4/ aIL4 mAb complexes at several time points as indicated at Results.

2.12 Expression of adhesion molecules

2.12.1 Cytofluorometric analysis of VLA-4 expression by LNC and SPL

SJL/J mice were immunized with MBP-CFA or with CFA on days 0 and 7 followed by an iv treatment of 1 mg of either aIFN or its control mAb GL113. One

or 2 days after disease onset, draining lymph nodes and spleen were removed, and prepared into single cell suspensions. Approximately 10^6 cells per tube were first incubated with 50-100 μl of culture supernatant from a rat B cell hybridoma producing rat anti-mouse VLA-4 Ab (a gift from Dr. B. Chan, UWO, Ont.). Following several washes, the cells were incubated with FITC-conjugated $F(ab)'_2$ goat anti-rat IgG (1:500) (Caltag Laboratories). The cells were then washed, and stained with either phycoerythrin-conjugated rat anti-mouse CD4 or CD8 Ab (1:20) (Cedarlane).

In all experiments, prior to the addition of any Ab, 20 μl of normal human serum was added to the cell pellet to prevent non-specific binding of immunoglobulin. All Ab incubation steps were carried out on ice, and were between 30-45 min long. Cells were analysed in either a FACScan cytofluorometer (Becton Dickinson, Mississauga, Ont.), or a Becton-Dickinson FACStar Plus Cell Sorter. Data were collected on at least 10^4 cells as determined by forward and orthogonal light scatter intensity. In all cases, plain PBS or PBS containing 0.01% sodium azide (Sigma) and 0.5% bovine serum albumin (Sigma) was used in the washes and as the diluent for Ab.

2.12.2 Immunohistochemical staining of brain sections

SJL/J mice were challenged with MBP-CFA followed by an iv treatment of 1 mg of either aIFN or the isotype-matched control Ab GI113. One or two days after disease onset, the mice were anaesthetized by an ip injection consisting of 9 mg valium and 8 mg ketamine per kg body weight. The animals were then perfused with 60 ml ice-cold 4% paraformaldehyde (Sigma) in PBS (PFA) through the left ventricle of the heart. The brains were removed, allowed to soak in PFA for

additional 2 hr at room temperature, and then in 20% sucrose in PBS overnight at 4°C. Tissues were snap-frozen in 2-methyl-butane (J.T. Baker Inc., Phillipsburg, NJ., USA) which had been frozen on liquid nitrogen. The frozen tissues were stored at -70°C until used.

Five to eight µm cryostat sections were cut, placed onto positively charged glass slides (Fisher Scientific, Ottawa, Ont.), and stored at -70°C until needed. The sections were processed and stained with Ab essentially as described by Stoll et al. (1993). Briefly, unless otherwise stated, all steps were carried out at room temperature. All incubations were performed in moist chambers with 50-100 µl reagent to cover the tissue section, followed by several washes in PBS. Slides were first allowed to reach room temperature, air-dried and fixed in acetone (BDH) for 10 min.The sections were incubated with 0.3% H_2O_2 in PBS (TM/MC Drug Trading Company Ltd., Scabourough, Ont.) for 15 min to inactivate endogenous peroxidases. Fc receptors were blocked by a 30 minute exposure to 5% normal goat serum (a gift of Dr. D. Percy, University of Guelph, Guelph, Ont.) in PBS. This was followed by an overnight incubation at 4°C with one of the following antibodies: (i) rat anti-mouse ICAM-1 (Serotect) (1/25 dilution or 40 µg/ml), (ii) rat anti-mouse CD4 or CD8 (Cedarlane) (100 µg/ml), (iii) rat anti-mouse VLA-4 (Cedarlane) (100 µg/ml), or (iv) rat anti-mouse VCAM-1 (200 µg/ml). The sections were then exposed to biotinylated goat anti-rat antibody (Jackson Immunoresearch Laboratories Inc.) (1/1000 dilution) in PBS containing 5% normal mouse serum for 90 min, followed by a 45 minute incubation with peroxidase-avidin complex in PBS (Jackson Immunoreasearch Laboratories, Inc.) (2 µg/ml). Diamino-benzidine (Sigma) was

added for 10 minutes, and the reaction was stopped by immersion in deionized distilled water. Nonspecific staining was checked by substituting the primary antibodies with PBS, or with an irrelevant rat Ab at the working dilution of the primary antibody. No nonspecific labelling was observed.

All sections were counterstained with hematoxylin as follows: The slides were immersed into Harris' hematoxylin (BDH) for 2-3 seconds followed by extensive washes in water. They were then dipped sequentially into acid alcohol (0.01% hydrochloric acid in 70% ethanol), ammonia water (5% ammonium hydroxide in water), 95% alcohol, absolute ethanol followed by 2-3 washes in xylene (Fisher Scientific). After the last wash, excess liquid was removed by absorbing the excess fluid with tissue paper, and the sections were mounted in Permount (Fisher Scientific).

2.13 Detection of IFNγ and TNF α/β in culture supernatants of activated lymph node cells

SJL/J mice were challenged with MBP-CFA once on day 0, or twice, on days 0 and 7. Following the challenge, they were also treated with 1 mg aIFN mAb iv Control mice were challenged only. On day 8 or 10 post-challenge, lymph node cells were isolated from the draining lymph nodes, and were grown at 3×10^6 cells/ml in culture medium in the presence or absence of 50 μg/ml MBP. The supernatants were collected 24 hr later, aliquoted and kept at -70°C until used.

2.13.1 IFNγ

The levels of IFNγ in culture supernanatants were determined by a capture ELISA according to the manufacturer of the anti-IFNγ Ab used (Cedarlane). Briefly,

wells of a high protein binding ELISA plate (Corning) were coated overnight at 4°C with 0.1 ml of 0.1 M sodium bicarbonate (pH 8.8) containing 2µg/ml capture anti-IFNγ mAb (18181D) (Cedarlane). The wells were washed extensively with ELISA buffer, followed by a 2 hr incubation at room temperature in blocking buffer (0.2 ml/well). After several washes, 0.1 ml volumes of ELISA buffer containing dilutions of culture supernantants or of mouse recombinant IFNγ (rIFNγ) (Cedarlane) were added to the wells. Following a 4-5 hr incubation at room temperature, the wells were washed, and incubated for 45 min with 0.1ml ELISA buffer containing 2 µg/ml biotinylated anti-IFNγ mAb (18112D) (Cedarlane). After several washes, they were incubated for another 30 min with 0.1 ml/well ELISA buffer containing 1/1000 dilution of SAAP (Jackson ImmunoResearch Laboratories Inc.). Following the washes, the wells were developed with p-nitrophenyl phosphate (Sigma) and read at 405 nm. The rIFNγ was used as a standard.

2.13.2 TNFα/β

The presence of TNF α/β in culture supernatants was determined by its ability to inhibit the growth of the TNF-sensitive cell line WEHI-164 (ATCC). Five to eight thousand WEHI-164 cells in 0.1ml volumes of culture medium were added to 3-4 replicate wells of a flat-bottomed 96-well plate (Nunc, Denmark). The wells also received 0.1ml of culture medium containing serial dilutions of supernatants. Control wells received 0.1 ml of culture medium only. Cultures were incubated at 37°C in 6% CO_2 for 48 hr. During the last 18 hr, the wells were pulsed with 1 µCi [methyl-^{3}H]thymidine (^{3}[H]TdR) (Dupont Canada-New England Nuclear, Montreal, Que.). At the time of harvesting, the medium in all wells was first removed, and

each well was washed once with 100 µl BSS. Fifty µl of BSS containing 0.05% trypsin (Sigma) and 0.53 mM EDTA (Fisher Scientific) were added to each well. After 5-10 minutes at 37°C, the cells were harvested with an automatic cell harvester (Skatron Co., Sterling, VA, USA). The results were expressed at percent inhibition which was calculated as: 1 - the ratio of disintegrations per minute (dpm) of cultures grown with supernatants to those grown in medium alone.

2.14 Detection of γδ TcR+ cells

SJL/J mice were injected with 20 x 10^6 MBP-activated lymph node cells as described in section 2.7. Except for control mice, all others were also injected iv with 1 mg aIFN mAb following cell transfer. One or two days after disease onset, the mice were sacrificed, the lymph nodes and spleen were removed, and prepared into single cell suspensions. Mononuclear cells in the CNS were collected by discontinuous density gradient centrifugation as described by Zeine and Owens (1992). Briefly, mice were anaesthetized with a mixture of ketamine and valium (see section 2.12.2), and perfused through the heart with PBS. The brains and spinal cords were then collected and homogenized in BSS. The nervous tissue was first centrifuged in BSS at 200 x g for 10 min at room temperature. It was resuspended in 4 ml of 70% Percoll (Sigma) in RPMI-1640 medium (4 spinal cords or 2 brains per 4 ml of 70% Percoll). This was overlaid by equal volumes of 37% and 30% Percoll, and the gradient was centrifuged at 700 x g for 15 min at room temperature. Mononuclear cells were collected from the 37%/70% interface, and were washed in RPMI-1640 containing 10% FCS.

After the last wash, 0.3 to 1 x 10^6 live cells per tube were incubated for 40

min on ice with phycoerythrin-conjugated hamster anti-mouse γδ TcR (Cedarlane), and the expression of γδ TcR was assayed by cytofluorometry as described above.

2.15 Determination of the effect of IFNγ on T cell activation by antigen-pulsed APC

2.15.1a Preparation of Concanavalin-A-stimulated spleen cell supernatants (RCAS)

Spleen cells from normal Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN, USA), at a concentration of 5 x 10^6 cells/ml, were stimulated in culture medium containing 2.5 μg/ml Concanavalin-A (Miles Scientific, Naperville, IL, USA). Supernatants were collected after 24 hr of incubation, and stored at -70°C until used.

2.15.1b Generation of antigen-specific short-term T cell lines

Donor SJL/J mice were challenged on day 0 with MBP-CFA. Ten to 12 days later, the draining lymph nodes were removed aseptically and processed into a single cell suspension. Cells were suspended at 6 to 8 x 10^6 cells/ml in complete medium containing 50 or 100 μg/ml MBP, and were incubated in 30 ml volumes in 75 cm^2 tissue culture flasks (Falcon, Lincoln Park, NJ, USA). After 4 days, live cells were collected by centrifugation over Ficoll-Hypaque as described in section 2.7, and were washed 3 times in BSS. The final pellet was resuspended at 10^6 cells/ml in culture medium containing 10% RCAS as a source of IL2. Irradiated (2500 rad) syngeneic spleen cells were used at 7 x 10^6 cells/ml as feeders. The cells were cultured at 37°C for 10 days after which live cells were again collected over Ficoll-Hypaque, washed and incubated at 1.5 x 10^6 cells/ml in culture medium containing

50 or 100 μg MBP and 7 x 10^6 feeder cells/ml. After 4 days, the cycle was repeated with 10 days in RCAS followed by 4 days in antigen. In all experiments, the T line cells were used at the end of the second or third passage out of RCAS.

T cell lines that were specific to KLH were generated in a similar way except that KLH was used as antigen.

2.15.2 Isolation of peritoneal exudate cells (PEC)

Peritoneal cells were obtained from 10- to 20-weeks old female SJL/J mice by sterile lavage with BSS. After 3 washes in BSS, the number of cells was adjusted to between 0.5 x 10^6 and 1 x 10^6 cells/ml in culture medium. In several experiments as indicated at Results, the PEC were cultured at 10^6 cells/ml in culture medium alone or in medium containing either murine recombinant IFNγ (rIFNγ) (Amgen, Thousand Oaks, CA, USA), aIFN or its isotype-matched control mAb J4.1, a mixture of rIFNγ and aIFN mAb, or a mixture of rIFNγ and J4.1. rIFNγ was used at 100 U/ml and mAb at 400 ng/ml. The cells were incubated in 8 to 10 ml volumes of culture medium in 25 cm^2 tissue culture flasks (Falcon) at 37°C in 6% CO_2 for 48 hr. At the end of the incubation period, both the nonadherent and adherent populations were collected, and washed in cold BSS. The adherent population was obtained by gentle scraping in ice-cold BSS. In selected experiments, freshly isolated PEC were cultured at 1 x 10^6 cells/ml in culture medium ovenight at 37°C. Following the incubation, nonadherent cells were discarded, and the adherent population was collected as described above.

2.15.3 Antigen (Ag) pulse and fixation of PEC

After the last wash, the pretreated or nontreated PEC were resuspended in

complete medium at 10^7 cells/ml in the presence or absence of 100 µg/ml MBP, or KLH. Cultures were incubated at 37°C on a shaking platform for 2 hr, after which time the cells were washed 3 times in BSS. In several experiments, following the antigen pulse, the PEC were fixed in 0.5% paraformaldehyde (Sigma) for 1 min. The reaction was stopped by the addition of 100 µl of an ice-cold solution of 6% glycine (BDH). The cells were then washed 4 times, and were used in proliferative assays as antigen-pulsed, fixed APC. This procedure does not affect the ability of the APC to deliver the co-stimulatory signals.

2.15.4 Isolation of thymocytes (THYM) and splenocytes (SPL)

The thymuses and spleens of 5-7 weeks old normal SJL/J mice were removed aseptically, and prepared into a single cell suspension. After the last wash, the cells were resuspended in culture medium. Both the THYM and SPL were pulsed with MBP under similar conditions as described for PEC (section2.15.3).

2.15.5 Pretreatment of T cells

Between 1-2 x 10^6 T line cells per ml were grown for 2 hr at 37°C in culture medium alone or in medium containing rIFNγ, rIFNγ plus aIFN, or rIFN plus the control mAb J4.1. The cells were then collected and washed prior to culture. All mAb and rIFNγ were used at 400 ng/ml and 100 U/ml, respectively.

2.15.6 Proliferative assays

Between 10^4 and 5 x 10^4 Ag-pulsed or unpulsed PEC were added in 0.1 ml volumes of culture medium to 4 or 6 replicate wells of flat-bottomed microtiter plates (Nunc, Denmark). In experiments where SPL or THYM were used as a source of APC, 5 x 10^5 cells were used. Nonfixed PEC, SPL or THYM were irradiated (2500

rads) prior to plating. 2.5 x 10^4 T line cells were then added in 0.1 ml volumes of culture medium to each well. In some experiments, at the time of plating, rIFNγ and/or aIFN mAb or the isotype matched mAb were added to cultures. The final concentrations of the lymphokine and mAb in cultures were 100 U/ml and 400 ng/ml, respectively. Cultures were incubated at 37°C in 6% CO_2 for 96 hr. During the last 18 hr, they were pulsed with 1 μCi/well of ^{3}[H]TdR (Dupont Canada-New England Nuclear). The magnitude of the proliferative response was expressed as stimulation index (SI), ie., average dpm of culture with Ag-pulsed PEC / average dpm of cultures with unpulsed PEC, and as Δdpm ± standard error (SE), i.e., the difference between the average dpm of cultures with antigen-pulsed PEC and that of cultures with unpulsed PEC.

2.15.7 Cytofluorometric analysis

Approximately 10^6 live cells per tube were incubated with the appropriate dilution of either rat anti-mouse CD4 (L3T4), or rat anti-mouse CD8 (Ly-2) mAb (1:500) (Cedarlane) (section 2.15.7c), or with culture supernatant containing rat anti-mouse IL2 receptor Ab (300 μl) (ATCC) (section 2.15.10). Cells were washed twice, and incubated with FITC-conjugated $F(ab)_2$ goat anti-rat IgG (1:500) (Caltag). After the last wash, the cells were suspended in PBS, and propidium iodide was added to allow the gating out of dead cells. Background immunofluorescence was determined by staining cells with the FITC-conjugated Ab alone. Expression of surface Ia molecules was assayed by staining with FITC-labeled Y-3P (1:100) (sections 2.15.7a & b).

2.15.7a Ia expression by PEC grown with rIFNγ

Freshly isolated PEC from SJL/J mice were grown in 6 well plates (Falcon) at 2-3 x 10^6 cells/ml in culture medium only, or in culture medium containing various amounts of rIFNγ (Amgen) as indicated at Results. After 48 hr, both the nonadherent and adherent cell populations were collected, washed, and assayed for surface Ia expression as described above.

2.15.7b Ia expression by PEC grown in activated-T cell culture supernatant, and by PEC grown with T line cells

Between 0.6 to 1 x 10^6 MBP-specific T line cells were grown in 4 ml volumes of culture medium in the presence of 0.5 to 1 x 10^6 MBP-pulsed, X-irradiated PEC. All cultures were incubated in 6-well plates (Falcon). Twenty four hr later, the supernatant was collected. Two to 3 x 10^6 freshly isolated PEC were grown per ml of either culture medium or of the supernatant. After 4C hr, the PEC were collected, washed and assayed for Ia expression by cytofluorometry.

In separate cocultures of T line cells and MBP-pulsed PEC, the nonadherent cells were removed after 24 hr of incubation. The adherent cell population was collected, and assayed for Ia expression.

2.15.7c Phenotypic analysis of MBP-specific T cell lines

At the end of the 2nd or 3rd cycle in RCAS, live cells were isolated over Ficoll-Hypaque (section 2.7), and assayed for CD4 and CD8 expression as described in section 2.15.7.

2.15.8 Detection of IL3 produced by T line cells

T line cells and Ag-pulsed PEC were cocultured under various conditions as described at Results. Culture supernatants were collected at 24 hr, and assayed

for the ability to sustain the growth of the BALB/c-derived, IL3-dependent mast cell line MC-9 (ATCC) as described by Zhao et al. (1993). Briefly, 3-5 x 10^3 MC-9 cells were added to 4 replicate wells in 0.1ml volumes of culture medium. To each well, 0.1 ml volumes of culture medium containing dilutions of supernatants were added. In parrallel, MC-9 cells were also grown in various concentrations of murine recombinant IL3 (rIL3) (Genzyme, Cambridge, MA, USA). Proliferative responses were assayed 72 hr later. The concentrations of IL3 in tissue supernatants were calculated using the rIL3 as a standard.

2.15.9 Determination of cell viability by cytofluorometry

Between 1-2 x 10^6 T line cells/ml were incubated for 2 hr at 37°C in culture medium containing either rIFNγ only (100 U/ml) or rIFNγ plus 0.5-1 x 10^6 irradiated, MBP-pulsed adherent PEC. Control cultures contained T cells grown under the same conditions but without rIFNγ. Nonadherent cells were collected at various times as indicated at Results. After washing, the cells were stained with propidium iodide, and their viability was determined on a FACScan cytofluorometer (Becton-Dickinson).

2.15.10 Examination of the effect of pretreatment of T cells with IFNγ on IL2R expression.

MBP-specific T line cells were pretreated with rIFNγ as described in section 2.15.5. Between 1-2 x 10^6 cells were then cultured with 0.5-1 x 10^6 Ag-pulsed or unpulsed PEC in 4 ml volumes of culture medium. Nonadherent cells were collected at various time points as indicated at Results and washed. The expression of IL2R was determined by cytofluorometry after staining the cells with rat anti-mouse IL2R

Ab (ATCC), fc!lowed by FITC-conjugated $F(ab')_2$ goat anti-rat IgG, as described in section 2.15.7. In parallel, the T cells and Ag-pulsed or unpulsed PEC were also cutured in a 96-well plate, and the proliferative response was assayed at 96 hr. In these cultures, the ratio of T cells to PEC was similar to that in the corresponding cultures in which IL2R expression was determined.

2.15.11 Examination of the effect of pretreatment of T cells with IFNγ on calcium (Ca^{+}) flux

T line cells were incubated for 2 hr at 37°C in culture medium containing 100 U/ml rIFNγ. They were washed, and incubated with Indo 1-AM (Molecular Probes, Eugene, OR, USA) (10μg per 10^6 cells) for 45 min at 37°C. Residual dye was removed by extensive washing of the cells with RPMI-1640. The calcium flux exhibited by 3 x 10^6 Indo-loaded T lymphocytes was monitored on a Becton-Dickinson FACSstar Plus Cell Sorter for a few min prior to the addition of 6 x 10^6 MBP-pulsed PEC, and continued for 8-10 min after the PEC addition. Control T cells (nontreated) were handled similarly, except that they were not pretreated with IFNγ.

2.16 Statistical analysis

The clinical and histological scores were analysed by the Kruskal-Wallis st with multiple comparisons of the means (Zar, 1974). Where appropriate (eg. time of disease onset, duration of disease, amounts of IFNγ or TNF α/β produced by LNC from aIFN mAb-treated and nontreated animals, dpm, etc.), single factor analysis of variance (ANOVA) or Student's t-test was used (Zar, 1974).

Chapter 3: RESULTS

3.1 Effect of anti-IFNγ (aIFN) and anti-IL2 (aIL2) mAb treatment on the development of EAE in SJL/J mice

3.1.1 Active induction of EAE

Despite a number of reports showing that SJL mice are susceptible to EAE induction by MBP, in our hands, the disease was not readily inducible in this particular strain. Using the immunization protocol that is commonly used in a number of laboratories (Lublin, 1984; Moore et al., 1987), we first challenged SJL/J mice with an MBP-CFA emulsion consisting of 400 μg guinea pig MBP and 80 μg Mtb sc at the 4 sites draining the axillary and inguinal lymph nodes. This immunization protocol resulted in very low incidence of the disease (Table 1). Only 1 animal or 2% of the total 44 mice thus challenged developed a mild form of EAE on day 42 after the first challenge (day 42 post-challenge). All others remained clinically and histologically normal throughout the 4-8 month observation period.

With the use of overlapping synthetic peptides, Kono et al. (1988) have shown that the MBP peptide spanning amino acid residues 87-114 contains 2 encephalitogenic epitopes for the SJL mice: residues 87 to 98 (MBP_{87-98}) and 91 to 104 (MBP_{91-104}). The peptide MBP_{87-103} containing the first encephalitogenic epitope was available to us so we tested its ability to induce EAE in the SJL/J strain. Among the 12 animals that were challenged with this peptide in CFA, none developed any clinical or histological signs of the disease throughout the 3 to 5 months of observation period

Since several studies reported that Bp injection enhanced both incidence

and severity of EAE, probably due to its ability to increase blood brain barrier permeability (Munoz et al., 1981; Linthicum et al., 1982; Linthicum and Frelinger, 1982; Yong et al., 1993), we treated our mice with Bp following the MBP challenge. In the first attempt, a total of 16 mice were challenged with MBP-CFA once on day 0. The animals were then given 10^{10} paraformaldehyde-fixed Bp organisms iv immediately following the challenge and again 48 hr later. Under this protocol, however, only 1 of 16 mice or 6% developed EAE during the 4-7 month period of observation. Again, this mouse showed only mild symptoms which did not appear until day 40 post-challenge (Table 1). In the next set of experiments, a total of 28 mice were challenged with MBP-CFA on days 0 and 7. The animals were also given Bp following the challenge on day 0 and 48 hr later. Among the 28 mice thus challenged, 9 or 32% showed mild symptoms of EAE which appeared at about 2 weeks post-challenge. The rest of the animals remained clinically and histologically healthy during the 4-7 month observation period. Several mice were also given the Bp injection immediately following and 48 hr after the second MBP-CFA challenge, but all mice died within 2 days after the last Bp injection (data not shown).

A number of studies have reported that EAE in the mouse can be induced upon challenging the animals with spinal cord homogenates (Brown and McFarlin, 1981; Brown et al., 1982). Since whole Sc homogenate may contain additional encephalitogenic antigens besides MBP, attempts were made to examine whether both disease incidence and severity in SJL/J mice could be enhanced by CNS challenge. A total of 21 mice were challenged with an emulsion containing 10 mg syngeneic Sc homogenate and 200 µg Mtb (Sc-CFA) on days 0 and 7. Despite

reports demonstrating that this protocol was capable of inducing high incidence of EAE (Brown and McFarlin, 1981; Brown et al., 1982), in our hands, only 7 of 21 of thus challenged animals or 33% exhibited EAE manifestations around day 25 post-challenge (Table 1). All these mice developed hind leg paralysis, and survived the acute episode. Several mice also underwent relapses during this study period (2 to 6 months) (data not shown). Histological examination of CNS tissue revealed lesions consisting of cellular infiltrates and demyelination typical of EAE (data not shown). When mice were challenged twice with Sc-CFA on days 0 and 7 and treated with Bp on day 0 and 48 later, similar EAE incidence and severity were observed. During the 3-4 month study period, 7 of 23 animals or 30% developed EAE by around day 19 post-challenge (Table 1). Three animals developed hind leg paralysis, and 4 quadriplegia. All survived the first acute episode, and developed a few relapses during the observation period (data not shown).

These results showed that, in our hands, among several immunization protocols studied, the most effective one was the challenge with Sc-CFA either once or twice and with or without Bp injection. MBP, on the other hand, did not readily induce the disease in this strain, even when the animals received Bp.

3.1.2 aIFN and aIL2 mAb treatment in passive EAE

Despite the difficulty in inducing active EAE in the SJL/J mouse by MBP-CFA or even by Sc-CFA, the passive transfer of 20×10^6 lymphoid cells was consistently successful. Therefore, we first examined the role of IFNγ and IL2 in this model by determining whether treatment with aIFN and/or aIL2 mAb would exert any effect in the development of passive EAE. In the experiment presented in figure (Fig.) 1,

Table 1

Active Induction of EAE in SJL/J Mice

Immunization protocol	Incidence of EAE[a]	MDO ± SE[b]	MCS[c]
2x MBP-CFA[d]	1/44 (4-8)	42	2.0
2x $MBP_{87\text{-}103}$-CFA	0/12 (3-5)	---	---
1x MBP-CFA + Bp[e]	1/16 (4-7)	40	2.0
2x MBP-CFA + Bp	9/28 (4-7)	15.6 ± 2.3	2.7
2x Sc-CFA[f]	7/21 (3-6)	25.0 ± 4.7	3.1
2x Sc-CFA + Bp	7/23 (3-4)	19.3 ± 3.6	3.6

[a] Number of mice that developed EAE over the total. The numbers in brackets indicate the length of the observation period in months.

[b] Mean day of disease onset ± standard error.

[c] Average maximum clinical scores of all mice that showed signs of disease.

[d] SJL/J mice were injected with an emulsion consisting of either 400 µg MBP plus 80 µg Mtb or of 100 µg $MBP_{87\text{-}103}$ plus 200 µg Mtb as described in Materials and Methods. Each animal was challenged either once (1x) on day 0 or twice (2x) on days 0 and 7.

[e] Each animal was given an iv injection containing 10^{10} paraformaldehyde-fixed organisims of (Bp) on days 0 and 2.

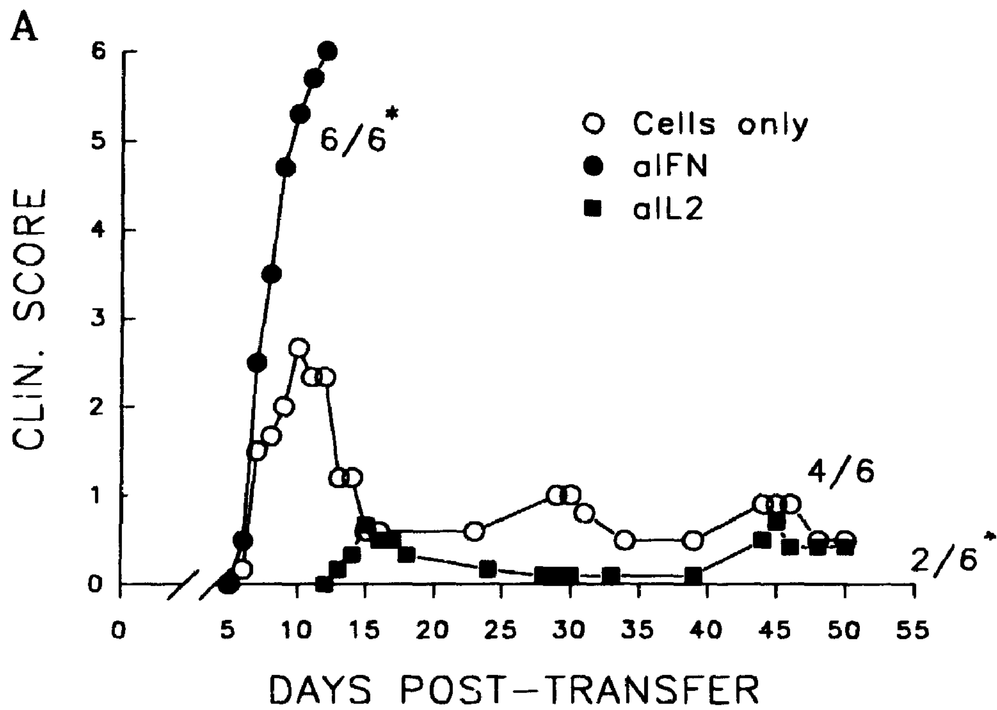
[f] SJL/J mice were injected with an emulsion consisting of 10 mg syngeneic spinal cord homogenate and 200 µg Mtb as described in Materials and Methods. The animals were challenged on days 0 and 7.

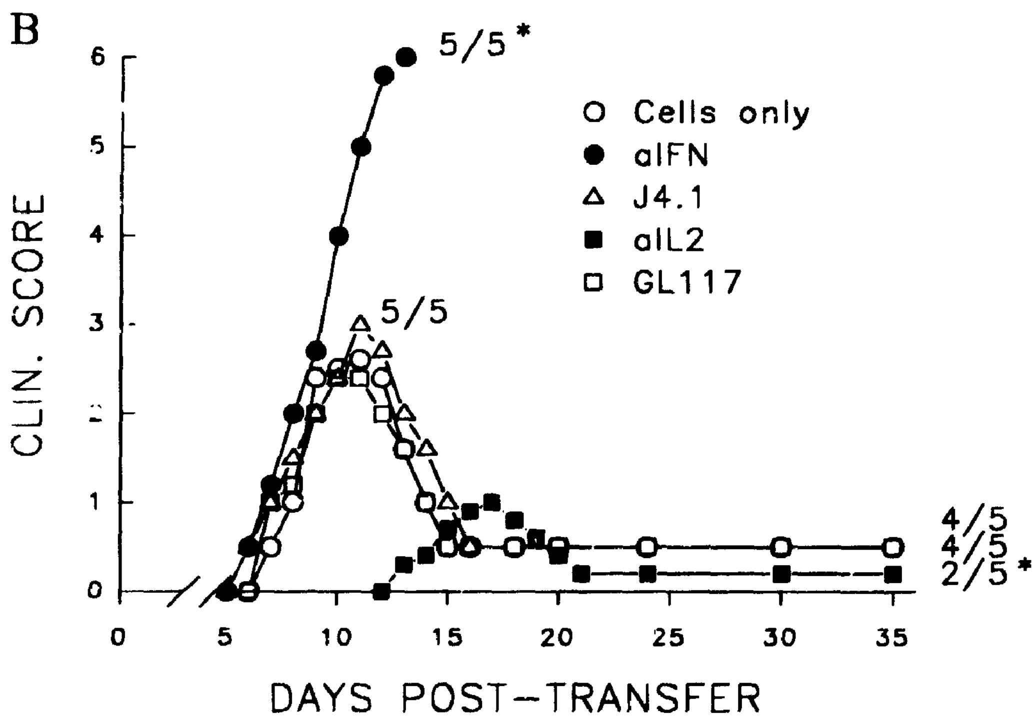
three groups of SJL/J mice were included: (i) mice that were given 20 x 10^6 cells only, (ii) mice that were injected with a mixture of either cells and 1 mg aIFN mAb or (iii) of cells and 1 mg aIL2 mAb. The Ab were mixed with the cells just prior to the transfer.

As shown, the transfer of MBP-activated cells alone determined a moderate form of EAE in four of six recipients with mean maximum clinical score (mean max. clin. score) of 2.8, and disease onset was around day 7 post-transfer. These mice also developed 2 mild relapses on days 29 and 45 post-transfer. In contrast, the transfer of cells mixed with aIFN mAb induced a severe form of EAE with lethal outcome for all recipients within a week after disease onset. On the other hand, the transfer of cells mixed with aIL2 mAb determined a mild form of EAE (mean max. clin. score of 0.7). The disease onset was significantly delayed ($p < 0.05$ as determined by single factor analysis of variance), and only two of six recipients developed overt disease. Severity of EAE in these mice was also significantly reduced when compared to animals that received cells only ($p < 0.05$). These 2 animals started a relapse on day 44 post-transfer which was equivalent in intensity to that observed in recipients of cells alone.

Similar results were observed when the experiment was repeated, as shown in Fig.1B. The transfer of MBP-activated LNC alone determined a mild form of EAE in 4 of 5 animals with an average maximum clinical score of around 3. In contrast, the inclusion of aIFN mAb to the transfer drastically enhanced EAE, resulting in death of all recipients, as observed in the previous experiment. The disease-enhancing effect was due specifically to aIFN mAb since similar treatment with an

Fig. 1 Time course of passively induced EAE in SJL/J mice treated with mAb against IFNγ or IL2. Lymph node lymphocytes from MBP-CFA sensitized donors were incubated with 100 μg/ml of MBP for 96 hr at 37°C. **(A)** Each recipient was injected ip with 20 x 10^6 live cells mixed with either 1 mg anti-IFNγ mAb (aIFN) (●), 1 mg anti-IL2 mAb (aIL2) (■), or in medium alone (cells only) (○). **(B)** SJL/J mice were treated as in (A). The animals were given a mixture of MBP-activated lymph node cells and 1 mg of either aIFN (●), its isotype-matched control mAb J4.1 (△), aIL2 mAb (■) or its isotype-matched control GL117 (□). Control animals received the cells only (○). Results are expressed as the average clinical score of all mice in the group on each day. In both (A) and (B), the number of animals with clinical EAE over the total number of animals in each group is indicated. The asterisks indicate groups of animals with clinical scores that were significantly different from those of animals receiving cells in medium alone ($p < 0.05$ as determined by the Kruskal-Wallis nonparametric test with multiple comparisons of the means).

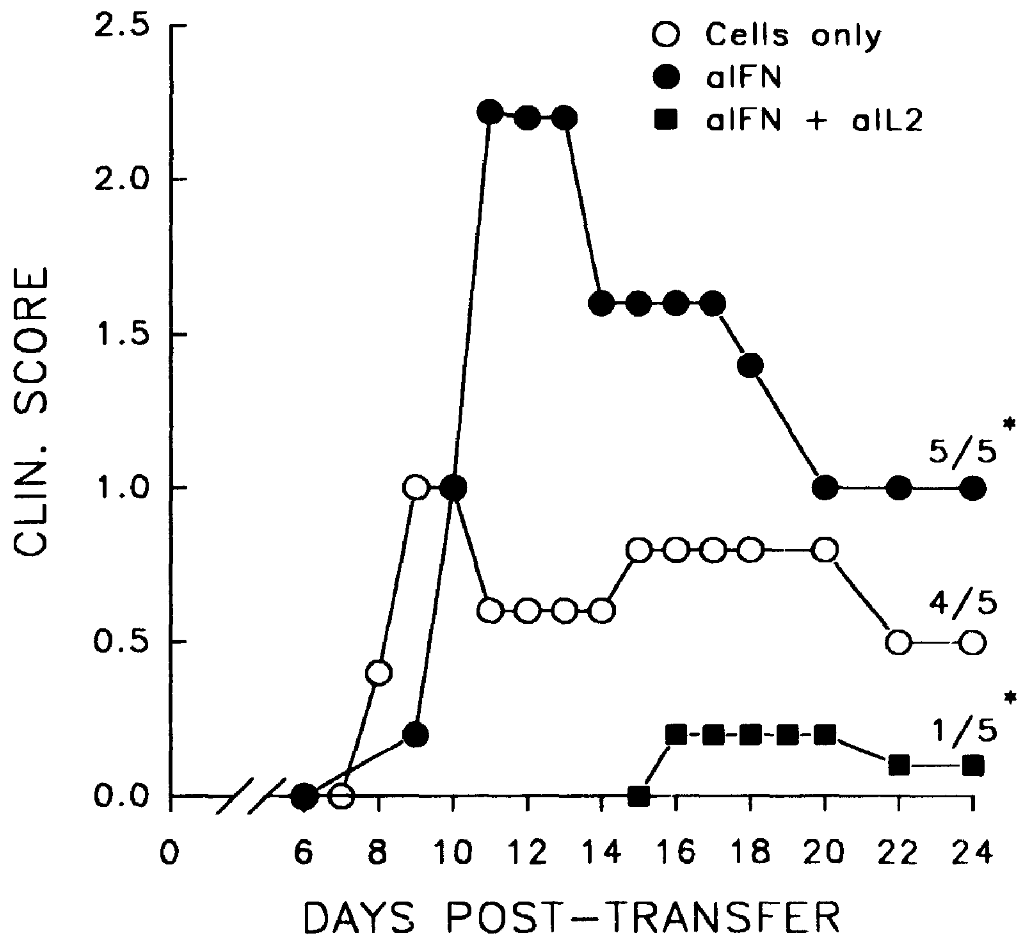

A
Cells only
aIFN
aIL2
6/6*
4/6
2/6*
CLIN. SCORE
DAYS POST-TRANSFER


B
Cells only
aIFN
J4.1
aIL2
GL117
5/5*
5/5
4/5
4/5
2/5*
CLIN. SCORE
DAYS POST-TRANSFER

isotype-matched control mAb (J4.1) was without effect. As seen above, the transfer of a mixture of MBP-activated cells and aIL2 mAb again significantly reduced both disease severity and incidence. Only 2 of 5 animals in this group showed signs of EAE. In addition, whereas in all other groups, the animals started to show signs of disease by day 6-7 post-transfer, disease onset in mice treated with aIL2 mAb was around day 13-14 post-transfer. The disease-inhibitory effect was attributable to the aIL2 mAb since the disease in the group receiving a mixture of cells and the isotype-matched control mAb (GL117) was not different from that of control. No relapses were observed during the 35 day observation period in either group.

The inhibitory effect of aIL2 mAb on the passive transfer of EAE suggested the possibility that the inclusion of aIL2 to the aIFN mAb treatment might lead to a reduction in disease incidence and/or severity. As shown in Fig. 2, the inclusion to aIFN mAb in the transfer of 20×10^6 MBP-activated cells led to a more severe form of EAE in all animals (mean max. clin. score of 2.3). On the other hand, the transfer of cells along with both aIFN and aIL2 resulted in a drastic reduction in disease development ($p < 0.05$) when compared to the recipients of cells plus aIFN mAb, or to recipients of cells alone. Furthermore, the only recipient that developed clinical symptoms in this group exhibited only a droopy tail (clin. score of 1). In addition, the disease onset was delayed for a week (day 16 post-transfer) in comparison to the other two groups. Although the disease in this experiment was milder than that in Fig. 1, the overall pattern remained the same. The difference in disease severity may simply reflect variations in the encephalitogenicity of the lymphoid cells isolated from different donors at different times.

Fig. 2 Effect of the treatment with a mixture of anti-IFNγ and anti-IL2 mAb on the passive induction of EAE. Each animal was injected ip w. 20 x 10^6 MBP-activated lymphocytes mixed with either 1 mg aIFN (aIFN) (●), a mixture of 1 mg aIFNγ and 1 mg aIL2 mAb (aIFN + aIL2) (■), or in medium alone (cells only) (○). Data are expressed as the average clinical score of all mice in the group on each day. The number of animals with clinical EAE over the total number of animals in each group is indicated in the graph. The asterisks mark groups of animals with clinical scores that were significantly different from those of animals receiving cells alone
($p < 0.05$).



2.5
2.0
1.5
1.0
0.5
0.0
CLIN. SCORE
Cells only
aIFN
aIFN + aIL2
5/5*
4/5
1/5*
0
6
8
10
12
14
16
18
20
22
24
DAYS POST-TRANSFER

CNS lesions in mice undergoing EAE are characterized by cellular infiltrates, and demyelination (Raine et al., 1980; Lᵢown et al., 1982). To examine whether aIL2 and/or aIFN mAb treatment had any effect on histopathological modifications, the CNS of mice in these groups was examined histologically. Animals that developed EAE were sacrificed either at the moribund state, or right after the peak of the disease. In passively-induced EAE, no substantial pathological differences between the groups of mice receiving the MBP-activated cells alone or cells with either aIFN, aIL2 or with a mixture of both mAb were observed (Table 2). Mice with clinical score of 6 (No. 130) and 3 (No. 132) had similar histological lesions. At times, a mouse with clinical score of 3 (No. 134) had spinal cord lesions which were more severe in terms of extent of infiltrates and necrosis than mice with scores of 5 (Nos. 49, 52, and 54), or of 6 (No. 130). The only clear difference was between mice that showed no clinical abnormality (No. 131, 133, and 135) and all the other mice with overt disease. In all forms, widespread lesions were observed in both the brain and spinal cord, especially in the white matter (Fig. 3). Heavy meningeal and perivascular inflammatory infiltrates consisting of mononuclear cells and macrophages were found throughout the hemispheres, brain stem, basal ganglia, cerebral peduncles, and also in the thoracic spinal cord. In addition, focal areas of necrosis with dense inflammatory infiltrates as well as myelin loss were seen in the cortex, medulla, brain stem, and also at the thoracic and lumbar levels of the spinal cord. The lesions in the cord were quite focal, and involved particularly the anterior white matter as well as the parasylvian tissue in the dorsal columns. In all groups, clinically normal animals showed no or very mild pathological abnormality. Similarly,

Table 2

Histological Scores of SJL/J Mice Treated with aIFN and/or aIL2 mAb at the Time of Cell Transfer

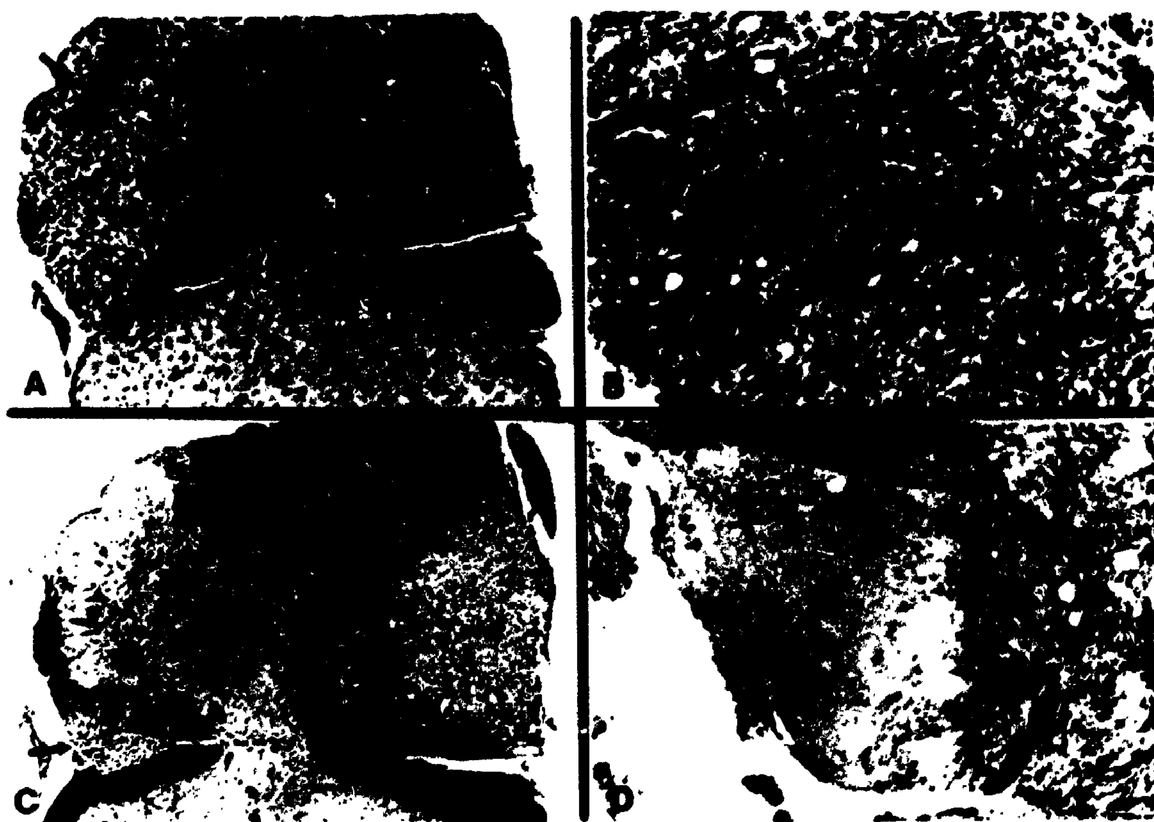
Treatment[a]	Animal No.[b]	Brain[c]		Spinal cord[c]	
		Infil.	Necrosis	Infil.	Necrosis
None	130 (6)	1.5	1.3	3.0	2.3
	131 (0)	0.4	0	0.1	0
aIL2	127 (3)	1.9	1.3	1.8	1.7
	129 (3)	0.7	0	1.0	1.2
aIFN	49 (5)	0.4	0	2.5	2.0
	52 (5)	1.2	1.0	2.2	1.0
	54 (5)	1.2	1.0	2.6	1.4
aIL2 + aIFNγ	132 (3)	1.6	0.7	2.7	1.9
	133 (0)	0	0	0.3	0
	134 (3)	2.0	1.7	3.5	4.0
	135 (0)	0	0	0	0

[a] Groups of SJL/J mice were injected with 20×10^6 MBP-activated lymph node cells in medium alone, or mixed with either 1 mg of aIFN, aIL2 or with a mixture of both mAb.

[b] Animals were sacrificed between days 12 and 18 post-transfer. The clinical score of individual animals at the time of sacrifice is indicated in brackets.

[c] Pathological changes were scored separately for cellular infiltrates (Infil.) and necrosis in the brain and spinal cords, on a scale of 0 to 4.

Fig. 3 Histology of SJL/J mice treated with anti-IFNγ and anti-IL2 mAb at the time of cell transfer. **(A)** Spinal cord section showing one large and two smaller areas of cellular infiltrates (arrows) in the white matter of a mouse receiving MBP-activated lymphoid cells, aIFN and aIL2 mAb on day 0. Maximum clinical score was 3 (day 18 post-transfer, H&E, x54). **(B)** Spinal cord section showing large mononuclear cell infiltrate of the white matter, in a mouse treated as in (A). Maximum clinical score was 3 (day 18 post-transfer, H&E, x108). **(C)** Spinal cord section showing one large and one smaller area of necrosis (arrows), in a mouse treated as in (A). Maximum clinical score was 3 (day 18 post-transfer, Solochrome R, x54). **(D)** Spinal cord section showing a necrotic area with complete loss of myelin, in a mouse receiving MBP-activated lymphoid cells and aIFN mAb on day 0. Maximum clinical score was 5 (day 12 post-transfer, Solochrome R, x216).


A
B
C
D

histological examination of actively challenged animals indicated that mice with no clinical signs of EAE had no pathological abnormality while those with clinical scores had widespread lesions (data not shown).

3.1.3 aIFN and aIL2 mAb treatment in active EAE

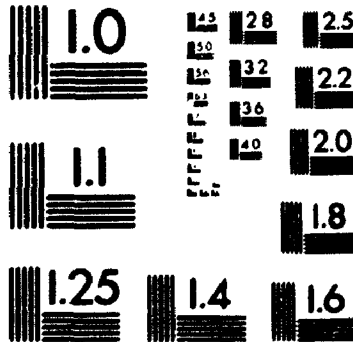
In 1988, Billiau and colleagues reported that treatment of mice with anti-IFNγ Ab in a mouse strain which is partially resistant to EAE (C57Bl/6) resulted in high incidence of disease after challenge with whole spinal ccrd in CFA. Since in our hands, as shown in Table 1, SJL/J mice do not develop EAE readily upon MBP-CFA, MBP_{87-103} -CFA or Sc-CFA challenge, and since our results in Fig. 1 and 2 demonstrated that aIFN mAb exerted a disease-enhancing effect in the passive induction of EAE, the question arose as to whether treatment with aIFN Ab would lead to higher incidence and/or more severe EAE in SJL/J mice challenged with MBP-CFA or with the encephalitogenic peptide MBP_{87-103}-CFA. To investigate this possibility, we challenged mice with MBP-CFA or with MBP_{87-103}-CFA on days 0 and 7, and treated them each with an iv injection of 1 mg aIFN mAb on the same days. An isotype-matched mAb J4.1 was used as a control for the aIFN mAb. As seen in Fig. 4A. 7 of 8 mice that were challenged with MBP_{87-103}-CFA and were given the aIFN mAb developed severe EAE. Disease onset was around day 14 post-challenge, and only one mouse survived after the first episode. This effect was due to the aIFN mAb treatment since EAE was not observed in the 7 animals that were challenged with MBP_{87-103}-CFA and treated with the control mAb (J4.1). None of the 8 mice that were similarly challenged but were not injected with any mAb showed signs of the disease. Similarly, after treatment with aIFN mAb of mice challenged

with MBP-CFA, signs of EAE appeared by day 14, and by day 19, all had died or had to be sacrificed *in extremis* (Fig. 4B). In contrast, consistent with earlier experiments, mice that were challenged with MBP-CFA only, failed to exhibit any sign of EAE over the 30-day observation period. The observation that aIFN mAb treatment resulted in 100% incidence of EAE is significant since similar treatment with the isotype-matched control mAb was without effect . EAE was not observed in any of the mice that were similarly challenged with pigeon cytochrome-C in CFA and treated with aIFNmAb (data not shown). Pigeon cytochrome-C was chosen as a negative control for MBP since this peptide was found to be immunogenic in the SJL/J mouse (data not shown), and since it has similar molecular weight and overall charge properties as MBP.

The observation that the administration of aIL2 mAb strongly inhibited the clinical manifestations of the passively induced EAE, even when it was mixed with a disease-enhancing dose of aIFN mAb (Fig. 1 and 2) raised the question as to whether aIL2 mAb could alleviate the effect of aIFN mAb in actively-induced EAE. To address this question, we included groups of mice that were treated with either aIL2 mAb alone, or a mixture of aIFN mAb and aIL2 mAb. In each case, the isotype-match mAb GL117 was used as a control for the anti-IL2 mAb. As indicated in Fig. 4B , when 1 mg of aIL2 mAb was injected along with aIFN mAb, the disease was almost as severe as in the aIFN mAb-treated group (mean clin. score of 5), indicating that under these conditions, the aIL2 mAb treatment had no significant protective effect on the aIFN mAb-induced exacerbation of active EAE. It must be noted, however, that eventually, two of six mice in this group recovered by day 36

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT



PRECISION℠ RESOLUTION TARGETS

post-challenge (data not shown), whereas all the mice treated with either aIFN mAb alone or with anti-IFNγ mixed with GL117 died, or had to be sacrificed by day 19 or 26 post-challenge, respectively.

Taken collectively, the results presented in Fig. 4 suggested that the exacerbation of actively-induced EAE by treatment with aIFN mAb was attributable to the neutralization of IFNγ. This could not be substantially reduced by the concomitant administration of aIL2 mAb. This notion was further supported by statistical analysis of clinical scores showing that there were no significant differences among these two groups of animals, ie. those treated with aIFN mAb alone and those with a mixture of aIFN and aIL2 mAb.

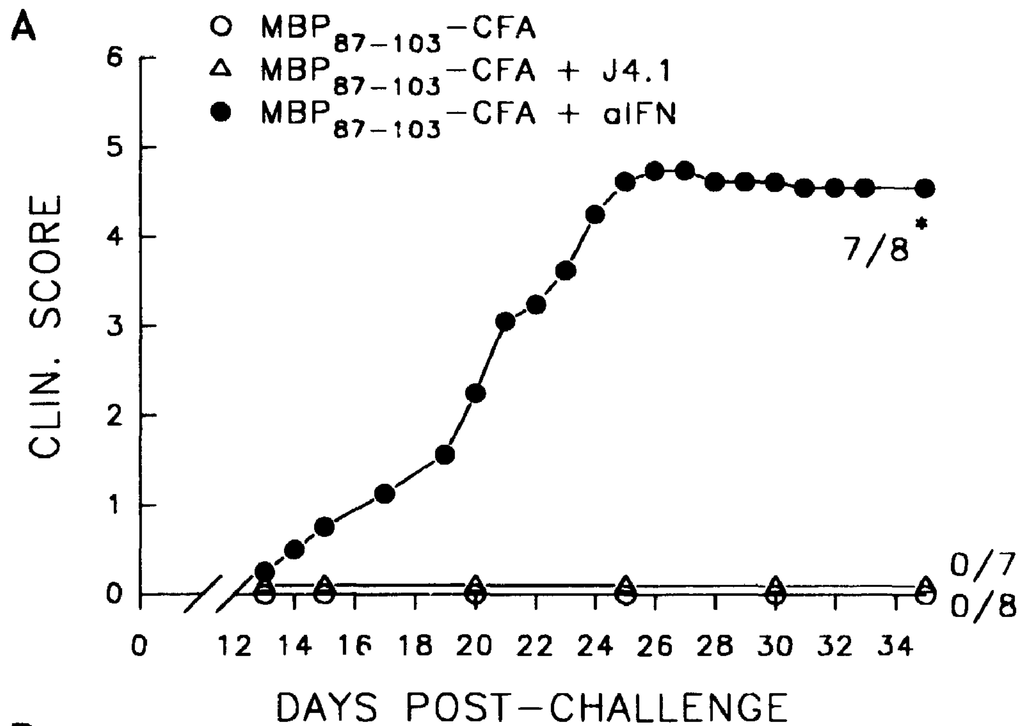
3.2 Effect of aIFN mAb treatment on the development of EAE in resistant mouse strains: A/J, BALB/c, C3H/HeJ, AKR, NZW and DBA/2

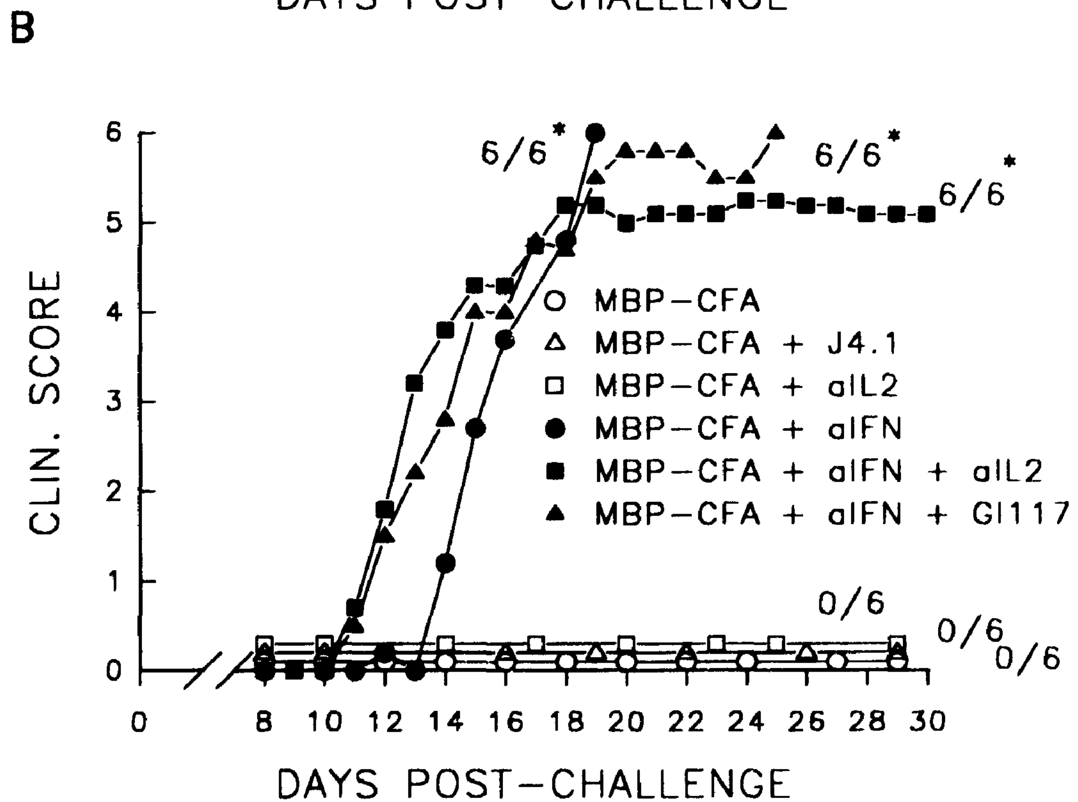
Several strains of mice have been considered fully or partially resistant to EAE (William and Moore, 1973; Gasser et al., 1975; Bernard, 1976; Raine et al., 1980; Teitelbaum and Arnon, 1981). Since our results and those by Billiau et al. (1988) indicated that administration of anti-IFNγ antibodies exacerbated EAE in the susceptible strain of mice (SJL/J) as well as in the partially resistant strain (C57Bl/6), we wished to determine whether EAE could be induced after treatment with aIFN mAb in refractory strains.

3.2.1 aIFN mAb treatment in active EAE

Mice of various strains were challenged with MBP-CFA on days 0 and 7, followed by aIFN mAb treatment. As shown in Fig. 5A, between 80 to 100% of A/J, BALB/c and C3H/HeJ mice that received the mAb developed severe EAE. In the

Fig. 4 Effect of anti-IFNγ and anti-IL2 mAb treatment on the active induction of EAE in SJL/J mice. Groups of SJL/J mice were immunized with an emulsion containing either 100 μg MBp$_{87-103}$ plus 200 μg Mtb **(A)**, or 400 μg MBP plus 80 μg Mtb **(B)** on days 0 and 7. On the same days, the animals were treated with an iv injection of either aIFN (●), its isotype-matched control mAb J4.1 (△), or aIL2 (□). Other groups of MBP-CFA challenged animals received a mixture of either aIFN and aIL2 mAb (■) or of aIFN and the isotype-matched control mAb for aIL2 (GL117) (▲). All mAb were given at 1 mg per mouse. Control animals were challenged with either MBP$_{87-103}$-CFA or MBP-CFA but not treated with any mAb (○). The results are expressed as the mean clinical score of all animals in the group on each day. The number of mice with clinical EAE over the total in each group is indicated. The asterisks mark groups of animals with clinical scores that were significantly different from those of control mice ($p < 0.05$).

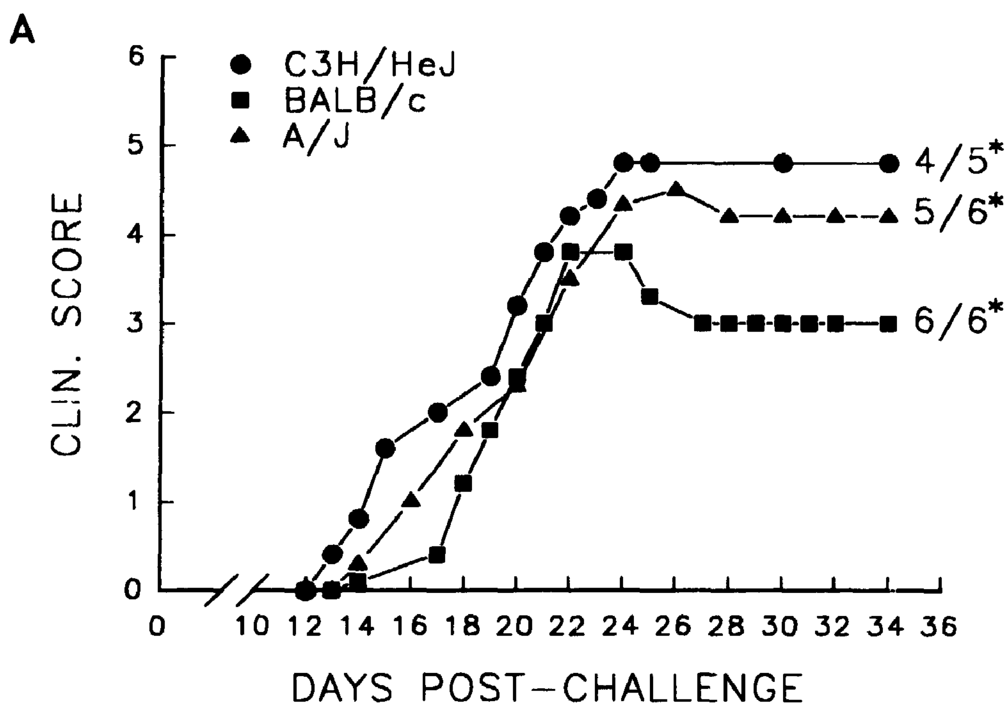

A
MBP_{87-103}–CFA
MBP_{87-103}–CFA + J4.1
MBP_{87-103}–CFA + aIFN
CLIN. SCORE
DAYS POST–CHALLENGE
7/8*
0/7
0/8
0 1 2 3 4 5 6
0 12 14 16 18 20 22 24 26 28 30 32 34


B
MBP–CFA
MBP–CFA + J4.1
MBP–CFA + aIL2
MBP–CFA + aIFN
MBP–CFA + aIFN + aIL2
MBP–CFA + aIFN + GI117
CLIN. SCORE
DAYS POST–CHALLENGE
6/6*
6/6*
6/6*
0/6
0/6
0/6
0 1 2 3 4 5 6
0 8 10 12 14 16 18 20 22 24 26 28 30

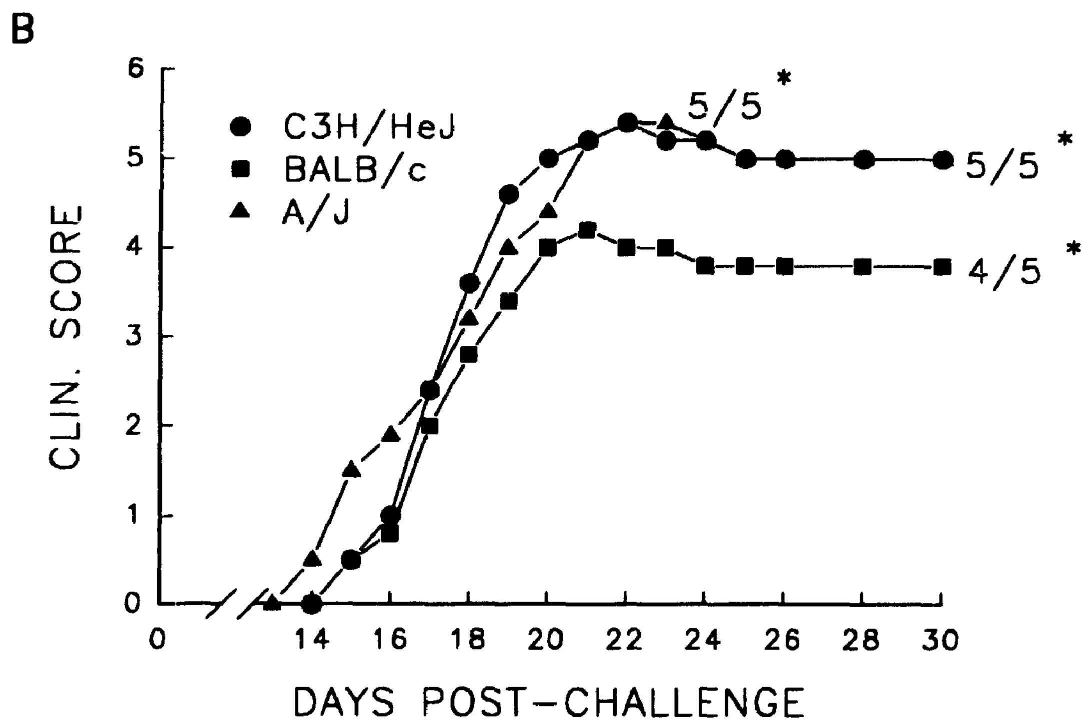
C3H/HeJ mice, overt disease started at around day 13 post-challenge, and peaked on day 25, at which time all mice that developed symptoms had died. A similar disease course was observed in the A/J strain in which 4 of 5 sick animals died or had to be sacrificed *in extremis*. In the BALB/c mice, the disease onset was later (day 17 post-challenge) when compared to that in the other two strains (day 13 and day 14 in the C3H/HeJ and A/J mice, respectively), and the clinical manifestations were less severe. However, the disease peaked earlier (day 21-22). Three mice that survived recovered clinically, and were observed for 60 days during which no relapsing episodes were detected. In contrast to the aIFN mAb-treated groups, MBP-CFA challenged animals of all these 3 strains that did not receive anti-IFN γ mAb appeared completely normal throughout the 60 day observation period (data not shown). When the experiment was repeated, similar patterns of disease time-course and severity were observed (Fig. 5B).

In order to confirm that IFNγ does play a role in preventing the development of EAE in refractory strains, 3 additional resistant mouse strains were included: DBA/2, NZW and AKR. Mice were challenged with MBP-CFA on days 0 and 7, followed by aIFN mAb treatment. Control mice were not given any mAb. As shown in Fig. 6, a similar disease-enhancing effect of aIFN mAb treatment on the active induction of EAE was observed in the DBA/2 and NZW. Four of 5 aIFN mAb-treated DBA/2 mice showed signs of EAE beginning on day 17 post-challenge, and by day 27, three of four animals that developed EAE had died. In the NZW mice, clinical manifestations of EAE appeared on day 20 post-challenge in 3 of 5 MBP-CFA challenged plus mAb-treated animals, and were less severe than in the other strain

Fig. 5 Active induction of EAE in C3H/HeJ, A/J and BALB/c mice treated with anti-IFNγ mAb. Mice of the C3H/HeJ (●), A/J (▲) and BALB/c (■) strains were immunized with 400 μg MBP in CFA on days 0 and 7, and treated on the same days with 1 mg aIFN mAb iv. All mice that were challenged with MBP-CFA and were not treated with aIFN (controls) failed to develop EAE, and were not included in the figure (5 or 6 mice of each strain). The data represent mean clinical score on each day for all animals in the group. For each group, the number of mice with clinical EAE over the total number of animals is indicated. Groups of animals with clinical scores that were significantly different ($p < 0.05$) from control groups are marked with an asterisk. Panels A and B represent two separate expreriments.



A
C3H/HeJ
BALB/c
A/J
4/5*
5/6*
6/6*
CLIN. SCORE
DAYS POST-CHALLENGE



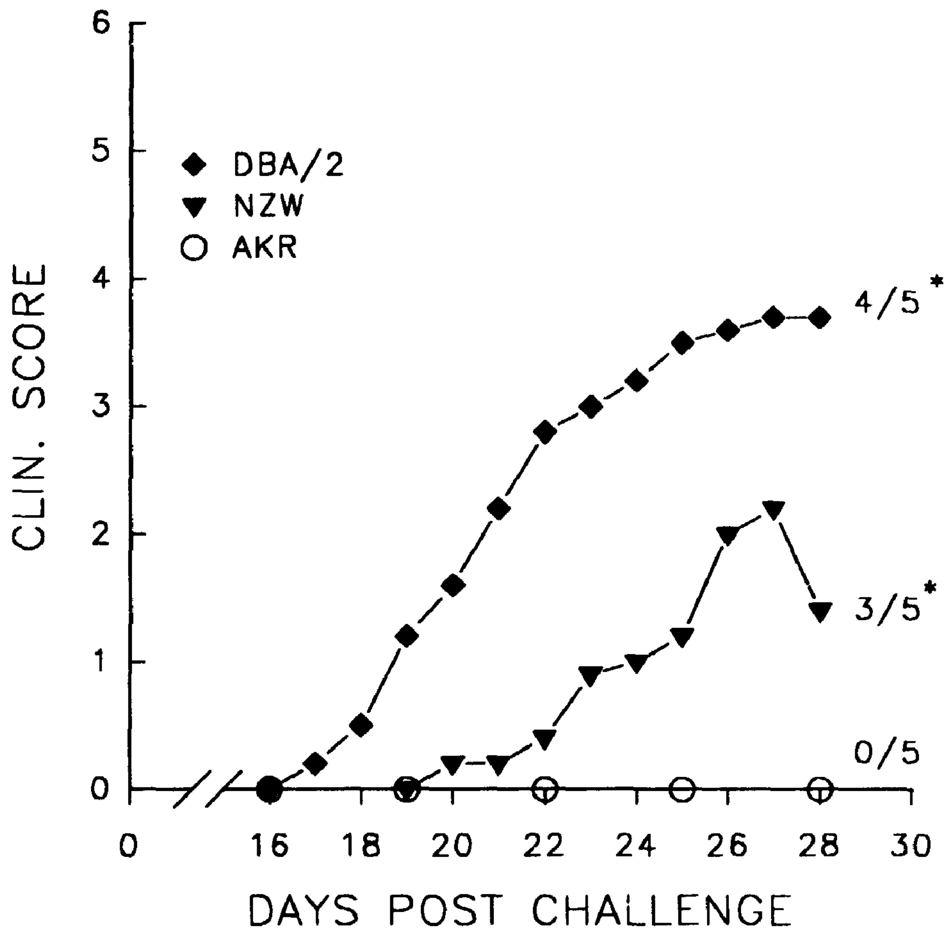
B
C3H/HeJ
BALB/c
A/J
5/5 *
5/5 *
4/5 *
CLIN. SCORE
DAYS POST-CHALLENGE

(max. clin. score of 4). By day 28, the animals started to recover. During the 58 day of observation period, none of the DBA/2 and NZW that survived underwent relapses (data not shown). Under similar conditions, none of the aIFN mAb-treated AKR mice developed EAE. It should be noted that none of the animals that were challenged with MBP-CFA but did not receive aIFN mAb showed signs of EAE, regardless of strain (data not shown).

To examine the histopathology in EAE mice of resistant strains, animals that developed clinical overt EAE in the active induction were sacrificed either when moribund, or between days 18 and 23 after challenge. All animals with clinical EAE had wide spread histological lesions characterized by extensive meningeal and perivascular inflammatory infiltrates with or without demyelination. Fig. 7A is a spinal cord section of an A/J mouse showing several areas of extensive cellular infiltrates. In addition, some lesions of EAE were also characterized by focal areas of necrosis and demyelination, as seen in the spinal cord section of a BALB/c mouse shown in Fig. 7D. In contrast, when clinically normal animals, regardless of whether they were in the control or in the mAb-treated groups were examined, no pathological changes were found (Fig. 7C).

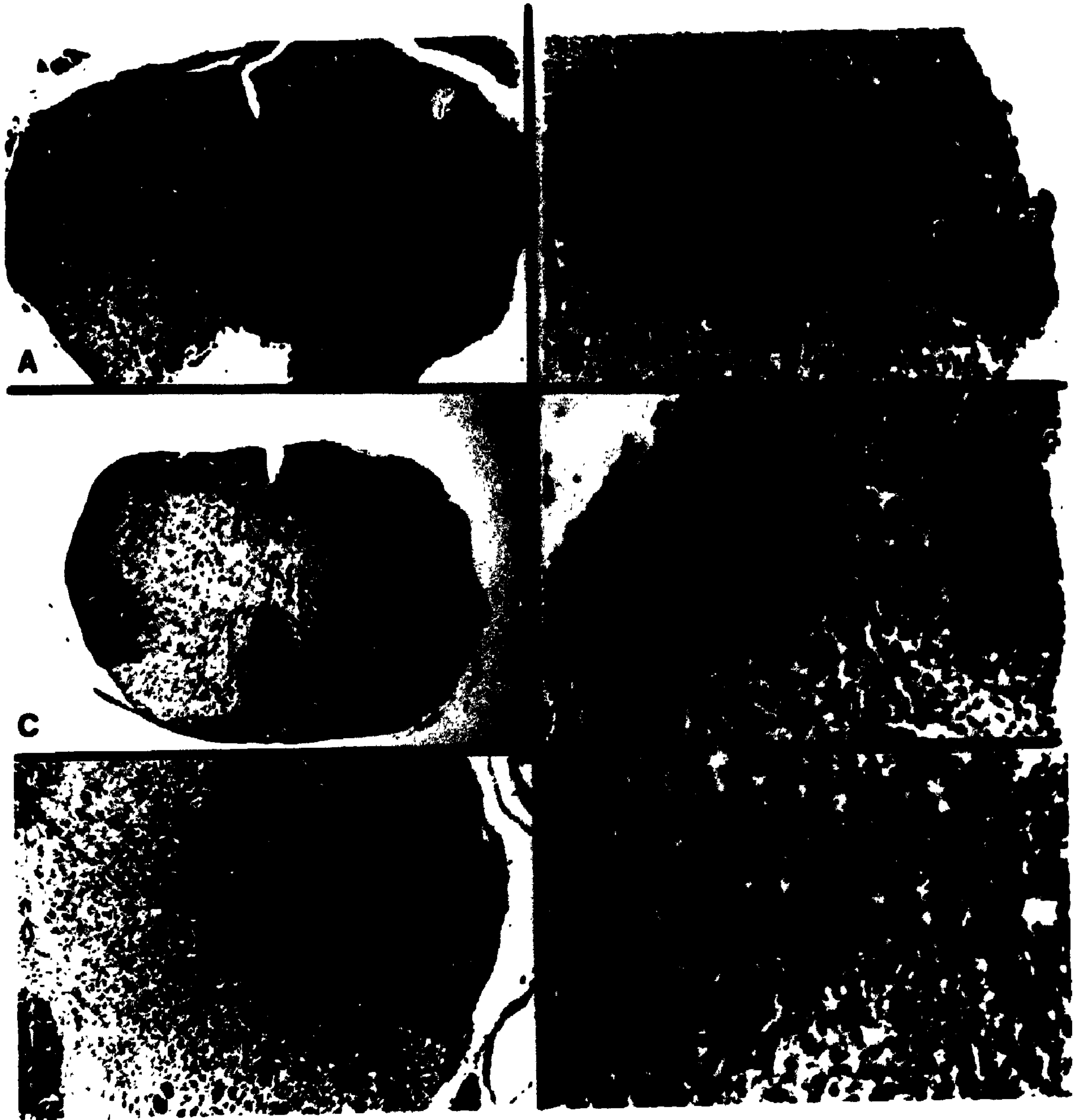
Since encephalitogenic MBP epitopes have been identified for susceptible strains of mice (Kono et al., 1988; Fritz et al., 1983b and 1990) but not for any of the resistant mouse strains studied here, and since the encephalitogenic peptide MBP_{87-103} was available to us, we tested the ability of this peptide to induce EAE in 3 mouse strains: A/J, C3H/HeJ and BALB/c with or without aIFN mAb treatment. The dosage and schedule of peptide immunization and aIFN mAb injection used

Fig.6 Active induction of EAE in the DBA/2, NZW and AKR mice treated with anti-IFNγ mAb. See Fig. 5 for details. The data represent mean clinical score on each day for DBA/2 (◆), NZW (▼) and AKR (○) mice. Animals that were challenged with MBP-CFA without aIFN mAb failed to develop EAE, and were not included in the figure (5 animals of each strain).



CLIN. SCORE
DAYS POST CHALLENGE
DBA/2
NZW
AKR
4/5*
3/5*
0/5
0
1
2
3
4
5
6
0
16
18
20
22
24
26
28
30

Fig. 7 Histology of EAE-resistant mouse strains treated with anti-IFNγ mAb. **(A)** Spinal cord section of an A/J mouse showing several areas of cellular infiltrates in the white matter (arrows). Clinical score at sacrifice (day 18) was 5 (H&E, x56). **(B)** Brain section of the mouse in (A) showing extensive invasion of the parenchyma by large nuclear infiltrates (H&E, x141). **(C)** Spinal cord section of an untreated A/J mouse sacrificed on day 20. The animal had no clinical manifestation, and the histological section appears normal (H&E, x22.4). **(D)** Spinal cord section of a Balb/c mouse showing a large area of demyelination, and a zone of residual normal myelin (arrows). Clinical of the animal at sacrifice (day 23) was 4 (Solochrome-R, x176). **(E)** Spinal cord section of a Balb/c mouse showing two focal areas of demyelination (arrows). Clinical score at sacrifice (day 21) was 5 (Solochrome-R, x56). **(F)** One of the two demyelinating foci from (E) at higher magnification (x281). Mice used in this figure were all challenged with MBP-CFA on days 0 and 7. Treated mice (A, B, D, E and F) received 1 mg aIFN mAb on the same days.

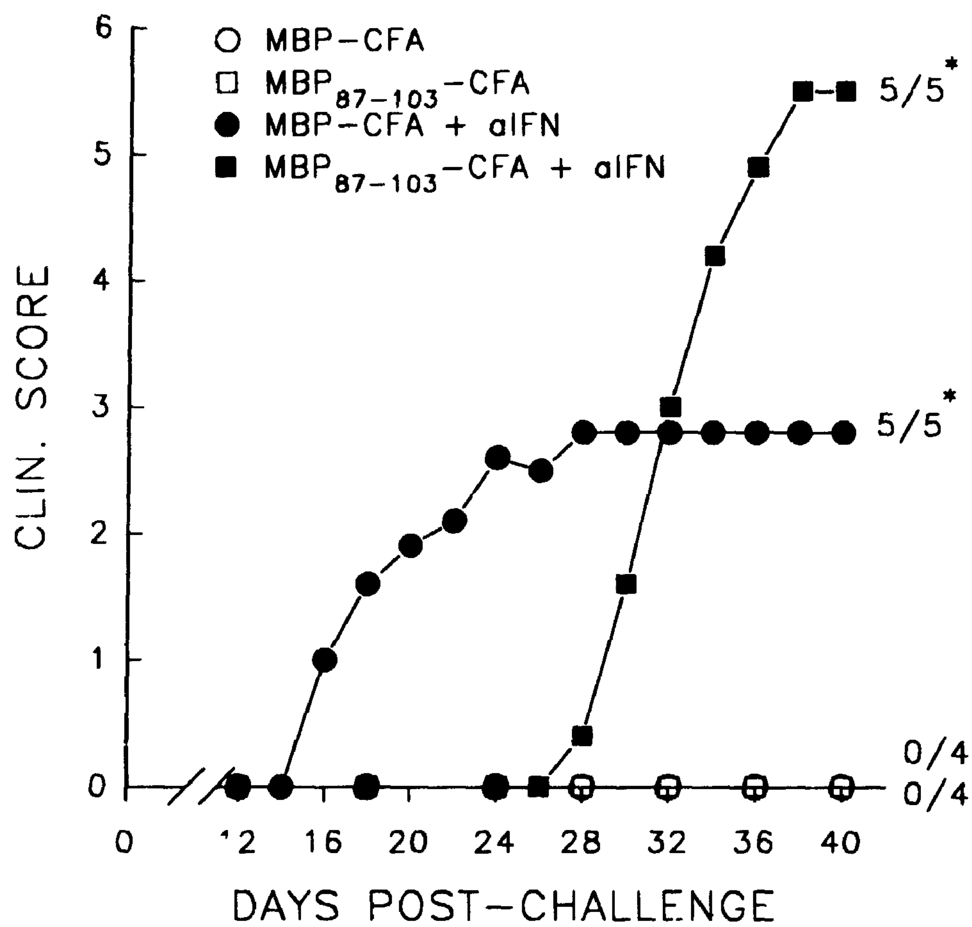

A
C

here were those found to induce EAE in the SJL/J (Fig. 4). Of the 3 strains tested, MBP_{87-103} was found to be encephalitogenic only for the BALB/c (Fig. 8) but not the A/J and C3H/HeJ (data not shown). Severe EAE was detected in all BALB/c mice challenged with whole MBP-CFA, or with MBP_{87-103}-CFA, followed by aIFN mAb treatment but not in mice that did not receive the mAb. Mice challenged with MBP_{87-103} exhibited more severe clinical signs (mean max. clin. score of 5.5) than mice challenged with whole MBP (mean max. clin. score of 2.8). However, in the former case, disease onset was later (day 28 post-challenge in comparison to day 16).

3.2.2 aIFN mAb treatment in passive EAE

From Fig. 5 and 6, it is evident that treatment with aIFN mAb following MBP-CFA challenge can overcome disease resistance to actively-induced EAE in 5 of 6 refractory strains. To examine whether the mAb had similar effect in the passive induction of EAE, mice of 3 strains, A/J, BALB/c and C3H/HeJ were given a mixture of 20 x 10^6 syngeneic MBP-activated lymph node cells and aIFN mAb. Control animals were given the cells only without any mAb. As can be seen in Table 3, EAE could not be passively transferred in the A/J and BALB/c mice, as none of these animals developed disease following the cell transfer. On the other hand, clinical signs of EAE could be detected in four of six C3H/HeJ recipients. However, these mice developed only a mild form of EAE with maximal clinical score of 0.67, and disease onset on around day 18 post-transfer. In contrast, the inclusion of aIFN mAb in the transfer shortened the time of disease onset in the C3H/HeJ by about 10 days. Both disease incidence and severity were increased. 100% of mice developed EAE with an average max. clin. score of 2.4. A similar disease-

Fig. 8 Effect of anti-IFNγ mAb treatment on actively induced EAE in BALB/c mice. Mice were challenged with either MBP-CFA (●), or MBP_{87-103} -CFA (■) on days 0 and 7 followed by an iv injection of 1 mg aIFN mAb. Control animals were challenged with MBP-CFA (○), or MBP_{87-103}-CFA (□) but were not treated with aIFN mAb. Data are expressed as the mean clinical score of animals in the group on each day. The number of mice with clinical EAE over the total number of animals in each group is indicated. Groups with clinical scores that were significantly different from those of control mice are marked by an asterisk ($p < 0.05$).



○ MBP−CFA
□ MBP_{87-103}−CFA
● MBP−CFA + aIFN
■ MBP_{87-103}−CFA + aIFN
CLIN. SCORE
0
1
2
3
4
5
6
5/5*
5/5*
0/4
0/4
0
12
16
20
24
28
32
36
40
DAYS POST−CHALLENGE

enhancing effect of aIFN mAb was observed in the A/J strain. Overt EAE was observed in two of four A/J recipients of cells plus aIFN mAb between days 28-29 post-transfer, with average max. clin. score of 2.8 which was significantly higher than that of A/J recipients of cells alone. These observations were further substantiated by statistical analysis showing that the effect of aIFN mAb treatment on the mean day of disease onset and severity was significant in both the C3H/HeJ and A/J mice ($p < 0.05$). On the other hand, similar treatment had no effect on the passive induction of EAE in the BALB/c mice. None of the animals developed EAE during the 55 day observation period following the cell transfer, regardless of whether or not aIFN mAb was included. Even the transfer of 10^8 MBP-activated lymphoid cells plus aIFN mAb failed to induce EAE in this strain (data not shown).

3.3 Effect of treatment with various amounts of aIFN mAb on the development of EAE in SJL/J mice

In all experiments performed so far, the disease-enhancing effect of aIFN mAb was observed in animals receiving 1 mg of the mAb following the MBP-CFA challenge or at the time of passive transfer. It was, therefore, of interest to examine whether the mAb was effective at lower doses.

3.3.1 Active EAE

A titration of the aIFN mAb indicated that in the active induction of EAE, treatment with even 100 µg mAb resulted in enhanced disease (Fig. 9). In this particular experiment, SJL/J mice were challenged with MBP-CFA on days 0 and 7 followed by the administration of either 1 mg, 300 µg or 100 µg aIFN mAb. EAE was detected in all animals. The disease course of mice receiving 300 µg mAb was

Table 3

Effect of aIFN mAb on Passive Induction of EAE in Resistant Strains of Mice

Strain	Treatment[a]	EAE/Total[b]	MDO ± SEM[c]	MCS[d]
C3H/HeJ	Untreated	4/6	18.0 ± 3.5	0.67
	Treated	6/6	8.8 ± 0.8	2.4
A/J	Untreated	0/4	NA[e]	0
	Treated	2/4	29.0 ± 5.0	2.8
BALB/c	Untreated	0/5	NA	0
	Treated	0/5	NA	0

[a] Lymph node lymphocytes from MBP-challenged donors were incubated with 50 μg/ml MBP for 96 hr. Syngeneic recipients were each injected ip with either 20×10^6 cells alone (untreated) or 20×10^6 cells plus 1 mg aIFN mAb (treated).

[b] Number of mice with clinical signs of EAE over the total.

[c] Mean day of onset ± standard error of the mean.

[d] Average maximal clinical scores of all mice in a group.

[e] Not applicable. None of the animals showed signs of EAE over the 55 day observation period.

Values which are significantly different from those of control (untreated) animals are underlined ($p < 0.05$).

similar to that of recipients of 1 mg mAb. In both groups, the animals started to show signs of EAE on days 13-14 post-challenge, and all had died of disease by day 20. The disease in animals that received 100 μg mAb was less severe (max. clin. score of 4), and all animals survived. However, these mice never fully recovered, and underwent a relapse starting on around day 29 post-challenge. In contrast, none of the 4 animals that were challenged with MBP-CFA only, developed EAE.

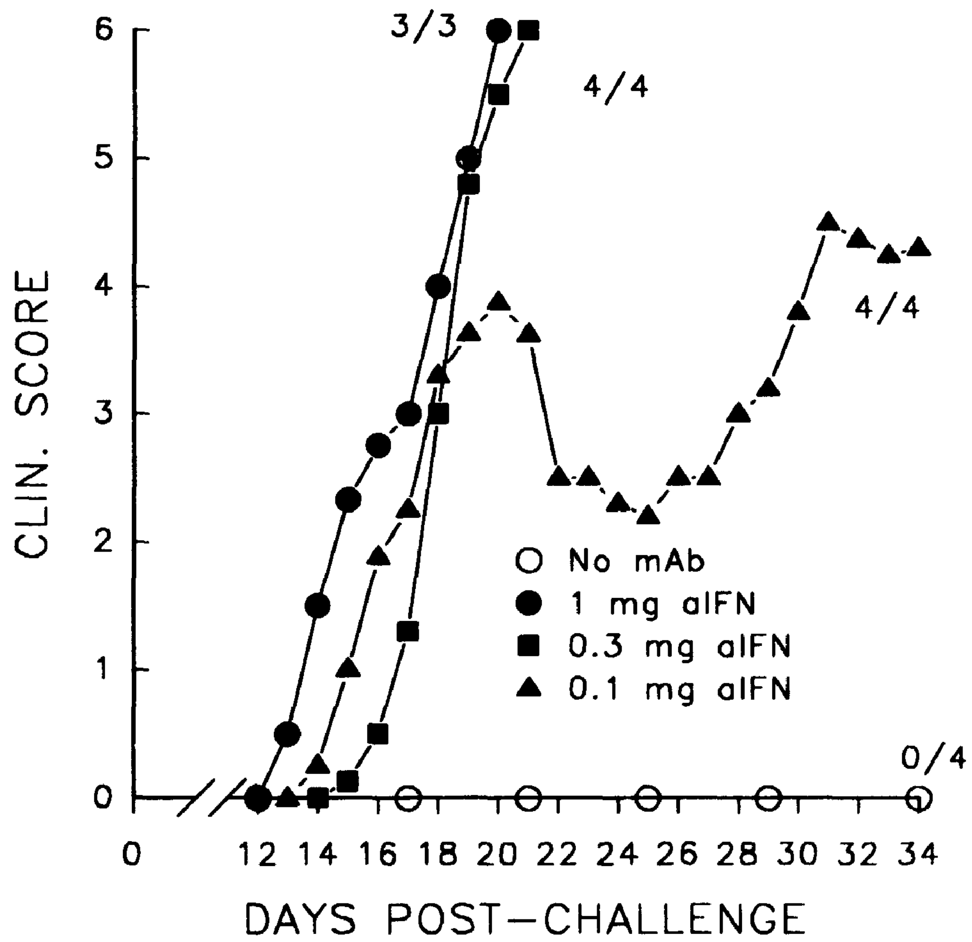
3.3.2 Passive EAE

In contrast to active disease, in the passive transfer of EAE, only 1 mg but neither 300 μg nor 100 μg aIFN mAb determined severe EAE (Fig. 10). All recipients of cells plus 1 mg anti-IFNγ died within 6 days of disease onset. On the other hand, disease severity was similar in recipients of cells alone and of cells plus 300 μg or 100 μg aIFN mAb (max. clin. score of 3.5 to 4 in all three groups). It should be noted, however, that disease duration was significantly longer in recipients of cells plus either 100 μg or 300 μg aIFN mAb. Animals in these two groups remained sick for about a week at which time the control mice (ie. recipients of cells alone) had recovered.

3.4 Effect of aIFN mAb treatment at various time points after active challenge or after passive transfer of EAE.

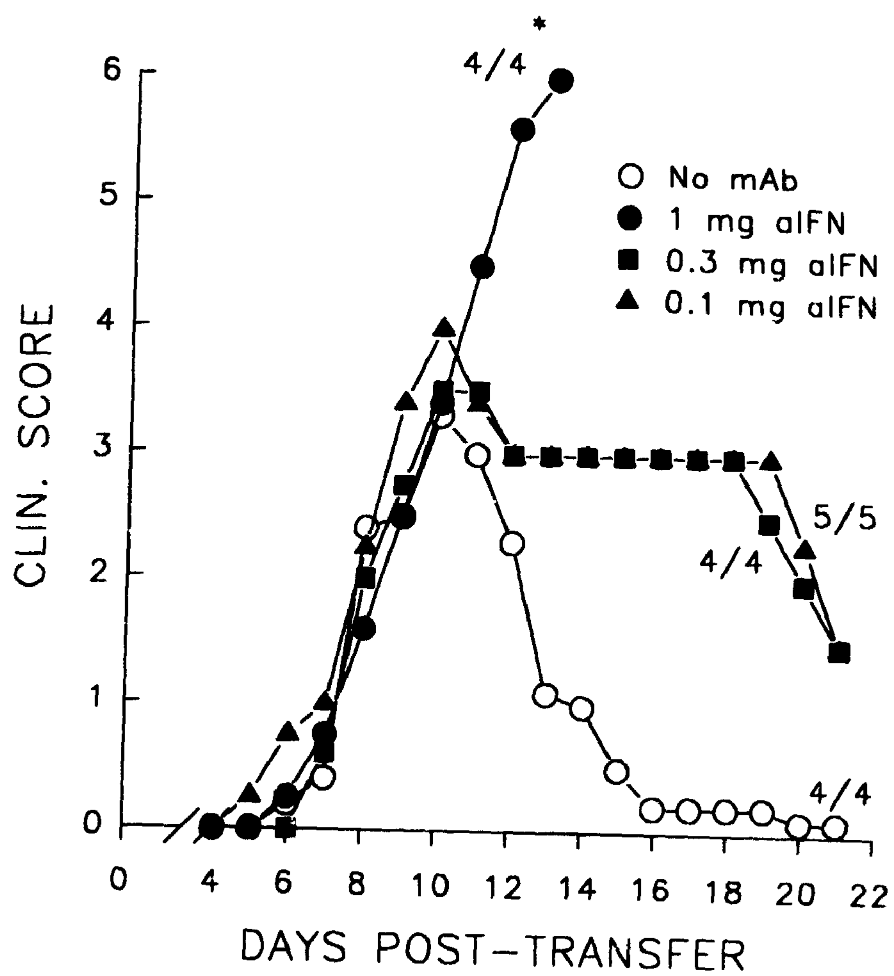
The results presented so far illustrate that aIFN mAb enhances EAE not only in the susceptible mouse strain but also in the refractory strains. Jacob et al. (1989) reported that in adjuvant arthritis, aIFN mAb had either a disease-dampening or disease-potentiating effect depending on the time of mAb administration. Thus,

Fig. 9 Effect of treatment with various amounts of anti-IFN mAb on actively induced EAE. SJL/J mice were challenged with MBP-CFA on days 0 and 7. Following each challenge, the animals were given an i.v injection of either 1 mg (●), 0.3 mg (■), or 0.1 mg (▲) aIFN mAb. Control mice were challenged but received no mAb (○). The number of animals with overt disease over the total number of animals in each group is indicated. All groups showed clinical scores that were significantly different from those of control animals ($p < 0.05$).



3/3
4/4
4/4
0/4
CLIN. SCORE
DAYS POST-CHALLENGE
0
1
2
3
4
5
6
0
12 14 16 18 20 22 24 26 28 30 32 34
No mAb
1 mg aIFN
0.3 mg aIFN
0.1 mg aIFN

Fig. 10 Effect of treatment with various amounts of anti-IFNγ mAb on passively induced EAE. Each animal received ip a mixture of 20 x 10^6 MBP-activated cells plus either 1 mg (●), 0.3 mg (■), or 0.1 mg (▲) aIFN mAb. Control animals were given 20 x 10^6 MBP-activated cells only (○). The number of mice with clinical EAE over the total number of mice in each group is indicated. The asterisk marks the group of animals with clinical scores that were significantly different from those of control animals ($p < 0.05$).



CLIN. SCORE
DAYS POST-TRANSFER
No mAb
1 mg aIFN
0.3 mg aIFN
0.1 mg aIFN
4/4
*
5/5
4/4
4/4
0
1
2
3
4
5
6
0
4
6
8
10
12
14
16
18
20
22

experiments were performed to establish whether the timing of aIFN mAb administration was also critical in determining the disease outcome in EAE.

3.4.1 aIFN mAb treatment at various time points after active challenge

SJL/J mice were treated with the mAb at various time points following the MBP-CFA challenge. As shown in Fig. 11, a single iv injection of 1 mg aIFN mAb on day 0 appeared to be the most effective when compared to the same amount of mAb given on day 7, 14 or 21. Overt EAE (clin. score of between 3 and 4) was observed in three of five recipients of aIFN mAb on day 0 while none or only one of five mice developed the disease following mAb administration at the other time points. Furthermore, the time of disease onset (day 12 post-challenge), the peak of disease (day 15 post-challenge) and severity in these 3 animals were almost the same as those of the animals treated with two doses of aIFN mAb on days 0 and 7. However, since disease incidence was different in the two groups, and since the animals receiving one mAb injection on day 0 had variable disease onset, the average clin. scores of the 2 groups on any given day appeared different. When the mAb was given on day 7 or 14, clinical signs were not detected until days 25 to 34 post-challenge.

3.4.2 aIFN mAb treatment at various time points after cell transfer

We next examined the effect of aIFN mAb administration at different time points after the transfer of MBP-activated lymphoid cells. Groups of mice were injected with either cells alone or with cells plus 1 mg aIFN mAb on day 0, day 4 or day 7 after the cell transfer. The results in Fig. 12 demonstrated that the disease was drastically potentiated only when aIFN mAb was given on day 0 ($p < 0.05$). By

day 6 post-transfer, all mice in this group developed EAE that was more severe than that in the controls (ie. recipients of cells alone) (mean max. clin. scores of 5.2 vs 2.8). In contrast, Ab administration on day 4 or 7 post-transfer had no effect on disease severity. Statistical analysis further confirmed that there was no significant difference among these 3 groups. Taken collectively, the results from Fig. 11 and 12 indicate that the potentiating effect of aIFN mAb in EAE is exerted early in both the active and passive induction of disease.

3.5 Effect of aIFN mAb neutralization at various time points in the passive and active induction of EAE

To gain more insight into the mechanism whereby IFNγ regulates EAE, we started to investigate at what stage in disease induction IFNγ (or aIFN mAb) exerts its effect. To address this question, we biotinylated the aIFN mAb, and treated the mice with the biotinylated preparation. Avidin was then injected at various times in order to remove the biotinylated mAb and neutralize its effect. The ability of the biotinylated aIFN (bt-aIFN) mAb to enhance EAE was not affected by biotinylation since animals injected with bt-aIFN mAb developed a form of EAE that was comparable to that of animals treated with the unbiotinylated antibody (data not shown).

3.5.1 aIFN mAb neutralization in passive EAE

As seen in Fig. 13, the administration of bt-aIFN mAb following the cell transfer strongly enhanced EAE. All recipients in this group died within 4 to 5 days after disease onset. The increase in disease severity was due to the bt-aIFN mAb since the administration of the biotinylated form of an isotype-matched Ab was

Fig. 11 Effect of anti-IFNγ mAb treatment at various time points after active challenge. Groups of SJL/J mice were challenged with MBP-CFA on days 0 and 7. They were treated with an iv injection of 1 mg aIFN mAb on day 0 (△), day 7 (◇), day 14 (□), or day 21(▽). One group of animals received mAb on both days 0 and 7 (●). The data are presented as the average clinical score of all animals in the group on each day. The number of mice with clinical EAE over the total number of mice in each group is indicated.

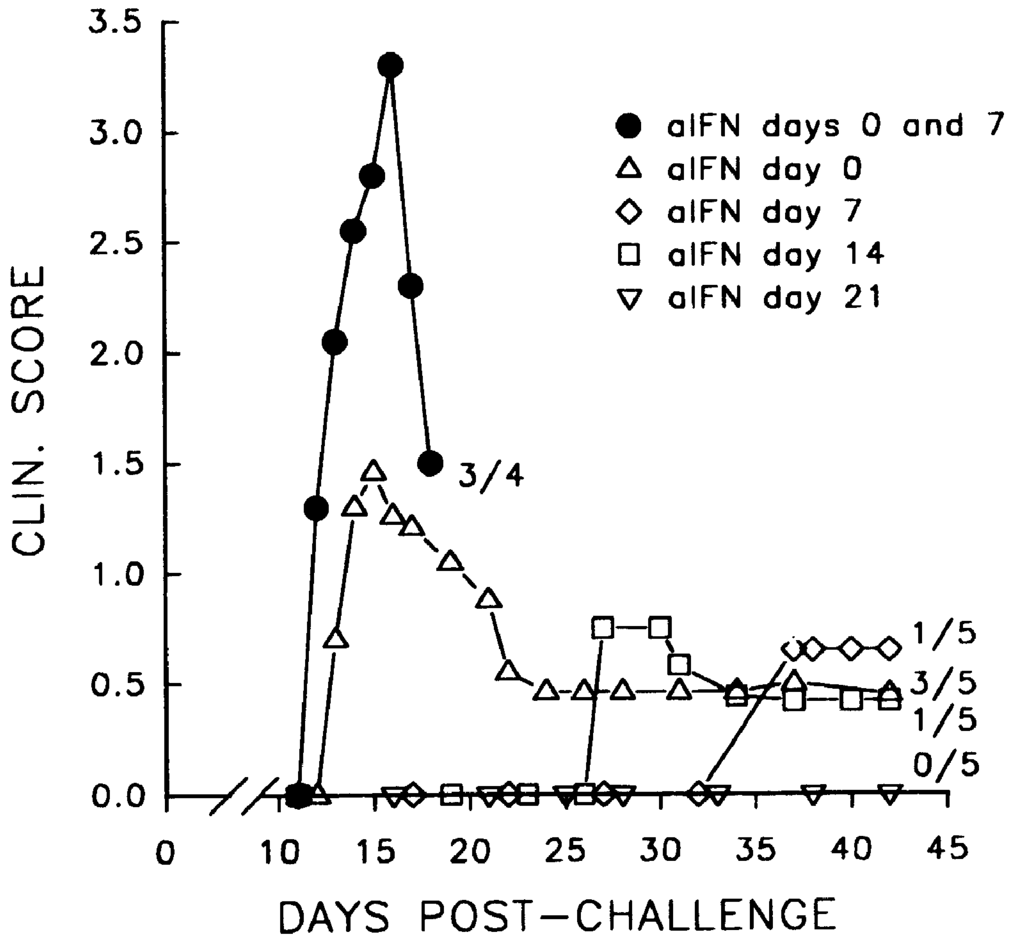
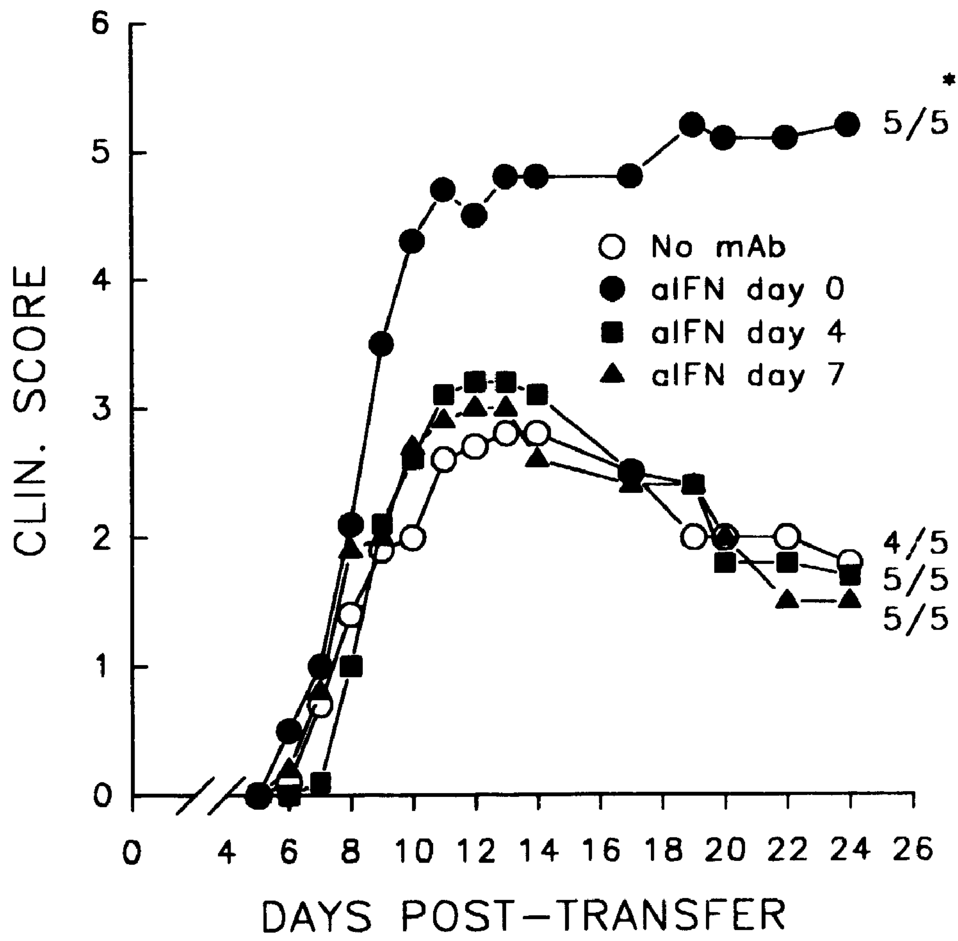

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CLIN. SCORE
aIFN days 0 and 7
aIFN day 0
aIFN day 7
aIFN day 14
aIFN day 21
3/4
1/5
3/5
1/5
0/5
0
10
15
20
25
30
35
40
45
DAYS POST-CHALLENGE

Fig. 12 Effect of anti-IFNγ mAb treatment at various time points after the transfer of MBP-activated lymphoid cells. Groups of SJL/J mice were injected ip with 20 x 10^6 live MBP-activated lymphoid cells. One mg aIFN mAb was either mixed with the cells (day 0) (●), or was given iv on day 4 (■), or on day 7 post-transfer (▲). Control animals received the same number of MBP-activated cells in medium alone (○). Data are presented as the average clinical score of all animals in the group. The number of animals that developed EAE over the total number of animals is indicated. The asterisk marks the group of animals with clinical scores that were significantly different from those of the controls ($p < 0.05$).


CLIN. SCORE
DAYS POST-TRANSFER
No mAb
aIFN day 0
aIFN day 4
aIFN day 7
5/5
*
4/5
5/5
5/5
0
1
2
3
4
5
6
0
4
6
8
10
12
14
16
18
20
22
24
26

without effect (data not shown). The observation that the disease-enhancing effect of bt-aIFN mAb was abolished when avidin was given immediately (ie. within 1 hr) following the mAb injection confirmed that the avidin could effectively neutralize the bt-aIFN mAb. In all mice that received the avidin at 24, 48 or 96 hr after the administration of bt-aIFN mAb, the disease was comparable to that in mice treated with bt-IFNγ without any avidin injection. All of the animals in these 3 avidin treated groups died or had to be sacrificed *in extremis* by day 10 to 12 post-transfer (5 animals per group). The observation that the disease-potentiating effect of bt-aIFN mAb was eliminated when the avidin was given within 1 hr (time 0) but not after 24hr suggested that aIFN mAb, and by implication IFNγ, exerts its effect early and within 24 hr after its administration since its removal after 24 hr was unable to reverse its effect.

In the experiments performed so far, aIFN mAb was given at the time of cell transfer. We investigated next whether the EAE enhancing effect of aIFN mAb could be observed if the Ab was given before the cell transfer. Six groups of mice were included in these experiments (Table 4). It can be seen that the treatment with bt-aIFN mAb either 24 hr prior to (-24 hr) or at the time of cell transfer determined EAE exacerbation. In these groups, all recipients died of the disease (mean max.clin. score of 6) while the recipients of cells alone developed only moderate EAE (mean max. clin. score of 2.8). In contrast, the enhancing effect of the bt-mAb was abolished when avidin was given immediately (ie. within 1 hr) following the mAb injection (mean max. clin. score of 3). It is interesting to note that when aIFN mAb was given at -24 hr, the injection of avidin at time 0, ie.

Fig. 13 Effect of anti-IFNγ mAb removal at various time points after cell transfer. Lymph node cells from MBP-CFA sensitized SLJ/J mice were incubated with 50 μg/ml MBP for 96 hr. Groups of 5 syngeneic recipients were given 20 x 10^6 MBP-activated live cells ip, and 1mg biotinylated anti-IFNγ mAb (bt-aIFN) iv immediately following the cell transfer (day 0) (●). Several groups of animals were also treated iv with avidin (Av.) (2 mg/mouse) at various time points as indicated. Control animals were injected with cells alone (○). EAE incidence was 100% in all groups. The results are expressed as the mean clinical score of all animals in a group on each day. The asterisks indicate groups of animals with clinical scores that were significantly different from those of controls ($p < 0.05$).

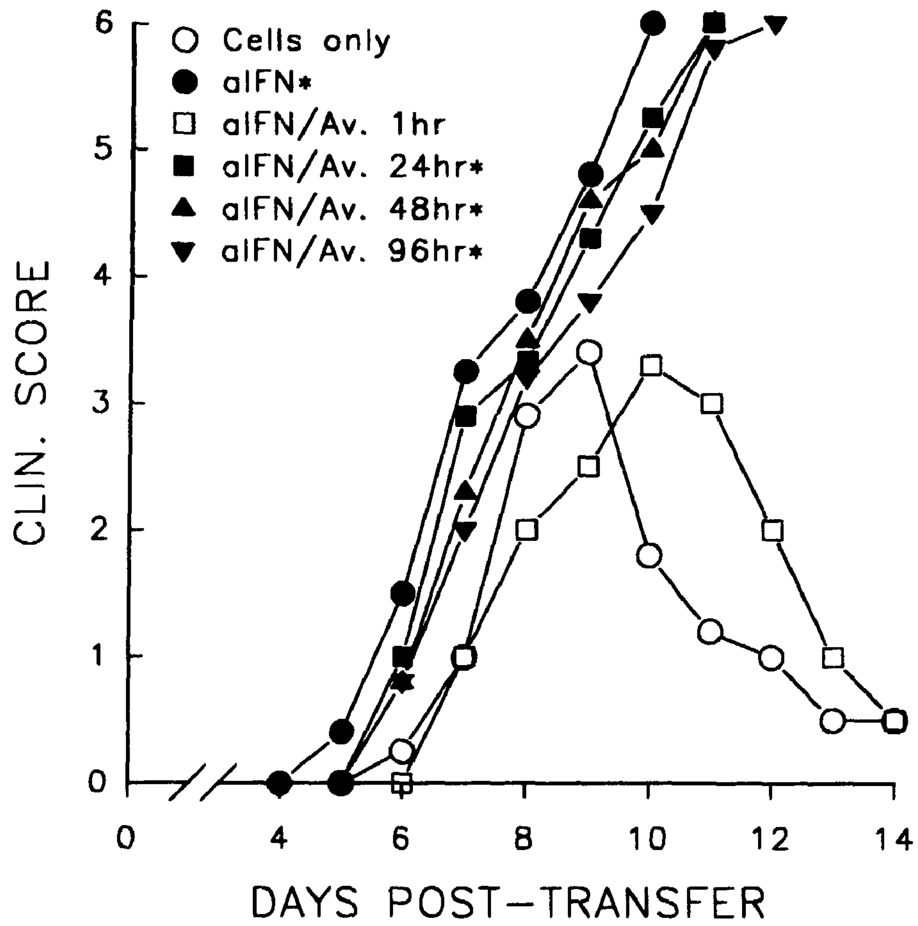

Cells only
aIFN*
aIFN/Av. 1hr
aIFN/Av. 24hr*
aIFN/Av. 48hr*
aIFN/Av. 96hr*
CLIN. SCORE
DAYS POST-TRANSFER
0
1
2
3
4
5
6
0
4
6
8
10
12
14

Table 4

Effect of aIFN mAb Treatment on Passive EAE when Given before Cell Transfer

Treatment[a]		EAE/Total[b]	MCS[c]
aIFN mAb[d]	Avidin[e]		
None	None	3/4	2.8
Day 0	None	4/4	<u>6.0</u>
-24 hr	None	4/4	<u>6.0</u>
-24 hr	-23 hr	4/4	3.0
-24 hr	Day 0	4/4	<u>5.5</u>
-24 hr	+24 hr	4/4	<u>5.5</u>

[a] Lymph node lymphocytes from MBP-CFA sensitized SJL/J mice were incubated with 50 μg/ml MBP for 96 hr at 37°C. Each syngeneic recipient was injected ip with 20 x 10^6 live cells at time 0.

[b] Number of animals with overt EAE over the total number. In all mice, disease onset was around day +7.

[c] Mean clinical scores. Underlined values differ significantly from those of untreated group (recipients of cells alone).

[d] Each animal received 1 mg of bt-aIFNγ mAb iv either on the day of cell transfer (time 0) or 24 hr before (-24 hr).

[e] Each mouse received 2 mg avidin in saline, iv at the times indicated. For mice receiving bt-aIFN mAb and avidin on the same day, the avidin was given approximately within 60 min after the antibody.

immediately before the cell transfer, failed to abolish the disease-potentiating effect of bt-aIFN mAb. This observation further supported the notion that aIFN mAb acts early, and that 24 hr between the time of mAb and avidin administration is sufficient for the Ab to exert its disease-potentiating effect.

Enhanced disease severity in recipients of bt-aIFN mAb at -24 hr was attributable to the neutralization of IFNγ since similar treatment using the biotinylated isotype-matched control mAb was without effect (Fig. 14A). In this experiment, the mice were injected with the bt-aIFN or its bt-control mAb 24 hr prior to the cell transfer (-24 hr), and with avidin at various times thereafter. Control mice received the cells alone without any mAb or avidin (group a). As observed previously, when the bt-aIFN mAb was given at -24 hr without avidin , the disease was significantly potentiated. All mice died by day 14 post-transfer (group b). It should be noted that in this case, avidin was not given, and hence, it is likely that the Ab was still present at the time of cell transfer. On the other hand, the injection of the biotinylated isotype-matched Ab was without effect (group c).The disease-enhancing effect was abolished when avidin was given shortly following the mAb injection (within 1 hr), confirming the ability of avidin to neutralize the bt-Ab (group d). Animals that received the bt-aIFN mAb at -24 hr, and the avidin 12 hr later (-12 hr) (group e) developed EAE which was not different from the control (mean max. clin. score of 3.3 in both groups). This observation suggested that during the 12 hr period after its administration, avidin could efficiently neutralize the bt-aIFN mAb. Otherwise, if sufficient amounts of bt-mAb were still present at the time of cell transfer, one would expect to see some disease enhancement.

Since the results in Fig. 14A suggested that avidin could effectively neutralize the bt-aIFN mAb within 12 hr of its administration, it was of interest to narrow down the time window required for avidin to work. To examine this parameter, mice were given the bt-mAb at -24 hr and avidin at -12 hr, -8 hr, -3 hr or 0 hr relative to the cell transfer. It can be seen from Fig. 14B that the administration of bt-aIFN mAb without the avidin treatment exacerbated EAE significantly (group b) as all animals died within a week from disease onset, whereas recipients of cells alone developed much milder symptoms (group a) (mean. max. clin. score of 3). As seen previously, when bt-aIFN mAb was given at -24 hr and avidin -12 hr (group c), the mice developed EAE which was comparable to that in the control, confirming that 12 hr were sufficient for avidin to neutralize the bt-mAb efficiently before the cell transfer. In the next two groups, the bt-mAb was given at -24 hr and avidin at either -8 (group d) or -3 hr (group e) relative to the cell transfer. No statistically significant difference in disease severity was observed in these mice when compared to the controls (group a). However, disease duration in groups d and e was significantly longer than that in the control ($p < 0.05$). In the control group, disease started around day 6, peaked between day 8 and 10, and the animals started to recover by day 11. On the other hand, mice receiving avidin at -8 hr or -3 hr showed signs of EAE which persisted until day 40, when the animals were sacrificed. These results suggested that neither 3 or 8 hr were sufficient for the avidin to neutralize completely the exacerbating effect of the bt-aIFN mAb. They were consistent with the fact that sufficient aIFN mAb was left behind at the time of cell transfer to change the evolution of the disease from an acute, remitting episode

to a protracted form. It appears therefore, that avidin requires between 8 to 12 hr to abolish the disease-enhancing effect of the bt-aIFN mAb. The observation that disease was not enhanced in group c where bt-aIFN mAb was given at -24 hr and avidin at -12 hr raised two possibilities that either this 12 hr period was not long enough for the bt-anti-IFN mAb to fully exert its effect, or that the Ab had to act directly on the transferred cells. In the last group of animals, the bt-IFN mAb was given at -24 hr and avidin immediately preceding the cell transfer (0 hr) (group f). Thus, the mice were not allowed any time between the avidin injection and cell transfer, and when transferred, the cells found an environment depleted of IFNγ. Under these conditions, the disease was strongly enhanced, indicating that within a period of 24 hr between the time of its administration and removal, the bt-aIFN mAb could fully exert its effect. Furthermore, this observation also demonstrated that the Ab acted during a time span covering 24 hr prior to cell transfer, thus quite early in disease induction.

Taken collectively, the results from Fig. 14A and B can be summarized as follows: (i) Upon its administration, a period of between 8 and 12 hr is required for avidin to remove the bt-aIFN mAb efficiently (Fig. 14A group e, 14B groups c, d and e). (ii) Within a period of 24 hr after its injection, aIFN mAb can fully exert its disease-enhancing effect (Fig. 14B, group f). (iii) The bt-aIFN mAb acts early in disease induction, during a time period spanning 24 hr prior to cell transfer (Fig 14B, group f). The next question was as to whether the mAb could complete its effect prior to the cell transfer or whether its presence at the time of transfer was essential for its disease-enhancing effect. In the next experiment, we investigated

the ability of bt-aIFN mAb given 48 hr before cell transfer to exacerbate the disease even when its activity was neutralized 12 to 24 hr prior to cell transfer. Groups of mice were treated with bt-aIFN mAb at -48 hr followed by avidin at -24, -18hr or -12 hr relative to cell transfer. The results in Fig. 14C show that the administration of avidin at either 18hr or 12 hr before cell transfer (groups d and e) could not interfere with the disease-exacerbating ability of the bt-aIFN mAb, since the clinical signs of mice in these two groups were not different from those of mice receiving the bt-aIFN mAb alone (group b). However, when avidin was injected 24 hr prior to cell transfer (group c), the aIFN mAb effect was completely abolished, since the course and intensity of the disease were identical to those in the group receiving MBP-activated cells alone (group a). Since in all three instances avidin had sufficient time to neutralize the bt-mAb effect (see Fig. 14A, B), the disease exacerbation after avidin administration at -18 and -12 hr can be explained by assuming that at the time of cell transfer, the biological effect of IFNγ was still sufficiently neutralized. Since 24 hr are sufficient for the Ab to exert its disease-exacerbating effect (Fig. 14B), the failure of the aIFN mAb to exacerbate disease when avidin was administered 24 hr before cell transfer (group e) can be explained only by postulating that this time period but neither 12 hr nor 18 hr period was sufficient to allow normally produced IFNγ to reach biologically effective levels in the recipients. These experiments were repeated with similar results (data not shown).

The results in Fig. 14D further strengthen the hypothesis that within a period of 24 hr following the avidin injection prior to cell transfer, the animals were able to restore IFNγ levels. As can be seen, treatment of bt-aIFN at -72 hr without any

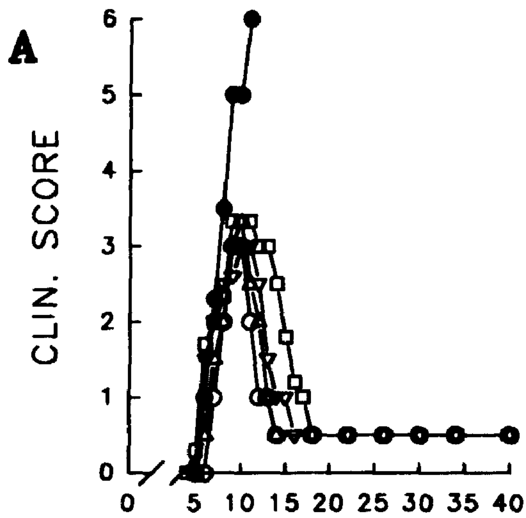
avidin injection resulted in enhanced EAE (group b). In contrast, animals treated with bt-aIFN mAb at -72 hr and avidin at -24 hr developed disease manifestations that were comparable to those of recipients of cells only (mean max. clin. score of 3) (group b vs group a), thus supporting the possibility that the 24 hr period following the avidin injection prior to the cell transfer was sufficient for the animals to replenish the IFNγ level to biologically effective amounts. On the other hand, all animals that were injected with bt-aIFN mAb at -36 hr and avidin at -12 hr (group d) developed severe EAE and died within a week after onset, thus, consistent with the possibility that 12 hr was not sufficient for the mice to build up the IFNγ level.

The results obtained so far can thus be summarized as follows: (i) aIFN mAb can fully exert its effect within 24 hr of its administration. (ii) The aIFN mAb exacerbates EAE only if administered either before or at the same time of the cell transfer but not later. (iii) When administered prior to cell transfer, the disease-exacerbating effect still persists for up to 18 hr even after the deliberate removal/neutralization of the bt-IFNγ mAb. (iv) Removal of the Ab between 18 hr and 24 hr prior to cell transfer results in the restoration of the disease pattern observed in untreated recipients of MBP-activated cells.

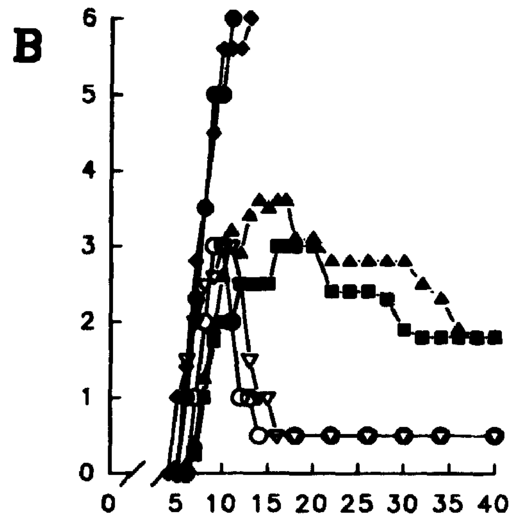
The neutralization of the bt-aIFN mAb by avidin occurs by virtue of biotin-avidin complex formation. To determine whether or not the biotin-avidin complexes themselves contributed nonspecifically to disease modulation, groups of mice were treated with a biotinylated isotype-matched mAb (bt-GL113), or with a mixture of bt-GL113 and nonbiotinylated aIFN mAb, 48 hr before cell transfer followed by avidin 24 hr before cell transfer (Fig. 15). The disease pattern of these mice was

Fig. 14 Effect of anti-IFNγ mAb removal at various time points. SJL/J mice were injected ip with 20×10^6 syngeneic, MBP-activated lymph node cells at time 0. Except for the control animals which received the cells only (○), all others were treated iv with 1 mg bt-aIFN or biotinylated control mAb (bt-GL113) at either -24 hr (in A and B), -48 hr (in C), or at -72 or -36 hr (in D). Avidin (2mg/mouse) was given iv just prior to cell transfer (time 0) or at various time points as indicated. Incidence of EAE was 80-100% in all groups (5-6 animals/group). The data represent the mean clinical score of all animals in the group on each day. The asterisks indicate groups of animals with clinical scores which were significantly different from those of control mice ($p < 0.05$). The letters in brackets are the group numbers by which the animals are referred to in Results.

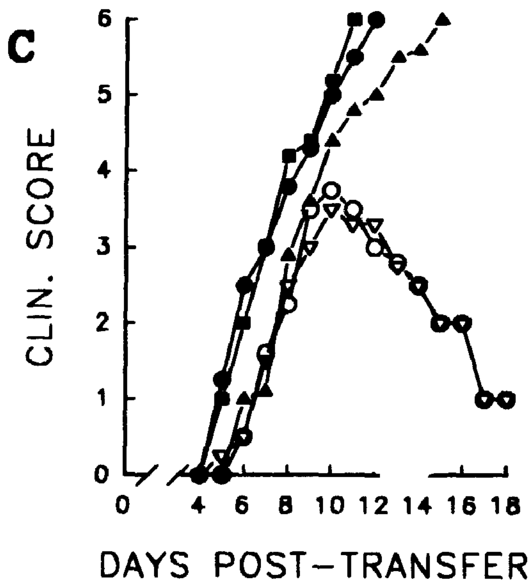
O Cells only (a)
● bt-aIFN/No Av. (b)*
△ bt-GL113/No Av. (c)
□ bt-aIFN/Av. -23hr (d)
▽ bt-aIFN/Av. -12hr (e)



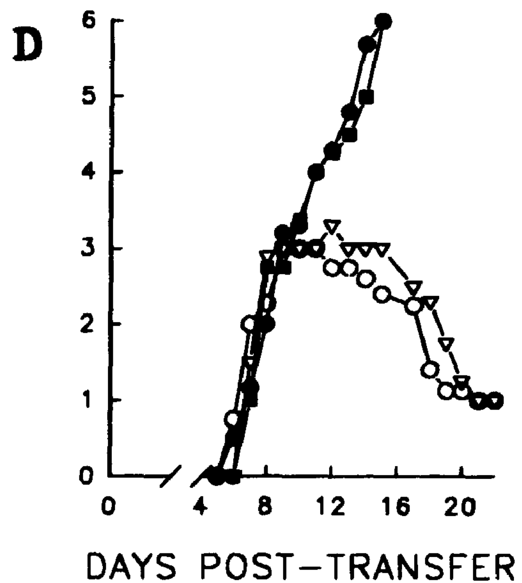
O Cells only (a)
● No Av. (b)*
▽ Av. -12hr (c)
■ Av. -8hr (d)
▲ Av. -3hr (e)
◆ Av. 0hr (f)*



O Cells only (a)
● No avidin (b)*
▽ Av. -24hr (c)
▲ Av. -18hr (d)*
■ Av. -12hr (e)*



O Cells only (a)
● bt-aIFN -72hr (b)*
▽ bt-aIFN -72hr/Av. -24hr (c)
■ bt-aIFN -36hr/Av. -12hr (d)*

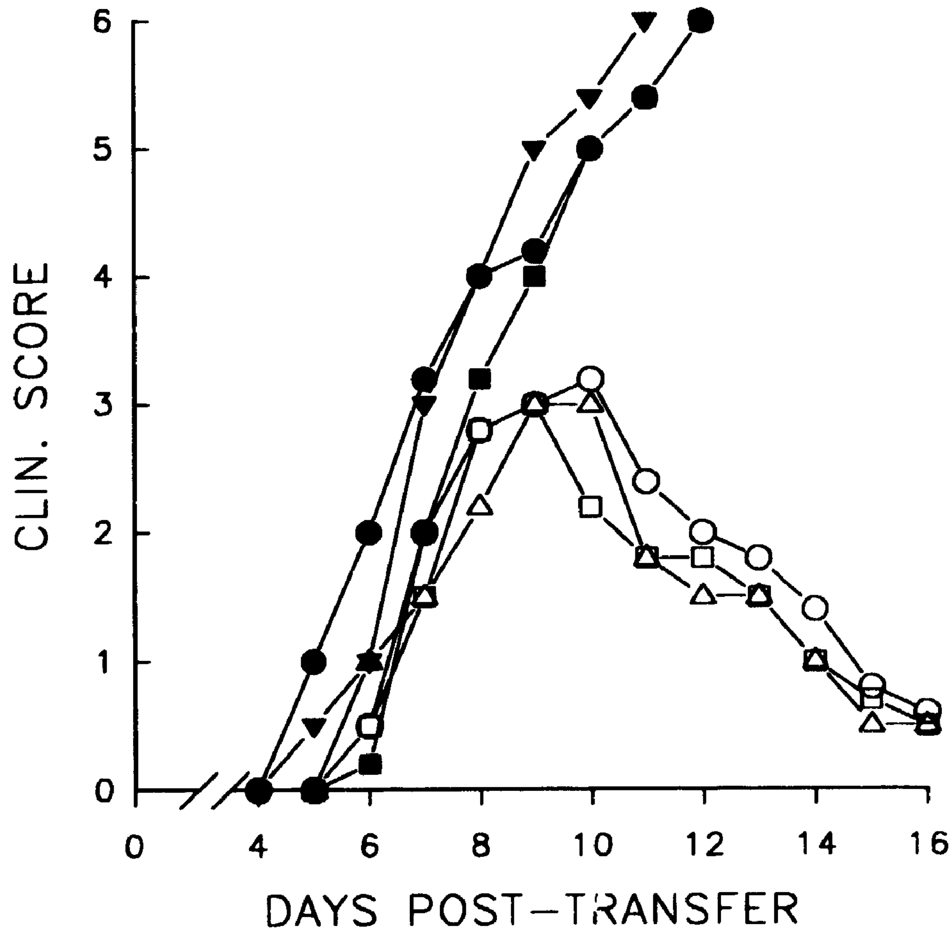


compared to that of mice receiving bt-aIFN mAb with or without avidin. Consistent with previous results, the injection of bt-aIFN mAb at -48 hr drastically potentiated EAE (group b) while recipients of cells alone developed a mild form of EAE (group a) (mean max. clin. score of 3.2). This enhancing effect was abolished when avidin was injected within 1 hr after the mAb (group c). As in Fig. 14C, all 4 mice that received the bt-anti-IFNγ at -48 hr and avidin at -24 hr (Fig. 15, group d) exhibited disease that was comparable to that of controls (group a). Animals receiving a mixture of unbiotinylated aIFN plus the bt-isotype matched control Ab at -48 hr, and avidin at -24 hr (group e) exhibited exacerbated disease indicating that the presence of complexes formed between the bt-GL113 and avidin did not affect the ability of nonbiotinylated aIFN to potentiate EAE. Similarly, avidin given in the absence of bt-Ab was without effect since the disease course of mice receiving bt-aIFN only at -48 hr and of those receiving nonbiotinylated aIFN at -48 hr and avidin within 1 hr later were essentially the same (groups b and f).

3.5.2 aIFN mAb neutralization in active EAE

Groups of mice were injected each with 300 μg bt-aIFN mAb following MBP-CFA challenge on days 0 and 7. They were also treated with avidin (600 μg/mouse) at various time points thereafter (Fig. 16). Control mice were challenged with MBP-CFA only. As can be seen, except for the aIFN mAb-treated animals receiving avidin within 1hr after the mAb injection, all other treated animals that received avidin at any later time developed severe EAE. There was no statistically significant difference in disease severity between recipients of avidin at 12 hr, 24 hr or 48 hr after the bt-aIFN mAb injection. On the other hand, none of the challenged but

Fig. 15 Effect of anti-IFNγ or its control mAb removal at various time points. SJL/J mice were injected ip with 20×10^6 MBP-activated lymph node cells at time 0. Forty eight hours prior to cell transfer, groups of mice were given iv 1 mg nonbiotinylated anti-IFNγ (aIFN), biotinylated anti-IFNγ (bt-aIFN) or aIFN plus bt-GL113. Avidin (2 mg/mouse) was then given iv at various time points as indicated. EAE incidence was 100 % in all cases (4-5 animals/group). The results are presented as the mean clinical score on each day for all mice in the group. The asterisks indicate groups of animals with clinical scores that were significantly different from those of controls (recipients of cells only) ($p < 0.05$). The letters in brackets indicate the group numbers by which the animals are referred to in Results.



Cells only (a)
bt-aIFN (b)*
bt-aIFN/Av. -47hr (c)
bt-aIFN/Av. -24hr (d)
aIFN + bt-GL113/Av. -24hr (e)*
aIFN/Av. -47hr (f)*
CLIN. SCORE
0
1
2
3
4
5
6
0
4
6
8
10
12
14
16
DAYS POST-TRANSFER

nontreated animals developed EAE. These results demonstrate that in the active induction of EAE, a period of 12 hr between the aIFN mAb and avidin injections was sufficient for the mAb to fully exert its disease-enhancing effect.

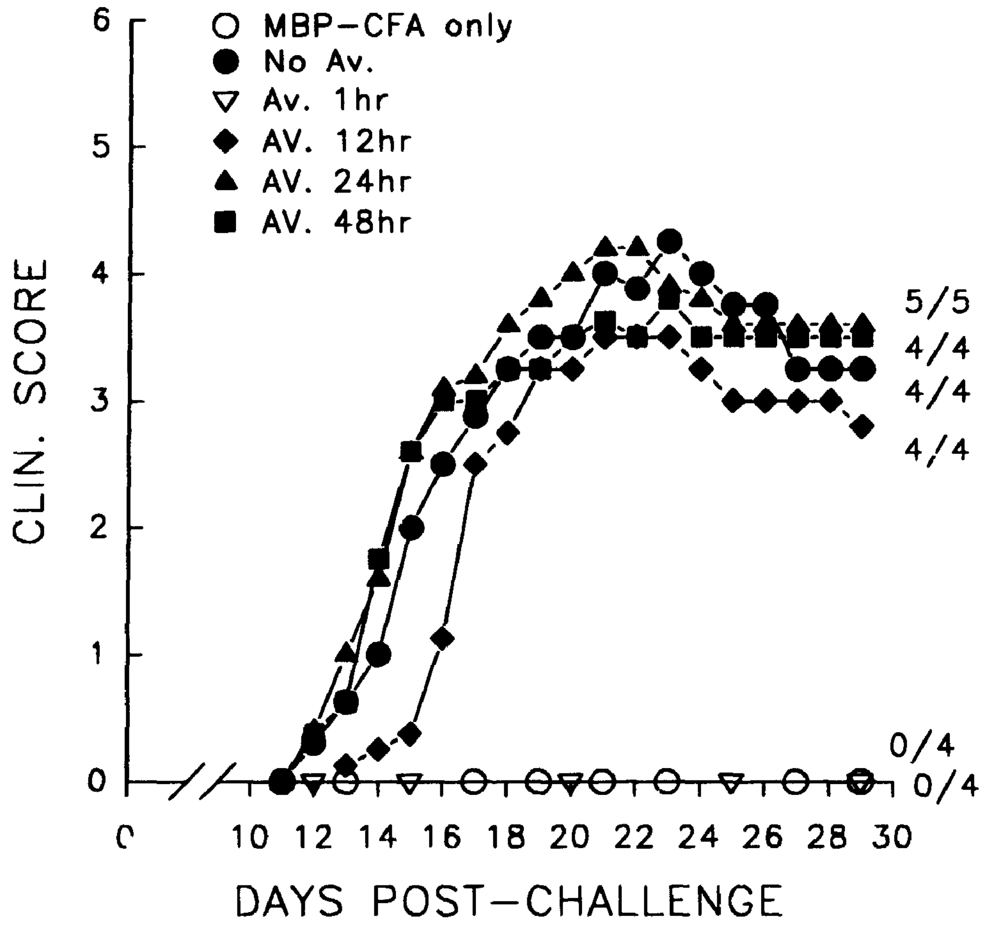
3.6 Expression of VLA-4, VCAM-1 and ICAM-1 molecules in MBP-CFA challenged, aIFN mAb-treated or nontreated SJL/J mice

A number of adhesion molecules and their ligands have been shown to be involved in orchestrating recruitment of lymphocytes to inflammatory sites. Among these is the $\alpha 4\beta 1$ intergrin or the VLA-4 antigen which has been considered the key in the entry of cells into the CNS (Baron et al., 1993; Tanaka et al., 1993). The migration of encephalitogenic T cells into the CNS appears to depend on the increased expression of the integrins VLA-4 and LFA-1 on T cells, and of their ligands such as VCAM-1 and ICAM-1 on the endothelial cells of the BBB. It was reported by Yednock et al. (1992) that EAE could be prevented by treatment with Ab against VLA-4. In addition, Baron et al. (1993) demonstrated that encephalitogenic T cell clones expressed much higher levels of VLA-4 than nonencephalitogenic clones. The above findings raised the possibility that in our case, aIFN mAb enhanced EAE through increased expression of VLA-4 and/or of its ligands, and hence, facilitated the entry of inflammatory cells into the CNS.

3.6.1 VLA-4 expression in the lymph nodes and spleen from aIFN mAb-treated and nontreated animals

Active EAE was induced in mice by MBP-CFA challenge followed by aIFN mAb injection on days 0 and 7. One or 2 days after disease onset, mice were sacrificed, and splenocytes and draining LNC were assayed for VLA-4 expression

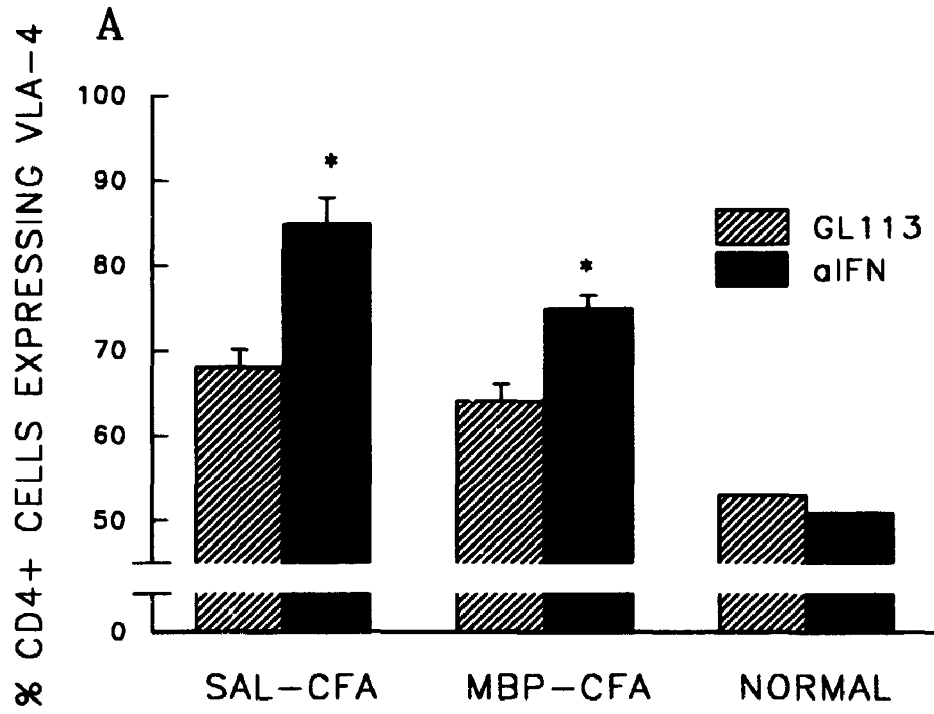
Fig. 16 Effect of anti-IFNγ mAb removal at various time points after active challenge. SJL/J mice were challenged with MBP-CFA followed by an iv injection of 300 μg bt-anti-IFNγ mAb on days 0 and 7 (time 0). The animals were then treated iv with 600 μg avidin 1 hr (▽), 12 hr (◆), 24 hr (▲), or 48 hr (■) after the mAb injection. Control mice were challenged with MBP-CFA only (○). The number of mice with clinical EAE over the total number is indicated in each group. The results represent the mean clinical score of all animals in the group on each day. All groups of animals exhibited clinical scores that were significantly different from those of controls ($p < 0.05$).

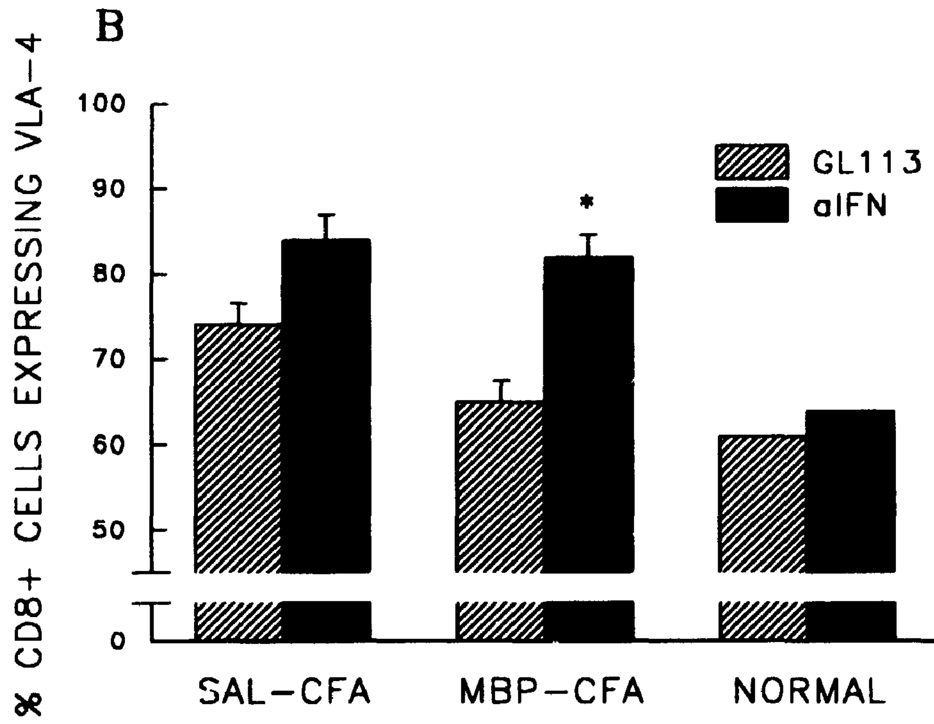


MBP-CFA only
No Av.
Av. 1hr
AV. 12hr
AV. 24hr
AV. 48hr
CLIN. SCORE
0
1
2
3
4
5
6
0
10
12
14
16
18
20
22
24
26
28
30
DAYS POST-CHALLENGE
5/5
4/4
4/4
4/4
0/4
0/4

by flow cytometry. Control mice were challenged with either MBP-CFA followed by treatment with the isotype matched control mAb, or with CFA only (saline-CFA) followed by treatment of aIFN or its control mAb. In all cases, both the experimental and control animals were sacrificed on the same day and assayed similarly. As can be seen in Fig. 17A, roughly 53% of $CD4^+$ cells expressed VLA-4 in the LNC from normal mice. The percentage of $CD4^+$ expressing VLA-4 increased in mice challenged with MBP-CFA or with CFA alone (saline-CFA). Sixty four and 68% of the $CD4^+$ population was found to express the VLA-4 antigen in mice challenged with MBP-CFA and treated with the control mAb GL113 mAb, and in mice injected with saline-CFA and GL113, respectively. Interestingly, in both MBP-CFA or CFA challenged mice, administration of anti-IFN mAb appeared to increase significantly the percentage of $CD4^+$ cells expressing the VLA-4 molecules above that seen in the controls. Up to 85% of the $CD4^+$ cells expressed VLA-4 in the saline-CFA plus aIFN mAb-treated mice in comparison to 68% in the animals receiving saline-CFA plus the control mAb (p value of 0.043). Similarly, 75% of the $CD4^+$ population was shown to be positive for VLA-4 in the MBP-CFA plus aIFN treated mice in comparison to 64% in the animals receiving the control mAb (p value of 0.048). The percentage of $CD8^+$ cells expressing VLA-4 molecules in the LN of the same mice is shown in Fig. 17B. Sixty one percent of the $CD8^+$ cells was found to express the VLA-4 antigen in the LN of normal mice. The percentage of $CD8^+$ cells expressing VLA-4 was slightly increased following the MBP-CFA or CFA challenge plus the isotype control mAb (65% and 74%, respectively). As in the case of $CD4^+$ cells, in the animals challenged with MBP-CFA plus aIFN mAb treatment, up to 82% of the

Fig. 17 Expression of VLA-4 molecules by lymph node cells isolated from anti-IFNγ mAb-treated and nontreated mice. SJL/J mice were challenged with either MBP-CFA or CFA alone (sal-CFA) on days 0 and 7 followed by an iv injection of 1 mg of either aIFN mAb (solid bars) or of its isotype-matched control mAb (GL113) (hatched bars). One or two days after disease onset, draining lymph node cells were isolated and assayed by 2-colour cytofluorometry for either CD4/VLA-4 or for CD8/ VLA-4 expression as described under Materials and Methods. Lymph node cells from a normal SJL/J mouse were also similarly analysed. The data were obtained from two mice of each treatment, and expressed as the percentage of $CD4^+$ (A) and $CD8^+$ (B) cells expressing the VLA-4 marker. The asterisks indicate groups of aIFN mAb- treated animals with % cells expressing VLA-4 which was significantly different from that of nontreated animals ($p < 0.05$ as determined by Student's t-test).


A
% CD4+ CELLS EXPRESSING VLA-4
100
90
80
70
60
50
0
GL113
aIFN
*
*
SAL-CFA
MBP-CFA
NORMAL

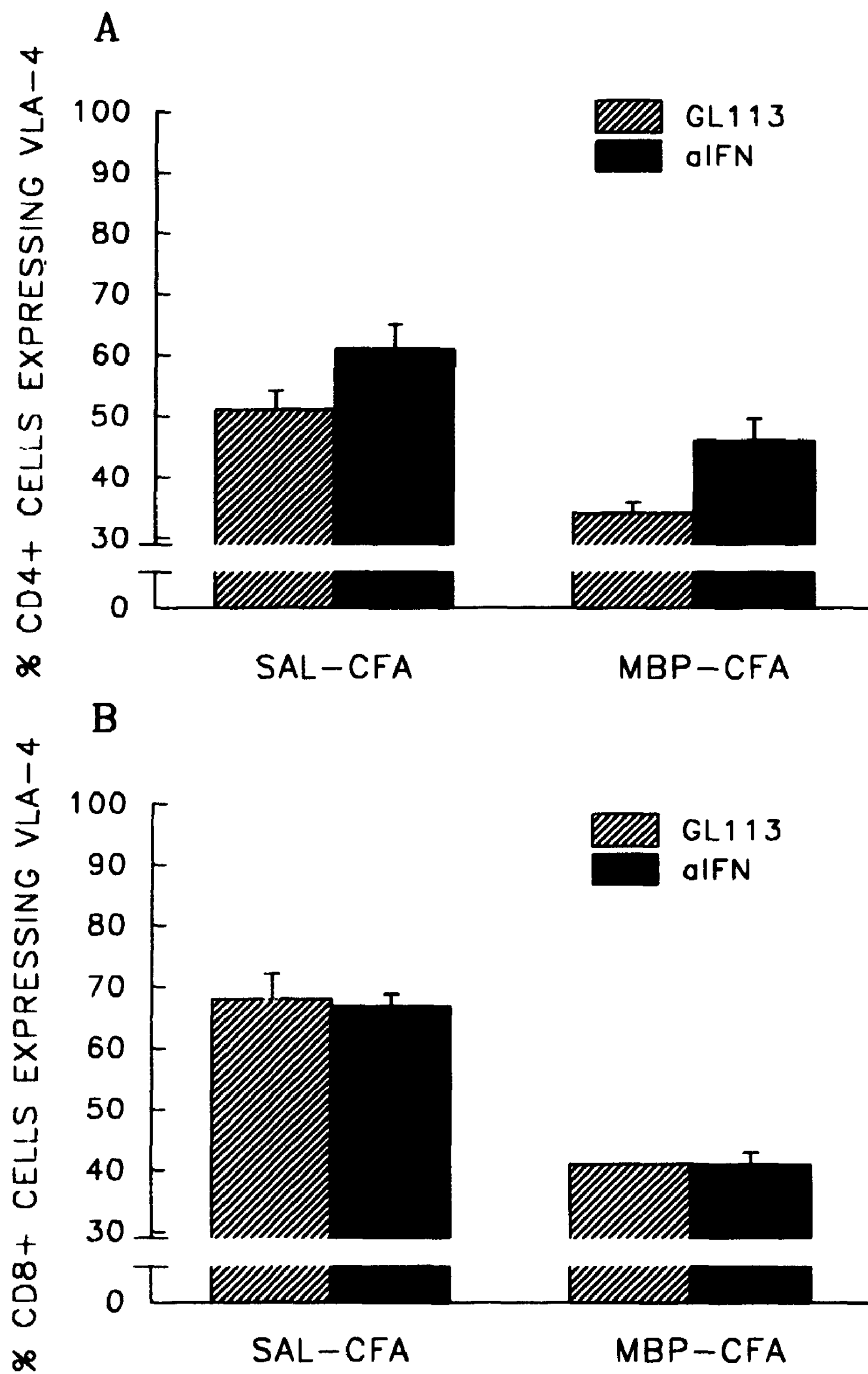

B
% CD8+ CELLS EXPRESSING VLA-4
100
90
80
70
60
50
0
GL113
aIFN
*
SAL-CFA
MBP-CFA
NORMAL

CD8$^+$ population expressed VLA-4 in comparison to only 65% in the animals treated with the control mAb (p value of 0.038). In the animals challenged with CFA followed by aIFN mAb injection, 84% of the CD8$^+$ cells expressed VLA-4 in comparison to 74% in the animals treated with the control mAb. However, this difference was not statistically significant (p value of 0.125).

Overall, the spleen of the same mice contained fewer VLA-4$^+$ cells than lymph nodes. In the animals challenged with MBP-CFA plus aIFN mAb, 46% of the CD4$^+$ population was shown to express the VLA-4 molecules in comparison to 35% in the animals treated with the isotype-matched control mAb (Fig. 18A). Similarly, the percentage of CD4$^+$ splenocytes expressing VLA-4 increased from 51% in the animals challenged with sal-CFA plus control Ab to 61% in the animals receiving aIFN mAb. However, these increases were not statistically significant (p values of 0.190 and 0.093 in the MBP-CFA and Sal-CFA cases, respectively).

In the CD8$^+$ splenocyte population, the percentage of VLA-4$^+$ cells was not affected by the aIFN mAb treatment in either the MBP-CFA or sal-CFA challenged groups (Fig. 18B). Regardless of whether the animals had been given the control or aIFN mAb, about 69% of the total CD8$^+$ splenocytes from CFA challenged animals expressed VLA-4$^+$. Similarly, in the spleen of MBP-CFA challenged and isotype-control mAb-treated or aIFN mAb-treated animals, approximately 40% of the CD8$^+$ population expressed the VLA-4 antigen. In summary, the data from Fig. 17 and 18 demonstrated that after aIFN mAb treatment, the percentage of VLA-4$^+$ cells was increased in the CD4$^+$ and in the CD8$^+$ populations in the lymph nodes, but not in the spleen.

Fig. 18 Expression of VLA-4 molecules by spleen cells isolated from anti-IFNγ mAb-treated and nontreated mice. The spleen cells from the same animals used in Fig. 17 were isolated, and the expression of CD4 and VLA-4 or CD8 and VLA-4 molecules was assayed and expressed as described in Fig. 17.


A
% CD4+ CELLS EXPRESSING VLA-4
100
90
80
70
60
50
40
30
0
GL113
aIFN
SAL-CFA
MBP-CFA
B
% CD8+ CELLS EXPRESSING VLA-4
100
90
80
70
60
50
40
30
0
GL113
aIFN
SAL-CFA
MBP-CFA

3.6.2 Immunostaining for VLA-4, VCAM-1 and ICAM-1 of brain tissues from aIFN mAb-treated and nontreated animals

Inflammatory cells were identified in frozen sections of brain by staining with the anti-CD4 or anti-CD8 mAb. As shown in Fig. 19B, massive infiltrates consisting of $CD4^+$ cells were present in the brain of SJL/J mice with clinical EAE (clin. score of 3) following MBP-CFA challenge and aIFN mAb treatment. The majority of the cells were $CD4^+$. The cells often accumulated around or near to blood vessels forming foci in the white matter, one of the characteristic histopathological features of EAE. There were also foci formed further away from the vessels as the cells invaded into the parenchyma (not shown). Anti-CD8 Ab staining of a serial section of the same brain shown in Fig. 19B revealed that these infiltrates also contained $CD8^+$ cells (Fig. 19C). However, this represented a minor population in comparison to the $CD4^+$ cells. As can be seen in Fig. 19D, staining with the anti-ICAM-1 Ab revealed that the cells within the inflammatory foci are positive for these molecules. Furthermore, endothelial cells of the blood vessels also expressed ICAM-1. Similarly, the endothelial cells were also found to express VCAM-1 (Fig. 19E). It is interesting to note that cells that appeared to be in the process of entering the CNS were found to be in contact with the endothelial cells of the blood vessels (Fig. 19D and E). This is consistent with the notion that contact between inflammatory cells and vascular endothelial cells through adhesion molecule-ligand interactions is the initial critical step in leukocyte trafficking (Raine et al., 1990b). When anti-VLA-4 mAb was used, virtually all infiltrated cells were found to express the VLA-4 molecules (Fig. 19F). Since the same section was not stained with both CD4 and

VLA-4 or CD8 and VLA-4, it is not possible to determine whether the same cell expressed both molecules. It should be mentioned that in all cases, a positive result was due to specific binding of Ab to the appropriate molecules since staining with an irrelevant Ab was found to be negative (Fig. 19A).

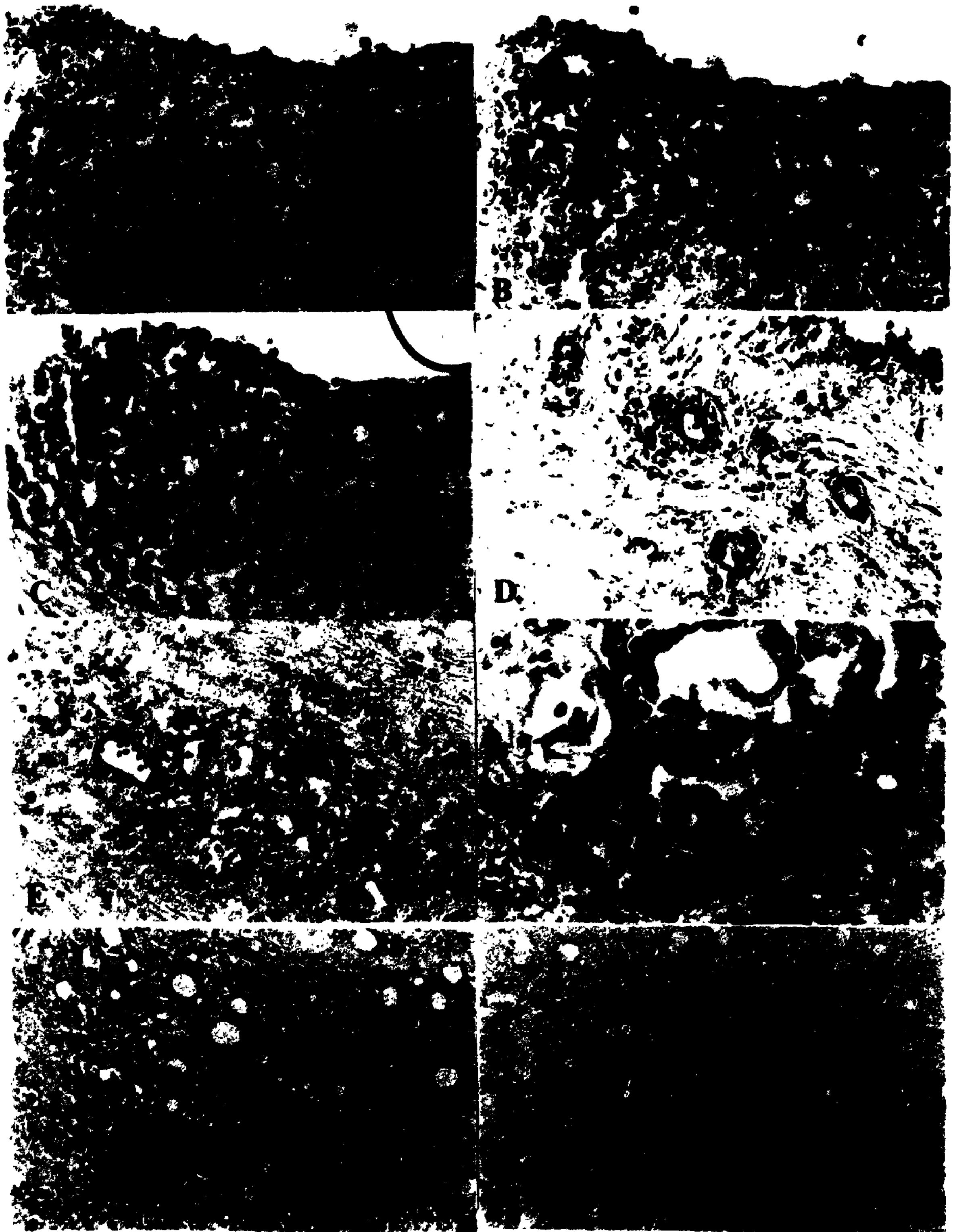
The above observations from brain sections of MBP-CFA challenged and aIFN mAb-treated animals were in contrast to those challenged with MBP-CFA and treated with the isotype-matched control Ab GL113. The animals had no clinical signs of EAE, and showed no abnormal histology. No $CD4^+$, $CD8^+$ or VLA-4^+ cells were found in the brain of these mice (Fig. 19G). Blood vessels were found void of infiltrating lymphocytes, and were stained only very faintly or not at all with ICAM-1 (Fig. 19G) or VCAM-1 (Fig. 19H). Taken together, the immunocytochemical studies shown here demonstrated increased expression of VLA-4, VCAM-1 and ICAM-1 in the CNS of MBP-CFA challenged and aIFN mAb-treated animals in comparison to those treated with the control mAb. These observations were thus consistent with the increased VLA-4 expression by $CD4^+$ lymph node cells from treated animals, as shown before (Fig. 17 and 18).

3.7 IFNγ and TNF α/β production by LNC from MBP-CFA challenged, aIFN mAb-treated and nontreated SJL/J mice

The disease-enhancing effect of aIFN mAb treatment suggested that, contrary to other observations documenting increased IFNγ gene expression in the CNS lesions of mice undergoing EAE (Stoll et al., 1993; Reno et al., 1994a), this cytokine has EAE-inhibitory function, at least during the early phases of disease induction. On the other hand, numerous studies have implicated TNF α/β as a

Fig. 19 Photomicrographs of immunohistochemically stained cryostat brain sections of MBP-CFA challenged, aIFN mAb-treated and nontreated SJL/J mice. (A) Brain section of an aIFN mAb-treated mouse showing a large area of cellular infiltrates. The section was stained with an irrelevant rat mAb, J4.1. The clinical score at sacrifice (day 18 post-challenge) was 3 (x90). Brain sections of the same mouse were also stained with anti-CD4 (B), and with anti-CD8 (C) mAb (x90). The same area is shown in (A), (B) and (C). (D) Anti-ICAM-1 mAb staining of a brain section of the mouse in (A) showing an infiltrate of ICAM-1$^+$ cells around a blodd vessel which was also positive for ICAM-1 (x56). (E) Anti-VCAM-1 Ab staining of a brain section of the mouse in (A) showing several blood vessels expressing VCAM-1 (arrows) (x56). (F) Brain section of an aIFN mAb-treated mouse stained with anti-VLA4 Ab. Clinical score at sacrifice (day 18 post-challenge) was 3) (x225). (G) Anti-ICAM1 mAb staining of a brain section from a mouse treated with the control mAb GL113, and sacrificed on day 19 post-challenge. The animal had no clinical manifestations. The histological section appeared normal (x56). (H) Anti-VCAM-1 staining of a brain section from the mouse in (G) (x56).

SJL/J mice used in this figure were all challenged with MBP-CFA on days 0 and 7. They also received 1 mg of either aIFN or the isotype matched control mAb GL113, on the same days.


B
C
D
E

disease-enhancing cytokine in EAE (Selmaj and Raine, 1988; Powell et al., 1990; Ruddle et al., 1990; Kuroda and Shimamoto, 1991; Selmaj et al., 1991a; Kim et al., 1992; Santambrogio et al., 1993; Stevens et al., 1994). The administration of TNF-α was reported to potentiate EAE in rats, while the treatment with anti-TNF α/β mAb inhibited passive induction of EAE. Based on these studies, we hypothesized that the disease exacerbation observed after aIFN mAb treatment could be explained by a concomitant increase in TNF α/β production by cells from challenged and aIFN mAb- treated mice. It was thus of interest to examine the level of IFNγ and TNF produced *in vitro* by lymphocytes from animals immunized with MBP-CFA or immunized and treated with aIFN mAb.

3.7.1 IFNγ production

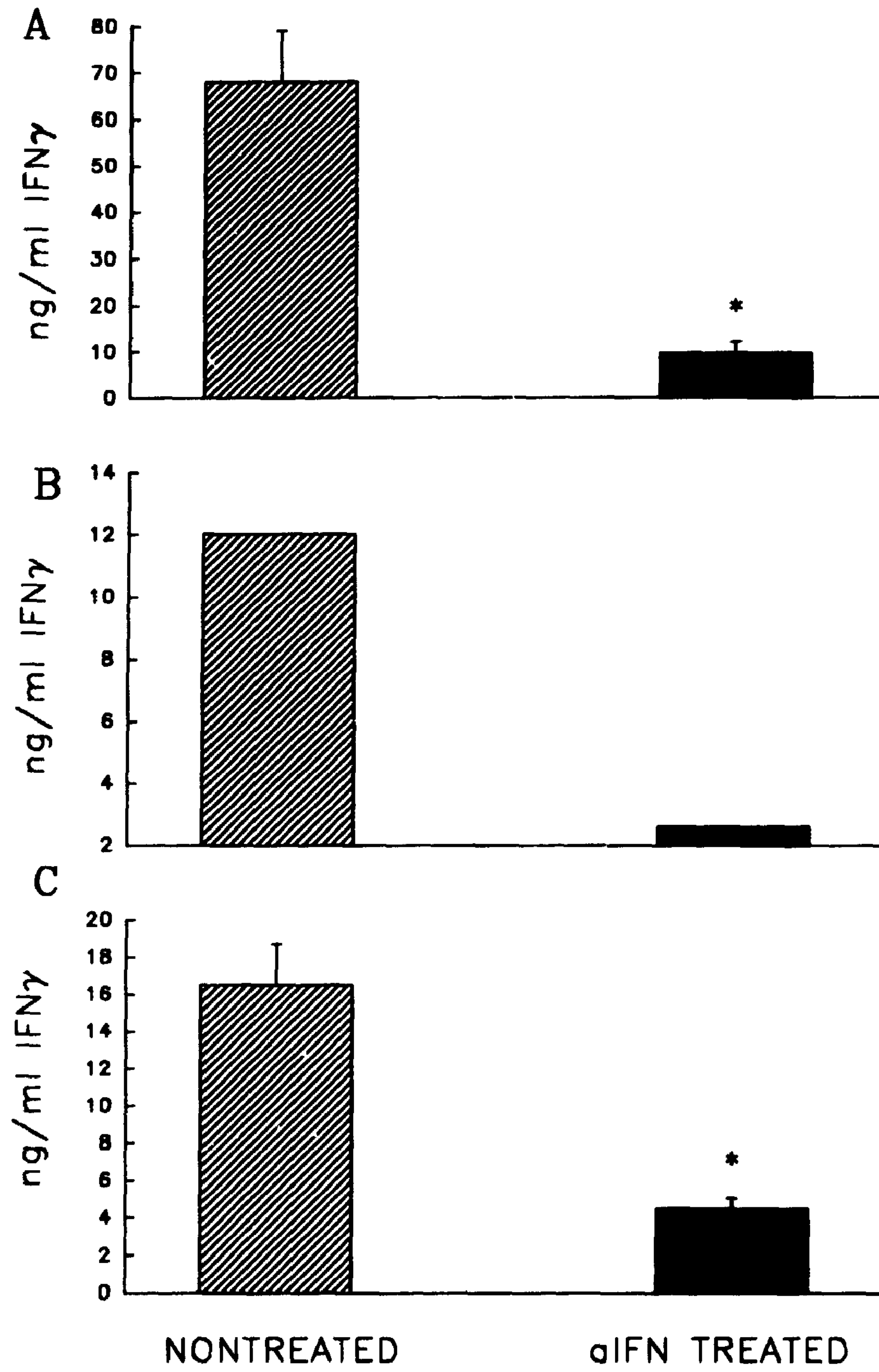
Ten days after immunization with MBP-CFA, the draining lymph nodes were prepared into a single cell suspension. The lymph node cells were grown with MBP as indicated in Materials and Methods, and IFNγ was determined in the 24 hr supernatants by capture ELISA using recombinant mouse IFNγ of known concentration as a standard. Background levels of IFNγ were obtained from cultures grown under the same conditions but in the absence of MBP, and were subtracted from cultures grown with MBP. As shown in Fig. 20A, in the presence of MBP, the amounts of IFNγ produced by LNC of two mice challenged with MBP-CFA were about 7 times higher than those produced by LNC of two mice challenged and treated with aIFN mAb. Cultures from the challenged, untreated mice produced 84 and 52 ng/ml IFNγ (mean value of 68 $\pm$ 11 ng/ml as shown in Fig. 20A) while cultures from challenged and aIFN mAb-treated mice only produced 13

and 6.5 ng/ml IFNγ (mean value of 9.8 ± 2.0 ng/ml as shown). Statistical analysis confirmed that the amount of IFNγ produced by the aIFN mAb-treated animals was significantly lower than that produced by the nontreated animals (p value of 0.035). In all cases, only negligible amounts of IFNγ were detected in similar cultures grown in the absence of MBP (data not shown).

Similar results were obtained when the cultures were initiated 8 days after challenge and mAb treatment (Fig. 20B), or when the mice were immunized twice (on days 0 and 7) following by aIFN mAb treatment on the same days (Fig. 20C). In the latter, cultures were initiated 10 days after the first immunization. In Fig. 20B, LNC from the challenged mouse produced 12 ng/ml IFNγ while those from the challenged and aIFN mAb-treated mouse produced only 2.6 ng/ml. In the experiments in Fig. 20C, 20, 17 and 12.5 ng/ml were detected in culture supernatant of LNC from 3 challenged but nontreated animals (mean value of 16.5 ± 2.2 ng/ml as shown), whereas only 5.6, 4.3 and 3.7 ng/ml (mean value of 4.5 ± 0.55 ng/ml) were produced by similar culture supernatants of LNC from aIFN mAb-treated mice. The difference in the amounts of IFNγ produced by LNC from aIFN mAb-treated and from nontreated animals was statistically significant (p value of 0.01).

It should be noted that in each of the 3 experiments presented in Fig. 20A, B and C, despite the clear differences in IFNγ production, the proliferative responses to MBP of cultures from all mice were comparable (data not shown), regardless of whether they had been treated or not with aIFN mAb. It appears therefore, that the ability of LNC to produce IFNγ in the presence of specific Ag

Fig. 20 IFNγ production by lymph node cells from anti-IFNγ mAb-treated and nontreated mice. SJL/J mice were immunized with an emulsion containing 400 µg MBP in CFA containing 80 µg Mtb on day 0 (A, B), or on days 0 and 7 (C). Following each challenge, the mice were either treated iv with 1 mg aIFN mAb (solid bars) or remained untreated (hatched bars). On day 8 (B) or day 10 post-challenge (A and C), lymphocytes were isolated from the draining lymph nodes, and grown at 3 x 10^6 cells/ml in 24-well plates in the presence or absence of 50 µg/ml MBP. The supernatants were collected 24 hr after the initiation of cultures, and were assayed for IFNγ levels by capture ELISA using mouse rIFNγ as a standard. The data (ng of IFNγ per ml of culture supernatants) were pooled from 2 animals/group and from 3 animals/group in (A) and (C), respectively. The data presented in (B) were obtained from 1 mouse/group. In all cases, the amounts of rIFNγ detected in the cultures of cells grown in the absence of MBP was subtracted from that of cultures containing MBP. The asterisks mark values that were significantly different from those of control (ie.nontreated) ($p < 0.05$).


A
80
70
60
50
40
30
20
10
0
ng/ml IFNγ
*
B
14
12
10
8
6
4
2
ng/ml IFNγ
C
20
18
16
14
12
10
8
6
4
2
0
ng/ml IFNγ
*
NONTREATED
αIFN TREATED

was reduced by the aIFN mAb treatment.

3.7.2 TNF production

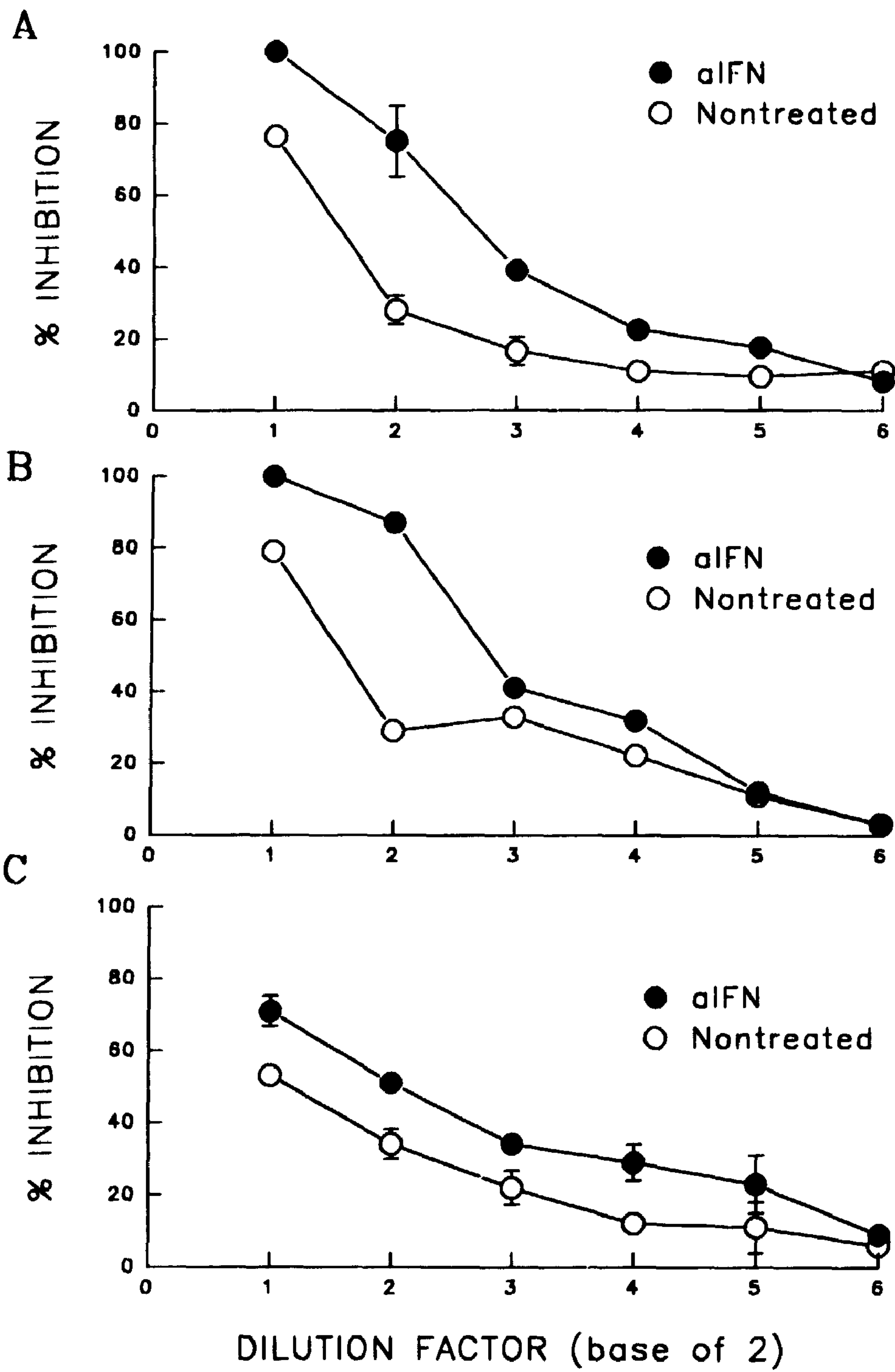
The same culture supernatants assayed for IFNγ as shown in Fig. 20 were also assayed for TNF α/β (Fig. 21). The presence of TNFα/β in the supernatants was tested by their ability to inhibit the growth of the sensitive cell line WEHI164. Since mouse rTNF was not available to us, it was not possible to determine this cytokine in quantitative terms. Instead, the culture supernatants were serially diluted, and the amounts of TNF were expressed as percent inhibition of WEHI164 cell growth. Inhibition by background TNFα/β levels (ie. inhibition by supernatants of cultures grown in the absence of MBP) was subtracted from inhibition by the corresponding culture supernantants grown with Ag. As seen in Fig. 21A, LNC of two mice challenged with MBP-CFA followed by aIFN mAb treatment produced higher amounts of TNF as reflected by higher percent of inhibition of WEHI164 cell growth. At dilutions 1:2, 1:4 and 1:8, culture supernatants of LNC from treated animals gave significantly higher percent inhibition of the WEHI164 cell growth than those from nontreated animals (p values of 0.018, 0.049 and 0.039, respectively). Similarly, culture supernatants of LNC removed 8 days after the MBP-CFA challenge also contained more TNF α/β in the mouse treated with aIFN mAb than in the nontreated animal (Fig. 21B). Since the same culture supernatants were used in both the IFNγ and TNF α/β assays, it appears that LNC from challenged mice treated with aIFN mAb produced less IFNγ but more TNF α/β in cultures in comparison to LNC from nontreated mice. Similar pattern of TNF α/β production was seen in mice challenged and treated with aIFN mAb on days 0 and 7, as

presented in Fig. 21C. At dilutions 1:2 and 1:4, culture supernatants from the three aIFN mAb-treated mice gave significantly higher percent inhibition of the WEHI164 cells than those from three nontreated animals (p values of 0.023 and 0.018, respectively). Thus, in all three experiments presented, significantly higher percent inhibition and hence, higher TNF α/β levels were detected in the supernatants of LNC from challenged and mAb- treated animals in comparison to challenged but nontreated animals.

3.8 Effect of aIFN mAb treatment on the number of $\gamma\delta$ TcR$^+$ cells in the lymph nodes, spleen and CNS

Early in 1994, a study by Dieli et al. reported that IL4 was an important mediator in the systemic transfer of DTH by T cell lines, and that $\gamma\delta$ TcR-expressing cells were the targets of its action. Since IFNγ is known to inhibit the production of IL4, and that these lymphokines antagonize each other in many aspects (Mond et al., 1986; Gautam et al., 1992), and since $\gamma\delta$ TcR$^+$ T cells have been implicated in both MS and EAE (reviewed by Raine, 1994ab), we next investigated as to whether in our case, neutralization of IFNγ by aIFN mAb would determine an increase of $\gamma\delta$ TcR$^+$ T cells. SJL/J mice were injected with MBP-activated lymphocytes followed by aIFN mAb. Two or three days after disease onset, the LNC, and splenocytes as well as mononuclear cells isolated from the brain and spinal cord of these mice were assayed for $\gamma\delta$ TcR-expressing cells by flow cytometry. Control mice received only the cells (Fig. 22A). A very low percentage of $\gamma\delta$ TcR$^+$ cells was detected in the lymph nodes (0.9%), spleen (1.6%) and CNS (1.3%), even when 20,000 instead of 10,000 cells were counted (data not shown). It should be noted that these low

Fig. 21 TNF-α/β production by lymph node cells from anti-IFNγ mAb-treated and nontreated mice. The same culture supernatants obtained in experiments presented in Fig. 20 were assayed for TNF α/β in a bioassay using the TNF α/β sensitive cell line WEHI-164. Five thousand WEHI-164 cells were grown in 4 replicate wells either in medium alone or in the presence of serial dilutions of the culture supernatants. The data are expressed as percent inhibition of growth of WEHI-164 cells grown in medium alone. Data were pooled from two aIFN mAb-treated (solid symbols), and two nontreated mice (open symbols) in (A), from one treated and one nontreated animal in (B), and from three aIFN mAb-treated and three nontreated mice in (C).


A
B
C
% INHIBITION
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80
60
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aIFN
Nontreated
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values were not unexpected since the population of γδ TcR-expressing cells represent only about 5% of the total T lymphocyte population. As seen in Fig. 22A, the absolute numbers of γδ TcR expressing cells in the lymph nodes, spleen and CNS did not appear to be affected by aIFN mAb treatment. The number of γδ TcR positive cells found in the same organs from nontreated animals were comparable to those of treated mice. Among the mice (2 aIFN mAb-treated and 2 nontreated) that were allowed to go through the disease course for 15 days, those that received cells and aIFN mAb exhibited more severe disease manifestations than those in the control group, thus confirming the enhancing effect of the mAb treatment (Fig. 22B). Taken collectively, the results presented in Fig. 22 suggest that while aIFN mAb enhances EAE, it does not appear to affect the γδ TcR-expressing cell population.

3.9 Role of IL4 in the development of actively induced EAE in SJL/J mice

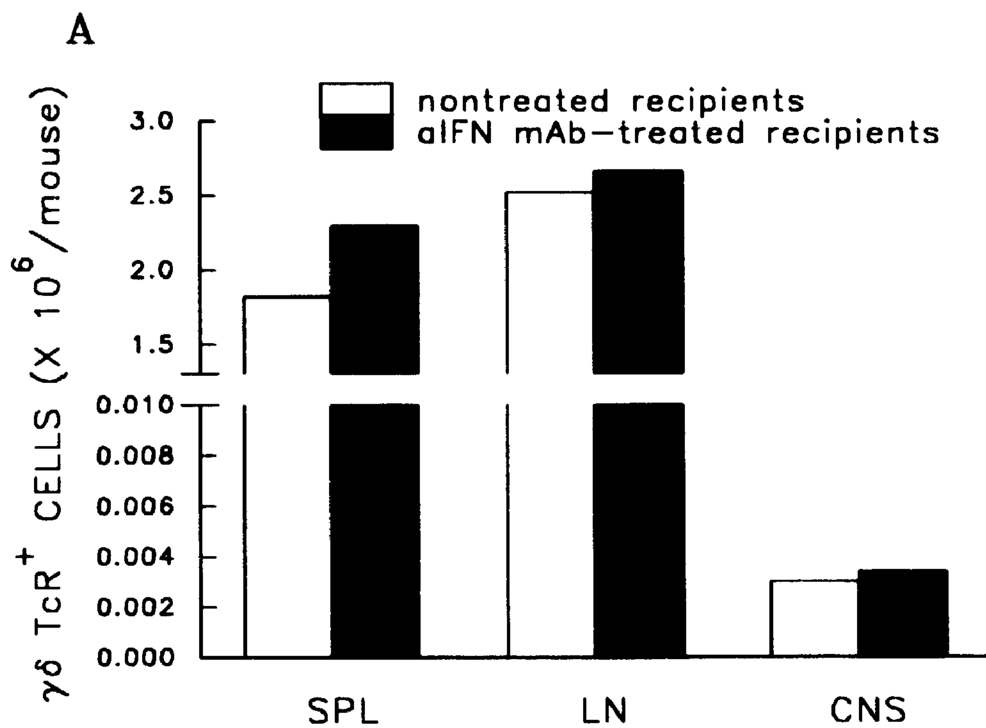
There is considerable evidence indicating that Th1 cells down-regulate Th2 cells through IFNγ while Th2 down-regulate Th1 cells through IL4 and/or IL10 (Swain et al., 1991; reviewed by Moore et al., 1993; Seder and Paul, 1994). Inasmuch that IFNγ appears to exert a dampening effect on EAE, and that IFNγ and IL4 antagonize each other in many respects (Mond et al., 1986; Gautam et al., 1992), it was of interest to examine whether or not IL4 had a disease-modulating role in EAE.

3.9.1 Effect of aIL4R mAb treatment

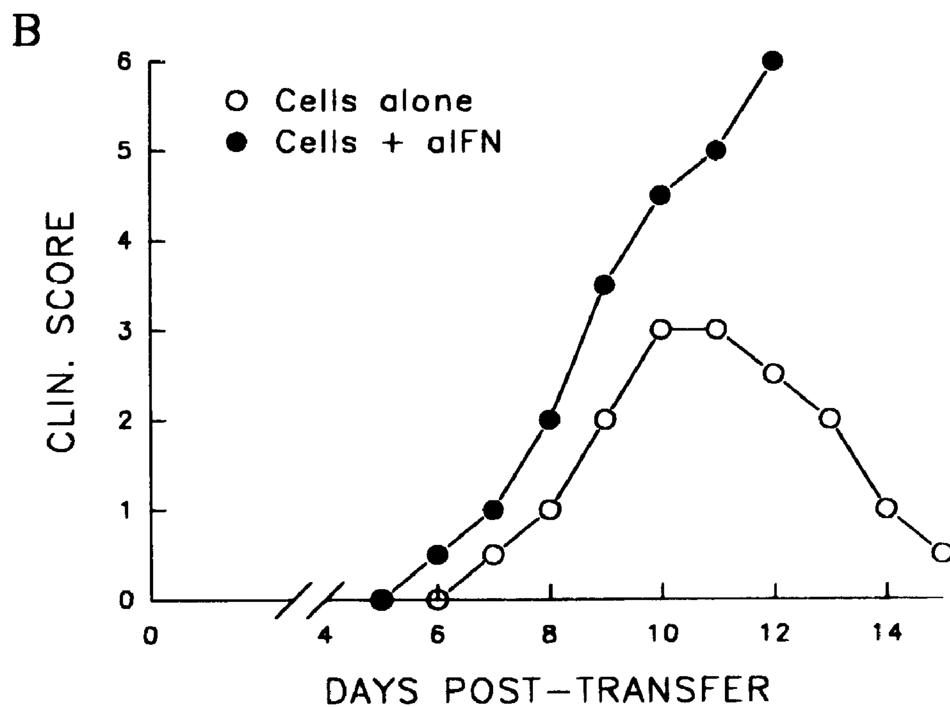
Using an approach similar to that for the role of aIFN mAb, we examined the effect of anti-IL4 receptor (IL4R) mAb on the development of EAE induced in SJL/J mice by MBP-CFA challenge plus aIFN mAb treatment. Four groups of animals

Fig. 22 Effect of anti-IFNγ mAb treatment on the numbers of γδ TcR^+ cells in the lymph nodes, spleen and CNS. SJL/J mice were injected with 20×10^6 syngeneic, MBP-activated lymph node cells on day 0, followed by 1 mg aIFN mAb (solid symbol). Control mice received the cells alone (open symbol). **(A)** Two or 3 days after disease onset (day 5-7), lymphocytes from lymph nodes and spleen as well as mononuclear cells from the CNS were pooled from mice within a group, and assayed for γδ TcR-expressing cells by cytofluorometry. The results are presented as the absolute number of γδ TcR^+ cells per animal. Two animals in each group were allowed to go through the whole 15 day course of disease **(B)**.

A



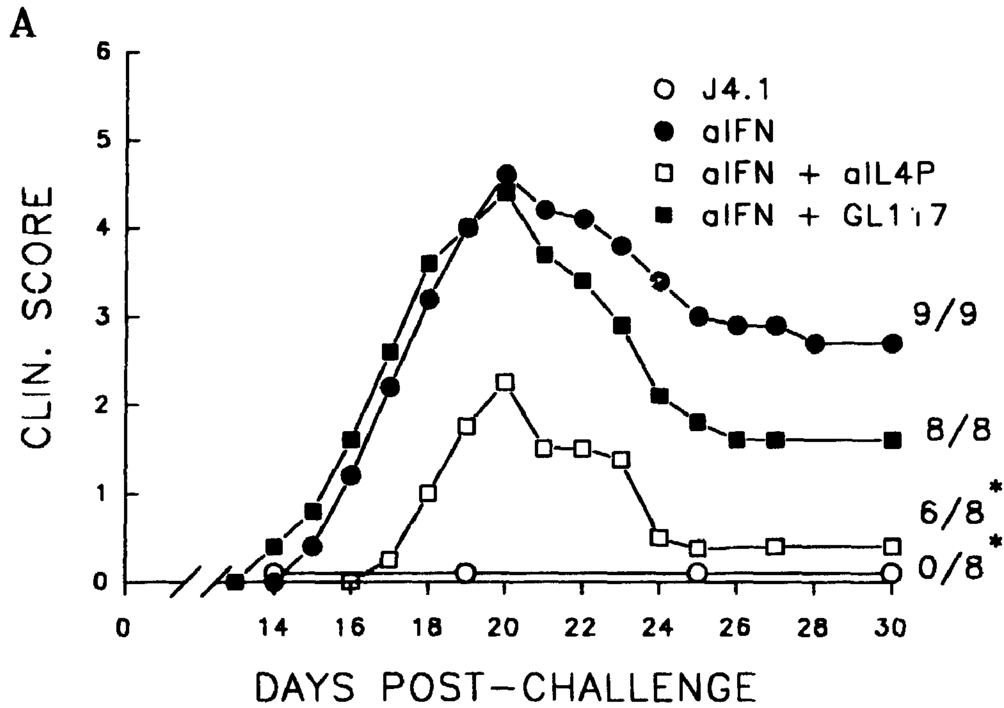
B



were included in the experiments presented in Fig. 23A. As observed previously, the injection of aIFN mAb following the MBP-CFA challenge on days 0 and 7 determined severe EAE in all animals. During the 30 day period of observation, in the MBP-CFA plus aIFN mAb-treated group, 2 of the 9 animals died of the disease. In contrast, none of the MBP-CFA challenged mice receiving the isotype-matched control mAb J4.1 showed any clinical signs. The interesting observation was that the disease induced by MBP-CFA plus aIFN mAb injection was significantly reduced by the inclusion of aIL4R mAb. Mild EAE was observed in 6 of 8 animals. The mean max. clin. score in this group was only 3 (hindleg paresis). This reduction in disease severity must be attributed to the anti-IL4R mAb since similar treatment using an isotype-matched control (GL117) could not alleviate the disease induced by MBP-CFA plus aIFN mAb treatment. All mice in this group developed EAE that was not significantly different from that in the animals challenged and treated with aIFN mAb alone. Similar results were obtained in a repeat experiment. As can be seen in Fig. 23B, EAE was significantly enhanced by the administration of aIFN mAb following MBP-CFA challenge on days 0 and 7. Incidence of disease was 100% with a mean max. clin. score of 4. In contrast, both disease severity and incidence were drastically reduced in MBP-CFA-challenged mice receiving a mixture of aIFN and aIL4R mAb. Only 1 of 5 animals developed a mild form of EAE (max. clin. score of 3). Similarly, only 1 of 5 challenged mice treated with aIL4R alone developed hindlimb paresis (clin. score of 3) during the 30 day period of observation. This is not surprising since the MBP-CFA challenge alone also failed, in our hands, to induce overt disease in this strain. Taken collectively, the results

Fig. 23 Effect of anti-IL4 receptor mAb on actively induced EAE in the SJL/J mice. Groups of 5 mice were challenged with MBP-CFA on days 0 and 7. (A) Following each challenge, the animals were also injected iv with either 1 mg aIFN mAb (●), a mixture of 1 mg aIFN and 3 mg anti-IL4 receptor (aIL4R) mAb (□), or with a mixture of 1 mg aIFN and 3 mg of the isotype-matched control mAb for aIL4R mAb (GL117) (■). None of the animals that were challenged with MBP-CFA and treated with 1 mg of the isotype-matched control Ab for aIFN (J4.1) (○) developed EAE. (B) Following each challenge, the animals were injected iv with either 1 mg aIFN mAb (●), 3 mg aIL4R mAb (■), or with a mixture of 1 mg aIFN mAb and 3 mg aIL4R mAb (▲). The number of animals with EAE over the total number in each group is indicated. Groups of mice with clinical scores that were significantly different from those of animals receiving aIFN alone is indicated by the asterisks ($p < 0.05$).

A



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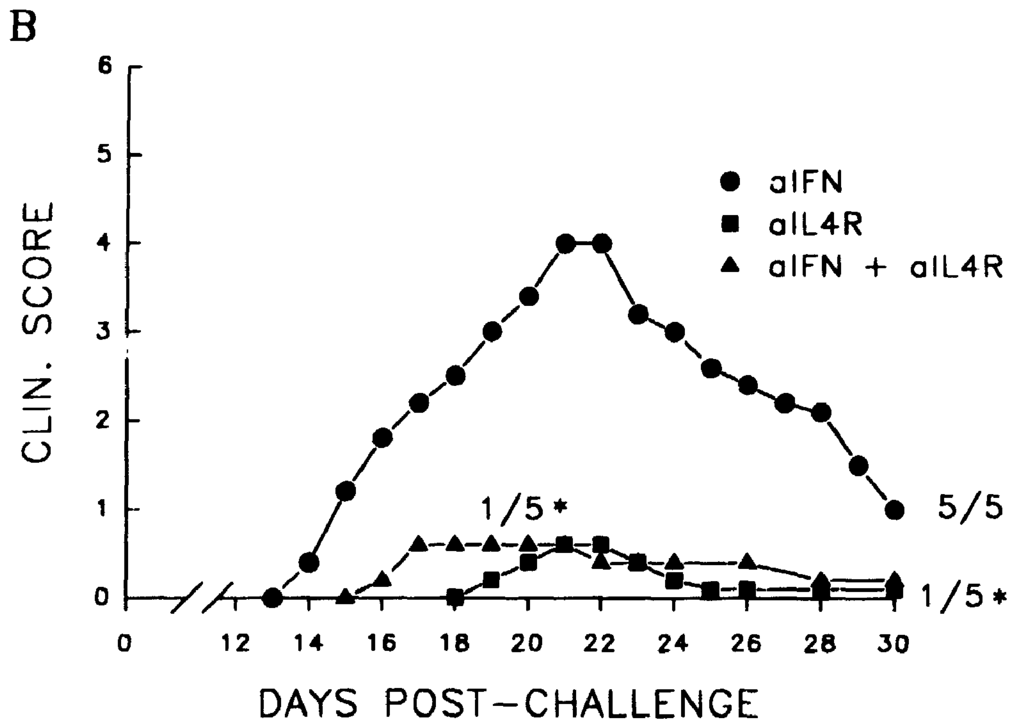


from Fig. 23 suggest that IL4 plays an opposite role to IFNγ in the development of EAE in SJL/J mice, as it appears to have a disease-potentiating effect.

3.9.2 Effect of IL4/aIL4 Ab complex treatment

At the time when this part of the study was being carried out, it was reported that the administration of IL4/anti-IL4 Ab (aIL4 Ab) complexes at specific lymphokine to Ab ratios led to increased lymphokine retention in the circulation, and thus, improved its biological effectiveness (Finkelman et al., 1993). Since our data suggested a role for IL4 in the induction of EAE, and since our source of IL4 was limited, we used this approach to further confirm the role of IL4 in the development of actively-induced EAE. Based on the observations that, in our hands, EAE was not readily inducible in SJL/J mice by MBP-CFA challenge in the absence of aIFN mAb, and that aIL4R mAb appeared to dampen the disease induced in this way, we hypothesized that administration of IL4 would enhance EAE induction. To test this hypothesis, mice were given IL4 as IL4/aIL4 Ab complexes, at several IL4/aIL4 mAb ratios as indicated in Table 5. As shown in Exp. 1, severe EAE was observed in all mice receiving aIFN mAb following the MBP-CFA challenge on days 0 and 7. Mice in this group started to show signs of disease by day 17 post-challenge, and only 1 animal survived by the end of the 45 day observation period (max. clin. score of 3). In contrast, none of the MBP-CFA challenged animals treated with the isotype-matched mAb (J4.1) developed EAE during the same 45 day period. Interestingly, severe EAE was observed in 2 of 4 challenged animals treated repeatedly with IL4/aIL4 mAb at 1.3/ 1.0 molar ratio (max. clin. score of 4 in each mouse). The treatment started on day -1 and continued every third day thereafter until day 21.

It must be noted however, that disease onset was delayed until day 36 post-challenge.

In Exp. 2, again, the injection of aIFN mAb following the MBP-CFA challenge on days 0 and 7 led to severe EAE. All 6 animals started to show signs of disease by 2 weeks after the challenge, and all died of the disease by day 20. In contrast, only 1 of 4 animals challenged with MBP-CFA alone developed a droopy tail (clin. score of 1) by day 31 post-challenge. On the other hand, as in the previous experiment, both EAE severity and incidence were enhanced in mice treated with IL4/aIL4 mAb complexes. In this particular group, the mice were given IL4/aIL4 mAb at 2.6/ 1 molar ratio on day -1 and every 3 days thereafter to day +21. Mean day of disease onset was around day 34.7 $\pm$ 6.2 post-challenge, and the mean max. clin. score in this group was around 2.

In the next experiment (table 5, Exp 3), the animals were treated with the complexes in a IL4/aIL4 mAb ratio of 1.6 /1 on days -1, +1, +6 and +8. As shown, 3 of 4 mice treated this way developed severe EAE, and two died of the disease by day 42 post-challenge. However, as before, disease onset was delayed. It must be noted that none of the challenged animals that were not given the IL4/aIL4 mAb complexes developed EAE over the 50 day period of observation.

Taken collectively, the results in Table 5 showed that administration of IL4 in the form of IL4/aIL4 mAb complexes could significantly enhance both incidence and severity of actively-induced EAE, strongly suggesting a disease-exacerbating role for IL4 in the disease development.

3.10 Effect of IFNγ on Ag-specific T cell line activation in response to Ag-

[a] SJL/J mice were challenged with MBP-CFA on days 0 and 7 followed by the injection of 1 mg of either aIFN or the isotype-matched mAb J4.1.

[b] Number of mice that developed EAE over the total number in the group.

[c] Mean day of disease onset ± standard error.

[d] Mean maximum clinical scores of all mice in the group.

[e] Not applicable. None of the animals showed any signs of disease throughout the study period of 45 days and 50 days in Exp. 1 and Exp. 3, respectively.

[f] Begining on day -1 and every 3 days thereafter until day 21, each animal was injected either ip or iv with a mixture containing 10 μg IL4 and 60 μg anti-IL4 mAb (aIL4) which had been allowed to react for 20-30 minutes prior to injection (1.3 moles of IL4 / 1 mole of mAb).

[g] Begining on day -1 and every 3 days thereafter until day 18, each animal was injected with a mixture containing 60 μg IL4 and 180 μg aIL4 mAb which had been allowed to react for 20-30 minutes prior to injection (2.6 moles of IL4/ 1 mole of mAb).

[h] On the day before and after each MBP-CFA challenge, each animal was injected with a mixture of 10 μg IL4 and 50 μg aIL4 mAb which had been allowed to react for 20-30 minutes prior to injection (1.6 moles of IL4/1 mole of mAb).

Values that are significantly different from those of control animals (MBP-CFA + J4.1 in Exp. 1; MBP-CFA challenge only in Exp. 2 and 3,) are underlined ($p < 0.05$).

Table 5

Effect of IL4/aIL4 mAb Complex Treatment on Active EAE in SJL/J mice

Treatment[a]	Incidence of EAE[b]	MDO ± SE[c]	MSC[d]
Exp.1			
MBP-CFA + aIFN	5/5	17.4 ± 2.4	5.4
MBP-CFA + J4.1	0/5	NA[e]	0
MBP-CFA + IL4/aIL4[f]	2/4	36.5 ± [illegible]5	2.0
Exp. 2			
MBP-CFA + aIFN	3/3	14.3 ± 0.5	6
MBP + CFA	1/4	31	0.25
MBP-CFA + IL4/aIL4[g]	3/3	23.3 ± 6.2	2.30
Exp. 3			
MBP-CFA	0/6	NA	0
MBP-CFA + IL4/aIL4[h]	3/4	34.7 ± 2.2	4.0

pulsed accessory cells.

IFNγ is a multifunctional cytokine whose biological activities have been well documented. Aside from being a potent activator of macrophages (Billiau and Dijkmans, 1990; Young and Hardy, 1990; Borden, 1992; Farrar and Schreiber, 1993), other effects include induction of class II MHC expression by various cell types (Wong et al., 1984; Zhang and Michael, 1990; Mantergazza et al., 1991) thus enhancing their ability to present Ag and to induce T cell activation, and the production of inflammatory cytokines such as IL1 and TNF (Arenzana-Seidedos et al., 1985; Nedwin et al., 1985). On the other hand, studies from several laboratories (Billiau et al., 1988; Voorthuis et al., 1990; Lublin et al., 1991; Kalman et al., 1992), as well as our own suggest that IFNγ has an anti-inflammatory function as well. In an attempt to resolve this paradox, we proposed to investigate certain aspects of IFNγ *in vitro*. We first examined the function of IFNγ in T cell activation by APC. In this series of experiments, short-term, MBP-specific T cell lines and PEC from SJL/J mice were used as a source of antigen specific T lymphocytes and as APC, respectively (see Materials and Methods).

3.10.1 Effect of IFNγ on the ability of PEC to induce Ag-specific proliferation by T cell lines

To confirm earlier reports that IFNγ enhances the ability of APC to induce T cell activation, PEC were isolated and grown in medium containing IFNγ, washed, pulsed with MBP for 2 hr and then fixed with 0.5% paraformaldehyde prior to coculture with the T line cells. Proliferative responses were assayed as a measure of T cell activation.

As seen in Fig. 24, treatment of the PEC with IFNγ significantly enhanced their ability to induce T cell proliferation ($p < 0.05$). Both dpm (69,320) and SI (127) values were increased more than 13 fold over the controls (dpm = 5,370, SI = 6). The enhancing effect was attributable to IFNγ since this was abolished by the addition of aIFN mAb, but not by the isotype-matched mAb J4.1. Similar results were obtained in 2 repeat experiments. As shown in Exp.1 of Table 6, again, T cell proliferation was significantly increased when IFNγ-treated, fixed PEC were used. Δdpm were increased from 19,264 to 272,948, and SI values from 8 to 74. The inclusion of aIFN mAb in the pretreatment of PEC abolished this enhancing effect as the response was reduced to the control level (Δdpm = 17,617, SI = 6.9). A similar enhancing effect of IFNγ was observed when a KLH-specific T cell line was used (Exp. 2 Table 6). In this case, the KLH-specific T line cells responded quite well to the nontreated, KLH-pulsed and fixed PEC. Δdpm and SI values were 38,472 and 21, respectively. On the other hand, the proliferative response of these T cells was remarkably enhanced when grown with KLH-pulsed and fixed PEC that had been pretreated with IFNγ (Δdpm = 163,800, SI = 79). Obviously, the enhancing effect on T cell proliferation was due to the pretreatment of PEC by IFNγ since the inclusion of aIFN mAb in the pretreatment abolished the enhancing effect. Taken collectively, the results in Fig. 24 and Table 6 are consistent with earlier findings (Beller,1984) that treatment of APC with IFNγ enhances their ability to present antigen and induce T cell activation.

Furthermore, in agreement with others (Beller, 1984; Wong et al., 1984; Cao et al., 1989; Mantergazza, 1991), it was observed that IFNγ pretreatment induced

Fig. 24 Effect of IFNγ on the ability of PEC to induce antigen-specific proliferation by T cell lines. PEC isolated from SJL/J mice were cultured at 10^6 cells/ml for 48 hr in medium containing aIFN or its isotype-matched control (J4.1) mAb, a mixture of rIFNγ and aIFN mAb, or a mixture of rIFNγ and J4.1mAb. Ab and rIFNγ were used at 400 ng/ml and 100 U/ml, respectively. The PEC were then washed and pulsed for 2 hr with 100 µg/ml MBP or were kept in medium alone. All PEC were washed and fixed with 0.5% paraformaldehyde following the antigen-pulse. Proliferative responses of cultures containing 2.5×10^4 T cells and 2×10^4 PEC were determined as described in Materials and Methods. Solid and hatched bars represent the average dpm $\pm$ standard error (SE) of six replicate cultures with unpulsed (background dpm) and MBP-pulsed PEC, respectively. The numbers in parentheses represent the stimulation index (SI) which is the ratio of the average dpm of cultures with pulsed-PEC to background dpm.

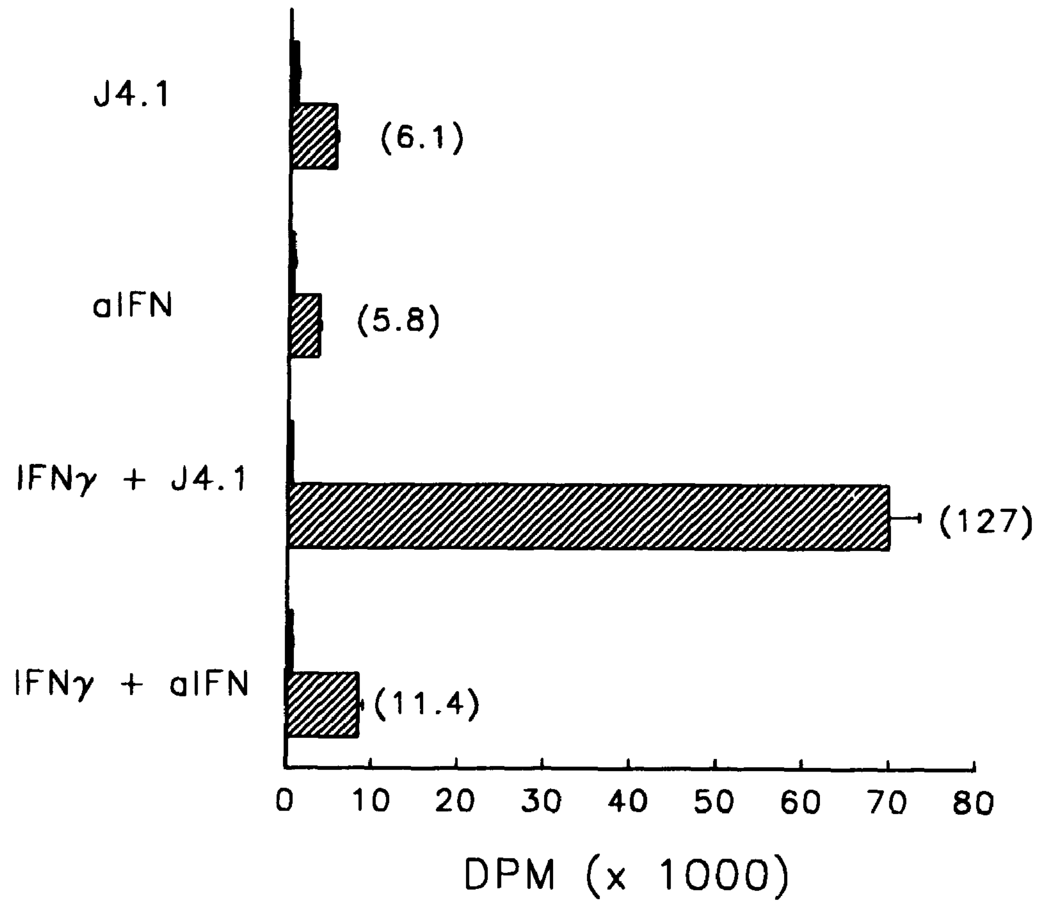

LYMPHOKINE and/or
mAb ADDED
J4.1
(6.1)
aIFN
(5.8)
IFNγ + J4.1
(127)
IFNγ + aIFN
(11.4)
0
10
20
30
40
50
60
70
80
DPM (x 1000)

Table 6

Effect of IFNγ on the Ability of PEC to Induce T Cell Proliferation to Antigen

Lymphokine and/or mAb added[a]	Δdpm ± SE[b]	SI[c]
Exp. 1		
J4.1	19,264 ± 3,012	8
aIFNγ	22,492 ± 2,226	7.6
IFNγ + J4.1	272,948 ± 20,064	74
IFNγ + aIFNγ	17,617 ± 1,920	6.9
Exp. 2		
None	38,472 ± 2,413	21
IFNγ + J4.1	163,800 ± 12,261	79
IFNγ + aIFNγ	70,564 ± 7,432	21

[a] Freshly isolated PEC were cultured with either J4.1, with aIFN mAb, with a mixture of aIFNγ mAb and IFNγ, or with a mixture of J4.1 and IFNγ. In all cases, mAb was used at 400 ng/ml and IFNγ at 100 U/ml. After 48 hr, the PEC were washed, and pulsed with either 50 μg/ml MBP (Exp. 1) or with 100 μg/ml KLH (Exp. 2). 3 x 10^4 Ag-pulsed or unpulsed PEC were cultured in 4 duplicate wells with 2 x 10^4 T line cells of appropriate Ag specificity (ie. MBP-specific T line cells in Exp. 1, and KLH-specifc T cells in Exp. 2). T cell proliferation was assayed 96 hr later.

[b] Δdpm ± standard error. Δdpm was calculated as the difference between the average dpm of T cells grown with Ag-pulsed PEC and that of cultures grown with unpulsed PEC. Background proliferation which represents the average dpm of 4 replicate cultures of T cells grown with unpulsed PEC ranged from 2,752 to 3,739 in Exp.1, and from 1,832 to 2,714 in Exp. 2.

[c] Stimulation index was calculated as the ratio of the average dpm of T cells grown with Ag-pulsed PEC to that of cultures grown with unpulsed PEC.

increased class II MHC expression on the PEC surface. As can be seen from Fig. 25B, after 2 days in medium alone, only 31% of the PEC expressed class II MHC. The percentage of class II MHC positive PEC had increased to 59% after they were grown in the presence of IFNγ for the same length of time. In addition, the fluorescence intensity of the IFNγ-treated population was also increased in this case, indicating an increase in the number of MHC class II molecules per cell.

To examine the amount of IFNγ required for increased class II MHC expression, PEC were isolated from SJL/J mice and incubated for 48 hr in either medium alone or in medium containing various concentrations of rIFNγ. At the end of the incubation period, both the adherent and nonadherent cell populations were assayed for Ia^s expression by FACS analysis. It can be seen from Fig. 26 that the effect of rIFNγ on the expression of Ia^s by the PEC is dose-dependent. In the presence of 100 U/ml rIFNγ, about 80% of the PEC expressed class II MHC in comparison to only 11% in the PEC population grown in medium alone. Up to 56% of PEC grown with 10 U/ml rIFNγ were Ia^s positive. In addition, the relative fluorescence intensity ot PEC grown at these high concentrations of rIFNγ (100 and 10 U/ml) was also enhanced indicating an increase in the number of Ia^s molecules per cell. The smallest amount of rIFNγ tested that could induce a detectable increase (from 11 to 28%) in Ia^{s+} cells was 0.5 U/ml , but the relative fluorescence intensity was not changed.

3.10.2 Effect of exogenous IFNγ on T cell proliferation in response to Ag-pulsed PEC

In contrast to the enhancement of T cell activation observed in Fig. 24 and

Fig. 25 Effect of IFNγ on induction of class II MHC expression by PEC. PEC were freshly isolated from SJL/J mice, and grown either in medium alone (**B**) or in medium containing 100 U/ml rIFNγ (**C**) for 48 hr. Both the adherent and non-adherent cells were then collected, stained with FITC-labelled anti-class II MHC (Ia^s) antibody, and assayed for Ia^s expression by cytofluorometry. The percentage of positive cells is indicated in the figure. Background fluorescence is presented in (**A**) (ie. PEC that were not exposed to anti-Ia^s Ab).

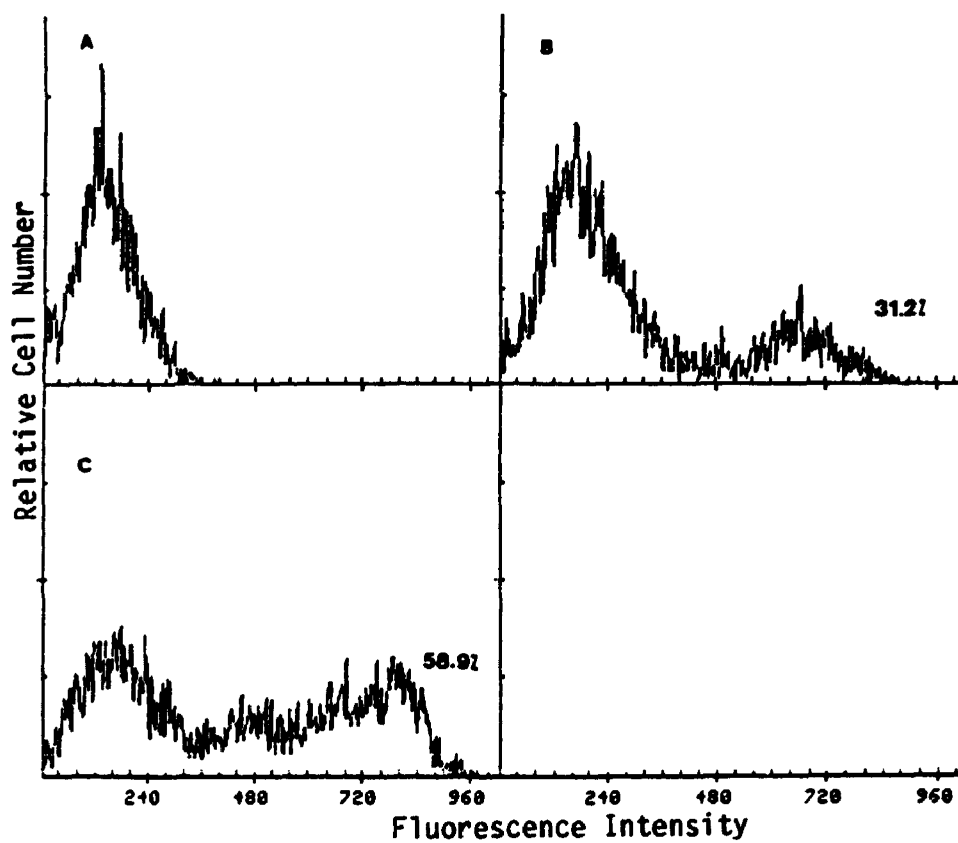

A
B
31.2%
C
58.9%
Relative Cell Number
240
480
720
960
240
480
720
960
Fluorescence Intensity

Fig. 26 Titration of the effect of rIFNγ on the expression of class II MHC by PEC. Freshly isolated PEC from SJL/J mice were grown for 48 hr either in medium alone (F) or in medium containing various concentrations of rIFNγ (B-E). At the end of the incubation period, the cells were collected, stained with FITC-labelled anti-Ia^{s} Ab, and analysed by cytofluorometry.The concentrations of rIFNγ and the percentages of positive cells are indicated in the figure. Panel (A) represents background fluorescence (ie. PEC that were not exposed to anti-Ia^{s} Ab).

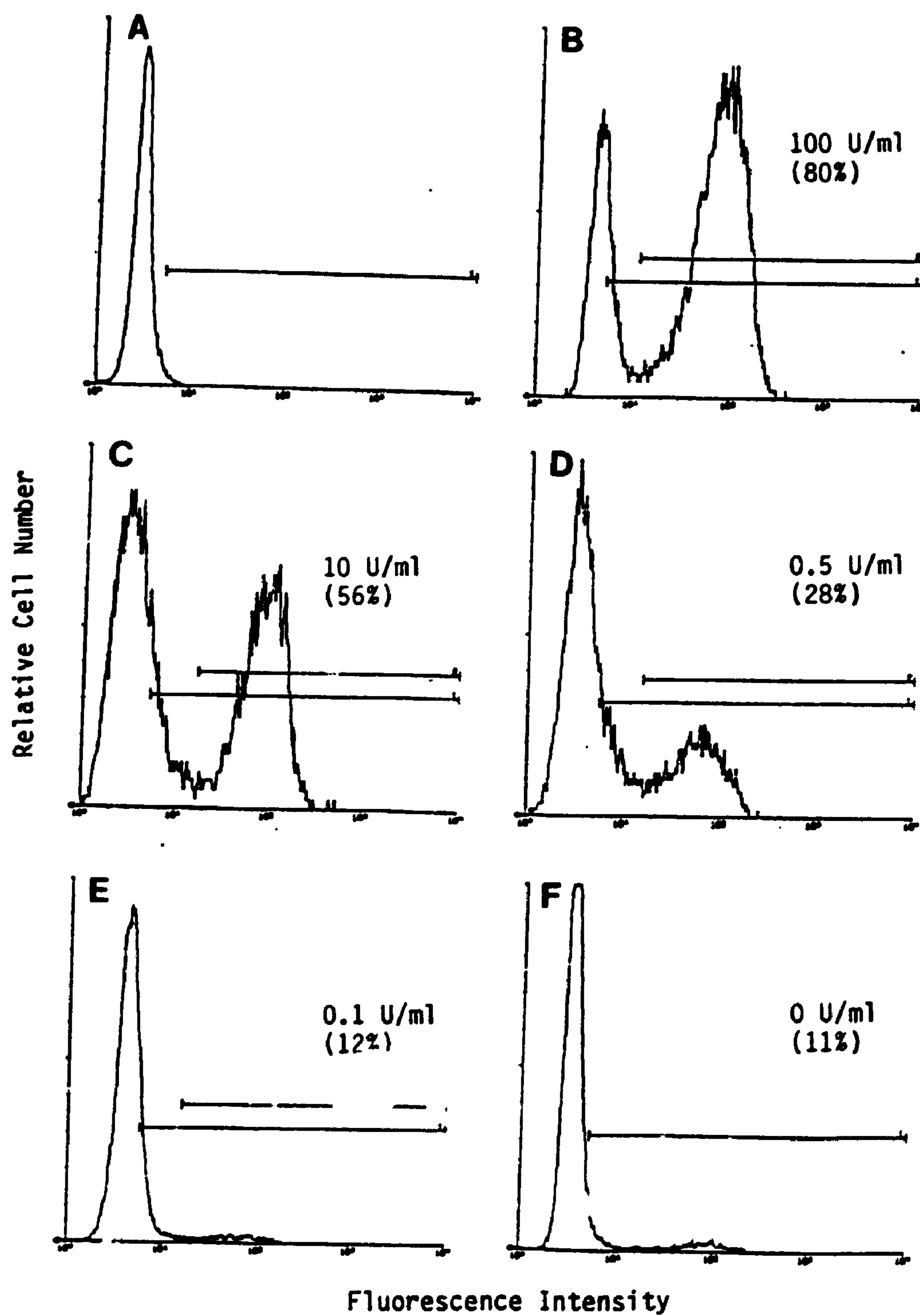
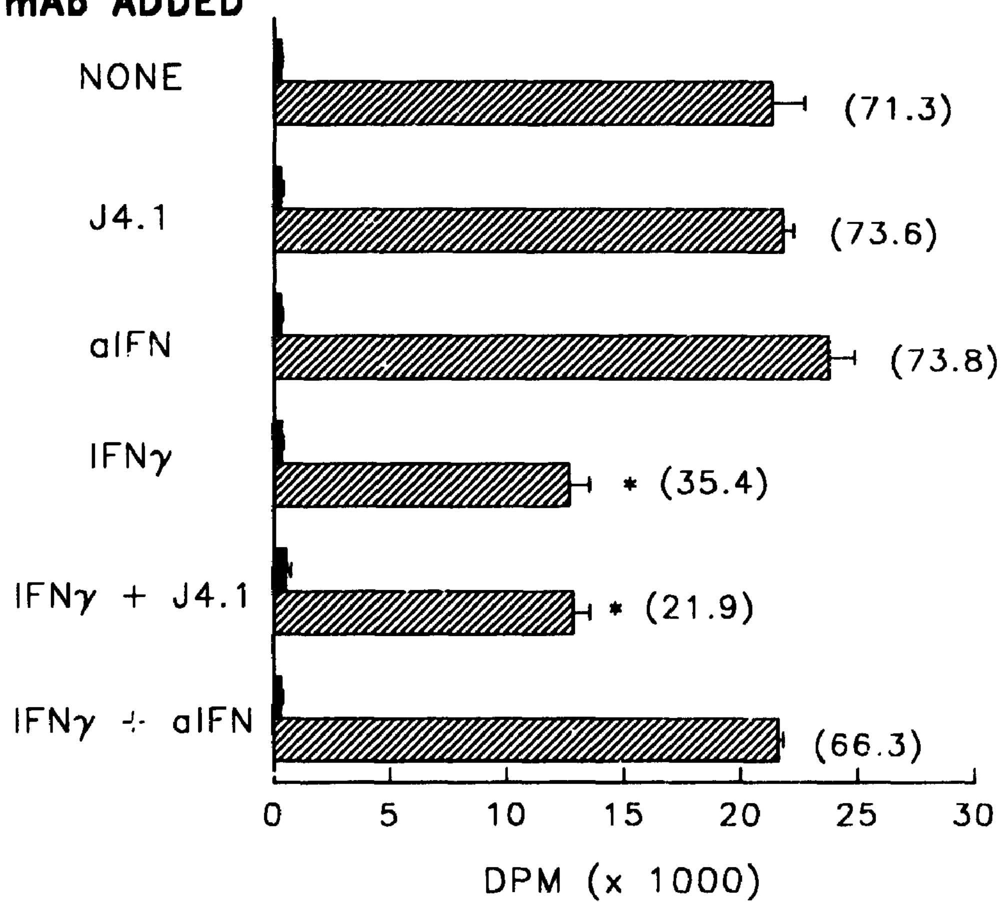

A
B
100 U/ml
(80%)
C
10 U/ml
(56%)
D
0.5 U/ml
(28%)
E
0.1 U/ml
(12%)
F
0 U/ml
(11%)
Relative Cell Number
Fluorescence Intensity

Table 6, the addition of 100 U/ml of rIFNγ at the start of PEC-T cell cultures had a clear inhibitory effect on T cell proliferation (Fig. 27). It can be seen that in the absence of exogenous IFNγ, the T cells could respond relatively well to the MBP-pulsed PEC (dpm = 21,381, SI = 71). The addition of either anti-IFNγ or the isotype-matched Ab was without effect (SI = 74 in both cases). On the other hand, the addition of rIFNγ to the cocultures significantly reduced T cell proliferation ($p < 0.05$). Both dpm and SI values were reduced by about 50% (dpm from 21,381 to 12,317, and SI 71 to 35). This inhibitory effect was attributable to IFNγ since the addition of aIFN but not the control mAb restored the response (dpm = 21,261, SI = 66). A similar inhibitory effect by IFNγ on T cell proliferation was observed in 2 repeat experiments (Table 7). In Exp. 1, the response to MBP-pulsed PEC of this particular cell line was quite high with Δdpm and SI values of 81,799 and 44, respectively. On the other hand, the Δdpm and SI were significantly reduced to 27,721and 18, respectively, in the cultures to which rIFNγ was added. The inclusion of anti-IFNγ mAb but not of the control mAb restored the response to the control level. In Exp. 2, again, the addition of IFNγ to the PEC-T cell cocultures significantly reduced T cell proliferation in response to the MBP-pulsed PEC ($p < 0.05$). In this case the T cell proliferative response was decreased by about 3 fold, with Δdpm being reduced from 115,289 to 36,150, and SI from 32 to 11. Again, the inclusion of aIFN but not of the control mAb restored the response, as Δdpm and SI values were brought back up to 103,304 and 31, respectively. It should be noted that in all cases where anti-IFNγ mAb was added alone to the cocultures, T cell proliferation was not affected. This observation will be discussed in section 3.10.6.

Fig. 27 Effect of exogenous IFNγ on antigen-specific T cell proliferation. Thirty six thousand nontreated, X-irradiated, MBP-pulsed or unpulsed PEC were cultured in 6 replicate wells with 2.5×10^4 MBP-specific T line cells. Anti-IFNγ mAb, J4.1 and rIFNγ were added to the cultures at the time of plating. The final concentrations of mAb and rIFNγ in cultures were 400 ng/ml and 100 U/ml, respectively. Proliferative responses of cultures containing unpulsed PEC (solid bars) and those of MBP-pulsed PEC cultures (hatched bars) are expressed as dpm $\pm$ SE. SI values are indicated in the parentheses. The asterisks mark cultures with dpm values that were significantly different from those of control cultures (ie. no mAb and/or IFNγ added) ($p < 0.05$ as determined by ANOVA).



LYMPHOKINE and/or mAb ADDED
NONE
J4.1
aIFN
IFNγ
IFNγ + J4.1
IFNγ + aIFN
(71.3)
(73.6)
(73.8)
* (35.4)
* (21.9)
(66.3)
0
5
10
15
20
25
30
DPM (x 1000)

Table 7

Effect of Exogenous IFNγ on T Cell Proliferation in Response to Ag-pulsed PEC

Lymphokine and/or mAb added[a]	Δdpm ± SE[b]	SI[c]
Exp. 1		
None	81,799 ± 8,049	44
IFNγ	25,721 ± 2,273	18
IFNγ + J4.1	30,855 ± 1,690	20
IFNγ + aIFNγ	68,582 ± 5,243	39
Exp. 2		
J4.1	115,289 ± 12,208	32
aIFNγ	115,080 ± 10,876	29
IFNγ + J4.1	36,150 ± 2,282	11
IFNγ + aIFNγ	103,304 ± 7,933	31

[a] 3×10^4 nontreated, X-irradiated, MBP-pulsed, or unpulsed PEC were cultured in 4 replicate wells with 2×10^4 MBP-specific T line cells. Anti-IFNγ, J4.1 mAb and rIFNγ were added at the start of the culture. The final concentrations of mAb and rIFNγ in cultures were 400 ng/ml and 100 U/ml, respectively.

[b, c] For details, see table 6.

Values that are significantly different from those of control cultures (ie. cultures to which no IFNγ and/or mAb was added in Exp. 1; cultures to which J4.1 mAb was added, Exp. 2) are underlined ($p < 0.05$ as determined by ANOVA).

3.10.3 Effect of exogenous IFNγ on T cell proliferation in response to Ag-pulsed splenocytes or thymocytes

Inasmuch that our data showed that the addition of IFNγ to cocultures of T line cells and Ag-pulsed PEC inhibited the T cell proliferation, the question arose as to whether the same treatment would have any effect on the response of T cells to other types of conventional APC such as splenocytes (SPL) or thymocytes (THYM). To examine this question, rIFNγ was added to cocultures of MBP-specific T line cells and either MBP-pulsed SPL or THYM. As demonstrated by the results from Table 8, in Exp. 1, the T line cells responded to the Ag-pulsed SPL with Δdpm and SI values of 26,574 and 6.6, respectively. However, the proliferative response was significantly reduced in cultures to which rIFNγ was added (Δdpm = 12,464, SI = 3.7) ($p < 0.05$). As before, the inclusion of aIFN but not of the control mAb abolished the inhibitory effect of rIFNγ (Δdpm = 29,667 ,SI = 6.6). A similar inhibitory effect of rIFNγ was observed when the T cells were stimulated with Ag-pulsed THYM (Exp.2). The response of T cells in the presence of exogenous rIFNγ gave reduced Δdpm from 43,502 to 9,419, and SI from 14 to 4. This inhibition was attributable to the rIFNγ since the inclusion of aIFN mAb restored the response (Δdpm = 38,904, SI = 12.4) while the isotype matched mAb was ineffective (Δdpm = 10,516, SI = 4). In summary, the results presented in Fig. 27 and Tables 7 and 8 demonstrated that IFNγ had the ability to inhibit T cell proliferation to Ag presented by three types of APC: PEC, splenocytes and thymocytes.

3.10.4 Effect of pretreatment of T cells with IFNγ on their proliferative response to Ag-pulsed PEC

Table 8

Effect of Exogenous IFNγ on T Cell Proliferation in Response to Ag-Pulsed Splenocytes andThymocytes

Lymphokine and/or mAb added[a]	Δdpm ± SE[b]	SI[c]
Exp. 1: SPL		
None	26,574 ± 1,854	6.5
IFNγ	12,464 ± 1,794	3.7
IFNγ + J4.1	14,552 ± 1,817	3.3
IFNγ + aIFNγ	29,667 ± 2,110	6.6
Exp. 2: THYM		
None	43,502 ± 4,948	14
IFNγ	9,419 ± 1,128	4
IFNγ + J4.1	10,516 ± 1,284	4
IFNγ + aIFNγ	38,904 ± 4,380	12.4

[a] 5 x 10^5 nontreated, X-irradiated, MBP-pulsed, or unpulsed splenocytes (SPL) (Exp.1) or thymocytes (THYM) (Exp. 2) were cultured in 4 replicate wells with 2 x 10^4 MBP-specific T line cells. Anti-IFNγ, J4.1 mAb and rIFNγ were added to the cultures at the time of plating. The final concentrations of mAb and rIFNγ in culture were 400 ng/ml and 100 U/ml, respectively.

[b, c] For details, see table 6.

Values that are significantly different from those of control cultures (ie. cultures to which no IFNγ and/or mAb was added) are underlined ($p < 0.05$).

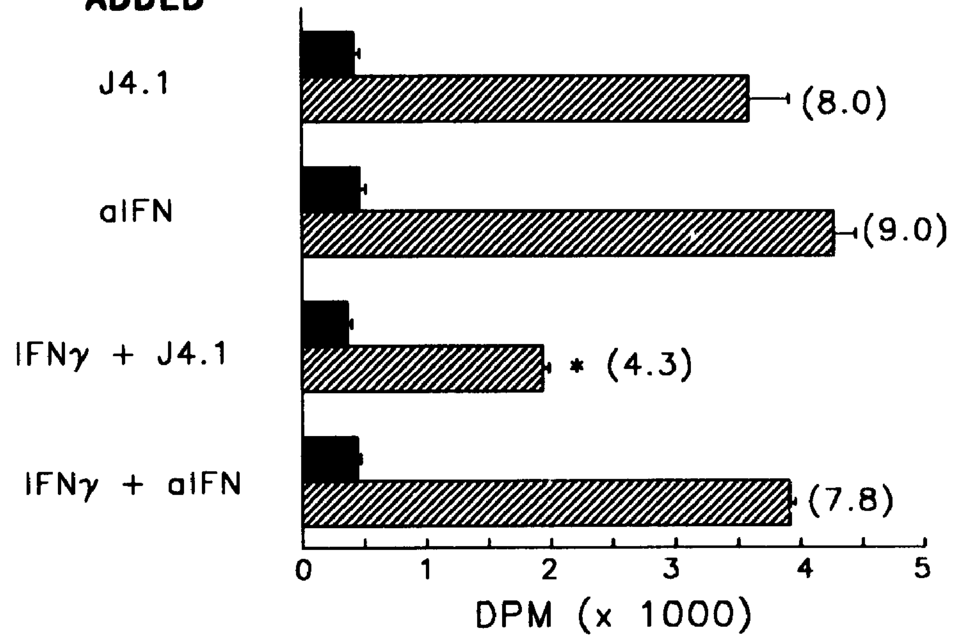
The finding that exogenous IFNγ added to cocultures at the time of plating suppressed antigen-specific T cell proliferation was surprising, especially since pretreatment of PEC with this lymphokine significantly potentiated the response. The question arose as to whether IFNγ acted directly on the T cells themselves to inhibit their proliferation or indirectly through the PEC population. Thus, T cells were first incubated with rIFNγ for 2 hr, washed and cultured with the X-irradiated, MBP-pulsed, or unpulsed PEC. Cultures of untreated T cells to which rIFNγ was added at the time of plating with the antigen-pulsed PEC (time 0) were included as controls (Fig. 28). As already seen in Fig. 27, the addition of a mixture of IFNγ and J4.1 mAb to the cocultures significantly inhibited the T cell proliferation (Fig. 28A). Δdpm value was reduced from 3,176 to 1,577, and SI from 8.0 to 4.3 ($p < 0.05$). This inhibition was abolished when aIFN mAb instead of J4.1 was added as the response was restored to the control level. In this case, Δdpm value was brought back up to 3,467 and SI value to 7.8. Again, the addition of aIFN mAb alone to the cocultures was without effect (Δdpm = 3,805, SI = 9.0).

In Fig. 28B, pretreatment of T cells with rIFNγ in the presence of J4.1 mAb significantly reduced their antigen-specific proliferation ($p < 0.05$). The Δdpm and SI values in these cultures were reduced by about 50-70% (Δdpm = 1,600, SI = 2.7) when compared to cultures of T cells pretreated with J4.1 alone (Δdpm = 3,002, SI = 9.9), or with aIFN mAb alone (Δdpm = 3,350, SI = 8.6). The inhibitory effect was due to IFNγ pretreatment of the T cells since the inclusion of aIFN mAb in the pretreatment abolished the inhibition. Both the Δdpm and SI values were brought back to the levels of untreated cultures (Δdpm = 3,466, SI = 7.8). It should be

Fig. 28 Effect of pretreatment of T cells with IFNγ on their proliferative response to antigen-pulsed PEC. Ten thousand nontreated, X-irradiated, MBP-pulsed or unpulsed PEC were cultured with 2.5 x 10^4 MBP-specific T line cells in 4 replicate wells. Proliferative responses are expressed as dpm ± SE, and SI values are indicated in parentheses. The asterisks mark cultures with dpm values that were significantly different from those of control cultures ($p < 0.05$). Solid and hatched bars represent the dpm ± SE of cultures containing unpulsed and MBP-pulsed PEC, respectively. **(A)** aIFN mAb and rIFNγ were added to the cultures at the time of plating (time 0). Control cultures received J4.1 mAb. The final concentrations of mAb and rIFNγ in cultures were 400 ng/ml and 100 U/ml, respectively. **(B)** The T cells were preincubated for 2 hr in medium containing 100 U/ml rIFNγ and 400 ng/ml of either J4.1 or aIFN mAb prior to culturing with the PEC (pretreated). Control T cells were preincubated with 400 ng/ml of J4.1 mAb only. The T cells were washed to remove residual rIFNγ and mAb, and were added to the PEC in cultures.

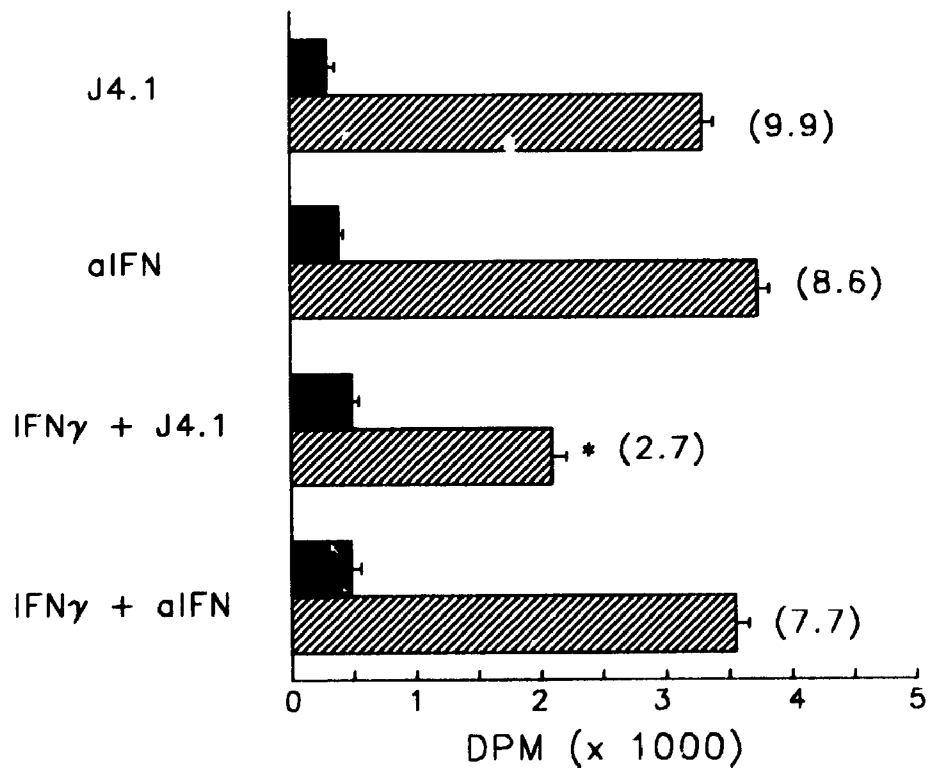
A

LYMPHOKINE and/or mAb ADDED



B

TREATMENT of T CELLS



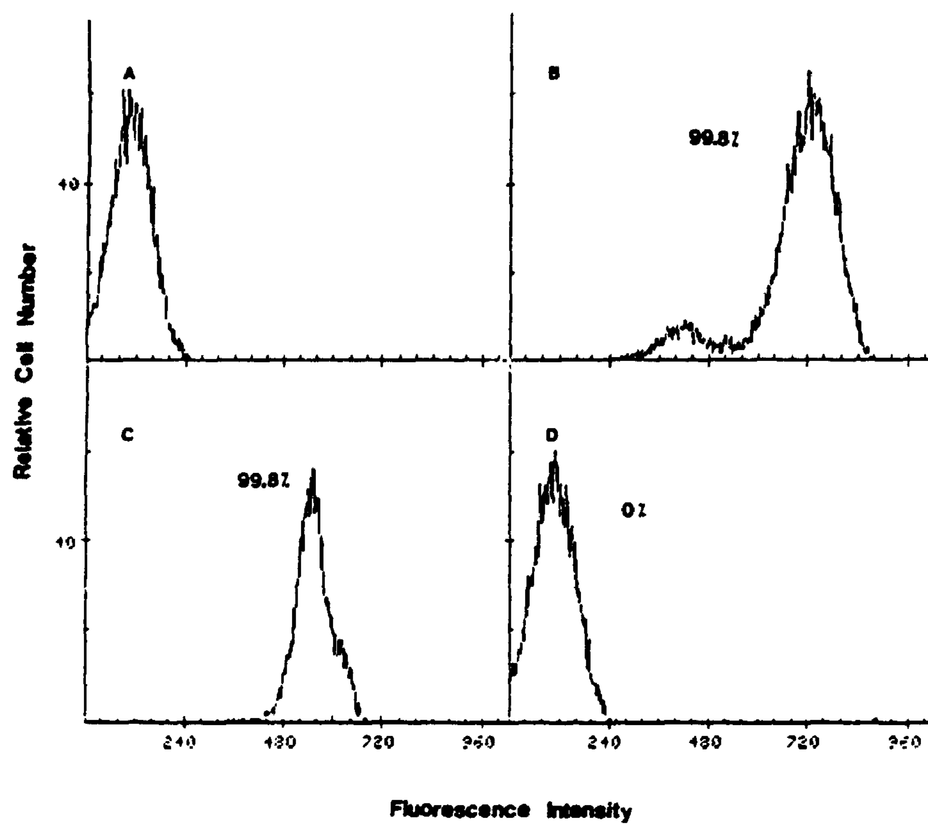
mentioned that 2 hr was chosen as the period for IFNγ-pretreatment of the T cells since in preliminary studies, 2 hr was found to be the minimum required time for optimal effect (data not shown).

The observation that the antigen-specific T cell proliferation was reduced following pretreatment of T cells with rIFNγ suggested that IFNγ suppressed T cell proliferation in response to antigen by acting directly on the T cell themselves. This observation was further supported by cytofluorometric analysis showing that the MBP-specific T cell preparations were comprised of only T lymphocytes, and that no feeder cells which were present in the propagation of the T line cells were carried over. As shown in Fig. 29, at the time of their use in the experiments, 99.8% of the T cell population were positive for the pan-T cell marker Thy 1.2 (Fig. 29B), and the same percentage of cells was positive for the CD4 marker (anti-L3T4) (Fig. 29C). None of the cells were positive after staining with anti-Lyt-2 antibody (CD8) (Fig. 29D).

3.10.5 Effect of IFNγ on IL3 production by T cell lines in response to Ag-pulsed PEC

It appears that IFNγ acts directly on the T cells themselves to inhibit their proliferation in response to antigen-pulsed APC. Upon activation by Ag-pulsed APC, the T cells can release a number of lymphokines. As mentioned in the Introduction, Th1 cells produce IL2, IL3, IFNγ, and LT while Th2 cells produce IL3, IL4, IL5 and IL10. Since our T cell population was a T cell line and not a clone, and since IL3 is produced by both T cell subpopulations, we examined next the level of IL3 produced as an indicator of T cell activation. Thus, 24 hr supernatants from

Fig. 29 Phenotypic analysis of MBP-specific T line cells. Cells were isolated and incubated in medium alone **(A)**, anti-Thy 1.2 **(B)**, anti-L3T4 **(C)**, or anti-Lyt-2 **(D)** mAb (see Materials and Methods). The percentage of positive cells is indicated in the figure.


A
B
C
D
99.8%
99.8%
0%
40
40
240
480
720
960
Relative Cell Number
Fluorescence Intensity

cultures containing MBP-pulsed PEC and IFNγ-pretreated T cells, or from cultures of Ag-pulsed PEC and T cells that were cocultured in the presence of rIFNγ were assayed for IL3. As can be seen in Table 9, Exp. 1, the amount of IL3 produced by the treated T cells was significantly less than that produced by nontreated cells. 211 ng/ml and 549 ng/ml IL3 were produced by the IFNγ treated and nontreated T cells, respectively. This decrease in IL3 production was consistent with a decrease in the proliferative response of cultures containing IFNγ-pretreated T cells. Δdpm values decreased from 31,979 to 14,818, and SI from 11 to 5.6. The inhibitory effect on both IL3 production and T cell proliferation was due to the pretreatment of T cells with IFNγ since only the inclusion of aIFN but not of the control mAb was able to neutralize this effect. When Ag-pulsed, fixed PEC were used, the T cell responses were lower but the same pattern of inhibition by rIFNγ-pretreatment of T cells was observed (Table 9, Exp. 2).

A similar pattern of reduction in IL3 production was also observed in cocultures containing T cells and Ag-pulsed PEC, to which 100 U/ml exogenous rIFNγ was added (Table 9, Exp. 3). The addition of IFNγ resulted in decreased amounts of IL3 from 510 ng/ml to 239 ng/ml. In addition, the proliferative responses of T cells in these cultures was also inhibited by the addition of rIFNγ, as Δdpm values were reduced from 29,498 to 11,815, and SI from 10 to 4.9. A similar pattern of inhibition of IL3 production and T cell proliferation were observed in a repeat experiment. As can be seen in Table 10, the addition of rIFNγ to the cocultures reduced the amounts of IL3 produced from 842 to 391 ng/ml, the Δdpm from 76,507 to 27,695, and the SI from 14 to 5.9. Similarly, the pretreatment of T cells

[a] 2.5×10^4 MBP-specific T line cells were grown with 4.0×10^4 MBP-pulsed or unpulsed, X-irradiated PEC in 4 replicate wells. In Exp. 1, the T cells were treated with either J4.1 or aIFNγ mAb alone, a mixture of rIFNγ and J4.1 or a mixture of rIFNγ plus aIFNγ mAb prior to being added to the PEC. In Exp. 2, the T cells were pretreated with either rIFNγ or with rIFNγ plus aIFNγ mAb, and were cultured with paraformaldehyde-fixed PEC. In Exp. 3, either J4.1 or aIFNγ mAb alone, a mixture of rIFNγ and J4.1 or a mixture of rIFNγ and aIFNγ mAb was added to the cocultures at the time of plating. In all experiments, the final concentrations of mAb and rIFNγ were 400 ng/ml and 100 U/ml, respectively. Supernatants were collected 24 hr after culture initiation, and were assayed for IL3. Similar cultures were carried out in parallel, and their proliferative responses were determined at 96 hr.

[b] Δdpm ± SE. Stimulation index is in brackets. Values that are siginificantly different from those of control cultures (first set of cultures listed in each experiment above) are underlined ($p < 0.05$).

[c] The presence of IL3 in culture supernatants was tested by the ability to sustain the growth of MC/9 cells as described in Materials and Methods. rIL3 of known concentration was used as a standard. The level of IL3 in each supernatant was obtained by subtracting the amount of IL3 in the supernantant of cultures containing unpulsed PEC from that of the corresponding cultures containing Ag-pulsed PEC.

Table 9

Effect of Treatment of T Cells with IFNγ on their Ability to Produce IL3 in Response to Ag-Pulsed PEC

Cultures [a]	Δdpm ± SE (SI)[b]	IL3 Production[c] (ng/ml)
Exp. 1		
J4.1-treated T cells + Ag-pulsed PEC	34,083 ± 3,507 (10.5)	549
rFNγ + J4.1-treated T cells + Ag-pulsed PEC	14,818 + 919 (5.6)	211
aIFN mAb treated T cells + Ag-pulsed PEC	31,979 ± 2,865 (10.4)	527
rIFNγ + aIFN mAb -treated T cells + Ag-pulsed PEC	32,029 ± 2,998 (12)	552
Exp. 2		
T cells + Ag-pulsed (fixed) PEC	4,120 ± 312 (7.3)	120
rIFNγ-treated T cells + Ag-pulsed (fixed) PEC	1,891 + 89 (3.4)	47
rIFNγ + aIFNγ mAb-treated T cells + Ag-pulsed (fixed) PEC	3,998 ± 410 (8.1)	101
Exp. 3		
T cells + Ag-pulsed PEC	28,073 ± 2,289 (11.3)	527
T cells + Ag-pulsed PEC + J4.1	29,498 ± 2,875 (10.2)	510
T cells + Ag-pulsed PEC + rIFNγ + J4.1	11,815 + 1,412 (4.9)	239
T cells + Ag-pulsed PEC + aIFNγ mAb	24,635 ± 2,098 (12.8)	503
T cells + Ag-pulsed PEC + rIFNγ + aIFNγ mAb	29,864 ± 2,817 (11.0)	545

3 of/de 3

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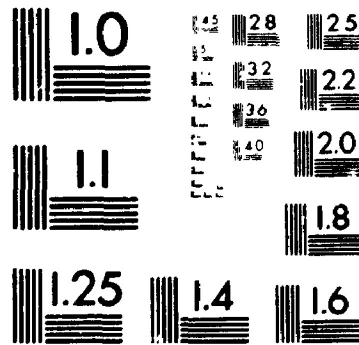


PRECISION℠ RESOLUTION TARGETS

Table 10

Effect of IFNγ on IL3 Production by T Cells in Response to Ag-Pulsed PEC

Cultures [a]	Δdpm ± SE (SI)[b]	IL3 Production[c] (ng/ml)
T cells + Ag-pulsed PEC	76,507 ± 6,912 (14.0)	842
T cells + Ag-pulsed PEC + rIFNγ + J4.1	27,695 + 1,075 (5.9)	391
T cells + Ag-pulsed PEC + rIFNγ + aIFN	75,917 ± 5,308 (16.0)	876
rIFNγ + J4.1-treated T cells + Ag-pulsed PEC	30,112 + 3,105 (6.1)	317
rIFNγ + aIFNγ-treated T cells + Ag-pulsed PEC	74,898 ± 7,117 (5.9)	819

[a,b,c] For details, see Table 9.

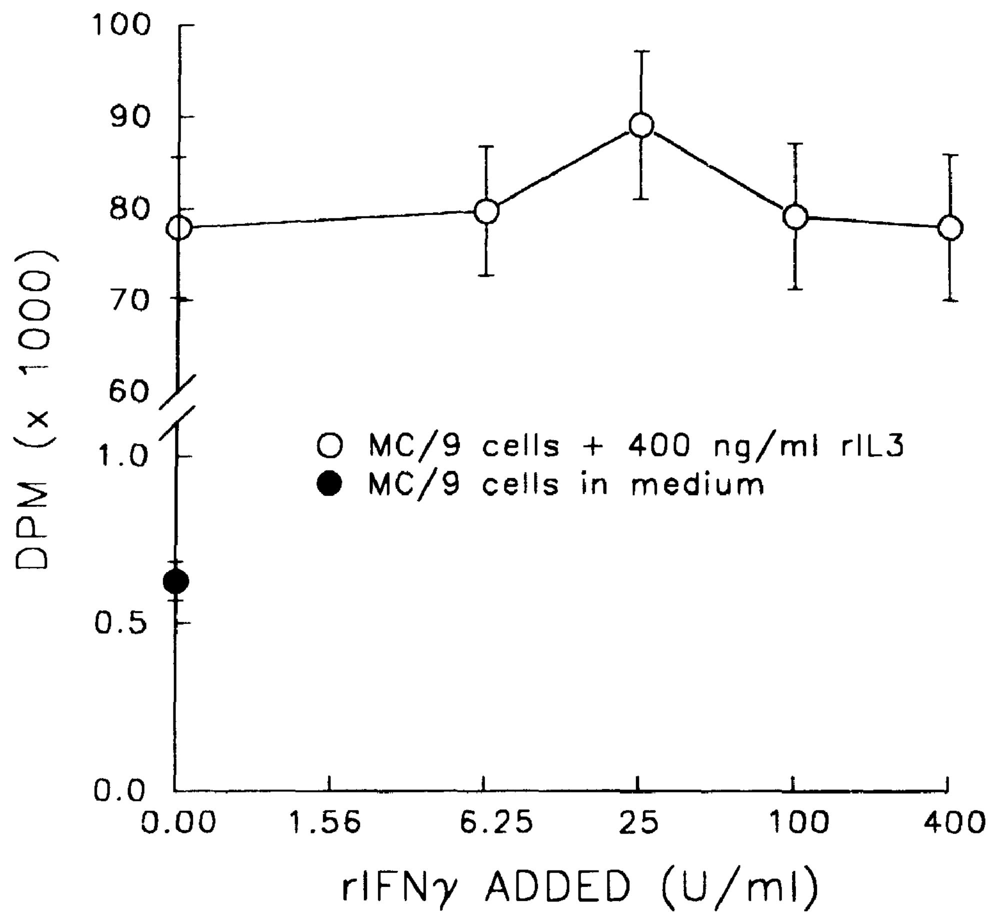
with IFNγ resulted in a reduction of IL3 levels from 842 to 317 ng/ml, the Δdpm from 76,507 to 30,112, and the SI from 14 to 6.1. In all cases, the decrease in IL3 production and T cell proliferation was attributable to IFNγ since aIFN but not the control mAb J4.1 could eliminate the inhibition.

To ensure that the reduction in IL3 (Tables 9 and 10) production, which was reflected by a reduction in the ability of the culture supernatants to induce MC/9 cell proliferation, was not due to a direct inhibitory effect of IFNγ added to cocultures, the MC/9 cells were grown in the presence of 400 ng/ml rIL3 and various concentrations of rIFNγ. As seen in Fig. 30, rIFNγ had no effect on the MC/9 cell growth. Taken collectively, these results demonstrated that IFNγ inhibited T cell activation by Ag-pulsed PEC, as measured by both T cell proliferation and IL3/cytokine production.

3.10.6 Cytofluorometric analysis of Ia+ expression on PEC grown in activated T cell supernatant

In our experiments, 400 ng/ml of aIFN mAb could easily neutralize 100 U/ml of exogenous rIFNγ. Yet, the addition of this mAb to cocultures of antigen-pulsed, X-irradiated PEC and T cells did not have any effect on the T cell response (Fig. 27, and 29, Table 7), even when the amount of mAb was increased to 10 ug/ml (data not shown). Possible explanations include: (1) Insufficient or no IFNγ was produced by the T line cells; (2) IFNγ was produced but did not have any detectable effect on T cell proliferation; (3) Endogenously produced IFNγ was not accessible to the aIFN mAb and therefore, could not be neutralized; or (4) IFNγ was produced by the T line cells, and was neutralized by the anti-IFNγ mAb, but effects similar to those of IFNγ

Fig. 30 Effect of rIFNγ on MC/9 cell growth. Five thousands MC/9 cells were grown in triplicate wells in medium containing 400 ng/ml rIL3 and various concentrations of rIFNγ (○). MC/9 cells were also grown in medium alone (●). Proliferative responses were assayed 48 hr later, and expressed as dpm ± SE.


100
90
80
70
60
1.0
0.5
0.0
DPM (x 1000)
0.00
1.56
6.25
25
100
400
rIFNγ ADDED (U/ml)
MC/9 cells + 400 ng/ml rIL3
MC/9 cells in medium

were exerted by other cytokines present in the cultures. To determine whether IFNγ was released by the T cells as a result of activation by the MBP-pulsed PEC, we tested the supernatants of these T cell cultures for their ability to increase MHC-class II expression on normal PEC in the absence or presence of aIFN mAb. Thus, the T cells were incubated with MBP-pulsed, irradiated PEC for 24 h, after which time the supernatant was collected. Freshly isolated PEC were then incubated for 48 h in this supernatant with or without the addition of 400 ng/ml aIFN mAb. As indicated in Fig. 31, up to 81% of the PEC population grown in the T cell-supernatant expressed Ia molecules while only 53% were Ia$^+$ in cultures grown in medium. This increase in Ia expression was due specifically to IFNγ since this effect was abolished by anti-IFNγ mAb.

3.10.7 Cytofluorometric analysis of Ia$^+$ expression on PEC grown with T line cells

The question arose then as to whether this endogenously produced IFNγ had a similar Ia-inducing effect on the MBP-pulsed PEC in the T-PEC cocultures, and whether the effect would be abolished by the addition of aIFN mAb. Thus, T line cells were incubated with MBP-pulsed PEC for 24 hr. The nonadherent cells were discarded, and the adherent population was collected, washed and assayed for Ia expression by cytofluorometry. As shown in Fig. 32, the antigen-pulsed PEC that had been grown with T cells were induced to express an increased number of Ia molecules, as indicated by an increase in fluorescence intensity and in the number of Ia$^+$ cells. However, Ia expression was not affected by the addition of a mixture of aIFN and aIL4R mAb. The addition of aIL4R antibody was predicated by

Fig. 31 Cytofluorometric analysis of Ia expression by PEC grown in activated-T cell culture supernatant. T line cells were grown with MBP-pulsed, irradiated PEC for 24 hr at which time the supernatant was collected. Freshly isolated PEC were grown either in complete medium or in the presence of T cell supernatant. In selected experiments, 400 ng/ml of anti-IFNγ mAb were added at the initiation of cultures. After 48 hr, the PEC were collected, washed, and assayed for Ia expression by cytofluorometry. The percentage of positive cells is indicated in brackets. Negative control refers to background fluoresence.

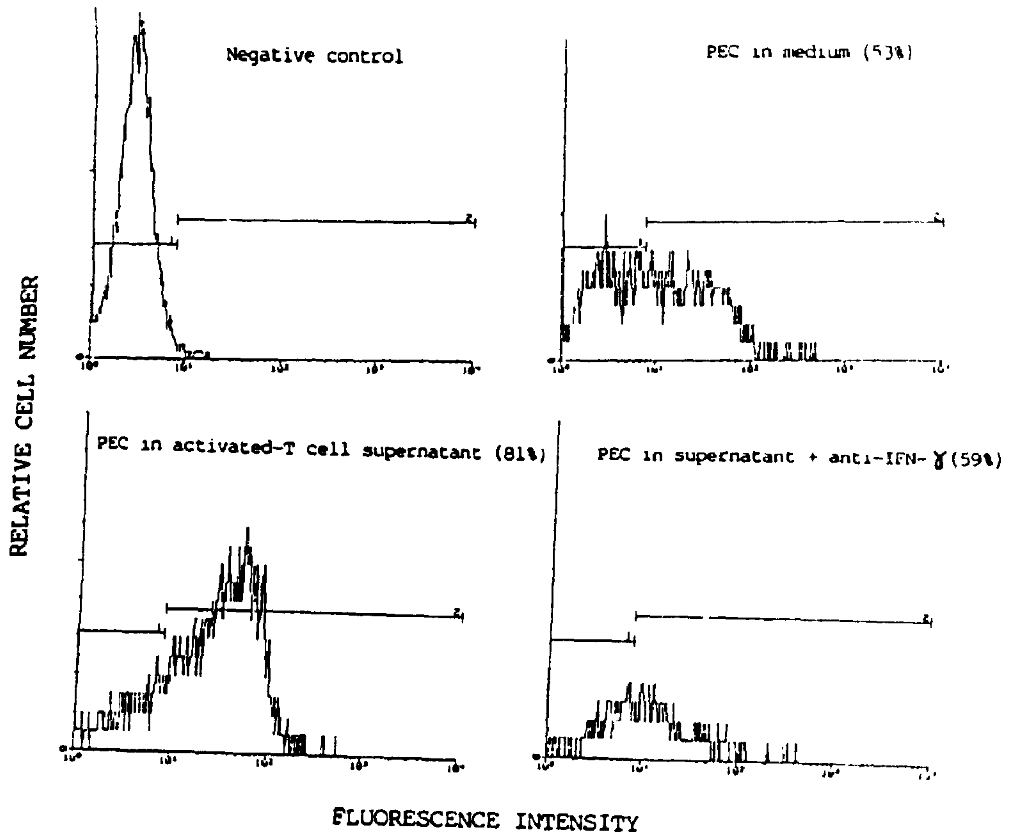
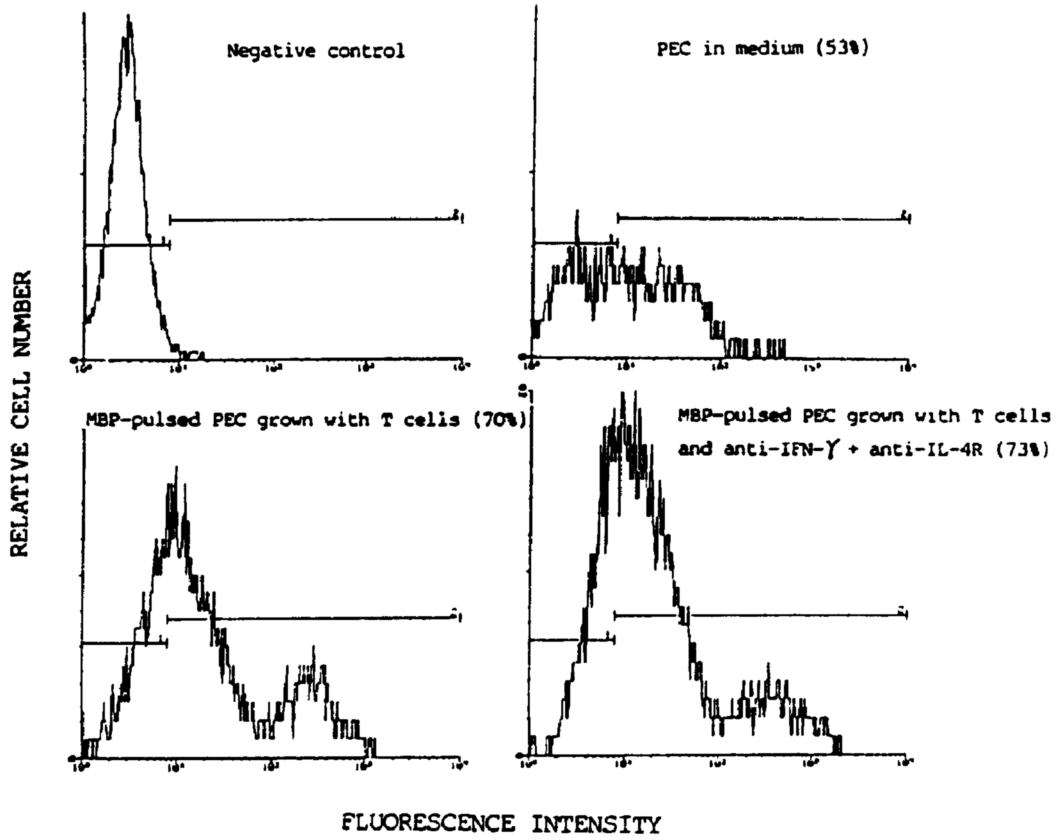

Negative control
PEC in medium (53%)
PEC in activated-T cell supernatant (81%)
PEC in supernatant + anti-IFN-γ (59%)
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY

Fig. 32 Cytofluorometric analysis of Ia expression by PEC grown with T line cells. MBP-pulsed PEC were grown with T line cells for 24 hr. After removing the nonadherent cells, the adherent cells were collected, washed, and stained for the presence of surface Ia molecules. In one experiment, 400 ng/ml aIFN and 10 µg/ml aIL4R mAb were added at the initiation of culture. The numbers in brackets indicate the percentages of Ia positive cells. Negative control refers to background fluorescence.


Negative control
PEC in medium (53%)
MBP-pulsed PEC grown with T cells (70%)
MBP-pulsed PEC grown with T cells
and anti-IFN-γ + anti-IL-4R (73%)
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY

the possibility that both IFNγ and IL4 may have contributed to increase in Ia expression by the APC (Cao et al., 1989).

Taken collectively, these results suggest that during the interaction between the antigen-pulsed PEC and T line cells, either most of the increase in Ia expression on the surface of the PEC was independent of both IFNγ and IL4, or these lymphokines were delivered across the membranes of the interacting cells in a way that rendered them inaccessible to the neutralizing effect of the antibodies.

3.10.8 Effect of exogenous IFNγ on T cell proliferation in response to IFNγ-pretreated, MBP-pulsed PEC

Since pretreatment of PEC with rIFNγ significantly enhanced T cell proliferation in response to Ag-pulsed PEC (Fig. 24 and table 6), it was of interest to determine whether the same pretreatment of PEC could counteract the inhibitory effect of IFNγ on the T cells. Thus, rIFNγ was added at time 0 to cocultures of T line cells and MBP-pulsed, IFNγ-pretreated or nontreated PEC. Since during the process of T cell activation, cytokines that are released may further affect the PEC, Ag-pulsed and fixed PEC were used in several cultures. The results presented in Table 11 can be summarized as follows:

(i) In the presence of exogenous IFNγ, the proliferative response of T cells to MBP-pulsed, nontreated PEC was again suppressed. The SI values were reduced from 176 to 52.

(ii) As seen in before, pretreatment of PEC with rIFNγ significantly enhanced their ability to induce T cell proliferation as the SI values were increased from 176 to 529.

Table 11

Effect of IFNγ on T Cell Proliferation in Response to IFNγ-Pretreated, Ag-Pulsed PEC

Pretreatment of PEC[a]	rIFN-γ added to cocultures[b]	SI
Irradiated PEC		
Nontreated	-	176
Nontreated	+	52
Treated	-	529
Treated	+	187
Fixed PEC		
Nontreated	-	12
Nontreated	+	1
Treated	-	31
Treated	+	4

[a] PEC isolated from SJL/J mice were cultured for 48 hr either in medium alone (nontreated PEC) or in medium containing 100 U/ml rIFNγ (treated PEC), were pulsed with MBP, and irradiated prior to coculture. In selected experiments, the PEC were fixed with 0.5% paraformaldehyde following the antigen pulse but were not irradiated. All PEC were then washed extensively prior to plating.

[b] 10^4 PEC were cocultured with 2.5×10^4 T line cells for 96 hr. In some experiments, rIFNγ was added at the time of plating, at a final concentration of 100 U/ml.

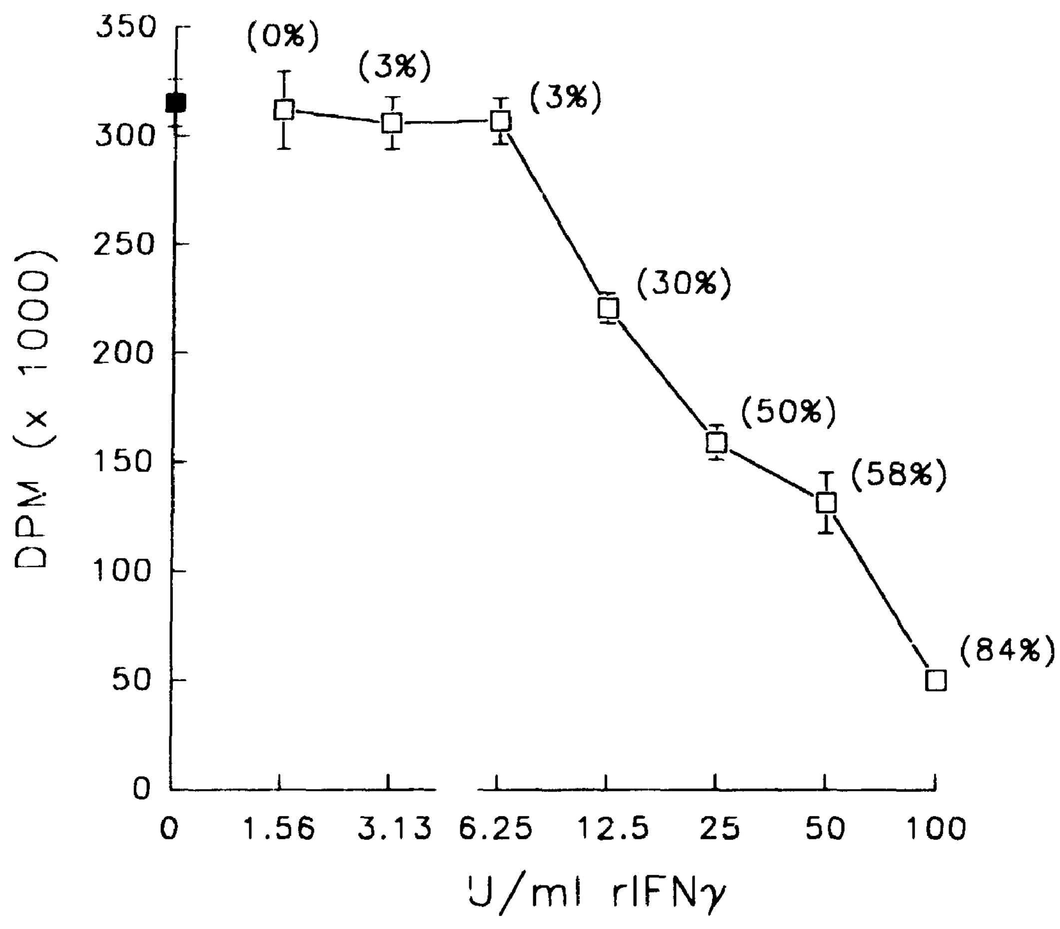
(iii) The addition of rIFNγ to cocultures of T line cells and IFNγ-pretreated PEC still inhibited the T cell proliferation. The SI value was reduced from 529 to 187.

(iv) The same pattern of the effect of IFNγ on T cell proliferation was observed when fixed PEC were used as APC.

3.10.9 Titration of inhibitory effect of IFNγ

From the studies described here it is evident that the addition of 100 U/ml IFNγ to the cocultures of T cells and Ag-pulsed PEC reduced T cell proliferation. On the other hand, the addition of IFNγ to the APC resulted in increased expression of MHC class II molecules and in a concomittant enhancement of T cell activation in response to Ag-pulsed, IFNγ-treated APC. One possible explanation for these opposing effects of IFNγ was that the amounts of IFNγ sufficient to increase Ag-presenting capability were too small to be inhibitory for T cell proliferation. To test this speculation, various amounts of IFNγ were added to the cocultures of T line cells and Ag-pulsed PEC, and the proliferative response of T cells was assayed as above. It can be seen from Fig. 33 that the inhibitory effect of IFNγ was dose dependent. In this experiment, the addition of 100 U/ml rIFNγ resulted in 84% reduction in dpm when compared to control cultures to which no rIFNγ was added. The degree of inhibition decreased with decreased amounts of IFNγ added. The lowest amount of exogenous IFNγ that still resulted in a detectable effect on T cell proliferation was 12.5 U/ml, at which point the proliferative response was reduced by 30%. The addition of 6.25 U/ml rIFNγ or lower was without effect. From Fig. 26, it is obvious that lower than 6.25 U/ml rIFNγ (0.5 U/ml) was still sufficient to

Fig. 33 Titration of inhibitory effect of IFNγ on antigen-specific T cell proliferation. Thirty thousand MBP-pulsed PEC were cocultured with 2.5 x 10^4 MBP-specific T line cells. rIFNγ was added to the cocultures at the start of the incubation period (□). Control cultures received no exogenous rIFNγ (■). Proliferative responses were assayed 96 hr later. The numbers in parentheses represent the percent inhibition of proliferation in comparison to the control.


350
300
250
200
150
100
50
0
DPM (x 1000)
(0%)
(3%)
(3%)
(30%)
(50%)
(58%)
(84%)
0
1.56
3.13
6.25
12.5
25
50
100
U/ml rIFNγ

upregulate class II MHC expression on APC.

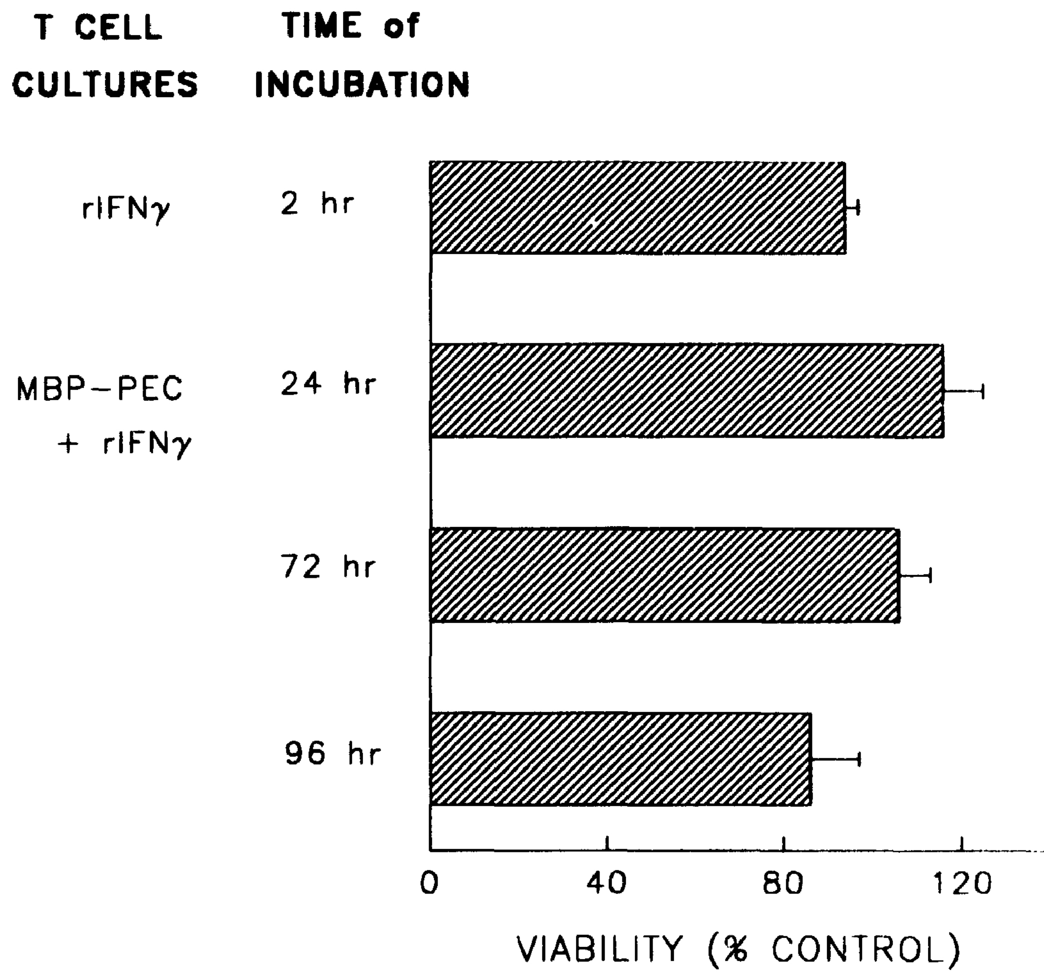
3.10.10 Viability of T cells pretreated with IFNγ

At the time of these studies, Liu and Janeway (1990) reported that IFNγ could induce programmed T cell death when the T cell receptors were cross-linked by anti-CD3 mAb in the absence of accessory cells. To determine whether, in our system, the reduction in T cell proliferation after treatment with rIFNγ was due to cell death, the viability of T cells in such cultures was examined by FACS analysis of propidium iodide-stained cells. As shown in Fig. 34, the viability of T cells after 2 hr incubation with rIFNγ or after various incubation periods with MBP-pulsed PEC and rIFNγ was virtually the same as that in control cultures which contained T line cells grown under the same condition but without rIFNγ. It should be noted that by tne end of 96 hr of incubation, the proliferative response of T cells grown with MBP-pulsed PEC and rIFNγ was only 65% of control cultures (data not shown) indicating that although the T cells were not killed, their ability to response to Ag-pulsed was reduced.

3.10.11 Effect of pretreatment of T cells with IFNγ on their expression of IL2R and calcium flux in response to Ag-pulsed PEC

Upon binding to the Ag-pulsed APC, a cascade of events occurs in the T lymphocytes, and proliferation is one of the end results. The question arose as to whether IFNγ affected earlier events in T cell activation. To address this issue, we examined the effect of IFNγ on the early events: the expression of IL2R on the T cells which occurs within hours, and the calcium flux which takes place within minutes, after contact with the Ag-pulsed APC.

Fig. 34 Determination of cell viability by cytofluorometry. T line cells were incubated in triplicate cultures in medium containing either rIFNγ (100 U/ml), or rIFNγ plus MBP-pulsed, X-irradiated PEC for various periods of time. In each case, control cultures contained T cells grown under the same conditions but without rIFNγ. At the time of the assay, the nonadherent cells were collected, washed, and their viability was determined after staining with propidium iodide.



T CELL CULTURES
TIME of INCUBATION
rIFNγ
2 hr
MBP–PEC + rIFNγ
24 hr
72 hr
96 hr
0
40
80
120
VIABILITY (% CONTROL)

3.10.11a IL2R expression

To examine the effect of IFNγ on the expression of IL2R, the T cells were first incubated with IFNγ for 2 hr, washed and cocultured with the MBP-pulsed adherent PEC. They were then collected at 24 and 96 hr, and assayed for IL2R expression by FACS analysis. As shown in Table 12, Exp. 1, 65% of the nontreated (control) T cell population expressed the IL2 receptor by 24 hr after the initiation of the cultures. By 96 hr, most of the receptors were lost since only 24% of these cells remained $IL2R^+$. In the IFNγ-pretreated T cell population, the percentage of IL2R expressing cells was significantly reduced. Only 44% and 9% of the treated population expressed IL2R at 24 and 96 hr, respectively. This corresponded to a decrease in the proliferative response of these treated cells. Δdpm was reduced from 34,491 as seen in the control culture to 19,541 in the treated population at the end of a 96 hr incubation period. The same pattern of reduced IL2R expression and proliferation in IFNγ-pretreated T cell population was observed when the experiment was repeated using a different MBP-specific T line cells (Table 12, Exp. 2). In this instance, only 55% and 11% of $IL2R^+$ cells were detected in the IFNγ treated lymphocyte population at 24 and 96 hr, respectively, in comparison to 78% and 33% in the nontreated population.

3.10.11b Calcium flux

One of the earliest detectable events in T cell activation is the calcium flux. To measure this, the T cells were first incubated with Indo-AM dye which is an ester taken up by all cells, and is known to bind to calcium. The amount of intracellular calcium is proportional to ratio of the bound vs free Indo. As seen in Fig. 35, within

Table 12

Effect of IFNγ on the Expression of IL2 Receptor by T Line Cells

Pretreatment of T cells[a]	% of IL2R$^+$ cells[b]		Proliferative response[c] (Δdpm)
	at 24 hr	at 96 hr	
Exp.1			
None	65	24	34,491 ± 2,642
IFNγ treatment	44	9	19,541 ± 1,721
Nontreated T cells grown with unpulsed-PEC	15	0	0
Exp.2			
None	78	33	41,273 ± 3,482
IFNγ treatment	55	11	28,571 ± 2,313
Nontreated T cells grown with unpulsed-PEC	27	8	0

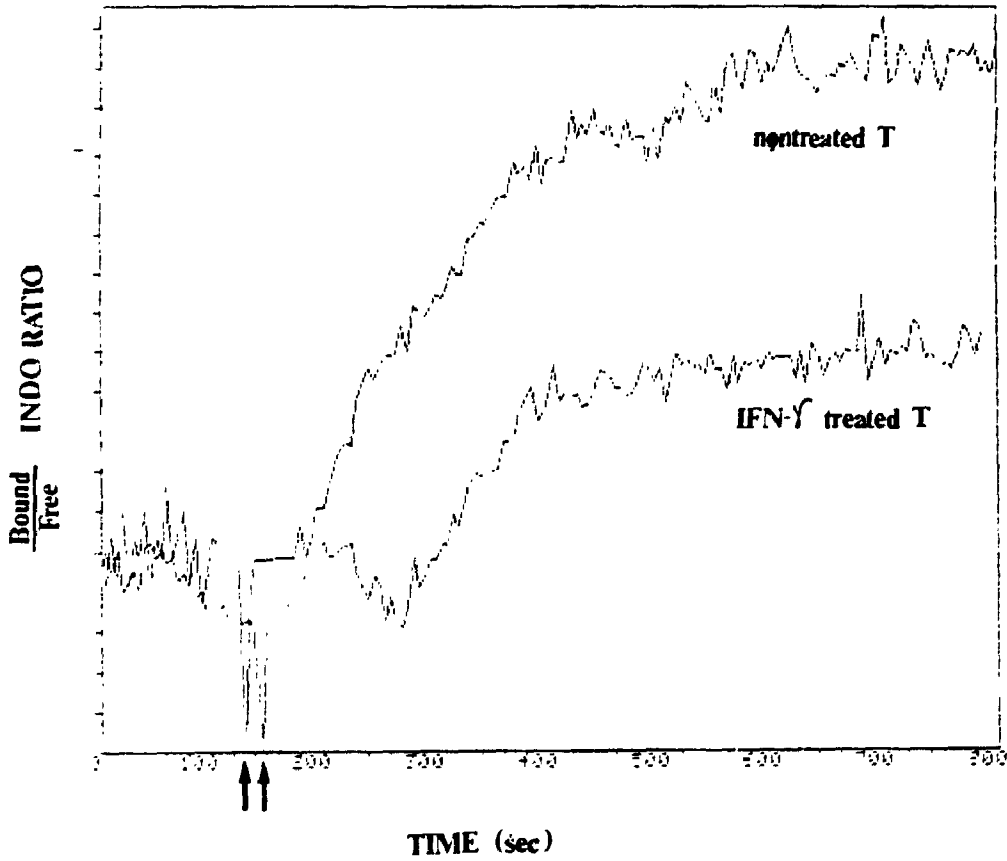
[a] MBP-specific T line cells were incubated at 37°C for 2 hr in either medium alone, or in medium containing 100 U/ml IFNγ. They were washed and cultured with MBP-pulsed or unpulsed (adherent) PEC.

[b] After either 24 or 96 hr of incubation, the T lymphocytes were removed, and assayed for IL2 receptor (IL2R) expression by cytofluorometry.

[c] Proliferative responses of the T cells in parallel cultures were determined at 96 hr and expressed as Δdpm ± SE.

seconds after the addition of MBP-pulsed PEC to the T cell cultures, there was a significant increase in the amount of calcium uptake by the cells. This calcium flux occurred in the T cell population only since the PEC were not stained with the Indo dye. The same pattern of calcium flux was observed in both control and IFNγ-treated cultures. The amounts of intracellular calcium increased quickly upon the addition of Ag-pulsed PEC, peaked at about 4-5 minutes after that, and stabilized for the next 7-8 minutes of observation. It must be noted, however that, in comparison to control ie. nontreated T cells, there was a drastic reduction in the levels of calcium uptake in T cells that were pretreated with IFNγ, suggesting that the inhibitory effect of IFNγ occurred very early in T cell activation.

Fig. 35 Effect of rIFNγ pretreatment of T cells on their Ca^{++} flux in response to the antigen-pulsed PEC. MBP-specific T line cells were incubated with 100 U/ml rIFN for 2 hr, washed, and loaded with Indo-AM dye as described in Materials and Methods. The arrows mark the time at which 6 x 10^6 MBP-pulsed PEC were added to 3 x 10^6 Indo-preloaded T line cells. The calcium flux was monitored for a period of about 13 min on a Becton-Dickinson FACSstar Plus Cell Sorter. Relative levels of intracellular calcium in the T cells were expressed as Bound/Free Indo ratios on an arbitrary scale. Control T cells (nontreated) were handled similarly, except that they were not pretreated with rIFNγ.


nontreated T
IFN-γ treated T
Bound/Free INDO RATIO
TIME (sec)

Chapter 4: DISCUSSION

4.1 Effect of aIFN and aIL2 mAb treatment on the development of EAE

Several studies have demonstrated that the chronic form of EAE can be readily induced in the SJL mice by the injection of MBP or of the encephalitogenic peptide MBP_{87-103} in CFA (Lublin,1984; Moore et al., 1987). However, we were unable to achieve high disease incidence and/or disease severity in these mice (Table 1). The reason for this discrepancy is not clear, but we cannot exclude the possibility of differences in the genetic background (ie. genetic drift) between our mice and those used by others, or differences in the potency of the guinea pig MBP preparations. The observation of low incidence of EAE in SJL/J mice challenged with the encephalitogenic MBP peptide was also reported by Sobel et al. (1990a). In our hands, the injection of spinal cord homogenate in CFA with or without Bp treatment resulted in the highest EAE incidence and severity (Table 1). Whereas MBP represents a single CNS antigen, the spinal cord homogenate consists of MBP and additional CNS antigens that may also contribute to encephalitogenicity. It has been well established that PLP is also encephalitogenic in the SJL mouse, and even more potent than MBP in disease induction (Sobel et al., 1990a). In addition, the galactocerebroside (GC), a sphingolipid that is enriched in myelin, has also been implicated in the pathogenesis of EAE based on the ability of anti-GC antibodies to mediate demyelination of nerve fibres *in vitro* (Dubois-Dalq et al., 1970) and *in vivo* (Brosnan et al., 1977; Lassmann et al., 1984). Myelin oligodendrocyte glycoprotein has also been reported to be encephalitogenic (Amor et al., 1994).

Several studies have reported that the administration of Bp along with the encephalitogenic challenge enhances both disease incidence and severity (Levine and Sowinski, 1973, 1974; Munoz and MacKay, 1984), and that Bp accentuates disease development through its ability to increase permeability of the BBB, thus causing "vascular leakiness" and allowing the entry of inflamed cells and mediators into the CNS (Munoz et al., 1981; Linthicum et al., 1982; Linthicum and Frelinger, 1982; Yong et al., 1993). Consistent with the enhancing effect of Bp, we observed increased EAE incidence and shortened disease onset in animals challenged twice with MBP-CFA and treated with Bp. However, under our experimental condition, Bp did not appear to have any effect on disease induction by one challenge with MBP-CFA or by one or two challenges with Sc-CFA. The reason for lack of effect by the Bp injection in these cases is not clear. Despite the difficulty in the active induction of EAE in this mouse strain, the disease can be readily induced by the transfer of MBP-activated lymph node cells (Pettinelli and McFarlin, 1981; Mokhtarian et al., 1984; Raine et al., 1984). In our hands, 80 to 100% animals developed EAE following the transfer of 20 x 10^6 MBP-activated mononuclear cells, with maximal clinical score of around 3 (hindleg paresis).

Phenotypic analysis has identified that encephalitogenic cells are $CD4^+$ and class II MHC restricted (Pettinelli and McFarlin, 1981). Upon activation, these cells produce a number of lymphokines such as IL2, IL3, LT, IFNγ , IL4, IL5, IL10 etc.... The role of each of these lymphokines in the pathogenesis of EAE has attracted much attention.

4.1.1 aIL2 and aIFN mAb treatment in the passive induction of EAE in SJL/J

mice

Although the main objective of this work was to investigate the role of IFNγ in EAE, we also examined the aIL2 mAb treatment in view of the fact that both IL2 and the expression of its receptor are crucial events in T cell activation and proliferation. Inhibition of T cell-mediated functions such as delayed-type hypersensitivity and graft rejection, and in several T-cell- and antibody-mediated autoimmune diseases was reported after *in vivo* treatment with aIL2R antibodies by a number of laboratories (Kirman et al., 1985; Kelley et al., 1986, 1988). In the Lewis rat, MBP-specific splenocytes cultured in the presence of MBP and aIL2R mAb exhibited markedly reduced proliferative responses and ability to transfer disease to naive syngeneic recipients (Hayosh and Swanborg, 1987). Based on these studies, it was suggested that in order to exhibit encephalitogenic property, the cells depend on IL2 for activation *in vitro* prior to the cell transfer. Although blocking the IL2R implies that the lymphokine itself is prevented from acting, the consequences of direct neutralization of IL2 by mAb had to be formally demonstrated. Our results showed that when administered to recipients, along with the cell transfer, the aIL2 mAb strongly inhibited both the clinical manifestations and disease incidence of passively transferred EAE in the SJL/J mouse (Fig. 1). In addition, in those animals with overt disease, the onset was also significantly delayed. It should be noted that while aIL2 mAb alleviated disease severity of the acute episode, it had no effect on relapses. It has been suggested that relapses in EAE require the reactivation of lymphocytes involved in the first attack, and/or of those that are specific for additional encephalitogenic antigen(s) generated as a

result of the first attack (Lehmann et al., 1992; Cross et al., 1993). Since the relapses occurred much later after the cell transfer and aIL2 mAb injection, it is possible that at the time of the relapse, most of aIL2 mAb, and hence IL2 neutralizing activity had been cleared.

It appears, therefore, that in order to exhibit full encephalitogenic activity, the cells must become reactivated *in vivo* and for this to occur, they depend on IL2 production. In support of this notion is the observation that treating cells with mitomycin C prior to transfer inhibited their ability to induce disease (Panitch, 1980). A role for IL2 in EAE induction is further supported by the findings by Rose et al. (1991) who demonstrated that depletion of IL2-utilizing cells by administration of human aIL2 Ab conjugated to a modified form of *Pseudomonas* exotoxin reduced EAE clinical manifestations, inflammation and demyelination in the CNS.

Whereas our results demonstrated that aIL2 mAb treatment alleviated the passively induced EAE, paradoxically, aIFN mAb treatment resulted in considerable disease exacerbation (see Fig. 36 for a summary of regulation by IL2 and IFNγ early in EAE induction). In most instances, while nontreated mice recovered from the disease, aIFN mAb-treated animals died or had to be sacrificed *in extremis*. This disease-enhancing effect was, however, completely neutralized by aIL2 mAb when the two mAb were given together at the time of cell transfer (Fig. 2). It is possible that since activation of encephalitogenic cells was inhibited by aIL2 mAb, the disease was prevented regardless of whether aIFN mAb was given or not.

The differences in clinical scores between the results presented in Fig. 1 and 2 can be explained by the fact that these experiments were carried out at different

times with lymphoid cells from different donors. Factors such as the virulence of the encephalitogenic T cells and the actual number of MBP-specific T cells isolated from the donors and reactivated *in vitro* in the presence of MBP could account for these differences. However, this does not mitigate the overall conclusion that treatment with aIFN mAb consistently exacerbated the passive transfer of EAE while treatment with aIL2 mAb reduced it.

The histological modifications observed are in line with those described by Moore et al. (1987) (Fig. 3). The most prevalent lesions was an extensive invasion of the white matter by mononuclear cells, to the point that the initial perivascular character of the infiltrates was no longer identifiable. Usually, massive infiltrates were associated with necrosis of the infiltrated white matter. Very extensive lesions were seen in mice exhibiting both severe (score of 5 or 6) and moderate clinical score (score of 3) (Table 2). The lack of a direct correlation between clinical and histological scores was not unexpected. First, both mice with clinical scores of 3 and 5 or 6 had paralysis, and only its extent was different. Secondly, lack of correlation between clinical and histological manifestations has been reported in both MS (Gilbert and Sadler, 1982) and in EAE (Paterson, 1982). It was observed that Lewis rats that were subjected to whole body irradiation prior to receiving MBP-activated LNC developed profound paralysis associated with intra-CNS deposits of fibrin, but unaccompanied by any evidence of cellular infiltrates. In addition, several protocols of EAE inihibtion have been shown to reduce clinical signs without any effect on histology (Swierkosz and Swanborg, 1975; Su and Sriram, 1991; Weinberg et al., 1994). It has been hypothesized that cellular infiltrates may be

secondary to the disease, and that fibrin-fibrinogen deposition and degradation, originally initiated by the effect of the inflammatory cells on the BBB, caused its "opening" of the BBB and allowed influx of fluid and inflammatory cells. Thus, clinical symptoms may correlate better with edema than with the cellular infiltrates in the CNS (Koh et al., 1992, 1993).

4.1.2 aIL2 and aIFN mAb treatment in the active induction of EAE in SJL/J mice

Our results also clearly indicated that aIFN mAb exerted a disease-potentiating effect in the development of actively induced EAE with both MBP-CFA and MBP_{87-103}-CFA (Fig. 4). However, whereas in passive EAE the disease-inhibitory effect of aIL2 mAb clearly neutralized the potentiating effect of aIFN mAb, this was not evident in active EAE. Although the addition of aIL2 to aIFN mAb treatment appeared to reduce severity of EAE (Fig. 4), there were no statistically significant differences in the clinical scores among these treated groups. It is possible that the failure of aIL2 mAb to dampen the effect of aIFN mAb during active induction might be overcome by increasing the dosage and/or the number of aIL2 mAb injections.

4.1.3 aIFN mAb treatment in the active induction of EAE in resistant mouse strains

Inasmuch that aIFN mAb treatment enhanced EAE in the susceptible mouse strain SJL/J, the question arose as to whether a similar treament would render resistant strains susceptible to the disease. Our results from experiments presented in Fig. 5 and 6 show that treatment with aIFN mAb following MBP-CFA challenge leads to overt disease in 5 mouse strains known to be refractory to EAE (Shaw et

al., 1992; Zhao et al., 1992). The disease induced by MBP-CFA and aIFN mAb in these resistant strains exhibits clinical and pathological manifestations typical of EAE and comparable to those seen in susceptible strains. Massive cellular infiltrations and demyelination were observed in diseased animals (Fig. 7). Taken together, these results suggest a disease-dampening role for IFNγ in both the susceptible and resistant mouse strains.

Induction of EAE and encephalitogenicity has been linked to the I subregion of the MHC (Fritz et al., 1985). The observations that either anti-T cell receptor or anti-Ia antibodies can inhibit EAE (Steinman et al., 1981; Zaller et al., 1990), and that MBP processing is required for disease induction (Cross et al., 1990b) support the notion that in suceptible animals, encephalitogenic T cells recognize and respond to a processed antigenic epitope in the context of the appropriate Ia molecule. EAE-permissive haplotypes include $H\text{-}2^s$, $H\text{-}2^u$, and $H\text{-}2^q$. However, our results demonstrate that mice of other haplotypes such as $H\text{-}2^d$ (BALB/c, DBA/2), $H\text{-}2^a$ (A/J), $H\text{-}2^k$ (C3H/HeJ) and $H\text{-}2^z$ (NZW) are rendered permissive by the administration of aIFN mAb. It is interesting to note that whereas aIFN mAb treatment significantly enhanced EAE in one $H\text{-}2^k$ strain (C3H/HeJ), it had no effect on the other (AKR). This observation supports the notion that susceptibility to EAE is not regulated solely by the MHC gene products, but that IFNγ and/or other factors are involved in disease resistance. In further support of this notion is the observation that SJL/J is susceptible to EAE induction whereas the B10. S strain is not even though both share the same $H\text{-}2^s$ haplotype (Korngold et al., 1986). Furthermore, several studies have suggested that susceptibility to EAE may reside

outside the MHC as high incidence of disease was seen in the SJL--->SJL and B10.S--->SJL bone marrow radiation chimeras, and low incidence in B10.S-->B10.S and SJL--->B10.S mice (Korngold et al., 1986; Lublin et al., 1986; Binder et al., 1993). It has also been suggested that genes for vaso-active amine sensitization may play a role in regulating susceptibility to EAE in mice (Linthicum and Frelinger, 1982).

As demonstrated in Fig. 8, EAE in the BALB/c mice could also be actively induced by MBP_{87-103}, the same peptide that is encephalitogenic for the SJL/J strain. However, in comparison to disease induced after aIFN mAb and whole MBP, the disease induced with this MBP peptide appeared later and was more severe. The reason for this is not clear. The intact MBP molecule may contain additional encephalitogenic epitopes for the BALB/c strain, and thus, may induce disease with a shorter time of onset. It is not known whether MBP_{87-103} is the major encephalitogenic epitope for the BALB/c, and hence, the question of why this peptide is a more potent inducer of EAE than the whole MBP molecule remains unanswered. In any case, the disease severity in the groups challenged by MBP_{87-103}-CFA followed by aIFN mAb treatment indicates that this peptide represents an encephalitogenic epitope for the SJL/J as well as for the BALB/c mice.

The observation that severe EAE was induced in the SJL/J plus 5 other strains following MBP-CFA and aIFN mAb injection raised the question as to whether the disease resistance may correlate with levels of IFNγ present. To our knowledge, there is no reliable ELISA system yet for the determination of serum levels of IFNγ since the lymphokine is susceptible to complement-mediated

inactivation in assay wells (van der Meide et al., 1991). Thus, IFNγ titers in sera are often determined by a plaque reduction assay using the L-929 cells with vesicular stomatitis virus as challenge virus (Shirahata et al., 1986). However, this assay does not distinguish between IFNγ and IFNα/β. Since neither anti-IFNα/β Ab nor the vesicular stomatitis virus was available to us, we were not able to determine differences, if any, in circulating levels of IFNγ among these strains. However, studies on IFNγ production by different strains suggest that disease resistance may not correlate with the levels of IFNγ present. Studies by De Gee et al. (1985) and by Shirahata et al. (1986) showed that there was no correlation between IFNγ levels in different strains and their susceptibility or resistance to African trypanosomes or to toxoplasma even though IFNγ has been shown to play a role in regulating susceptibility to both of these infections.

4.1.4 aIFN mAb treatment in the passive induction of EAE in resistant mouse strains

Among the three strains tested for passive induction, treatment with aIFN mAb enhanced EAE in the A/J and C3H/HeJ strains but failed to do so in BALB/c mice, suggesting that different regulatory mechanisms may be involved in EAE in various strains. In support of this is the report that cyclophosphamide markedly enhanced EAE development in the BALB/c but not in other strains (Arnon, 1982). Our results demonstrated that in the BALB/c mice, disease-preventing regulatory mechanisms can be overcome by a combination of active challenge and aIFN mAb, but not by the combined action of the transferred cells and the antibody. Consistent with this notion is the observation that EAE can be induced in a number of resistant

mouse strains by a combination of cell transfer and MBP-CFA challenge, and that neither the cell transfer alone nor the MBP-CFA challenge induces EAE (Shaw et al., 1992; Zhao et al., 1992).

4.1.5 Treatment with various amounts of aIFN mAb in the passive and active induction of EAE

Whereas severity of EAE in SJL/J mice was exacerbated in recipients of cells and 1 mg aIFN mAb, that in recipients of cells plus 300 or 100 μg mAb was of equal severity as in the controls (Fig. 10). However, the disease duration was much longer in mAb-treated animals than in controls. The reason for this effect is not clear but the observation raises the possibility that IFNγ may also play a role in disease recovery. In actively induced EAE, the treatment of challenged mice with 300 or 100 μg mAb appeared as effective at potentiating the disease as the treatment with 1 mg mAb (Fig. 9). Active induction of EAE involves the priming and activation of encephalitogenic cells. On the other hand, in the passive induction of EAE, the cells had been primed and activated *in vitro* prior to transfer. One can envisage that higher amounts of aIFN mAb may be required to regulate disease induced by already activated cells than for regulating priming and/or activation of naive resting cells. There is evidence that different requirements may operate for the regulation of activated and nonactivated cells (Byrne et al., 1988; Croft et al., 1994).

4.1.6 Treatment of aIFN mAb at various time points after active challenge or cell transfer

Our data clearly indicate that aIL2 mAb inhibits the passive induction of EAE

in the SJL/J mouse, and that aIFN mAb potentiates both the actively and passively induced EAE not only in the SJL/J but also in a number of other mouse strains, thus implying an enhancing role for IL2 and a dampening role for IFNγ in disease development. The anti-inflammatory role of IFNγ was of particular interest since IFNγ is notorious for its pro-inflammatory activities. The lymphokine is a potent macrophage activator (Farrar and Schreiber, 1993), and inducer of class II MHC expression on a variety of cell types including astrocytes and vascular endothelial cells in the CNS, both of which are known to play a role in presenting antigens to the inflammatory cells in the CNS (Fontana et al., 1984; McCarron, 1985, 1986; Massa et al., 1987). In addition, IFNγ induces the production and secretion of other inflammatory cytokines such as IL1 and TNF (Arenzana-Seidedos et al., 1985; Nedwin et al., 1985).

To examine more closely the effect of aIFN mAb in EAE, we first examined how the disease would be affected when the mAb was given at various time points following the cell transfer or the MBP-CFA challenge, since it was reported by Jacob et al. (1989) that in adjuvant arthritis, aIFN mAb had opposite effects on the disease development depending on the time of its administration. Our data suggest an early regulatory role for IFNγ in the development of EAE. In active EAE, the administration of aIFN mAb following each MBP-CFA injection, i.e. on days 0 and 7, resulted in the most severe form of EAE with the highest disease incidence in comparison to those receiving only one mAb injection (Fig. 11). Furthermore, disease severity as well as incidence decreased as the mAb was given later after the MBP-CFA challenge. It should be mentioned that in Fig. 11, the clinical scores

of animals treated with 1 mg dose of aIFN mAb on days 0 and 7 are lower than those in shown in Fig. 4. This difference is not unexpected since the experiments were done at different times on different batches of animals. In passive EAE, administration of aIFN mAb at the time of cell transfer exacerbated disease, whereas mAb administered on day 4 post-transfer or later failed to do so (Fig. 12). Thus, it appears that aIFN mAb must act early in disease induction, and is required at the time of both MBP-CFA challenges in order to exacerbate disease. Inasmuch that EAE is a model for MS, it is interesting to note that MS patients treated with IFNγ showed exacerbation of the disease (Panitch et al., 1987). The reason for this discrepancy is not clear, but it may be connected with the fact that the treatment was long after overt disease appearance and the patients had undergone already at least two overt clinical episodes.

4.1.7 Effect of aIFN mAb removal at various time points

To gain more insights into the time at which aIFN mAb is required, we used biotinylated mAb which would allow its removal at will by the injection of avidin. In the passive induction of EAE where aIFN mAb was given at the time of transfer, the injection of avidin after 24 hr or later was unable to alleviate the effect of the mAb, suggesting again that aIFN mAb acts early, and that a period of 24 hr between the mAb and avidin injections was sufficient for the mAb to exert its effect. It is worth noting that whereas the clinical scores in recipients of mAb at -24 hr and avidin at -8 hr or -3 hr were comparable to that of controls, disease duration was significantly longer ($p < 0.05$) (Fig. 14). This raised the possibility that when avidin was given only 8 or 3 hr prior to the cell transfer, not enough mAb was removed by the time

of cell transfer to completely neutralize its disease-exacerbating effect. Thus, small amounts of the mAb still present at the time of cell transfer affected the duration but not the severity of the disease. In line with this is the earlier observation that treatment of only 300 or 100 μg aIFN at the time of cell transfer resulted in longer disease duration without affecting disease severity (see section 4.1.5)

Our results from Fig.14 suggest that aIFN mAb was able to act even prior to the cell transfer, and thus, implying that the mAb exerts its effect on the environment in which the transferred cells operate. However, this does not exclude the possibility that the mAb has also an effect on the transferred cells.

As mentioned in Results, disease in recipients of aIFN mAb at -48 hr and avidin at -12 hr or -18 hr was exacerbated, whereas that in recipients of aIFN mAb at -48 hr and avidin -24 hr was not, even though in both cases, the mAb was allowed sufficient time to act (Fig. 14). It should be noted, however, that in these groups, the time period between the removal of aIFN mAb by avidin and cell transfer was different. This observation raised the possibility that within 24hr after aIFN mAb removal, the animals may be able to revert to the pre-mAb-treated status, and thus, would develop a conventional disease course upon cell transfer. Consistent with this notion is the observation that EAE in mice treated with bt-aIFN at -36 hr and avidin -12 hr was enhanced while that in animals treated with mAb at -72 hr and avidin -24 hr was not (Fig. 14). One possible explanation is that during this "recovery" period, the biologically effective levels of IFNγ were restored such that upon transfer, the cells find a normal environment. As mentioned above, this assumption is difficult to test in the absence of a reliable ELISA assay to measure

serum level of IFNγ. Alternatively, but not mutually exclusive is the possibility that whatever effect the mAb has on the host cells, such as increased expression of cell adhesion molecules, is reversible by the time the cells are transferred, and the animals reacquire their original disease susceptible state.

Taken together, the results from Fig. 13, 14 and Table 4 indicate that at least two important factors may be involved in determining whether or not the aIFN mAb treatment would affect the disease development in the passive induction of EAE: (i) the time period that the mAb is allowed to be present and to exert its effect, and (ii) the time period between the removal of the mAb and cell transfer.

In the active induction of EAE, a period of 12 hr between the aIFN mAb and avidin injections was sufficient for the mAb to enhance the disease (Fig. 16). This period was shorter than that required for the mAb to affect disease severity of passively induced EAE. Taken collectively, these observations suggest that active induction of EAE is more sensitive to regulation by IFNγ such that small changes affecting this regulation will allow disease development. This is consistent with the observation that even 100 μg of the mAb was sufficient to enhance active but not passive induction of EAE (see section 4.1.5). Similarly, the observation that, in the absence of aIFN mAb, active induction of EAE in the SJL/J and other mouse strains is much more difficult than the passive induction is consistent with this notion.

Inasmuch that IL12 is a potent inducer of IFNγ (Trinchieri, 1993), it is interesting to note that *in vitro* activation of PLP-specific LNC in the presence of IL12 enhanced their ability to induce disease, and that administration of Ab against IL12 following the transfer of PLP-activated LNC prevented EAE (Leonard et al.,

1995). It should be pointed out, however, that the *in vitro* effect of IL12 was found to be independent of increased IFNγ production. In the case of anti-IL12 Ab treatment, one may argue that when given *in vivo*, the Ab should be able to reduce IFNγ production, and hence, should enhance rather than inhibit EAE. Since the effect of aIFN mAb had to occur early in the induction phase, it is possible that in the case of anti-IL12 Ab treatment, a reduction in the level of IFNγ due to neutralization of IL12 occurred too late to have an effect. Besides, *in vivo*, the disease-enhancing effect of IL12 may not neccessarily operate through an increase in IFNγ level.

4.2 Effect of aIFN mAb treatment on the expression of VLA-4 molecules by lymph node cells and splenocytes, and of VLA-4, VCAM-I and ICAM-1 in the brain

In our studies and in those of others, we have seen that systemic administration of aIFN Ab exacerbates the disease suggesting a down-regulatory role for IFNγ. Our data further showed that the aIFN mAb can exert its effect early, at a time when the T cells could not have interacted with the BBB. This implies that systemic IFNγ is preventing the triggering of lymphocytes involved in disease induction, and/or, is affecting their ability to interact with, and cross the BBB into the CNS. A number of studies have demonstrated that increased expression of adhesion molecules and their ligands correlated with cell entry into the CNS (Cannella et al., 1990, 1991; Raine and Cannella, 1992; Raine et al., 1990b), thus suggesting a role for adhesion molecules and their ligands in the binding of inflammatory cells to the endothelial cells of the BBB at the time of entry. Two of the

most studied molecules are LFA-1 and its ligand ICAM-1. However, results on blocking entry of lymphocytes into the CNS, and hence EAE, by treatment with antibodies against ICAM-1 and/or LFA-1 have been inconsistent. One study reported that treatment with anti-ICAM-1 Ab inhibits EAE (Archelos et al., 1993), while others showed that the treatment had no effect (Canella et al., 1993; Willenborg et al., 1993), or that treatment with anti-LFA-1 Ab worsened the disease (Welsh et al., 1993). Recently, the role of the VLA-4 molecule and its ligand VCAM-1 has received increasing attention. Binding of SJL T cell clones specific for MBP to SJL brain microvessel endothelial cells was reported to be inhibited by Ab against VLA-4 or against its ligand (Tanaka et al., 1993). In addition, encephalitogenic T cell clones expressed high levels of VLA-4, whereas non-encephalitogenic clones did not, and the pathogenicity of these T cell clones can also be inhibited by mAb specific for VLA-4 (Baron et al., 1993). It has also been shown that treatment with Ab against VLA-4 prevented EAE development in rats (Yednock et al., 1992). Another study showed that VLA-4-negative T cell clones can induce EAE only if the host is pretreated with Bp and irradiated prior to cell transfer (Kuchroo et al., 1993). It was suggested that, in this case, pretreatment with Bp would increase permeability of the BBB, and thus facilitate migration of T cells into the CNS, thereby circumventing the requirement for VLA-4 expression. Our data from Fig. 17 and 18 showed that the percentage of $CD4^+$ cells expressing VLA-4 in the lymph nodes and was significantly higher in animals challenged with CFA or with MBP-CFA, and treated with aIFN mAb in comparison to that of challenged animals treated with the control Ab.

The brain tissue from animals treated similarly as those assayed for VLA-4 by FACS analysis described above were examined by immunohistochemistry for the expression of various adhesion molecules such as ICAM-1, VCAM-1 and VLA-4. Expression of these adhesion molecules were detected in the brain of animals challenged with MBP-CFA and treated with aIFN mAb while brain sections from animals treated with the control mAb were not stained or stained very faintly with ICAM-1 and VCAM-1, and not at all with VLA-4. It is possible that the expression of these molecules is enhanced by the presence of infiltrated cells, for example, by the cytokines released. In our control animals, since there was no cellular infiltration, the immunohistochemical staining technique used here may not be sensitive enough to detect the low expression of these molecules. The increased expression of VLA-4, ICAM-1 and VCAM-1 following the aIFN mAb treatment supports the notion that aIFN mAb acts early to enhance EAE by providing conditions either directly or indirectly for increased expression of adhesion molecules on the lymphocytes and on the BBB, thus, facilitating the entry of cells into the CNS.

4.3 Effect of aIFN mAb treatment on the production of IFNγ and TNF α/β by lymph node cells

Our data demonstrated that LNC obtained from MBP-CFA challenged and aIFN mAb-treated mice produced relatively lower amounts of IFNγ than LNC from challenged but nontreated animals. This observation is consistent with that reported by Mu and Sewell (1994). In the 3 experiments presented here, IFNγ production in the mAb-treated animals was reduced by as much as 70 to 85% in comparison to

nontreated animals, thus, excluding the possibility that aIFN mAb treatment enhanced EAE through induction of more IFNγ production. On the other hand, LNC from aIFN mAb-treated mice produced significantly more TNF-α/β than LNC from challenged but nontreated animals. Since the WEHI164 cells are inhibited by both TNF-α and β, and since anti-TNF-α- and anti-TNF-β-specific antibodies were not available to us, it is not known whether the difference seen here occurred in the production of TNF-α or β or in both. In any case, these results demonstrated that aIFN mAb treatment inhibited IFNγ production, while enhanced TNF α/β production by LNC. It is not clear as to whether the very cell that produces less IFNγ is compensating by producing more TNF α/β or distinct cells are involved. The observation that LNC from challenged and aIFN mAb-treated mice produced relatively higher levels of TNF α/β raises the possibility that aIFN mAb treatment enhances EAE indirectly through increased TNF α/β production which has been directly implicated as an inflammatory cytokine in EAE development. TNF α/β has been demonstrated to induce apoptosis in oligodendrocyte cultures, and mediate myelin damage (Selmaj and Raine, 1988; Selmaj et al., 1991a) as well as disruption of the BBB (Kim et al., 1992). A direct correlation was reported between TNF α/β production by MBP-specific T cell clones and their encephalitogenicity (Powell et al., 1990). Its direct impact on EAE was demonstrated by the observation that treatment of rats with human TNF-α exacerbated EAE while anti-TNF α/β antibodies protected mice form passive induction of EAE (Ruddle et al., 1990; Kuroda and Shimamoto, 1991). It is likely that enhanced VCAM-1 expression following the aIFN mAb treatment is secondary to the increase in TNF α/β

production since Barten and Ruddle (1994) reported that TNF can induce VCAM-1 expression, thus supporting the notion that aIFN mAb treatment enhances EAE through induction of increased TNF α/β production.

4.4 Effect of aIFN mAb treatment on the number of γδ TcR⁺ cells in the lymph nodes, spleen and CNS

The γδ TcR⁺ T cells have recently come under scrutiny in MS since they have been found in significant numbers in and around both the active and chronic MS brain lesions (reviewed by Raine, 1994ab). The observations that hsp 65⁺ oligodendrocytes were associated with γδ TcR expressing T cells, and that γδ TcR⁺ T cells could be stimulated by hsp to exhibit cytolytic activity on cultured oligodendrocytes suggested functional significance for these cells (Selmaj et al., 1991b; Freedman et al., 1991). Interestingly, in mouse EAE, γδ TcR⁺ T cells have also been implicated as they were detected in acute as well as in chronic lesions, and also in association with hsp 65 expression (reviewed by Raine, 1994ab). Recently, γδ TcR⁺ T cells were also shown to play a role in the DTH reaction. It was reported that under the influence of IL4, γδ TcR⁺ T cells enhanced the ability of αβ TcR⁺ T cells to mediate systemic transfer of DTH (Dieli et al., 1994). Inasmuch that our data demonstrated a disease-dampening role for IFNγ in EAE development, that IFNγ and IL4 are known to play opposite roles in many functions (Mond et al., 1986; Gautam et al., 1992), and that EAE is considered a manifestation of DTH response, we addressed the question as to whether aIFN mAb administration potentiated EAE by allowing increased IL4 levels, and hence, γδ TcR⁺ T cell activities. Our data, however, suggest that this does not appear to be the case. The

number of γδ TcR^+ cells in the CNS, spleen and lymph nodes was similar in both control and aIFN mAb-treated animals (Fig. 22). Studies on the role of γδ TcR^+ T cells in inflammation in general, and in MS and EAE have focused on those at the site of action, and thus, the lack of difference in the number of γδ TcR expressing cells in the CNS of treated and nontreated animals does not support the notion that aIFN mAb treatment enhances EAE by induction of increased γδ TcR^+ cell number. However, whether there is a difference in the function and/or activities of these γδ TcR^+ remains an open question.

4.5 Role of IL4 in the development of EAE in SJL/J mice

One of the known activities of IFNγ is its ability to down-regulate the development as well as the functions of the Th2 cell population. It has been demonstrated that IFNγ favours the differentiation of Th0 cells into the Th1 cells rather than the Th2 cells (Swain et al., 1991; Seder and Paul, 1994). Moreover, IFNγ has been reported to inhibit the proliferation and lymphokine production by the Th2 cells (Gajewski and Fitch, 1988). As already mentioned, Th1 cells are characterized by IL2, IL3, IFNγ and LT production whereas Th2 cells by IL3, IL4, IL5 and IL10. Since aIFN mAb administration resulted in reduced IFNγ production by LNC, it is possible that neutralization of IFNγ by aIFN mAb could contribute to a relative increase in the activities of Th2 cells, and their lymphokines, especially IL4 (Mu and Sewell, 1994).

4.5.1 Effect of aIL4R mAb treatment

Inasmuch that IFNγ seems to exert a dampening effect on EAE, we were interested in examining whether or not IL4 played any disease modulatory role. This

led to the experiments presented in Fig. 23 which showed that EAE exacerbation did occur after MBP-CFA challenge and aIFN mAb treatment, and that the effects of aIFN mAb administration could be partially overcome by concomitant administration of aIL4R mAb suggesting a role for IL4 in EAE development. Since a number of cell types express IL4R, such as mast cells, fibroblast, endothelial cells, T cells etc., it is not known which one was affected by this treatment.

4.5.2 Effect of IL4/aIL4 mAb complex treatment

The direct contribution of IL4 to disease induction was supported further by the observation that active induction of EAE was enhanced in the MBP-CFA challenged SJL/J mice by the administration of IL4/anti-IL4 mAb complexes (Table 5). It has been demonstrated by Finkelman et al. (1993) that IL4, when administered in the form of complexes with aIL4 mAb, has longer retention time in the circulation, and therefore, enhanced biological activities. As their studies pointed out, in order to have an effect, the lymphokine and mAb must be used at certain lymphokine to mAb molar ratios. At several molar ratios and injection schedules used here, IL4/anti-IL4 mAb treatment clearly determined EAE exacerbation. Both disease incidence and severity were clearly enhanced. The use of anti-IL4 mAb in all cases raised the possibility that the mAb rather than the IL4 contributed to enhanced disease. However, the injection of aIL4 mAb alone, even in amounts 15 times higher than that used here, following MBP-CFA challenge, was without effect (data not shown).

Taken collectively, the results presented in Fig. 23 and Table 5 support a role for IL4 in EAE development, and are in accordance with the hypothesis that

neutralization of IFNγ by aIFN mAb treatment may lead to increased IL4 activities, and hence, enhancing disease induction (see Fig. 36 for a summary of immuno-regulation early in EAE by IL2, IFNγ and IL4). It is interesting to note that under our experimental conditions, the disease induced by IL4/anti-IL4 mAb complexes was not as severe as that induced following aIFN mAb treatment. More severe EAE may be induced by other schedules and/or dosage of IL4/anti-IL4 mAb complex administration. Furthermore, aIFN mAb treatment may enhance EAE through more than one modality.

The observation that injection of IL4/anti-IL4 mAb complexes enhanced EAE raised the question as to whether aIFN mAb acted also through complexes of the IFNγ produced *in vivo* and the injected mAb, rather than by direct neutralization of IFNγ by the mAb. This possibility is unlikely, however, for several reasons. The production of cytokine/anti-cytokine mAb complexes did not enhance the *in vivo* effect for all cytokines, and the aIFN mAb used in our studies (XMG-6) failed to enhance the retention time and biological activities of IFNγ (Finkelman et al., 1993). Moreover, as reported in the case of IL4/aIL4 mAb complexes, the effect of IL4 was enhanced only when optimal molar ratios of IL4 and aIL4 mAb were used. These IL4/aIL4 mAb ratios range between 0.6/1 and 2/1. In our studies, doses of between 100 µg and 1mg aIFN mAb were effective, and It is unlikely that all these amounts were in the optimal range required for longer IFNγ retention. In addition, direct administration of IFNγ to mice resulted in reduced disease severity, and this did not require aIFN Ab (Billiau et al., 1988).

The suggestion that IL4 plays a role in EAE induction appears to contradict

studies demonstrating that EAE is inducible by the transfer of activated encephalitogenic Th1 cells (reviewed by Liblau et al., 1995; Baron et al., 1993; Rott et al., 1993). However, the observation that Th1 cells can transfer EAE does not exclude the possibility that Th2 cells can also cause disease or contribute to disease induction. Our finding on the effect of IL4 in EAE seems also inconsistent with reports demonstrating increased expression of IL10 mRNA during the recovery phase (Kennedy et al., 1992), and prevention of EAE by IL10 treatment (Rott et al., 1994). Those studies thus support a disease-dampening role for Th2 cells. However, they do not exclude a role for IL4 early in EAE induction. Treatment with IL4 was currently reported by Racke et al. (1994) to inhibit passive induction of EAE. It should be noted, however, that in their study, the cells were already primed, and activated prior to transfer and to the IL4 treatment, whereas in our studies, the cells had to be primed and activated *in vivo* under the influence of either aIFN plus aIL4R mAb, or of IL4/aIL4 mAb complexes. The issue as to which lymphokine(s) predominates at what stage of disease development mav be critical. IL4 may have a disease-dampening role while IFNγ a disease-exacerbating role at later stage in disease development, whereas they may play opposite roles early in disease induction.

As to how IL4 enhances EAE remains obscure. Like many other lymphokines, IL4 is a pleiotropic molecule with diverse functions on a variety of cell types. Its biological activities include stimulation of endothelial cells leading to enhanced adhesion of lymphocytes (Masinovsky et al., 1990; Thornhill et al., 1990; Galea et al., 1993). This property of IL4 may play an essential role in the

development of EAE since contact between the circulating immune cells and the BBB endothelial cells has been considered to be the first step leading to CNS invasion.

The current dogma is that EAE is a Th1 cell-mediated disease, based on the observations that encephalitogenic T cells express the Th1 cell lymphokine profile (Baron et al., 1993), and that treatment with pentoxifylline, which selectively suppresses the production of Th1- but not Th2-associated lymphokines, prevents the induction of EAE (Rott et al., 1993). The migration of encephalitogenic T cells into the CNS appears to depend on expression of adhesion molecules. IL4 has been reported to enhance, selectively, endothelial cell adhesiveness for T cell (Galea et al., 1993). Moreover, this lymphokine has been reported to be a potent modulator of VCAM-1 expression either alone (Smith et al., 1993), or in synergy with TNF-α (Briscoe et al., 1992). Our results are consistent with the notion that systemic IFNγ may suppress EAE by preventing the passage of Th1 cells through the BBB which may occur by interference with the ability of Th2 cells to upregulate adhesion molecule expression on the BBB endothelium and/or on the Th1 cells. In accordance with this notion, we observed that upon aIFN mAb treatment following the MBP challenge, the production of IFNγ by lymphocytes decreased significantly (see section 4.3). This may allow concomitant increase in IL4 production. On the other hand, TNF production was increased by the Ab treatment, and there was also an increase in the number of cells expressing VLA-4 (see section 4.2). Thus, it should be interesting to examine IL4 production by aIFN mAb-treated animals.

4.6 Effect of IFNγ on Ag-specific T cell activation by Ag-pulsed accessory

cells

We have observed that aIFN mAb and hence, IFNγ, acts early in the development of EAE. Collectively, these observations point towards two opposing functions of IFNγ. On one hand, IFNγ induces class II MHC expression on APC (reviewed by Farrar and Schreiber, 1993), thereby contributing to T cell activation. On the other hand, the exacerbating effect of aIFN mAb implies a suppressive role for this lymphokine. To gain more insights into the role of IFNγ, we examined the effect of IFNγ on T cell activation *in vitro* by Ag-pulsed APC. Our results demonstrate a two-sided effect for the lymphokine on the immune response. On one hand, IFNγ acts on the APC by enhancing their ability to induce Ag-specific T cell proliferation. On the other hand, IFNγ acts on the T cells themselves by inhibiting their response to the Ag-pulsed APC.

4.6.1 Effect of exogenous IFNγ on T cell proliferation in response to Ag-pulsed PEC

Our data presented in Fig. 24 and Table 6 are consistent with those by Beller (1984) demonstrating that IFNγ-pretreated PEC considerably enhanced antigen-specific T cell proliferation over the untreated controls. Since in this case the PEC were fixed and, hence, were not responsive to any lymphokine produced during coculture with the T cells, the enhancing effect should be attributed to the direct action of the rIFNγ on the PEC that occurred prior to fixation. This is further supported by the observation that the presence of aIFN mAb during the pretreatment abolished this enhancing effect. Similarly, IFNγ pretreatment of PEC significantly enhanced T cell proliferation in response to the pretreated, Ag-pulsed,

X-irradiated (nonfixed) PEC (Table 11). This appears to be at odds with earlier findings by Albina et al. (1991) that rIFNγ induced PEC to release nitric oxide radicals known to be inhibitory to T cells (Albina and Henry,1991). It must be noted, however, that in their system large numbers of peritoneal macrophages were used.

Several studies reported a function for IFNγ in promoting the expression of class II MHC on the APC surface (Beller, 1984; Cao et al., 1989; Mantergazza et al., 1991). In agreement with these studies, we have observed an increase in the number of cells expressing Ia molecules, as well as an increase in the number of Ia molecules per cell in the PEC population (Fig. 25 and 26). Since a number of nontreated PEC express Ia molecules constitutively, it is not surprising that fixed PEC that were not pretreated with IFNγ could also induce T cell responses to some extent.

The results obtained from cultures of IFNγ-pretreated T cells support the notion that IFNγ acted on the T cells themselves by inhibiting their proliferation in response to the Ag-pulsed PEC (Fig. 28). This is further supported by the observation that the addition of rIFNγ to cocultures of T line cells and rIFNγ-pretreated PEC still resulted in inhibition of T cell proliferation (Table 11). The inhibition was not due to toxic substances in the rIFNγ preparation since it was abolished by aIFN mAb. Furthermore, the results from Fig. 34 indicate that the reduction in T cell response was not due to T cell death, and that the T cells were inhibited from proliferation in response to the Ag-pulsed PEC but were not killed by IFNγ. Cytofluorometric analysis revealed that at the time of the experiments, the MBP-specific T cell preparations were composed of T cells only, thus excluding the

possibility that IFNγ might have acted indirectly in triggering an inhibitory pathway by contaminating cells (Fig. 29). Moreover, the observation that T cell proliferation was also reduced following the addition of IFNγ to the cocultures of T line cells and MBP-pulsed, fixed PEC further supports the notion that IFNγ acts directly on the T cells (Table 11). The PEC could not be activated by the IFNγ added or by any other lymphokine released by the T cells during cocultures since they were fixed and therefore, incapable of producing any inhibitory molecules. It should also be noted that in all cases where fixed PEC were used as APC, the T cell response was always lower than those in cultures containing live (unfixed) PEC. This is not unexpected since fixed PEC are unresponsive to IFNγ and/or other lymphokines released during the cocultures which may induce increased expression of accessory molecules that are known to be involved in T cell activation, such as LFA-1 (Molina and Huber, 1991), or the membrane-bound form of IL1 (Donnelly et al., 1990) or B7 (Linsley et al., 1991).

In addition to the PEC population, other types of conventional APC include dendritic cells, splenocytes and thymocytes. Our data from Table 8 indicate that the inhibitory effect of IFNγ was also observed when X-irradiated splenocytes or thymocytes were used as APC, and thus are consistent with the notion that the action IFNγ was on the T cells rather than the APC.

The degree of T cell proliferation as well as of IFNγ induced suppression varied among cultures. This may be explained by the fact that these experiments were carried out with different MBP-specific short-term T cell lines. Differences in reactivity between different lines could account for these variations. However, this

should not mitigate the validity of the results since the T cells responses were compared within and not between experiments. In most cases, the degree of inhibition was between 40-80 %. A higher concentration of rIFNγ may be needed to inhibit more strongly or completely the response. Alternatively, it is possible that not all T cells may be susceptible to the inhibitory effect of IFNγ (Gajewski and Fitch, 1988).

4.6.2 Effect of exogenous IFNγ on IL3 production by T cells in response to Ag-pulsed accessory cells

Activated T cells produce a number of lymphokines such as IL2, IL3, IL4, IL5, IL10, etc. Since our results demonstrated that IFNγ inhibits the T cell proliferation, the question arose as to whether lymphokine production was also affected. Our data on IL2 production were inconsistent and unconclusive since there was no correlation between the level of IL2 produced and the degree of proliferation (data not shown). The fact that the IL2 produced can also be taken up and utilized by the T cells in the cultures may explain the lack of correlation between proliferation and levels of IL2 in the supernatants. For this reason and because both Th1 and Th2 cells produce IL3, this lymphokine was examined instead. Our results demonstrated that pretreatment of T cells with rIFNγ not only inhibited their proliferation in response to the Ag-pulsed PEC, but also reduced IL3 production by these cells (Tables 9,10). Since in cases where the PEC were fixed, IL3 production was also reduced, this could not be attributed to IL3 being utilized by the PEC (Table 10). In addition, IL3 production was restored by the addition of anti-IFNγ but not by the isotype-matched control mAb. Thus, it appears that IFNγ acts on the T cells to

inhibit both their proliferation and lymphokine, or at least, IL3 production. It should be noted that since Ab against IL3 was not used in these cultures, and since the MC/9 cells may also be responsive to other cytokine(s) besides IL3, the amounts of IL3 showed here may have been over-estimated. However, this should not mitigate the overall conclusion that pretreatment of T cells with IFNγ or the addition of IFNγ to cocultures of T cells and Ag-pulsed PEC inhibited both the proliferation and cytokine production by the T cells.

4.6.3 Endogenous IFNγ production and its effect

Our results showed that IFNγ was produced by the activated T cells, and that this endogenously produced IFNγ might contribute to increased T cell responses through its ability to induce Ia expression on the Ag-pulsed PEC in the T-PEC cocultures (Fig. 31). In our studies, 400 ng/ml of aIFN mAb could neutralize 100 U/ml of exogenous rIFNγ. However, the addition of the mAb to cocultures of Ag-pulsed, X-irradiated PEC and T cells did not prevent the increase in MHC II expression on the PEC present in the cocultures (Fig. 32), even in the presence of up to 10 μg/ml mAb (data not shown). The failure of aIFN mAb to affect Ia expression on the PEC could be attributed to the fact that in the absence of IFNγ and/or IL-4 other factor(s) could induce increased Ia expression (Chang and Lee, 1986). It is known that the T cells and the Ag-pulsed PEC enter in contact due to multiple cell-cell interactions provided by Ag-Ia-TcR, CD4-Ia, CD2-LFA3, LFA1-ICAM-1 and CD28-B7 interactions (Linsley et al., 1990). These interactions on their own may account for increased Ia expression, and thus, the addition of aIFN mAb may not have any effect on the expression of Ia molecules by the PEC. Moreover,

delivery of IFNγ across the membranes of the interacting cells may render the lymphokine inaccessible to the mAb, thus protecting it from being neutralized. Kupfer and co-workers (Kupfer and Singer, 1989; Kupfer et al. 1991) showed that upon interaction with Ag-pulsed B cells, T cells exhibit polarized lymphokine synthesis and clustering of lymphokine receptors adjacent to the T-B cell contact area, suggesting directed secretion toward the bound B cell at the T-B bound membranes. It is also worth noting that the addition of aIFN mAb alone to cocultures of Ag-pulsed PEC and T cells failed to increase the T cell response over that observed in cocultures grown in the presence of the control mAb. One possible explanation is that the IFNγ produced by the T cells in cultures was present in an amount which, while sufficient to stimulate increased Ia expression, was below the level required to inhibit T cell activation. In support of this hypothesis is the observation that the doses required to induce increased Ia expression on PEC were much lower than those required to exert a detectable inhibitory effect on the T cell proliferation in response to Ag-pulsed PEC (Fig. 26 and 33).

4.6.4 Effect of exogenous IFNγ on IL2R expression and calcium flux by T cells in response to Ag-pulsed PEC

Upon coming into contact with Ag-pulsed APC, a cascade of events occurs leading to T cell activation and proliferation. The earliest detectable events involve increased turnover of phosphatidyl inositol and a rapid increase in cytoplasmic calcium concentration either through the opening of Ca^{++} channel in the plasma membrane or by mobilization of intracellular stores under the influence of inositol triphosphate. Whereas an increase in the expression of high affinity receptor for IL2

can be detected within hours, the Ca^{++} can be detected within minutes after the engagement of the T cells to the Ag-pulsed APC. Our data presented in Table 12 and Fig. 35 demonstrated that IFNγ exerted its inhibitory effect in T cell proliferation in response to Ag-pulsed PEC at a very early stage, such that both IL2R expression and Ca^{++} flux were affected. Since the T cell were not cloned, it is not known whether the inhibitory effect was selective on the Th2 cells, as reported by Gajewski and Fitch (1988).

Chapter 5: CONCLUDING REMARKS

Taken collectively, the work of this thesis demonstrated a disease-contributory role for IL2 in the development of EAE in the SJL/J mice, and a disease-inhibitory role for IFNγ in this mouse strain as well as in several other disease resistant strains. A disease-inhibitory function for IFNγ is further supported by preliminary experiments in which MBP-CFA challenged-IFNγ-knockout mice of the BALB/c strain (GKO) developed a lethal form of EAE with 100% incidence without the administration of aIFN mAb. In contrast, only 10% of the wild-type BALB/c developed a mild form of disease under similar conditions. These observations also indicate that IFNγ is not needed for EAE manifestations. As illustrated in Fig. 36 which summarizes the data presented, we propose that IFNγ may regulate EAE through multiple mechanisms, each of which may be operative at a different stage in the course of disease induction. As mentioned before, EAE is an inducible T cell-mediated autoimmune disease of the CNS, requiring the priming and activation of encephalitogenic cells. These cells must cross the BBB into the CNS, where inflammation and damage to the CNS occur. The data reported here support a disease down-regulatory role for IFNγ early in disease induction through its ability to act on the T cells themselves to inhibit their activation. In addition, IFNγ may interfere with the entry of cells into the CNS since administration of aIFN mAb enhanced VLA-4, VCAM-1 and ICAM-1 expression. The observation that LNC from aIFN mAb-treated animals produced more TNF-α/β is also consistent with the notion that IFNγ may further interfere with the entry of cells into the CNS by inhibiting the expression of adhesion molecules since TNF-α/β has been known

to induce expression of adhesion molecules and their ligands (Barten and Ruddle, 1994). Furthermore, IFNγ may modulate the disease by inhibiting the production of TNF-α/β which has been shown to mediate demyelination and apotosis of oligodendrocytes (Selmaj et al., 1988, 1991a), and to play a disease-enhancing role in EAE (Rudlle et al., 1990; Kuroda and Shimamoto, 1991; Santambrogio et al., 1993). Treatment of MBP-CFA challenged mice with a mixture of aIFN and aTNF mAb, of aIFN and aVLA-4 or aVCAM-1 mAb may further confirm this notion.

IFNγ has been known to inhibit the generation of Th2 cells and also their lymphokine production. The issue of IFNγ modulation of EAE through its ability to inhibit Th2 cell activation and/or the production of IL-4, which has been shown by the data presented here to play a role in disease development, remains to be further elucidated. This may bring about a re-evaluation of the role of Th2 cells in EAE, as the current dogma is that the disease is Th1 cell-mediated, based on the ability of Th1 cell clones to transfer disease. It would be interesting to examine the lymphokine profiles of lymphocytes from aIFN mAb-treated and from nontreated animals. IFNγ has been known to be inflammatory, and IL4 anti-inflammatory. The studies presented here, however, demonstrated that these lymphokines may play dual roles.

Inasmuch as EAE has been considered the best animal counterpart for MS, a human demyelinating disease of the CNS, the results presented here do not seem to be consistent with reports of exacerbation in MS patients treated with IFNγ. It must be noted that, however, treatment in MS can start only after diagnosis of clinical symptoms, whereas our studies concentrated on the function of aIFN/IFNγ

early in EAE development. The question of whether IFNγ plays an opposite role at later stages in EAE remains to be examined.

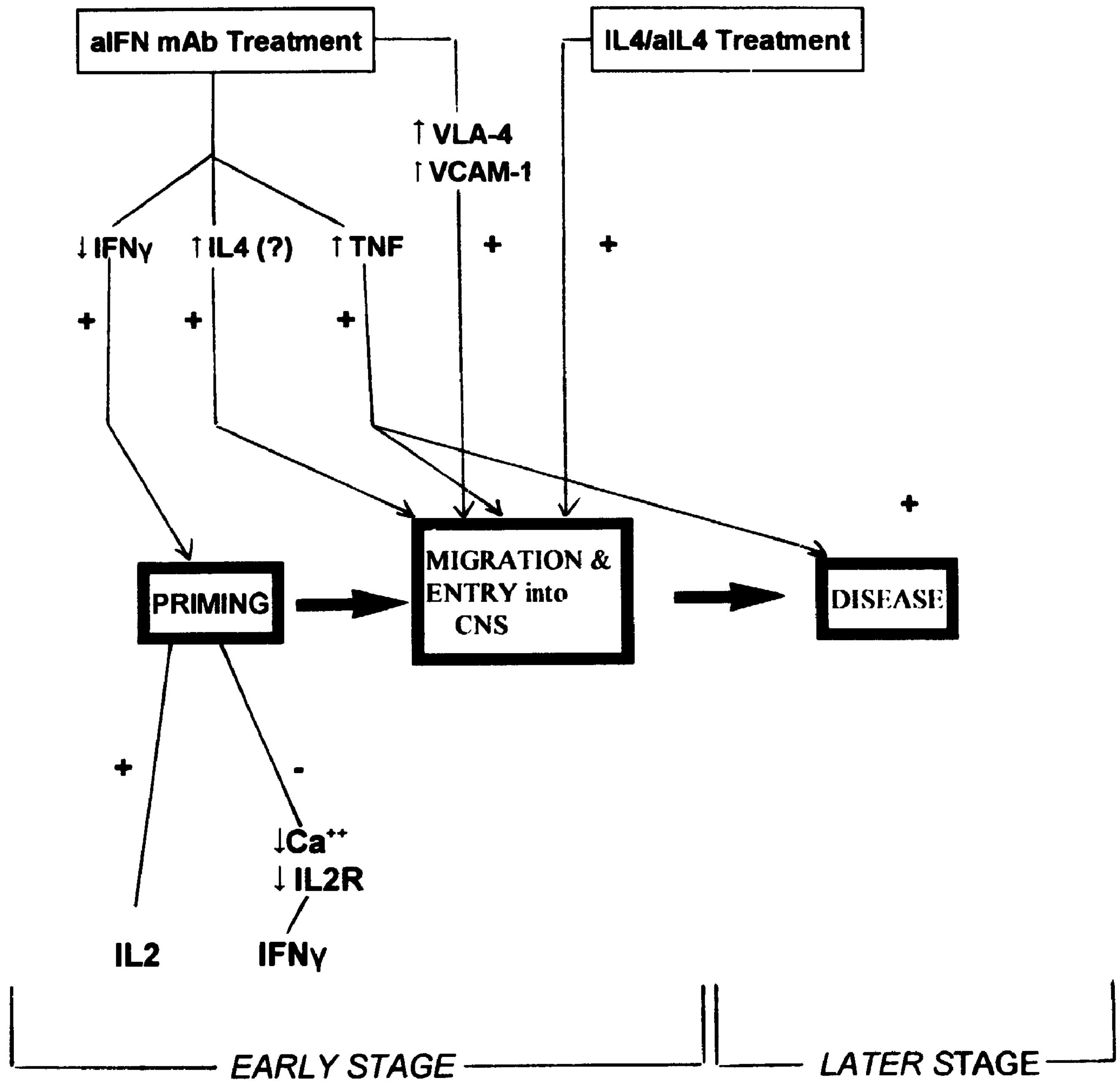


Fig. 36 Schematic diagram illustrating the regulation by IL2, IFNγ and IL4 early in the development of EAE. This figure summarizes the findings presented in the thesis, and illustrate a model for the immunoregulation of early stages in EAE induction by IL2, IFNγ and IL4. The plus and minus signs repre: ɪnt positive and negative effects, respectively The increase in IL4 levels following aIFN treatment was marked with a question mark since it was hypothesized based on lower IFNγ production, and was not actually determined.

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