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# CYTOKINE EXPRESSION AND REGULATION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

by

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# Cytokine expression in experimental allergic encephalomyelitis

# ABSTRACT

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease characterized by leukocytic infiltration of the central nervous system (CNS) and demyelination and remission/relapse. It is induced by $CD4^+$ T cells. We used reverse transcriptase/polymerase chain reaction to analyse T cell and cytokine gene expression in the CNS of SJL/J mice with myelin basic protein-induced EAE. Undetectable in normal CNS and cerebrospinal fluid, the expression of CD3, IL-2, IFN-γ, and TNFα increased in EAE, correlating with disease severity, then dropped to background levels during remission. IL-2 and IFN-γ were produced by $CD4^+$ $CD45RB^{low}$ T cells isolated fron LN and CNS. In contrast, TNFα was predominantly made by macrophages and microglia in the CNS. Purified microglia from normal CNS were induced to express TNFα by activated TH1 supernatant, suggesting that TNFα expression by cells in the CNS could be regulated by cytokines from infiltrating T cells. IL-4 was not detectable in total CNS or in isolated $CD4^+$ $CD45RB^{low}$ cells from the CNS, but was readily amplified from $CD4^+$ $CD45RB^{low}$ LN T cells. This suggests an enrichment of TH1 cells in autoimmune CNS.

To determine the effect of IFN-γ expression in the CNS, we produced transgenic mice using an IFN-γ cDNA downstream of an MBP promoter. Expression of the transgene was CNS-specific. MHC class I was induced in the CNS of transgenic mice. Transgenic animals that were backcrossed up to 5 generations with SJL/J did not develop spontaneous pathology. However, when they were immunized with MBP in adjuvant, the penetrance of EAE was greater, symptoms were more severe, and the duration of the first episode significantly longer than in non-transgenic littermates, suggesting a role for IFN-γ in the amplification and perpetuation of EAE.

# RESUME

L'encéphalomyélite allergique expérimentale (EAE) est une maladie autoimmune du système nerveux central (SNC) induite par les cellules T CD4$^+$. Chez la souris SJL/J, l'EAE peut être induite par immunisation avec la protéine basique de la myéline. Dans ce modèle, nous avons trouvé que l'expression des gènes de CD3, IL-2, IFN-γ et TNFα dans le SNC et le liquide céphalorachidien est directement corrélée à la sévérité de la maladie. Les niveaux d'expression de ces gènes chez des souris en rémission sont équivalents aux niveaux mesurés chez des souris normales. L'étude des sources cellulaires de ces cytokines dans le SNC a révélé que les ARNm d'IL-2 et d'IFN-γ sont transcrits par des cellules CD4$^+$, alors que le gène du TNFα est surtout exprimé par la microglie et les macrophages infiltrants. De plus, les cytokines produites par un clone Th1 induisent l'expression du gène du TNFα dans des cellules microgliales purifiées. Ces résultats suggèrent que, dans le SNC de souris atteintes d' EAE, l'expression du gène du TNFα est régulée par les cellules T infiltrantes, par le biais de leurs cytokines. L'absence d'expression du gène de l'IL-4 dans le SNC de souris atteintes d' EAE, alors que cette cytokine est abondamment transcrite dans leurs ganglions lymphatiques, indique une accumulation préférentielle de cellules T de type Th1 dans le SNC de ces souris.

Dans le but de définir le rôle de l'IFN-γ dans l'EAE, nous avons généré des souris transgéniques exprimant cette cytokine dans le SNC. Ces souris n'ont pas développé de manière spontanée une inflamation du SNC. En revanche, quand l'EAE est induite chez ces souris par immunisation avec la PBM, les symptômes sont plus sévères et la durée du premier épisode plus longue que chez des souris non-transgéniques de la même portée. Ceci suggère que l'IFN-γ est un facteur déterminant dans l'amplification et la progression de l'EAE, mais n'est pas, à lui seul, un facteur suffisant pour induire la maladie.

# ACKNOWLEDGEMENTS

I wish to thank all the people who made this work possible: my parents for their moral and financial support; my wife Brigitte for her unlimited patience and constant encouragement; little Tatiana whose first steps and words helped me forget many failed experiments.

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I would also like to mention all the people whose cheerful presence made five years of lab life seem so short: Johanne Poudrier and Diane Heath, for providing the musical background (all the leaves are brown....), and for their affection; Veronique Taupin for all the stimulating interactions; Rana Zeine, MD, PhD, for the interesting lunchtime conversations; Salimah Gillani, Michelle Krakowski, Ciro Piccirillo, Betsy Davidson, Vincent (Poursan) Dodelet, Martin (Tintin) Bourbonniere, Eustache (Boner) Paramithiotis, Ken Williams, JoAnne McLaurin, Annette Zerbib, and Nathalie Labrecque, for their friendship.

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# PREFACE TO THE THESIS

In accordance with the guidelines concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, I have opted to present the experimental portion of this thesis (Chapters 2-5, inclusive) in the form of original papers. A provision in the guideiines concerning thesis prepration reads as follows:

> *Candidates have the option, subject to the approval of their Department, of including as part of their thesis, copies of the text of a paper(s) submitted for publication, or a clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the study, when this review is appropriate, and (5) a final overall conclusion and/or summary.*
>
> *"Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (eg. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported in the thesis."*
>
> *"In the case of inclusion of manuscripts co-authored by the candidate and others the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make clear the responsibilities of the different authors of co-authored papers."*

I have included, as chapters of this thesis, four original papers, of which two have been accepted for publication, and two that have been submitted for publication. Chapters 2-5, inclusively, each contain an Abstract, Introduction, Materials and Methods, Results, and Discussion section. Chapters 3-5, inclusively, contain prefaces that serve as the connecting texts to bridge the manuscripts. A General Introduction and Concluding Chapter have also been included. References are listed at the end of every chapter.

The manuscripts, in order of their appearance in the thesis are:

1- Renno, T., R. Zeine, J.M. Girard, S. Gillani, V. Dodelet, and T. Owens. 1993. Selective Enrichment of Th1 CD45RB$^{low}$ CD4$^{+}$ T cells in autoimmune infiltrates in experimental allergic encephalomyelitis. Int. Immunol. In press

2- Renno, T., C. Piccirillo, J.Y. Lin, and T. Owens. TNFα expression by resident microglia and infiltrating leukocytes in the CNS of mice with experimental allergic encephalomyelitis: regulation by Th1 cytokines. Submitted.

3- Renno, T., S. Gillani, R.P. Sekaly, J.P. Antel, and T. Owens. Exacerbation of experimental allergic encephalomyelitis in transgenic mice expressing interferon-gamma in the central nervous system. Submitted.

4- Renno, T., J.Y. Lin, C. Piccirillo, J. Antel, and T. Owens. 1993. Cytokine production by cells in cerebrospinal fluid during Experimental Allergic Encephalomyelitis in SJL/J mice. J. Neuroimmunol. In press

I was responsible for all the research in Chapters 2 to 5, with the following exceptions:

Rana Zeine provided the FACS profile in Fig. 1, Chapter 2.

J.M. Girard and Salimah Gillani performed the cytokine immunostaining in Table 1, Chapter 2.

Salimah Gillani performed the class I MHC and myelin staining in Fig. 11, Chapter 4

Jia-You Lin extracted and characterized mouse CSF in Table 1, Chapter 5, and helped perfuse mice used in certain experiments described in Chapter 3.

Ciriaco Piccirillo performed PCR reactions in Fig. 2, Chapter 3, and in Figs. 3 and 4, Chapter 5.

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# CHAPTER 1

# GENERAL INTRODUCTION

Specific immune responses (IR) are generated when antigens (Ags) are recognized by T and B cells through their surface receptors. T and B cells are lymphocyte subsets that develop in the thymus and bone marrow, respectively. By virtue of allelic exclusion, each B cell expresses only immunoglobulin (Ig) receptors of only one specificity that recognize the native, 3-dimensional structure of protein Ags. The end result of the ligation of an Ig by its Ag is the production by the B cell (now called plasma cell) of soluble antibody (Ab) of the same specificity as its surface Ig. In most cases, Ab production requires, in addition to Ig ligation, help from T cells specific to the same Ag. Unlike B cells, T cells, also subject to allelic exclusion, recognize antigen in the form of processed peptides, in association with the products of the Major Histocompatibility complex (MHC).

T cells can be subdivided on the basis of their expression of the invariant CD4 or CD8 co-receptors. $CD4^+$ T cells recognize specific peptides in the context of class II MHC, whereas $CD8^+$ T cells recognize peptide in association with class I MHC. Class I MHC is expressed on all nucleated cells, usually associated with peptides that were generated through the catalytic pathway, whereas class II MHC is constitutively expressed only on certain cell types called antigen-presenting cells (APCs), normally in association with peptides generated, through the endosomal pathway, from exogenous Ags. Professional APCs, which include dendritic cells, macrophages, and B cells, also express costimulatory molecules without which a T cell cannot be primed. This requirement for co-stimulatory molecules on APCs, and the restricted cellular distribution of class II MHC means that primary activation of $CD4^+$ T cells will only occur in restricted conditions.

This is especially important given that $CD4^+$ T cells are the major cell type implicated in providing help to B cells, $CD8^+$ T cells, and macrophages, for Ab production, cytotoxicity, and phagocytosis/cytotoxicity, respectively. T cell help involves cytokine production.

(For review, see Fundamental Immunology, W.E. Paul, ed. 1989)

### Cytokines

Cytokines are secreted -and occasionally membrane-bound- mediators that are implicated in inflammation and haematopoietic development. The expression and regulation of some of these cytokines in autoimmune disease will be the focus of this thesis.

IL-2 is a 14-16 kD glycoprotein (by SDS-PAGE) (Robb et al., 1981) produced by antigen- or mitogen-activated T cells (Morgan and Ruscetti, 1976). Although its primary activity is the autocrine or paracrine stimulation of T cells, which it induces to grow and divide, other cell types express receptors to IL-2, namely natural killer (NK) cells, whose activity it potentiates (Seigel et al., 1987), and B cells (Hiroisho et 1992). Mice in which IL-2 expression was inactivated by gene targeting show normal thymocyte and peripheral T cell subset composition, but exhibit reduced polyclonal in vitro T cell responses and increased levels of serum IgG (Schorle et al., 1991).

IL-4, another T cell-derived cytokine, is a 12-15 kD glycoprotein (by SDS-PAGE) with a wide range of biological activities (Howard et al., 1982). IL-4 has been historically implicated as a growth factor for B cells (Howard et al., 1982). In vitro studies have shown IL-4 to be involved in thymocyte maturation (Lowenthal et al., 1988), in T cell growth (Sideras et al., 1988), and in the proliferation of a variety of haematopoietic precursors (Peschel et al., 1987). Studies in which the IL-4 gene has been inactivated by gene targeting have directly demonstrated the role of this cytokine in Ab isotype switching to IgG1 and IgE , but did not support the in vitro findings implicating this cytokine in thymocyte development and T cell function(Kuhn et al., 1991).

IFNs are divided into 2 types: type I IFN includes IFN-α and IFN-β, made by leukocytes and fibroblasts, respectively. These display antiviral properties (Trinchieri et al., 1985). Type II or immune interferon (IFN-γ) is primarily produced by antigen-activated T cells (Perussia et al., 1980). Murine IFN-γ is a 21-24 kD glycosylated polypeptide which acts through a widely distributed high affinity receptor (Trinchieri et al., 1985). IFN-γ plays a role in both the inductive and effector phases of the immune response. It is a potent activator of macrophages, in which it induces phagocytosis and other effector functions (Kelso et al., 1982). In addition to upregulating class I and class II MHC on the surface of APCs (Virelizier et al., 1984), IFN-γ can induce class II MHC in certain non-professional APCs (Massa et al., 1987), which might contribute to perpetuating the activation of $CD4^+$ T cells in tissues where APCs may not be readily available. Recently, experiments in which the IFN-γ gene was disrupted have pointed to an essential role for IFN-γ in inducing effector macrophage function and in the process of clearing intracellular pathogens from infected animals (Dalton et al., 1993).

The TNF family includes two proteins encoded by contiguous genes located within the MHC locus of both humans and mice. TNFα or cachectin is predominantly produced by activated macrophages either as a 17kD soluble factor (Beutler et al., 1986), or as a membrane-bound 26kD polypeptide (Kriegler et al., 1988). TNFβ or lymphotoxin is made mainly by activated T lymphocytes as a soluble 20-25 kD protein (Ruddle et al., 1967; Aggarwal et al, 1985). Both TNFα and lymphotoxin are found as homomultimers, and although they exhibit only a limited sequence homology (28-35%) at the amino acid level (Gray et al., 1984), the 2 molecules are functionally related since they act through the same receptor complex (Li et al., 1987). TNFα was originally identified as a cachexis-inducing factor produced by LPS-induced macrophages (Kawakami et al., 1982). It is now clear that this cytokine can be produced by most leukocytes. including T cells (Steffen et al., 1988). In addition to its toxicity to transformed cell lines, TNFα is expressed on the surface of cytotoxic T cells (CTL) whose activity can be blocked by anti-TNFα antibodies (Liu et al., 1989). Recent studies in which the gene encoding the TNF receptor has been inactivated have confirmed the role of TNF (α and β) in cellular toxicity and have directly implicated these cytokines in the clearing of intracellular bacterial infections (Rothe et al., 1993).

## Th1 v/s Th2

In mice, a functional dichotomy in cytokine production has been described for $CD4^+$ T cells, whereby IL-2 and IFN-γ are secreted by T helper (Th)1 (inflammatory) cells, and IL-4 is secreted by Th2 (B helper) cells (Mosmann and Coffman, 1989). TNFα is produced by both subsets. Original evidence for this dichotomy came from analysis of murine T cell clones, a large percentage of which secreted either IFN-γ or IL-4, but some of which were found to produce both these cytokines simultaneously. Interest in Th subtypes was enhanced when it was found that $CD4^+$ T cell populations in parasitized rodents and humans were biased towards production either of Th1 or Th2 cytokines, and that differential production of these cytokines correlated with resitance or susceptibility to certain pathogens (Locksley et al., 1987; Heinzel et al., 1989; Yamamura et al., 1991). Early attempts to identify the factors responsible for determining the Th subtype of $CD4^+$ T cells revealed differences in TCR-mediated signalling between Th1 and Th2 cells, at the level of phosphoinositide turnover (Gajewski et al., 1990). This led to the hypothesis that the TCR of a T cell determines, through differential signalling, whether a Th cell will be of one or the other subtype. Subsequent studies showed that T cells expressing the same transgenic TCR could secrete IFN-γ or IL-4, depending on the nature of the APC used, indicating an important role for APCs, rather than TCR, in this process (Seder al., 1992).

### Activation phenotypes

Regardless of the pattern of cytokines they produce, activated T cells can be identified phenotypically by examining the expression levels of certain surface molecules. For instance, signalling through the TCR induces the expression of the receptor for IL-2 on the surface of of T cells. Such an expression alone, however, does not necessarily indicate T cell activation, since it has been shown that anergized T cells (see below) can also express this receptor (Jenkins et al., 1987). One molecule whose expression levels have been associated with T cell activation is CD45R. Antigen stimulation of $CD4^+$ T cells *in vitro* induces the conversion of CD45R to low mol wt isoforms ($CD45RB^{high}$ to $RB^{low}$ in mice, and CD45RA to RO in humans) (Birkeland et al., 1989). T cells with this phenotype have been shown to be responsible for recall responses (Powrie and Mason, 1988), and to secrete elevated titers of cytokines upon *in vitro* activation, in contrast to naive $CD4^+$ T cells which express high mol wt isoforms of CD45R (Bottomly et al., 1989, Salmon et al., 1989).

### Tolerance

If Ag recognition invariably leads to T cell activation, then safeguards are needed to insure that IRs are not mounted against self Ags (self tolerance). Tolerance is obtained through the collective action of several mechanisms:

Clonal deletion. In the thymus, where immature T cells (thymocytes) develop, certain self Ags are expressed. Thymocytes that recognize these self Ags, in the context of self-MHC, with relatively high avidity, are clonally deleted (Kappler et al, 1987; MacDonald et al., 1988) by programmed cell death, or apoptosis (Shi et al., 1989;

Smith et al., 1989). Clonal deletion is not, however, sufficient to achieve tolerance to all self Ags. This may be due in part to the sequestration of certain self antigens in peripheral organs, away from the thymus. These potentially autoreactive thymocytes will thus escape deletion in the thymus and mature into peripheral T cells that will colonize the spleen and lymph nodes.

A mechanism that might deal with these autoreactive T cells in the periphery is clonal anergy. Anergy or inactivation is defined as the inability of a T cell to respond to antigenic stimulation by producing IL-2, and therefore by proliferating (Jenkins et al., 1987; Rammensee et al., 1989). Anergy is achieved when a signal is delivered as a result of TCR ligation by Ag/MHC, in the absence of a second, co-stimulatory signal. It has been shown that such a co-stimulatory signal is obtained upon the interaction of CD28, expressed on the surface of T cells, with its ligand, namely B7 or BB-1, on the surface of professional APCs (June et al., 1990). T cells that were thus rendered anergic will fail to be activated by subsequent encounter of with Ag/MHC, even on the surface of an APC. Given the inducibility of class II MHC on the surface of many non-APCs, this mechanism insures that autoreactive $CD4^+$ T cells will not be triggered simply as the result of the recognition of Ag/MHC on the surface of such cells (Burkly et al., 1989). It is noteworthy that clonal anergy has been observed in developing thymocytes, presumably as a result of inappropriate Ag presentation to thymocytes by thymic epithelial cells (Ramsdell and Fowlkes, 1990).

Peripheral deletion is yet another mechanism of tolerance induction. Indeed, it was shown that injection of adult mice with Mls-1 superantigen (SAg)-disparate cells leads to the proliferation, followed by the loss of SAg-reactive T cells (Jones et al., 1990). Finally, Ag-specific suppression (Germain and Benacerraf, 1981) and idiotypic networks (Jerne, 1974) have been postulated to play a role, although current thinking favors deletion/anergy models of tolerance induction.

### Autoimmunity

Despite these safeguards, functional autoreactive T and B cells exist (Sakagushi and Sakagushi, 1990; Milgrom and Witebski, 1962), and occasionally individuals develop autoimmune diseases. Some autoimmune diseases are characterized by widespread inflammation and by the presence of circulating autoantibodies. In most cases, it is not clear whether autoantibodies are responsible for the pathogenic process or simply one of its byproducts. Autoantibodies can exert their action in different ways. For instance, in systemic lupus erythematosus (SLE), where patients have high serum concentrations of anti-double stranded DNA Abs and commonly develop glomerulonephritis, it is believed that these Abs bind DNA in the circulation, followed by the deposition of the immune complexes thus formed in the basement membrane of the kidney, complement fixation, and eventually, tissue damage (Schwartz and Datta, 1989). Autoantibodies can also act by blocking an active site on their target, preventing the natural ligand(s) of that target from binding to it. This is the case in myasthenia gravis (MG), a disease characterized by weakness of skeletal muscles (Tzartos et al., 1991). Patients with MG have autoantibodies to the acetylcholin receptor. These Abs bind their target and prevent its binding of acetylcholin, which is required for triggering a muscle's action potential (Schonbeck et al., 1990).

Whatever their mechanism of action may be, autoantibodies (thymus-dependent ones, at least) would not be generated, and therefore would not be a factor in autoimmune diseases, without the contribution of the cellular compartment of the IR. This contribution is mainly in the form of $CD4^+$ T cell-produced cytokines that are necessary for B cell function. Cellular IRs have also been directly implicated in the pathogenesis of certain autoimmune diseases, including rhumatoid arthritis, juvenile diabetes, and multiple sclerosis (MS).

### Multiple sclerosis

MS is a disease of the central nervous system (CNS) that is characterized by myelin loss (demyelination), resulting in impaired nerve conductance and neurological symptoms. A striking feature of the disease is the relapsing/remitting course it often takes. Histologic analysis of an MS "plaque" shows T cells around the edge, whereas macrophages predominate in the center of the lesion (Hauser et al., 1986). Macrophages are thought to be an important effector cell type in the pathology of MS since they were shown to directly strip myelin from around axons (Raine, 1983), and because of their potential for cytotoxicity through the release of such substances as reactive oxygen species and TNFα (Nathan, 1987). The role of T cells in the pathogenesis of MS is less well defined. The autoantigen(s) in MS has not yet been identified, although different myelin components have been proposed to play that role. Myelin basic protein has received the most attention because MBP-reactive T cells have been identified in the blood of normal individuals (Burns et al., 1983), and with elevated frequencies in the blood and cerebrospinal fluid (CSF) of patients with MS (Allegretta et al., 1990, Olsson et al., 1990). CNS- and CSF-derived T cells exhibit a limited heterogeneity in the rearrangement of their TCRs (Wucherpfennig et al., 1992; Ben-nun et al., 1991; Oksenberg et al., 1990), suggesting an enrichment of T cells of certain specificities, presumably as a result of their proliferation in response to activation by autoantigen(s). The V-region biases vary between reports, possibly reflecting the genetic heterogeneity inherent in humans. Transfer of CSF from patients with MS into severe combined immunodeficiency (SCID) mice caused these mice to develop MS-like symptoms (Saeki et al., 1992). Given that the inflamed CSF contains predominantly T cells (Cashman et al., 1982), the data can be interpreted as supporting a role for T cells in the induction of MS, although this experiment requires validation.

The factors involved in susceptibility to MS have not been elucidated. There is evidence that genes encoding MHC antigens, especially class II Ags, are associated with increased incidence of the disease (Tiwari and Terasaki, 1985). Moreover, it was shown that a gene within the TCRβ complex or a closely linked locus influences the susceptibility to MS (Seboun et al., 1989). Taken together, these studies point to genetic factors in MS and to an association of the disease with ($CD4^+$) T cells. Studies on families confirm the influence of genetic factors. In one study, 26% of monozygotic twin pairs were concordant for MS, compared to 2.3% of dizygotic pairs, and 1.9% of nontwin sibling pairs (Ebers et al., 1986). However, the less than perfect concordance rate in monzygotic twins indicates that factors other than genetic (e.g. environmental), may be important in MS. The association of infectious agents such as viruses and bacteria with MS has been extensively investigated. However, there is no evidence to date implicating an infectious agent in the aetiology of the disease. Epidemiological data do show a strong geographical north-south gradient in the incidence of MS that cannot be explained simply on the basis of population genetics (Winn et al., 1989).

The study of early immune mechanisms involved in MS is complicated by the fact that patients are often diagnosed long after the initial insult, at a time when, due to the phenomenon of "epitope spreading" (Lehmann et al., 1992), it is difficult to interpret information on the Ag-specificity of early CNS-infiltrating T cells. Additional constraints are placed on the study of MS by the limited availability of

## EAE

Experimental allergic encephalomyelitis (EAE), an animal model for MS, has generated considerable interest in the past several decades.
Several features of EAE make it an attractive model for MS: First, like in MS, the course of EAE can be relapsing/remitting or chronic/progressive (Fritz and McFarlin, 1989; Baker et al., 1991). Second, although demyelination is not a key feature during the first episode of EAE, it is readily observed in mice with chronic EAE or in those that have experienced more than one bout of inflammation (Raine, 1983). Here also, the similarity to MS is striking, since early lesions in the human disease are more inflammatory than demyelinating (Raine, 1983). Finally, definition of the autoantigen in EAE makes the dissection of Ag-specific IRs possible, and the availability of imbred animal strains with varying degrees of susceptibily to EAE facilitates the study of genetic factors at play in autoimmune diseases of the CNS.

EAE has been traditionally induced [in a variety of animals, (mostly rodents, but also primates,)] by inoculation with crude CNS homogenate in complete Freund's adjuvant (CFA) (Kabat et al., 1946). Better definition of the autoantigens in these crude extracts has allowed the use of purified myelin proteins, such as MBP and proteolipid protein (PLP), and eventually defined short peptides from these proteins, in the induction of the disease (Tuohy etal., 1988; Fritz et al., 1990). The finding that EAE can be transferred into naive recipients by autoantigen- specific CD4$^+$ T cells, but not by CD8$^+$ T cells or B cells (Petinelli and Mc Farlin, 1981; Holda and Swanborg, 1983), and that the induction of the disease can be blocked by antibodies to CD4 or to class II MHC (Brostoff and Mason, 1984; Waldor et al., 1985; Sriram and Roberts, 1986 Sriram et al., 1987), has led to the now-widely accepted hypothesis that encephalitogenic peptides bind to class II MHC molecules and activate autoreactive CD4$^+$ T cells, which are therefore the mediators of EAE. Despite the apparent simplicity of this model, several issues need to be considered. First, what is the the site of primary activation of CD4$^+$ T cells, given the absence of MHC in normal CNS? Also, what are the requirements for encephalitogenicity of CD4$^+$ T cells and are these cells only inducers of the inflammatory process or are they also part (directly or indirectly) of the effector mechanisms leading to tissue damage? Another important question regards the factors that contribute to the susceptibility of certain strains of animals, but not others, to develop EAE. What mediates remission? Finally, what is the role of cytokines in EAE, what are their cellular sources, and how is their expression regulated?

In normal rodent CNS, in situ immunohistochemical staining has failed to demonstrate expression of either class I or class II MHC antigens. In vitro culture of CNS cells has however shown these antigens to be inducible on the surface astrocytes, microglia, and oligodendrocytes (class I only) (Yong and Antel, 1992). Class II MHC antigens can be readily detected immunohistochemically on microglia in the CNS of animals with EAE (Vaas et al, 1986). Both astrocytes and microglia can present Ag to previously activated CD4$^+$ T cell lines, in MHC class II-restricted manner (Frei et al., 1987; Sun and Wekerle, 1986). However, neither of these cell types is able to activate unprimed, naive CD4$^+$ T cells (Segdwick et al., 1991). This suggests that autoreactive T cells are primed in the periphery, before they migrate to the CNS, where their activation is then perpetuated by class II-bearing astrocytes and/or microglia. This is consistent with the observation that activated, but not resting, T cells are capable of crossing the blood-brain barrier (Hickey et al., 1991) (a highly specialized network of enthothelial cells with tight junctions, that controls the access of blood-borne factors into the CNS and preserves homeostasis in that tissue), and that activated (CD45RB$^{low}$) CD4$^+$ T cells predominate among infiltrating T cells in the CNS of mice with EAE (Zeine and Owens, 1992).

Activation of T cells leads, among other things, to the modification or upregulation of adhesion molecules (Springer, T.A., 1990). Several such molecules have been implicated in the traffic of T cells and in their ability to cross endothelia (Dustin and Springer, 1988). Of particular interest for EAE is the $\alpha 4\beta 1$ integrin, VLA-4, whose ligands are fibronectin and VCAM-1 (Raine and Canella, 1992). VLA-4 has been directly implicated in the binding of activated T cells to CNS endothelia (Yednock et al., 1992). Moreover, analysis of MBP-reactive T cell clones has correlated the expression of VLA-1 on these clones with their ability to enter the brain parenchyma and cause autoimmune disease (Baron et al., 1993). LFA-1 is another adhesion molecule expressed on the surface of T cells that has been shown to mediate adherence to endothelia through its interaction with its ligand ICAM-1. Anti-ICAM-1 Abs block the development of EAE (Baron et al., 1993).

In addition to the activation of their adhesion molecules, activated T cells produce cytokines. One important cytokine produced at the early stages of an inflammatory reaction is IFN-γ. Indeed, IFN-γ is a strong inducer of adhesion molecules on the surface of CNS endothelia, including VCAM-1 and ICAM-1 (Hughes et al., 1988). This is consistent with the observation that Th1 (IFN-γ-producing), but not Th2 clones, are encephalitogenic (Ando et al., 1989). Taken together, these experiments suggest a scenario whereby $CD4^+$ T cells encounter Ag in the periphery (LN), leading to their activation. Those clones expressing high levels of VLA-4 and LFA-1, and producing IFN-γ will upregulate VCAM-1 and ICAM-1 on the surface of BBB endothelia, bind to those receptors, and enter the CNS parenchyma. Two observations are potentially inconsistent with this scenario. First, injection of anti-IFN-γ Abs exacerbates, rather than protects from, EAE (Billiau et al., 1988; Duong et al., 1991). Second, some investigators have detected the Th2 cytokine, IL-4, in the CNS of animals with EAE (Merriil et al., 1992; Kennedy et al., 1992). *The issue of Th-1 v/s Th-2 cytokine expression will be experimentally addressed in chapter 2. Investigation of the potential role of IFN-γ in CNS pathology will be reported in chapter 4.*

TNFα is emerging as key player in EAE. When Abs to TNFα were administered after the adoptive transfer of MBP-specific T cells, they prevented the development of the disease, but did not prevent the transfer of EAE from TNFα-treated mice into naive recipients, and did not inhibit the proliferation of encephalitogenic MBP-reactive T cells in response to MBP (Selmaj et al., 1991). These data argue for a role of TNFα in the effector, rather than the inductive, phase of the disease. Moreover, TNFα is directly cytotoxic to oligodendrocytes (Selmaj and Raine, 1988). The cellular sources of TNFα in inflamed CNS remain poorly defined, although in vitro studies show the ability of cultured astrocytes and microglia to produce this cytokine (Righi et al., 1989; Lieberman et al., 1989), especially in response to IFN-γ (Frei et al., 1987). *These issues (cellular sources and cross-regulation of cytokines in EAE) will be addressed in chapter 3 of this thesis.*

The study of cytokines in EAE may not be only of interest for the understanding of the mechanisms underlying the disease. In MS, the only CNS-related compartment that is routinely available for testing is the CSF. Analysis of cytokines present in the fluid phase of the CSF have suggested correlation with disease activity in some studies (Sharief et al., 1991; Franciotta et al, 1989). Cytokine production by T cell clones generated from CSF in response to antigen has been reported (Benvenuto et al., 1991), as was cytokine production by individual CSF-derived T cells following short-term exposure to antigen in vitro (Olsson et al., 1990). However, a crucial question remains: does the immune status of the CSF compartment reflect that of the CNS parenchyma during disease, and therefore is the identification of cytokines in this compartment of useful diagnostic value? *In chapter 5, we take advantage of the availability of both CSF and CNS tissues in mice with EAE to address this question.*

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# CHAPTER 2

# SELECTIVE ENRICHMENT OF TH1 CD45RB$^{LOW}$ CD4$^{+}$ T CELLS IN AUTOIMMUNE INFILTRATES IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

# ABSTRACT

The cytokine effector status of CD4$^+$ T cells from LN and central nervous system (CNS) of SJL/J mice immunized with autoantigen in adjuvant for the induction of EAE was compared. CD4$^+$ T cells were FACS-sorted based on the levels of expression of the activation marker CD45RB. Low levels of expression of this surface marker are induced by antigen recognition and are associated with "effector" T cell function. Reverse transcriptase-PCR (RT-PCR) was used to analyse the expression of different T cell cytokine genes in the sorted populations. CD45RB$^{low}$ cells constituted a minority of CD4$^+$ cells in the LN and expressed elevated levels of IL-2, IFN-γ, and IL-4 messenger RNA (mRNA), whereas the CD45RB$^{high}$ CD4$^+$ population did not express detectable message for these cytokines under linear PCR conditions. By contrast to the LN, CD4$^+$ cells from the CNS were predominantly CD45RB$^{low}$ and expressed readily detectable levels of IL-2 and IFN-γ mRNA, but almost no IL-4 transcription could be detected. IL-4 mRNA levels in CNS were 100-250-fold lower than in LN. Also, IL-4 message could not be detected in the CNS one week after remission. A cytokine-specific immunocytochemical single cell staining technique was used to enumerate cytokine-producing cells in LN cell (LNC) populations and in CNS infiltrates. 1-5% cells in isolated LNC produced detectable IL-2 and IFN-γ. By contrast, the frequency of cytokine-producing cells stained in perivascular infiltrates in frozen sections from the brains of animals with active EAE was 10-fold higher. The frequency of cytokine-producing cells correlated closely with that of CD45RB$^{low}$-expressing cells in both the LN and the infiltrated brain. Our results confirm the functional significance of the CD45RB phenotype, and indicate an enrichment of Th1 cells in the CNS, which suggests an active selection process in the activation/accumulation of antigen-specific T cells at the site of inflammation.

# INTRODUCTION

Signalling through the T cell antigen receptor induces the *de novo* transcription of cytokine genes, and secretion of newly-translated cytokines (1,2). Antigen stimulation of CD4$^+$ T cells *in vitro* also induces the conversion of CD45 to low mol wt isoforms (3). T cells with this memory/effector phenotype secrete elevated titers of certain cytokines (eg. IL-4 and IFN-γ) upon *in vitro* activation, in contrast to naive CD4$^+$ T cells which express high mol wt isoforms of CD45 (4-7). A functional dichotomy in cytokine production has been described for CD4$^+$ T cells, whereby IL-2 and IFN-γ are secreted by Th1 (inflammatory) cells, and IL-4 is secreted by Th2 (B helper) cells (8). The strongest evidence for this dichotomy has come from analysis of murine T cell clones, but CD4$^+$ T cell populations in parasitized mice, and in rats and humans have also been shown to be biased towards production either of inflammatory (Th1) or helper (Th2) cytokines (9-11). Whereas it was originally thought that CD45R phenotypes might correlate with cytokine secretion patterns, current consensus is against any phenotypic definition.

T cell-derived cytokines are implicated in the induction of autoimmune inflammatory responses. The active lesions in clinical and experimental autoimmune inflammatory diseases such as adjuvant and rheumatoid arthritis, multiple sclerosis and EAE are characterized by the presence of T cells of the memory/effector phenotype (12-15), which correlates with elevated cytokine production (3,4). Elevated cytokine titers have been directly demonstrated in synovial fluid from patients with rheumatoid arthritis and in the cerebrospinal fluid of patients with multiple sclerosis and the brains of mice with EAE (16-19).

The phenotype and functional attributes of T cells immediately upon isolation must reflect their *in vivo* status. The commonly-used approach of isolating T cells and restimulating them *in vitro* not only removes them from their natural milieu, but experimentally perturbs their status. To better understand the role of cytokines in *in vivo* immune responses, it is important to analyze cells either in *situ* or without further *in vitro* stimulus. We have used FACS-sorting, RT-PCR and immunostaining to examine the production of IL-2 and IFN-γ mRNA by lymphocyte subpopulations in the LN and CNS of mice with EAE. Our results show enrichment for a memory/effector $CD4^+$ subset in the CNS relative to autoantigen-immunized LN, with correspondingly elevated frequencies and mRNA levels for IL-2 and IFN-γ. By contrast, whereas IL-4 mRNA was readily detectable in cells of similar phenotype in LN, it was undetectable in RNA from total CNS or from memory/effector T cells isolated from CNS. These results indicate that inflammatory infiltrates in the CNS contain selected and functionally distinct populations of activated cells from those in lymph nodes.

## MATERIALS AND METHODS

### Mice

Female SJL/J mice ( 5-8 weeks) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Cells from inguinal, brachial, para-aortic and axillary LN were used as LNC.

### Cell lines and mAbs

The following anti-cytokine mAbs (of rat origin) were used as culture supernatants; $IgG_{2a}$ anti-murine IL-2 (S4B6) (8), $IgG_1$ anti-murine IL-4 (11B11) (20) and $IgG_1$ anti-murine IFN-γ (R4-6A2) (21). These supernatants were inhibitory in cytokine-specific bioassays. Other rat mAbs included $IgG_{2b}$ anti-murine CD4 (GK1.5) (22) and $IgG_{2a}$ anti-murine $CD8_a$ (53-6.72) (23).

### EAE induction

EAE was induced by two subcutaneous injections 7 days apart, in the base of the tail and flank, of 500 µg rat spinal cord homogenate (RSCH) or 400 µg purified myelin basic protein (MBP) (Sigma, St. Louis, MO.) in CFA (Difco, Detroit, MI), containing 50 µg H37RA *M. tuberculosis* (Difco). Symptoms were first observed 14 days after the initial injection. The animals used for this study had severe EAE, corresponding to grades 3-4 (severe hind limb paresis and/or paralysis of one or both hind limbs) (24). Before removal of brains for histology, animals were anesthetized with chloral hydrate (3.5 g/Kg) (Fisher, Montreal, Qc.), then perfused through the heart with PBS or with paraformaldehyde (PFA) (Fisher, Montreal, Qc.) (4% in PBS).

## RT-PCR

Messenger RNA was isolated from FACS-sorted CNS cells and LNC using Quick-Prep$^{TM}$ (Pharmacia, Montreal). For PCR analysis, RNA from equivalent cell numbers was subjected to a one-step reverse transcription and amplification protocol. Briefly, mRNA was added to a tube containing 10 U avian myoblastosis virus reverse transcriptase (Pharmacia, Montreal, Qc.), 2.5 U Taq DNA polymerase (Gibco BRL, Montreal, Qc.), 20 U RNA Guard$^{TM}$ RNAse inhibitor (Pharmacia), 10 mM of each dNTP, 50 pmoles of each primer, and a buffer mixture consisting of 50 mM KCl, 100 mM Tris (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin. Primers used were as follows: CD3γ sense primer 5'-ATGGAGCAGAGGAAGGGTCTG-3', CD3g antisense primer 5'-TCACTTCTTCCTCAGTTGGTT-3', IL-2 sense primer 5'-TGCAGCTCGCATCCTGTCTCA-3', IL-2 antisense primer 5'-AGAAGGCTATCCATCTCCTCA-3', IFN-γ sense primer 5'-ACACTGCATCTTGGCTTTGC-3', IFN-γ antisense primer 5'-CGACTCCTTTTCCGCTTCCT-3', IL-4 sense primer 5'-ATGGGTCTCAACCCCCAGCTA-3', IL-4 antisense primer 5'-CTACGAGTAATCCATTTGCAT, actin sense primer 5'-TGGGTCAGAAGGACTCCTATG-3', Actin antisense primer 5'-CAGGCAGCTCATAGCTCTTCT-3'. Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler (Norwalk, CT) for 15 minutes at 50°C then amplified for 25 cycles (denaturation 1 min 94°C, annealing 2 min 60°C, extension 2.5 min 72°C). 40 µl per sample were run in 1% agarose gels in TAE buffer. DNA was transferred to Nytran membranes (Schleicher and Schull Inc., Keene, NH) by vacuum blotting (Pharmacia), and hybridized by incubation with $^{32}P$-labelled cDNA probes for 18 hours at 42°C in 50% formamide, 1 M NaCl, with 1.0% SDS and 10% Dextran Sulphate (25), then washed for 5 minutes at room temperature in 1 X SSC, 0.1% SDS, 10 minutes 1 X SSC 0.1 % SDS at 65°C, 10-15 minutes in 0.1 X SSC, 0.1% SDS at 65°C and 20 minutes in 0.1 X SSC, 0.1% SDS at 65°C. Probes were labelled by random priming 25 hg DNA with [a$^{32}$P]dCTP (5 µCi) (ICN, Mississauga, Ont.), using a kit (Gibco/BRL). Cytokine probes were cDNA inserts purified from plasmids described by

Kelso and Gough (26): IL-2, an N-terminal 337 base pair PstI-HindIII fragment, and IFN-γ, a 643 base pair PstI fragment. The actin probe was a purified amplification product. The CD3 cDNA used was a 700 bp HindIII + EcoRI fragment from clone pB10.AT3g-1 (27), kindly provided by Dr R.P. Sekaly (IRCM, Montreal).

### Histology

Brains and lymph nodes were postfixed following removal from mice by incubation for 1 hour in 4% PFA in PBS, then infused with 20% sucrose in PBS overnight at 4°C. Tissues were frozen in 2-methyl-butane on dry ice, and 10-12 μm sections cut using a cryostat. Sections were stained with haemotoxylin and eosin for evaluation of infiltration and for orientation of cytokine staining of adjacent sections. Infiltrated cerebellum was chosen for staining, because of the large number of infiltrates available per section. Sections were cut from brains of 4 mice with EAE. Between 5-15 perivascular infiltrates were scored per mouse. More than 3000 cells were counted altogether. As controls for cytokine staining, cytokine cDNA-transfected plasmacytomas (28) and cells of a Th1 clone were fixed with PFA after adhesion to poly-L-lysine-coated slides.

### Cytokine staining

Slides were dehydrated through a graded ethanol series, then endogenous peroxidases were neutralized by incubation in 0.3% hydrogen peroxide in methanol. Slides were incubated with 1% normal goat serum (Organon/Teknika Inc, Scarborough, Ontario) for 1 hour at room temperature, then for variable times (overnight or 1 hour, depending on the cell source and the determinant being detected) at 4°C with mAb supernatants, followed by two successive sequences of 2% goat anti-rat Ig (Organon/Teknika) and rat peroxidase-anti-peroxidase (rat PAP, Sternberger Monoclonals Inc, Baltimore, MD), diluted to 2% in PBS containing normal goat serum. Unlike staining for surface antigens such as Thy-1, CD4 and CD8, this 'double bridging' protocol was usually necessary for detection of cytokines in untransfected cells. With this modification, the immunoperoxidase staining technique was sufficiently sensitive to detect intracellular cytokines in both cell lines and lymph node cells. Diamino-benzidine (Sigma) at 600 µg/mL with 0.3% hydrogen peroxide in PBS was then added for 10 minutes, and the reaction stopped by immersion in distilled water. Slides were then dehydrated in ethanol before mounting in Permount (Fisher). Slides were photographed using a Nikkon photomicroscope equipped with Nomarski filters. In control stainings, the proportions of Con A-primed and anti-CD3 restimulated cells that stained with anti-Thy-1.2 (74.9 ± 12% ), anti-µ (10.4 ± 7%) and anti-MHC II (3.6 ± 1.7%) were comparable with proportions determined by FACS (88.0%, 4.1% and 1% respectively), and the specificity of cytokine staining was confirmed by staining a Th1 clone and cytokine cDNA-transfected plasmacytomas. In brain sections, staining with anti-CD8α (53-6.7, rat $IgG_{2a}$), the isotype control for anti-IL-2, was only slightly above background (Table 1).

### Isolation of lymphocytes from CNS.

Animals were perfused through the heart with PBS, then brains and spinal cords removed, dissociated through nylon mesh, centrifuged at 200g for 10 minutes and then the pellets were centrifuged on a discontinuous (30%:37%:70%) Percoll gradient at 500g for 15 minutes (15). Mononuclear cells were obtained from the 37%:70% interface, washed and analyzed by flow cytometry (15).

### Flow Cytometry.

Cells were stained by sequential incubations (20 minutes at 4°C) with primary mAbs, followed by FITC-coupled goat anti-rat IgG (Kirkegaard Perry Laboratories, Gaithersburg, MD). mAbs used were those listed already, plus two anti-CD45RB mAbs (23G2 (3), and C363-16A (5)), which gave equivalent staining results and could be used interchangeably. CD4$^+$ cells were analyzed by gating on cells that stained with PE-coupled anti-CD4 (Becton-Dickinson, Mountain View, CA). Dead cells were excluded by propidium iodide staining. Fluorescence was analyzed using a FACScan (Becton-Dickinson). Cells were sorted using a Becton Dickinson FACStar. Typically, sorting yielded $10^3$ to $10^4$ CNS cells, and $10^5$ LNC.

## RESULTS

### CD45RB$^{low}$ T cells are enriched in EAE CNS

Mononuclear cells were isolated by density gradient centrifugation from the CNS in which EAE was induced by immunization with RSCH and CFA. Approximately 10-20% of cells in this population were CD4$^{+}$. In previous studies (15), we have shown the great majority of CD4$^{+}$ cells within these populations to be $\alpha\beta$TCR$^{+}$ T cells. CD4$^{-}$ cells in the CNS included a small proportion of CD8$^{+}$ T cells (see below) but were greater than 90% non-T cells (29). The absolute number of CD4$^{+}$ T cells in the CNS correlated with disease severity. Flow cytometric analysis of these CD4$^{+}$ cells showed them to be strongly biased towards reduced expression of CD45RB (Fig. 1A). The majority of unprimed LNC express a high level of CD45RB and activated T cells express reduced levels of this marker. As previously reported for passively transferred EAE (15), CD4$^{+}$ cells expressing low levels of CD45RB were the major CNS-derived T cell population. This expression of a memory/effector or activated phenotype contrasted strikingly with antigen plus adjuvant-immunized LNC from the same animals, which were predominantly (>90%) CD45RB$^{high}$ (Fig. 1A). The same flow cytometric profiles were obtained regardless of whether EAE was induced with MBP or RSCH (compare Fig. 1A and 1B).

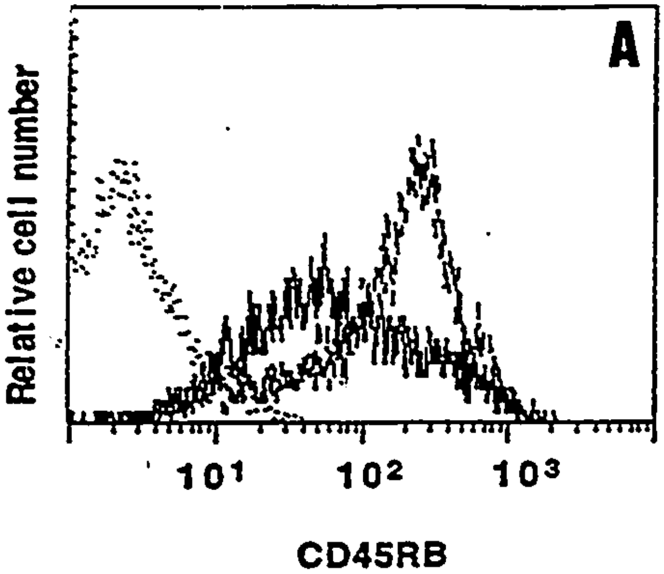
### IL-2 and IFN-γ are produced by CD45RB$^{low}$ T cells from EAE LN and CNS

CD4$^{+}$ cells that expressed either low or high levels of CD45RB were sorted from LNC (Fig. 2A and B), and CD4$^{+}$ (all of them CD45RB$^{low}$) cells were sorted from CNS isolates (Fig. 2C) of MBP/CFA-immunized mice. In the presorted population, the peak intensity of CD45RB-stained CD4$^{+}$ CNS cells was in channel 15, whereas the peak intensity of the presorted LN CD4$^{+}$ cells was in channel 100, with a shoulder

Figure 1

Phenotype of $CD4^+$ T cells in LN and CNS of mice with EAE.

Lymphocytes were isolated from LN and CNS of mice with EAE induced by immunization with RSCH+CFA (A) or with MBP+CFA (B). Cells were stained with anti-CD4 and anti-CD45RB mAbs and analysed by flow cytometry. Profiles show the expression of CD45RB on electronically gated $CD4^+$ populations. A. Solid line: CNS cells; broken line: LNC; dotted line: unstained CNS cells. B. CNS and LN populations are as indicated.


A
Relative cell number
10¹
10²
10³
CD45RB

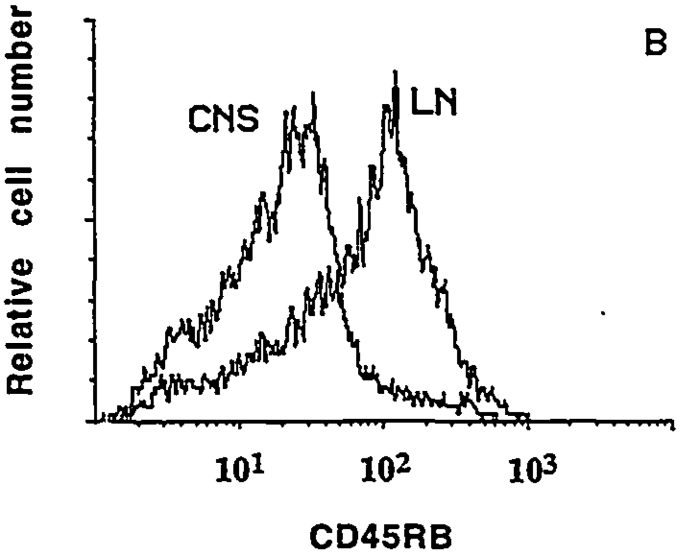
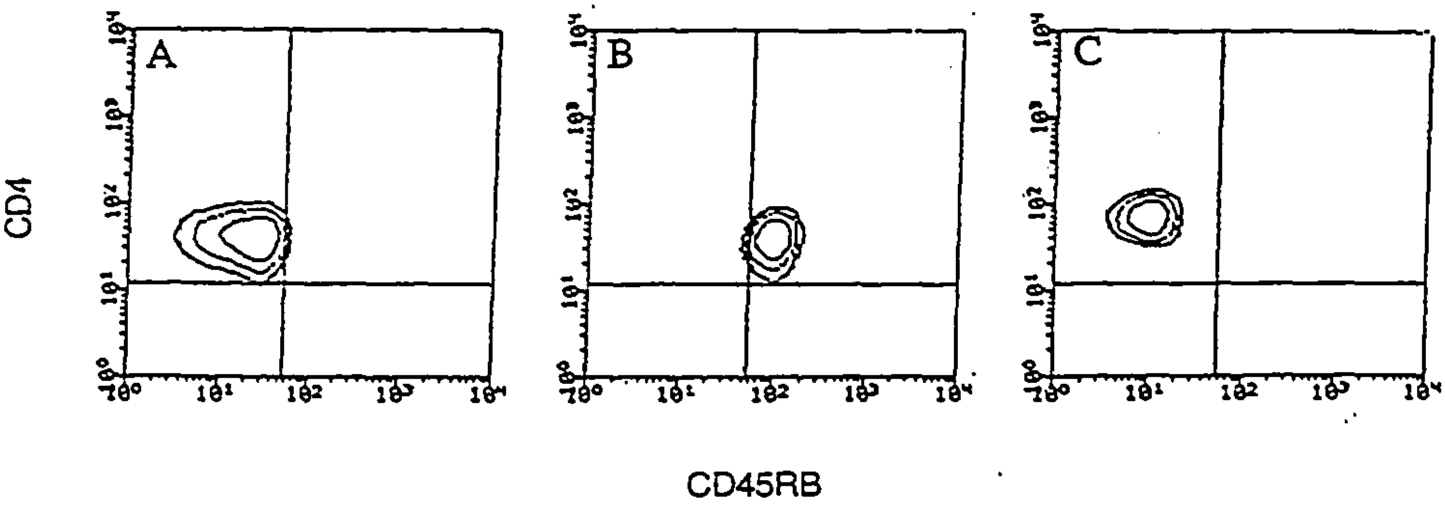

B
CNS
LN
Relative cell number
10^1
10^2
10^3
CD45RB

Figure 2.

FACS-sorting of $CD4^+$ LN and CNS cells from mice primed for EAE.

Lymphocytes were isolated from LN and CNS of mice immunized for EAE with MBP+CFA, and stained for the expression of CD4 and CD45RB. LNC were sorted into $CD4^+$ $CD45RB^{high}$ and $CD4^+$ $CD45RB^{low}$ populations. $CD4^+$ cells from the CNS were also sorted. The profiles show CD4 and CD45RB expression by the sorted cells. A: Sorted $CD45RB^{low}$ $CD4^+$ LNC. B: Sorted $CD45RB^{high}$ $CD4^+$LNC. C: Sorted $CD4^+$ CNS cells.


A
B
C
CD4
CD45RB
10⁰
10¹
10²
10³
10⁴

around channel 20 (Fig. 1B). A slightly higher CD4 staining intensity was observed on T cells in the CNS compared to LNC.

Semi-quantitative RT-PCR was then used to examine cytokine gene expression in the sorted populations. PCR reactions were carried out using 25 cycles, previously determined to be on the linear portion of the amplification curve, and titrations of input RNA yielded linearly-related signal intensities on Southern blots (not shown). The primers used spanned separate exons, and the bands obtained were of the appropriate molecular weight, to rule out amplification of contaminating genomic DNA. Significantly, the sorted LNC populations from animals with EAE differed markedly in their cytokine production, although CD3 message levels suggested an equivalent number of T cells in each sample and actin indicated equivalent RNA loading (Fig.3A). IL-2 and IFN-γ mRNA were strongly amplified from CD45RB$^{low}$ LNC, whereas message encoding these cytokines was barely detectable in CD45RB$^{high}$ LNC under these PCR conditions (Fig. 3B and C). Consistent with this, and with the predominance of the CD45RB$^{low}$ phenotype in CNS, mRNA for both these cytokines was readily detectable in CD4$^{+}$ T cells isolated from the CNS of mice with EAE (Fig. 3B and C). This directly demonstrates a functional relationship between memory/effector CD4$^{+}$ T cell phenotype and cytokine gene expression.

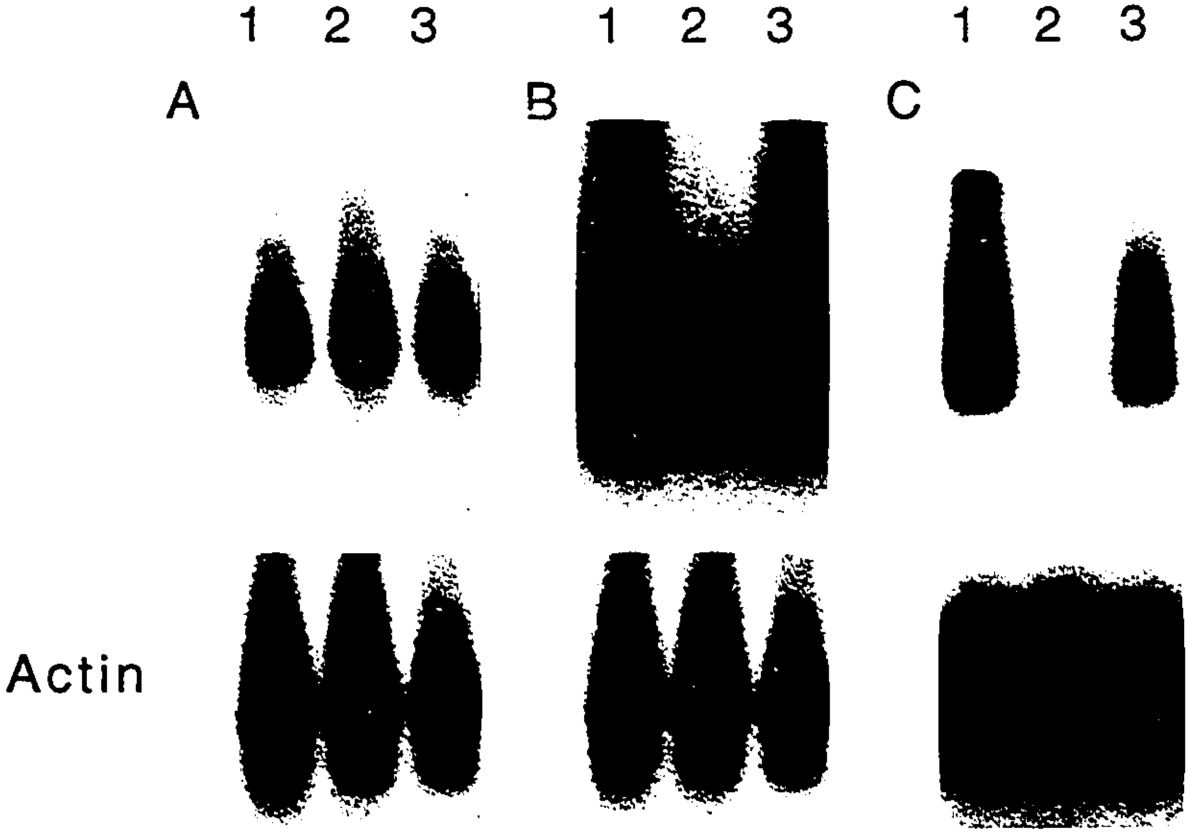
**Enrichment for IL-2- and IFN-γ- producing cells in EAE infiltrates**

IL-2- and IFN-γ-producing cells in autoimmune infiltrates in the CNS of mice with RSCH-induced EAE were enumerated in frozen sections that were stained using specific mAbs. The staining protocol was validated by showing specific staining of cell lines. Peroxidase substrate was localized to the cytoplasm and in many cells could be visualized in the nuclear cleft. Equivalent detail was not discernable in sections, but individual cells could be discriminated using Nomarski optics. 43.9 ± 4.9% (n=9) of

Figure 3.

**PCR analysis of cytokine gene expression in sorted CD4$^+$ CNS and LNC.**

RNA was extracted from the FACS-sorted populations described in Fig. 2, reverse transcribed, and amplified in a PCR reaction using primers and DNA probes specific for: CD3 (A), IL-2 (B), and IFN-γ (C). Actin-specific PCR and Southern blots were carried out in parallel to control for RNA loading. Lane 1: CD4$^+$ CD45RB$^{low}$ LNC; lane 2: CD4$^+$ CD45RB$^{high}$ LNC; lane 3: CD4$^+$ CNS cells.


1 2 3
1 2 3
1 2 3
A
B
C
Actin

## Table I.

Frequency of cytokine-producing cells in primed LN and CNS Infiltrates.

| Population | % Cytokine-positive cells | |
|---|---|---|
| | IL-2 | IFN-γ |
| PBS/CFA-primed LN | 6.3 ± 3.3 (2) | 3.1 ± 1.5 (2) |
| RSCH/CFA-primed LN | 4.6 ± 0.5 (2) | 1.3 ± 0.3 (3) |
| CNS Infiltrates in EAE cerebellum | 43.9 ± 4.9 (9) | 45.7 ± 4.4 (10) |

LNC from mice that had been primed either with PBS (line 1) or RSCH (line 2) in CFA were analyzed directly after isolation. Between 500-3000 cells were counted for each cytokine staining. Percentages of cytokine-staining cells have been corrected for staining in the absence of primary mAb. Cytokine-producing cells in infiltrates in the CNS of mice with EAE were counted under Nomarski optics. 63.5 ± 2.8% (n=8) of cells were $CD4^+$, and 6.6 ± 2.3% (n=3) were $CD8^+$, in agreement with previous reports (31). The staining protocol did not allow simultaneous identification of more than one determinant in infiltrates.

Values represent means ± SEM of separate experiments, the number of experiments is given in parentheses.

the infiltrating cells were IL-2-positive and 45.7 ± 4.4% (n=10) were IFN-γ-positive (Table 1). These numbers were significantly higher (almost 10-fold) than those obtained by staining immediately ex-vivo LNC from mice immunized with RSCH to induce EAE, where an average of 5.5% of *in vivo*-primed LNC stained with anti-IL-2 and 2.2% with anti-IFN-γ (Table 1). An average of 63.5 ± 2.8% (n=8) of cells stained in infiltrates were $CD4^+$(data not shown), whereas only 6.6 ± 2.3% (n=3) were $CD8^+$ (data not shown), in agreement with previous reports (30). The anti-CD8 mAb was of the same isotype as anti-IL-2. IFN-γ staining (rat $IgG_1$) was controlled by an anti-IL-5 rat $IgG_1$ mAb TRFK-5, which did not stain LNC or sections. In a separate study, we have shown that only $CD4^+$ cells isolated from CNS transcribe IL-2 or IFN-γ mRNA, and that $CD4^-$ cells do not (Renno et al, submitted for publication). This allows calculation that the IL-2 and IFN-γ-producing cells that we stained in infiltrates represent 71% of the $CD4^+$ population, which correlates closely with the proportion of those populations that expressed low levels of the CD45RB isoform (Figure 1). Similarly, the proportion of cytokine-producing $CD4^+$ cells in *in vitro*-activated LNC correlated well with that of cells which were $CD45RB^{low}$. Taken together, these results show an enrichment of IL-2- and IFN-γ-producing cells in autoimmune CNS, and demonstrate a strong correlation between detectable cytokine-production and the expression of low levels of the CD45RB marker *in vivo*.

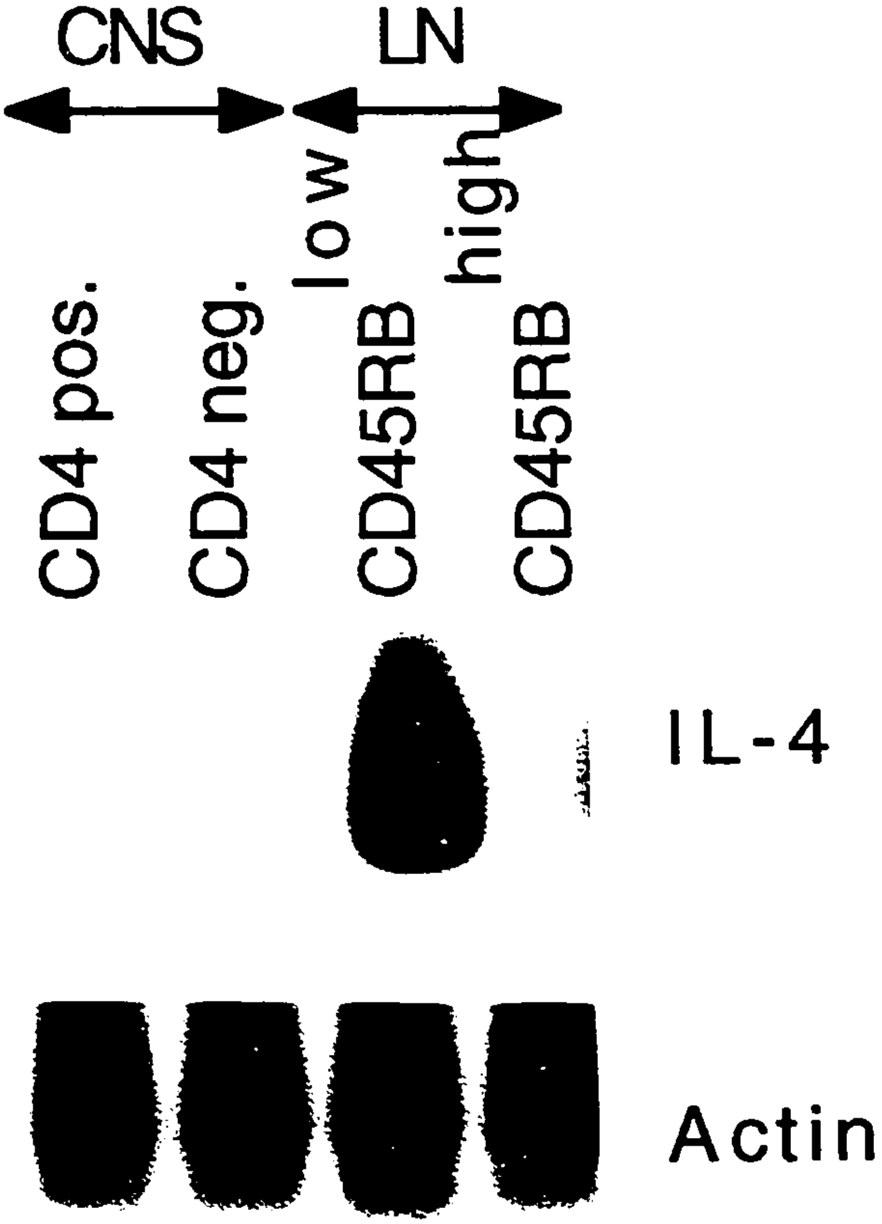
### IL-4 is strongly expressed in LN but almost undetectable in CNS

In striking contrast to IL-2 and IFN-γ, mRNA for IL-4 was undetectable in the same FACS-sorted $CD45RB^{low}$ $CD4^+$ CNS cells as described in Fig. 2, at linear PCR conditions. A faint band could however be detected in the original autoradiogram (but not in reproductions) when amplification was carried out for 40 cycles (Fig.4). This is consistent with weak IL-4 staining which was observed in a minority of EAE infiltrates in frozen sections (not shown). mRNA for IL-4 was also undetectable in

Figure 4.

PCR analysis of IL-4 gene transcription in sorted CNS and LNC.

RNA was extracted from the sorted populations described in Fig. 2, and from sorted CD4$^-$ CNS cells. RNA was reverse transcribed and amplified in a 40-cycle PCR reaction using IL-4- and actin-specific primers. PCR products were Southern blotted and hybridized with IL-4 and actin cDNAs, respectively.


CNS
LN
CD4 pos.
CD4 neg.
low CD45RB
high CD45RB
IL-4
Actin

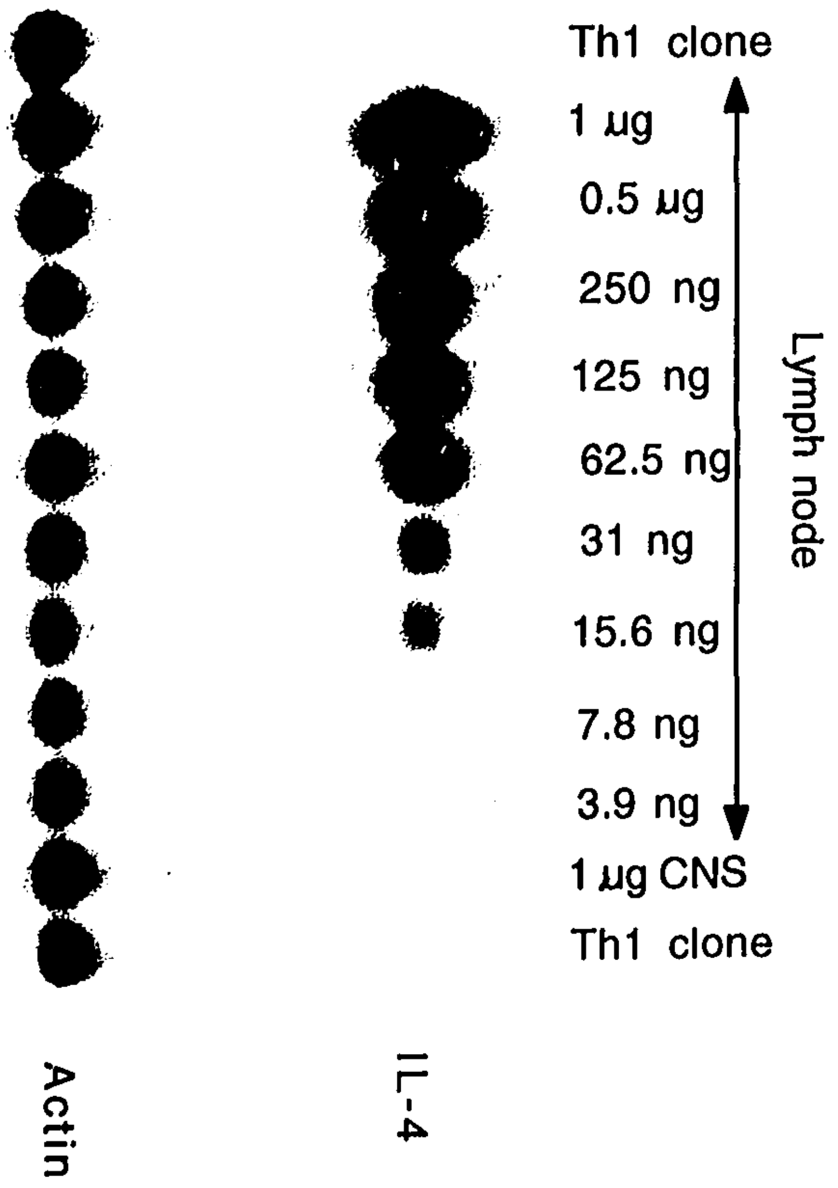
RNA isolated from total CNS (not shown) and in RNA from isolated CD4$^{+}$ CNS cells (Fig.4). Like IL-2 and IFN-γ, IL-4 mRNA was undetectable in CD45RB$^{high}$ LNC, but was readily amplified from CD45RB$^{low}$ CD4$^{+}$ LNC of animals immunized with EAE (Fig 4). To assess the relative levels of IL-4 mRNA, we titrated RNA from LN and compared RT-PCR detection to that from CNS. In Figure 5 it is clear that LN RNA contained at least 100-fold more IL-4 mRNA than CNS. PhosphorImager analysis extended this difference to 250-fold (not shown). The possibility that IL-4 might become expressed later during the disease was also addressed by RT-PCR analysis of total CNS RNA from mice immunized with RSCH+CFA. Up to several days after symptoms had remitted, IL-4 mRNA remained undetectable, even using 40 PCR amplification cycles (data not shown). IFN-γ and IL-2 mRNA were readily detectable in total CNS RNA during peak disease whether MBP or RSCH was used to induce disease, confirming the results of analysis of sorted cells.

The frequency of IL-4-producing cells in LNC isolated directly from mice immunized with RSCH+CFA was 0.9%, compared to 1.3% for IFN-γ and 4.6% for IL-2 (Table 1). We confirmed IL-4 production in these populations by RT-PCR. This lower frequency of IL-4-producing cells was nevertheless perfectly detectable by RT-PCR.

Figure 5.

Comparison of IL-4 mRNA levels in LN versus CNS.

RNA from LN from mice with severe EAE (grade 3, induced by immunization with MBP + CFA) was titrated two-fold from 1 mg. 1 mg of RNA from CNS from unprimed mice was added to each sample. RNA (1 mg) from whole CNS (brain + spinal cord) from the animal with EAE and a control of 1 μg RNA from a Th1 clone (E9.D4) were also assayed. RT-PCR was carried out using actin- and IL-4-specific primers, at 25 cycles. PCR amplimers were blotted to a membrane and then hybridized with radiolabelled probes for actin and IL-4.


Th1 clone
1 µg
0.5 µg
250 ng
125 ng
62.5 ng
31 ng
15.6 ng
7.8 ng
3.9 ng
1 µg CNS
Th1 clone
Lymph node
Actin
IL-4

## DISCUSSION

We have shown a profound distinction between CD45RB$^{low}$ CD4$^+$ populations in LN and in autoimmune CNS infiltrates, pointing to a highly selective process in the accumulation of inflammatory cells in the CNS. Although this study does not address cytokine production at a single cell level, it clearly shows distinct cytokine production patterns by different T cell populations in vivo. The abundance of mRNA for IL-2 and IFN-γ in CD45RB$^{low}$ CD4$^+$ CNS cells, and the very low levels of IL-4 transcripts in the same cell population, suggests that the infiltrating CD4$^+$ T cell populations in peak EAE were of the Th1 type. This contrasts with CD45RB$^{low}$ CD4$^+$ LNC from the same animals, which transcribed readily detectable levels of IL-2, IFN-γ, and IL-4.

Merrill et al. (31) and Khoury et al. (32) used immunostaining to show a high frequency of cells staining for IL-2 and IFN-γ, but a low frequency or absence of IL-4-producing cells in rodent CNS in active EAE. By contrast, Kennedy et al. used RT-PCR to analyze RNA from whole CNS and showed IL-4 mRNA in the spinal cord of mice with acute EAE (33). This discrepancy could have reflected differences in the sensitivity of these techniques. However, we have used both immunostaining and RT-PCR and we find both techniques to agree that IL-4 signals from the CNS are weak or non-existent. We could detect IL-4 in the CNS either as weak immunostaining, or as a weak signal by high cycle (non-linear) RT-PCR, distinct from the strong staining and PCR signals for IL-2 and IFN-γ. By using flow cytometry to sort T cell subsets we have been able to identify the source of cytokines as infiltrating CD4$^+$ memory/effector cells, which would otherwise require double staining. This finding is significant, since many cytokines can be produced by non-lymphoid cells, and RT-PCR from whole CNS tissue would detect cytokines from a variety of sources. It is difficult to assess the relative significance of weak bands in RT-PCR and weak staining of isolated cells in sections, and it remains formally possible that some of the cells stained in sections were not represented in FACS-sorted subpopulations, although our combined CD4 and cytokine frequency analysis argues for correspondence. Our data argue strongly against IL-4 production by CD4$^+$ T cells in EAE.

That Th1 CD4$^+$ cells should infiltrate to induce an inflammatory response is consistent with the described role for Th1 cytokines in delayed type hypersensitivity and inflammation (34), and with reports that encephalitogenic T cell clones are preferentially Th1 (35). The almost complete absence of Th2 at the same site is however striking, and suggests several possibilities. Either autoreactive Th2 cells are induced in the LN but do not enter the CNS, or Th2 cells enter the CNS, but do not become activated there. The possibility that Th2 cells do not enter the CNS was ruled out by our high cycle PCR, and by our immunostaining results and those of others (31, 32) that showed that activated Th2 cells were not absolutely excluded from the CNS. This argues against any prohibition of activated T cell (including Th2) entry to the CNS. In previous studies (15, and unpublished data) we showed that activated OVA-reactive T cells entered the CNS but neither accumulated or converted to CD45RB$^{low}$, there being no possibility of antigen recognition. Virtually all CNS CD4$^+$ T cells in this study were CD45RB$^{low}$ and therefore autoantigen-activated. The few CD45RB$^{high}$ T cells that were present would not be detected by our PCR analysis, which only detects the products of activated (CD45RB$^{low}$) T cells.

Another possibility is that Th phenotype correlates with antigen specificity, those T cells which recognize encephalitogenic peptides being exclusively Th1. This is unlikely since Seder et al. (36) have shown that T cells bearing a transgenic TCR recognizing a pigeon cytochrome C peptide could produce either IFN-γ or IL-4, depending on the culture conditions. Furthermore, we and others have isolated T cell clones that recognize encephalitogenic MBP peptides which produced IL-4 upon activation (Zeine and Owens, unpublished, and Sriram, personal communication). Seder et al showed that the presence of IL-4 during initial T cell priming induced T cells to produce IL-4 upon restimulation (36). Conversely, IFN-γ has been shown to inhibit the activation of Th2 cells (37). This mutually inhibitory ability of Th1 and Th2 cytokines would be difficult to reconcile with the detection of both Th1 and Th2 in the same LN, unless there was compartmentalization of effects in the LN that does not occur in the CNS. Finally, the nature of the antigen-presenting cell might influence or dictate the cytokine profile of a T cell. The differential cytokine production between T cells in the LN and the CNS may therefore reflect a difference in the APC's that are available in each compartment. For instance, dendritic cells, which have been shown to be the most potent inducers of IL-4 (36), are prevalent in the LN but absent from the CNS.

In this study, we have also used immunostaining with cytokine-specific mAbs to enumerate IL-2- and IFN-γ-producing cells within CNS infiltrates. The proportion of cells that produced cytokines was roughly 10-fold higher than that detected in adjuvant-primed LN, or LN from animals immunized with RSCH for EAE induction. About 63% of infiltrating cells were $CD4^+$. If we take into account that all of the IL-2 and IFN-γ mRNA in the CNS is transcribed by $CD4^+$ cells (Renno et al., submitted for publication), we can then calculate that about 70% of $CD4^+$ cells in CNS infiltrates were producing cytokines, in striking agreement with the proportion that express low levels of CD45RB. Previously-published data implicating the $CD45RB^{low}$ phenotype in cytokine secretion have used *in vitro* stimulation protocols, which although informative have left unanswered whether the situation *in vivo* corresponds exactly. Our experiments, by analyzing unmanipulated cells directly isolated from mice and sorted into CD45RB subsets, have for the first time directly confirmed that the CD45RB$^{low}$ phenotype has functional significance.

In conclusion, we have directly demonstrated that CD45RB$^{low}$, but not CD45RB$^{high}$, CD4$^{+}$ T cells, which are 10-fold enriched in the autoimmune CNS, are responsible for the expression of T cell-derived cytokines in the LN and CNS. Whereas IL-2 and IFN-γ were readily detectable both in the LN and CNS, IL-4 mRNA was abundantly expressed in the LN, but not the CNS of mice during active EAE. This pronounced bias towards the expression of Th1 cytokines in the CNS suggests an active selection process in the activation of antigen-specific T cells in the site of inflammation.

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# CHAPTER 3

## TNFα expression by resident microglia and infiltrating leukocytes in the CNS of mice with experimental allergic encephalomyelitis: regulation by Th1 cytokines

## Preface

In chapter 2, I demonstrated that CD45RB$^{low}$ CD4$^+$ T cells accumulate in the CNS of mice with EAE and produce IL-2 and IFN-$\gamma$, but not IL-4. This suggests the predominance of a DTH-like inflammatory response in the CNS of these animals. In this chapter, I investigate the cellular sources and the regulation of expression of the inflammatory cytokine, TNF$\alpha$, in the autoimmune CNS.

# ABSTRACT

The inflammatory cytokines IFN-γ and TNFα have been demonstrated in various autoimmune diseases, and are thought to participate in the induction and pathogenesis of disease. TFNα is a cytopathic cytokine which is cytotoxic for oligodendrocytes in vitro and has been implicated in the pathology of multiple sclerosis and its animal model experimental allergic encephalomyelitis (EAE). We used reverse transcriptase (RT)/PCR to study the kinetics, cellular source and regulation of cytokine gene expression in the central nervous system (CNS) of SJL/J mice with myelin basic protein (MBP)-induced EAE at different stages of the disease. Undetectable in normal CNS, the expression of CD3, IL-2, IFN-γ, and TNFα mRNA was readily detectable in the CNS of mice during peak disease, then coordinately dropped to background levels during remission. Analysis of cells isolated from the CNS of mice with acute EAE showed that the Th1 cytokines, IL-2 and IFN-γ, were produced by infiltrating $CD4^+$ T cells. In contrast, TNFα was predominantly transcribed by non-T mononuclear CNS cells, the majority of which were identified as microglia and macrophages by their CD45 and Mac-1 phenotype. Incubation of freshly-derived, adult microglia from normal, uninfiltrated, CNS with activated Th1 supernatant induced the production of TNFα mRNA, consistent with previous observations. Therefore TNFα is made both by CNS-resident microglia as well as by infiltrating macrophages during EAE, and this production is likely to be tightly controlled by cytokines secreted by infiltrating $CD4^+$ T cells.

## INTRODUCTION

EAE is an autoimmune disease characterized by leukocytic infiltration of the CNS and demyelination (1), and as such resembles the human disease multiple sclerosis (MS). EAE can be transferred into naive animals by neuroantigen-specific CD4$^+$ T cells (2), implicating this T cell subset in the induction of EAE. We have previously demonstrated that CD4$^+$ T cells that infiltrate the CNS in EAE exhibit phenotypic characteristics of effector T cells and that they produce the Th1 cytokines, IL-2 and IFN-γ (3). These and other cytokines, including the inflammatory mediator, TNFα, have been demonstrated in the CNS of rodents with EAE (4-6). TNFα is an important component of the inflammatory response in EAE, since anti-TNFα antibody prevents transfer of the disease (7). TNFα is also cytotoxic to oligodendrocytes in vitro (8). Moreover, a positive correlation exists between encephalitogenicity of T cell clones and their production of TNFα (9). Finally, TNFα was identified in MS lesions (10), and its concentration in the cerebrospinal fluid (CSF) of patients with MS were found to predict progression of the disease (11).

Cytokines can be expressed by more than one cell type. For instance, TNFα can be produced by T cells (12), macrophages (13), as well as in vitro-propagated fetal microglia (14, 15) and astrocytes (16). Given this redundancy and the potential importance of TNFα and other cytokines in EAE, we sought to identify cytokine-producing cells and the mechanisms that regulate their expression in the CNS of mice with EAE.

In the present study, we have investigated the cellular sources of the cytokines produced in the CNS of such mice. We find that IL-2 and IFN-γ are produced by CD4$^+$ T cells, whereas TNFα is expressed by the leukocytic infiltrate containing mostly macrophages and microglia. The fact that freshly-derived, adult microglia can be induced to express TNFα by Th1 cytokines, and the tight correlation between infiltration of activated T cells and cytokine production in vivo, implicate T cell cytokines in the regulation of TNFα expression by other leukocytes in EAE.

## MATERIALS AND METHODS

### EAE induction

EAE was induced by two subcutaneous injections 7 days apart, of 5-8 week-old female SJL/J mice (Harlan Sprague Dawley, Indianapolis, IN), in the base of the tail and flank, with 400 µg purified myelin basic protein (MBP) (Sigma, St. Louis, MO.) in CFA (Difco, Detroit, MI), containing 50 µg H37RA *M. tuberculosis* (Difco). Symptoms were first observed 14 days after the initial injection. Animals used for total RNA extraction had severe EAE, corresponding to grades 3-4 (severe hind limb paresis and/or paralysis of one or both hind limbs) (17), or had remitted from severe EAE. Before removal of brains and spinal cords, animals were anesthetized with chloral hydrate (3.5 g/Kg) (Fisher, Montreal, Qc.), then perfused through the heart with PBS.

### RT-PCR

Total RNA was isolated from homogenized CNS as described (18) and mRNA was isolated from FACS-sorted CNS cells using Quick-Prep™ (Pharmacia, Montreal). For PCR analysis, equivalent amounts of total CNS RNA (determined by spectrophotometry) or mRNA from equivalent cell numbers were subjected to a one-step reverse transcription and amplification protocol. Briefly, mRNA was added to a tube containing 10 U avian myoblastosis virus reverse transcriptase (Pharmacia, Montreal, Qc.), 2.5 U Taq DNA polymerase (Gibco BRL, Montreal, Qc.), 20 U RNA Guard™ RNAse inhibitor (Pharmacia), 10 mM of each dNTP, 50 pmoles of each primer, and a buffer mixture consisting of 50 mM KCl, 100 mM Tris (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin. Primers used were as follows: CD3γ sense primer 5'-ATGGAGCAGAGGAAGGGTCTG-3', CD3γ antisense primer 5'-TCACTTCTTCCTCAGTTGGTT-3', IL-2 sense primer 5'-TGCAGCTCGCATCCTGTCTCA-3', IL-2 antisense primer 5'-AGAAGGCTATCCATCTCCTCA-3', IFN-γ sense primer 5'-ACACTGCATCTTGGCTTTGC-3', IFN-γ antisense primer 5'-CGACTCCTTTTCCGCTTCCT-3', TNFα sense primer 5'-AGCACAGAAAGCATGATCCG-3', TNFα antisense primer 5'-CAGAGCAATGACTCCAAAGT-3', actin sense primer 5'-TGGGTCAGAAGGACTCCTATG-3', Actin antisense primer 5'-CAGGCAGCTCATAGCTCTTCT-3'. Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler (Norwalk, CT) for 15 minutes at 50°C then amplified for 25 cycles (denaturation 1 min 94°C, annealing 2 min 60°C, extension 2.5 min 72°C). 40 µl per sample were run in 1% agarose gels in TAE buffer. DNA was transferred to Hybond-N membranes (Amersham, Baie d'Urfe, Qc) by positive pressure blotting (Stratagene, Aurora, Ont.), and hybridized by incubation with $^{32}$P-labelled cDNA probes for 18 hours at 42°C in 50% formamide, 1 M NaCl, with 1.0% SDS and 10% Dextran Sulphate (19), then washed for 5 minutes at room temperature in 1 X SSC, 0.1% SDS, 10 minutes 1 X SSC 0.1 % SDS at 65°C, 10-15 minutes in 0.1 X SSC, 0.1% SDS at 65°C and 20 minutes in 0.1 X SSC, 0.1% SDS at 65°C. Probes were labelled by random priming 25 ng DNA with [a$^{32}$P]dCTP (5 µCi) (ICN, Mississauga, Ont.), using a kit (Gibco/BRL). Cytokine probes were cDNA inserts purified from plasmids: IL-2, an N-terminal 337 base pair Pst1-HindIII fragment (20), IFN-γ, a 643 base pair Pst1 fragment (20), and TNFα, a 1.5 Kb PstI-BamHI fragment of the mouse TNFα cDNA (21). The actin probe was a purified amplification product. The CD3 cDNA used was a 700 bp HindIII + EcoRI fragment from clone pB10.AT3g-1 (22), kindly provided by Dr R.P. Sekaly (IRCM, Montreal).

### Isolation of mononuclear cells from CNS.

Animals were perfused through the heart with PBS, then brains and spinal cords removed, pooled, dissociated through a wire mesh, centrifuged at 200g for 10 minutes and then the pellets were centrifuged on a discontinuous (30%:37%:70%) Percoll gradient at 500g for 15 minutes (23). Mononuclear cells were obtained from the 37% (1.048 g/ml):70% (1.086 g/ml) interface , washed and analyzed by flow cytometry.

### Flow Cytometry.

Cells were stained by incubation (20 minutes at 4°C) with FITC-coupled rat or hamster mAbs, after blocking with normal rat or hamster Ig, respectively. mAbs used were: anti-CD4 (GK1.5) (24), anti-Mac-1 (M1/70.15.11.5.HL) (25), and anti-CD45 (M1/89.18.7.HK) (25), and hamster anti-mouse CD3ε (145-2C11) (26). Dead cells were excluded by a combination of forward and side scatter. Fluorescence was analyzed on a FACScan using a LYSYS software (Becton-Dickinson). Cells were sorted using a Becton Dickinson FACStar. Typically, sorting yielded $10^3$ to $10^4$ CNS cells, and $10^5$ LNC.

### TNFα induction.

Mononuclear cells were isolated from normal uninfiltrated CNS, as described above. Microtiter wells containing $10^5$ cells were incubated overnight in duplicate in the presence or absence of supernatant from an activated Th1 clone (E9.D4) (27) at a final concentration of 40 U/mL IFN-γ and 0.006 U/mL IL-2. Cells from each treatment group were pooled, mRNA was extracted, and RT-PCR was carried out using TNFα and actin oligonucleotide primers. Southern blotting and PhosphorImager analysis were then performed. TNFa signals were normalized to their respective actin signals. Results shown represent the mean radioactivity from triplicate RT-PCR amplifications.

## RESULTS

### PCR amplification

To determine the conditions for PCR amplification, a fixed amount of RNA from infiltrated CNS was reverse transcribed and amplified using different primers for an increasing number of cycles. The signal was in the linear portion of the amplification curve up to 32 cycles. Twenty five-cycle PCR was arbitrarily chosen and used for amplification, after reverse transcription, of a titration of RNA from LN cells, using CD3-specific primers. A fixed amount of normal brain RNA was added in order to control for dilution effects that can occur when amplifying a rare message, such as T cell or cytokine message from total CNS tissue. To insure detectability of lower RNA concentrations, Southern blotting using a specific probe was performed and showed that the amplification product was directly and linearly proportional to the amount of input RNA (Fig. 1). The same PCR conditions were therefore used in subsequent experiments.

### CD3 and cytokine expression correlates with disease activity

To examine the expression of T cell and cytokine mRNA in relation to disease, perfused brain and spinal cord were obtained from control mice or mice that had been immunized with MBP in CFA to induce EAE. The immunized mice all had grade 3 EAE and were processed either during peak clinical disease or one week after symptoms were no longer detectable. Total RNA from pooled brain and spinal cord was extracted and subjected to RT-PCR and Southern blotting as described above. Fig. 2 shows that CD3 mRNA increases substantially during peak EAE, then sharply decreases to normal levels in remission, consistent with previous studies (28). We then compared the expression of IL-2, IFN-γ, and TNFα, as well as CD3, in a separate experiment. The radioactive signal was

Figure 1.

## PCR amplification is linear.

A titration of LN RNA was reverse transcribed and subjected to 25 cycles of PCR amplification using CD3-specific primers. A fixed amount (0.5 µg) of normal brain RNA was added to each tube before reverse transcription. The amplification product was electrophoresed on an agarose gel, transferred to a membrane, and hybridized to $^{32}$P-labelled CD3-specific cDNA. For comparison, 0.5 mg RNA from the CNS of mice with grade 1 (mild) or grade 3 (severe) EAE was included.

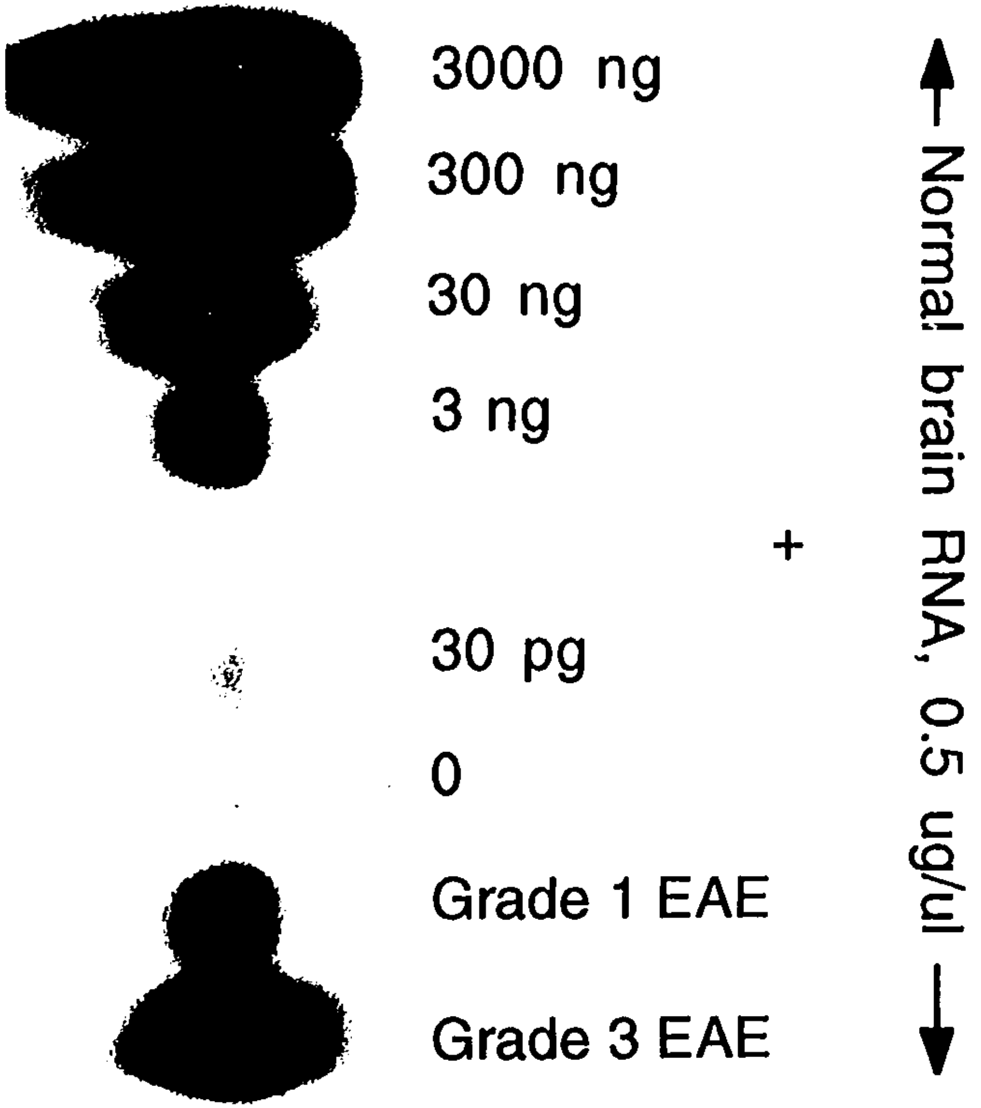
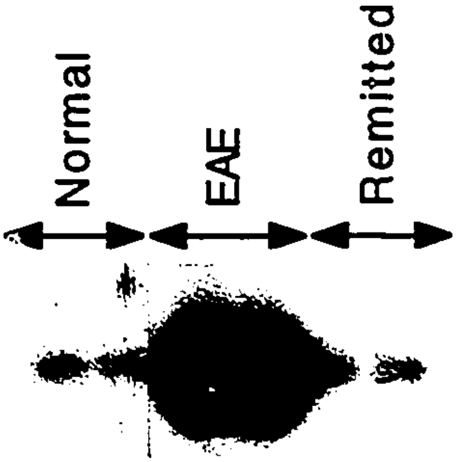

3000 ng
300 ng
30 ng
3 ng
+
30 pg
0
Grade 1 EAE
Grade 3 EAE
Normal brain RNA, 0.5 ug/ul

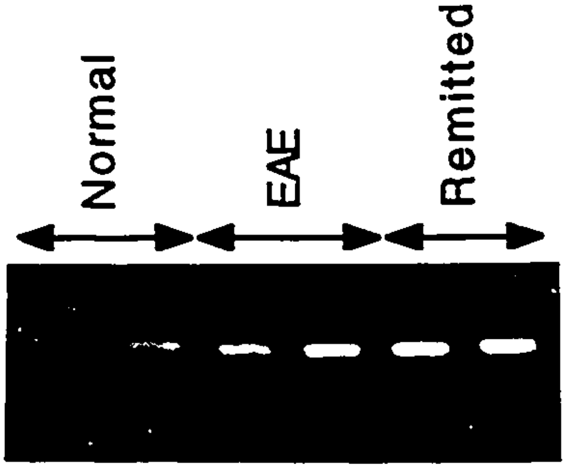
Figure 2.

T cell infiltration correlates with disease stage.

0.5 µg total RNA from the CNS of normal mice, from mice in peak EAE, or in remission, was subjected to RT/PCR using CD3-specific primers, and analysed as described in legend to Fig. 1. Corresponding actin amplification products were visualized using ethidium bromide staining. Each lane represents a separate mouse.



CD3



Actin

quantitated with a PhorphorImager, the values normalized to the actin signal, which was amplified and Southern blotted in an identical fashion, and expressed as arbitrary PhosphorImager Units. Fig. 3 confirms the CD3 result in Fig. 2, and shows that the kinetics of expression of IL-2, IFN-γ, and TNFα message correlated tightly with those of CD3. When brain and spinal cord were separately analysed, analogous results were obtained.

### Identification of cytokine-producing cells in the inflamed CNS

We wanted to directly establish which cell type(s) produce(s) IL-2, IFN-γ, and TNFα in inflamed CNS. At peak EAE, mice were perfused with PBS, their CNS tissue pooled, and mononuclear cells isolated from a discontinuous Percoll gradient. These cells were then sorted based on the levels of the CD4 marker that are expressed on T cells. Fig. 4A shows CD4 expression by the presorted mononuclear cell population, and Figs. 4B and 4C show the sorted populations. Messenger RNA was then extracted from the sorted cell populations and RT-PCR was performed. Fig. 5A shows that whereas CD4$^+$ cells expressed most if not all of the IL-2 and IFN-γ mRNA, TNFα message was predominantly expressed by CD4$^-$ cells. As expected , the great majority of the the CD3 message was synthesized by the CD4$^+$ fraction, since only a small proportion of infiltrating T cells are CD8$^+$ (23). RT-PCR analysis on sorted CD3$^+$ and CD3$^-$ cells from inflamed CNS showed that TNFα message was in the CD3$^-$ fraction (Fig. 5B), ruling out the possibility that it was produced by CD8$^+$ T cells in the CD4$^-$ fraction.

### Characterization of TNFα-producing cells in inflamed CNS

TNFα-producing mononuclear cells were then further analyzed by flow cytometry. The CD45 marker was used to discriminate between different cell populations (29). Virtually all cells isolated from normal, uninfiltrated CNS,

Figure 3.

Quantitative analysis of PCR amplification of T cell, IL-2, IFN-γ, and TNFα message from total CNS RNA.

RT/PCR were performed as described in the legend to Fig. 1 and in Materials and Methods. Radioactive bands on Southern blots of PCR amplification products were quantitated by PhosphorImager analysis. Each point represents a separate mouse and was normalized to the actin signal from the same experiment. Normal: unimmunized. G3: severe EAE. G3R: remitted from severe EAE.

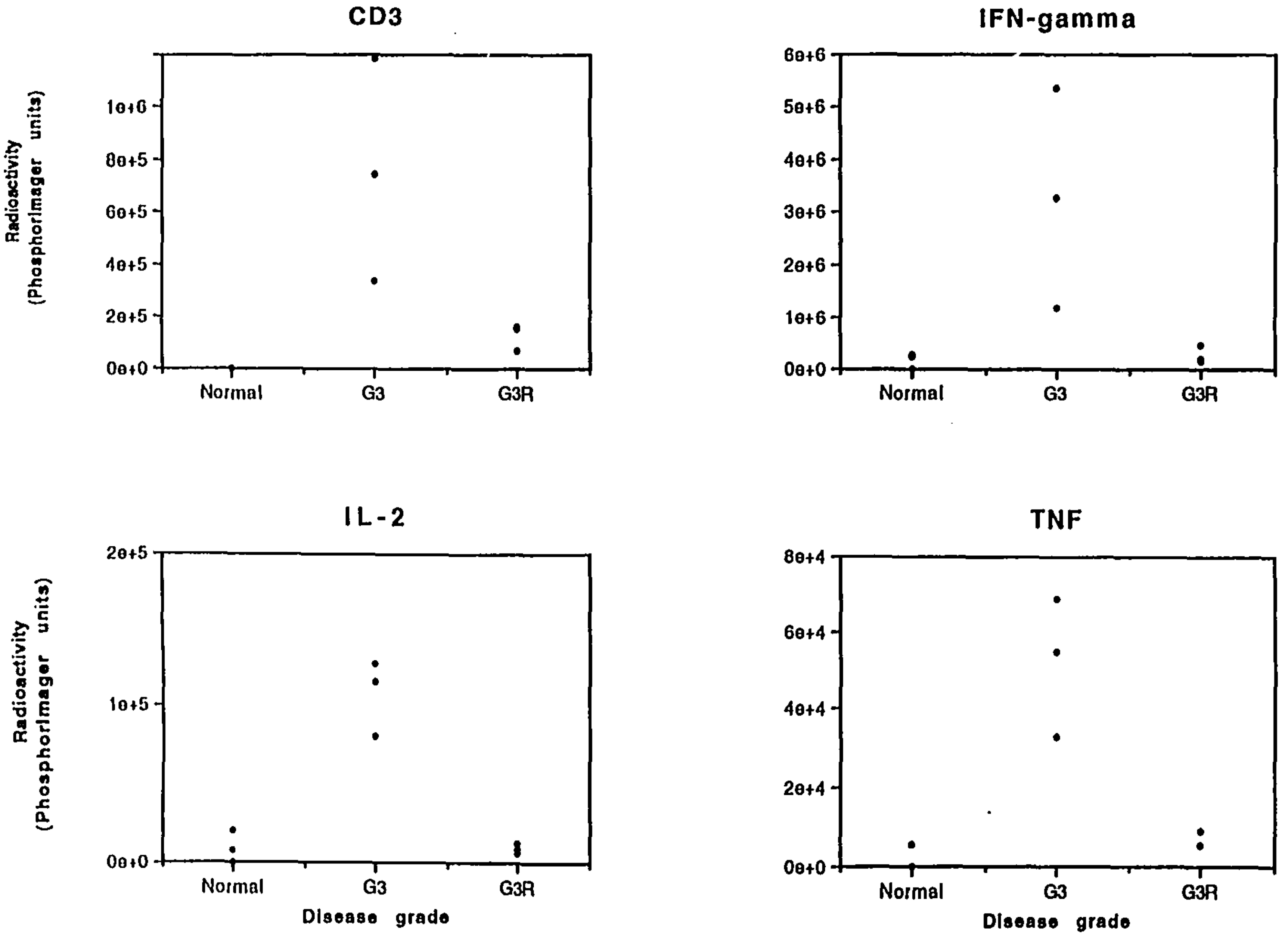

CD3
Radioactivity (PhosphorImager units)
1e+6
8e+5
6e+5
4e+5
2e+5
0e+0
Normal
G3
G3R
IFN-gamma
6e+6
5e+6
4e+6
3e+6
2e+6
1e+6
0e+0
Normal
G3
G3R
IL-2
Radioactivity (PhosphorImager units)
2e+5
1e+5
0e+0
Normal
G3
G3R
Disease grade
TNF
8e+4
6e+4
4e+4
2e+4
0e+0
Normal
G3
G3R
Disease grade

Figure 4.

FACS-sorting of CD4$^+$ T cells from the CNS of mice with EAE.

Mononuclear cells were isolated from the CNS of mice immunized for EAE as described in Materials and Methods, and were stained for the expression of CD4 using a PE-coupled anti-CD4 MAb. Cells were sorted into CD4$^+$ and CD4$^-$ populations using a Becton-Dickinson FACStar Plus. The profiles show CD4 expression by the unsorted (A), and sorted CD4$^-$ (B) and CD4$^+$ (C) cells.

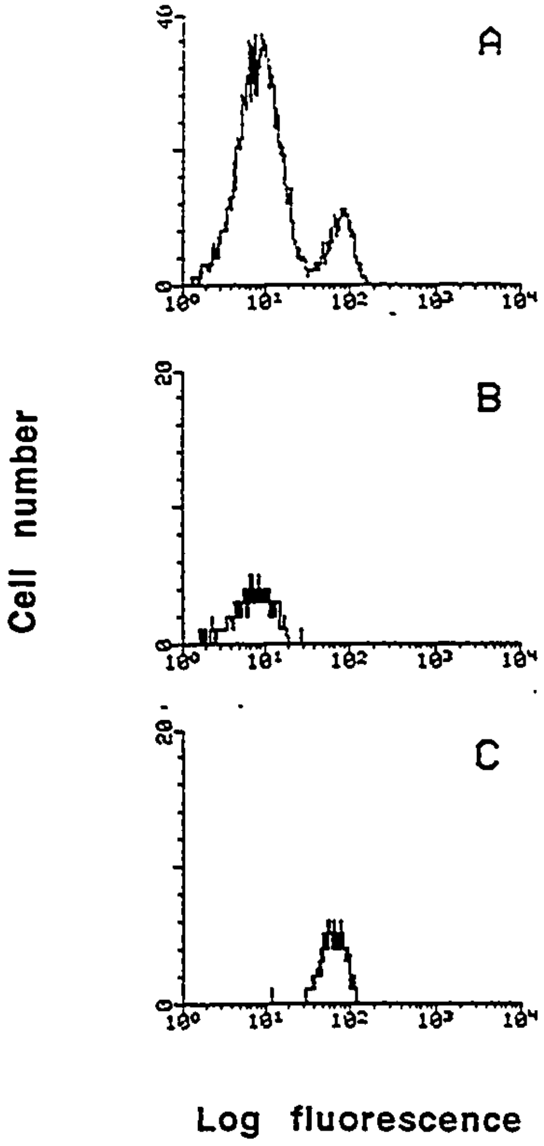
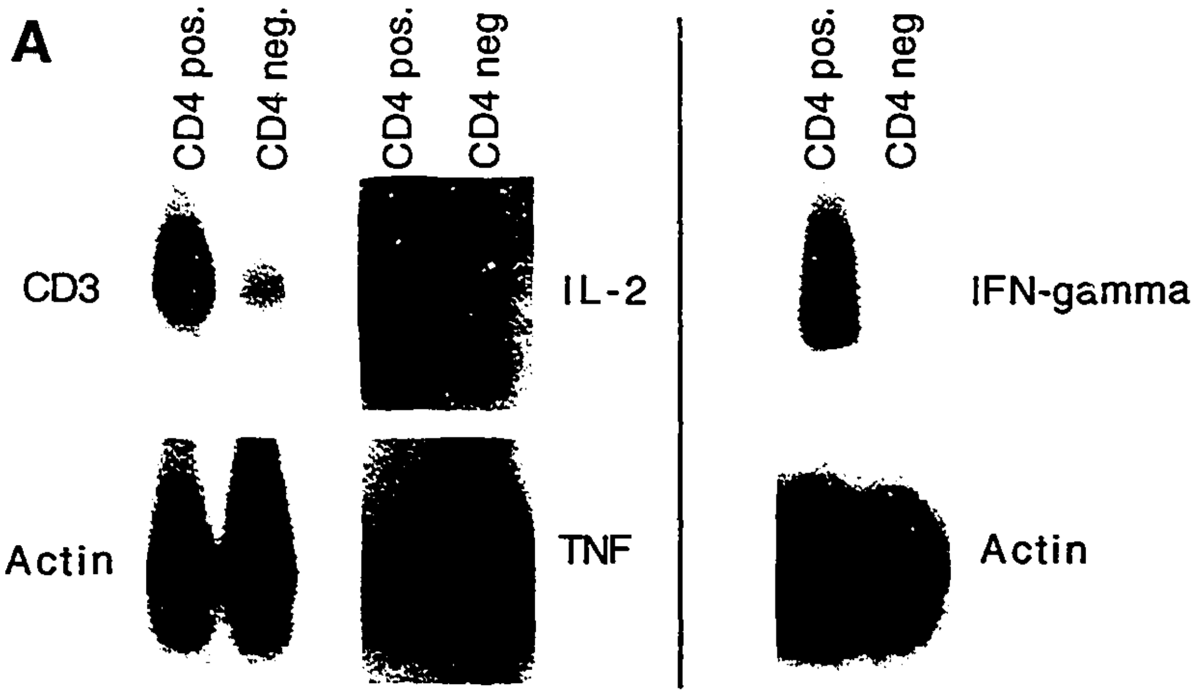
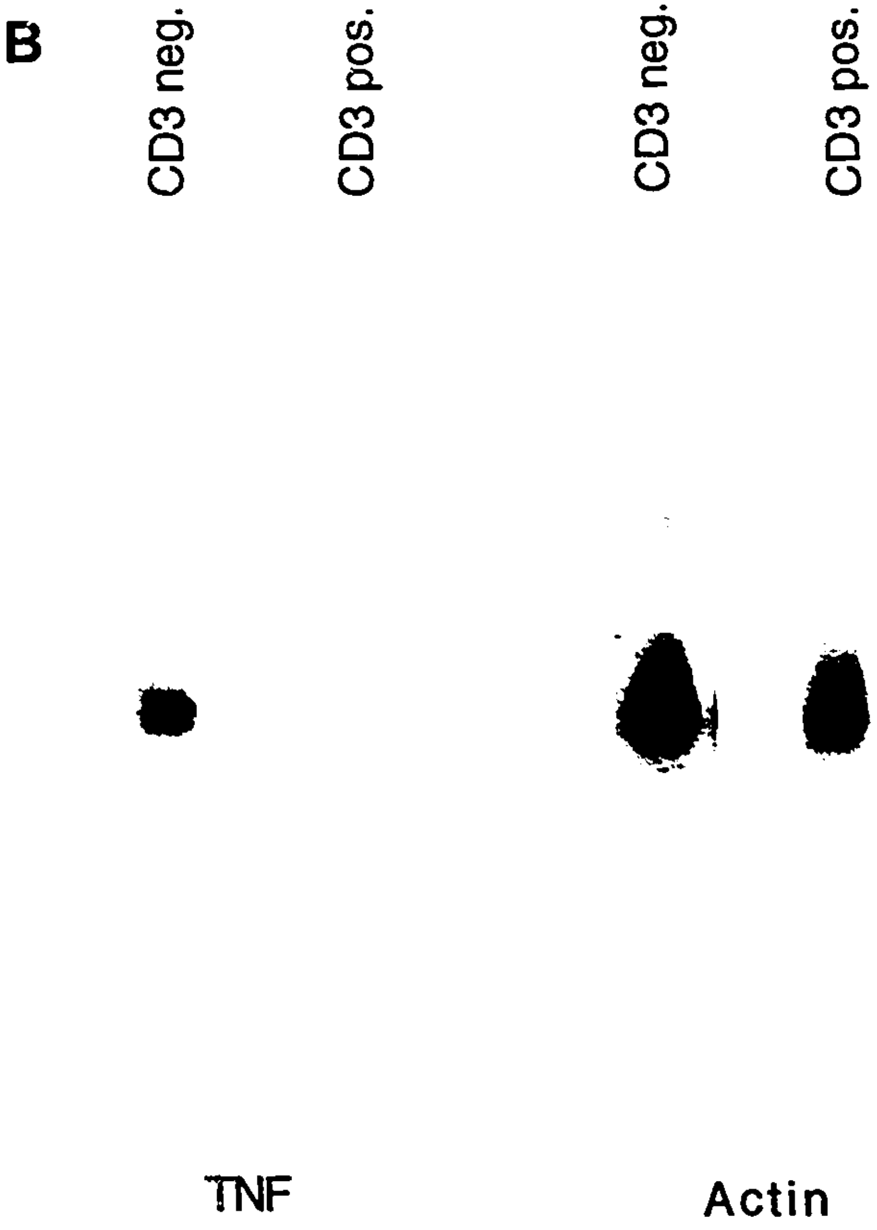

A
B
C
40
20
20
0
10⁰
10¹
10²
10³
10⁴
Cell number
Log fluorescence

Figure 5.

**$CD4^+$ T cells from EAE CNS produce IL-2 and IFN-γ, but not TNFα.**

Mononuclear cells were purified from the CNS of mice with EAE and cells were sorted based on the expression of (A) CD4 or (B) CD3. RNA was extracted, and RT/PCR and Southern blotting were performed using CD3, IL-2, IFN-γ, and actin-specific primers, as desribed above.


A
CD4 pos.
CD4 neg.
CD4 pos.
CD4 neg
CD4 pos.
CD4 neg
CD3
IL-2
IFN-gamma
Actin
TNF
Actin


B
CD3 neg.
CD3 pos.
CD3 neg.
CD3 pos.
TNF
Actin

expressed low levels of CD45 (Fig 6A ), and Mac-1 (Fig. 6B) , a characteristic phenotype of microglia. The weak CD45 staining contrasted with the high staining levels exhibited by splenocytes (Fig. 6C). Cells isolated from the CNS of mice with EAE were similarly characterized. In this case 3 populations were identified by CD45 staining (Fig. 6D): (i) a minority $CD45^-$ population, therefore of non-haematopoietic origin, (ii) a $CD45^{low}$ microglial population corresponding to that identified in control animals without EAE, and (iii) a majority population expressing high levels of CD45. Mac-1 staining indicated that the CNS population from mice with EAE contained $Mac\text{-}1^+$ infiltrating macrophages and activated microglia (Fig. 6E). Over 95% of the CNS-derived cells in EAE were $CD45^+$ (eg. hematogenous cells) (Fig. 6D), of which 20-30% are known to be T cells (23, and T.R., unpublished observations). Since 50% of total CNS cells were $Mac\text{-}1^+$ (Fig. 6E), and these did not include T cells, this allows calculation that the majority of cells in the TNFα-producing (eg. non-T) population are $Mac\text{-}1^+$ macrophages and microglia. This was supported by FACS-sorting $CD45^{high}$ and $CD45^{low}$ subsets fron the CNS of mice with EAE. In this sort, $CD4^+$ and $CD8^+$ cells were excluded, so the $CD45^{high}$ population did not contain T cells. RT-PCR analysis showed both populations to produce equivalent levels of TNFα message, per input RNA (controlled by actin mRNA levels) (Fig. 7).

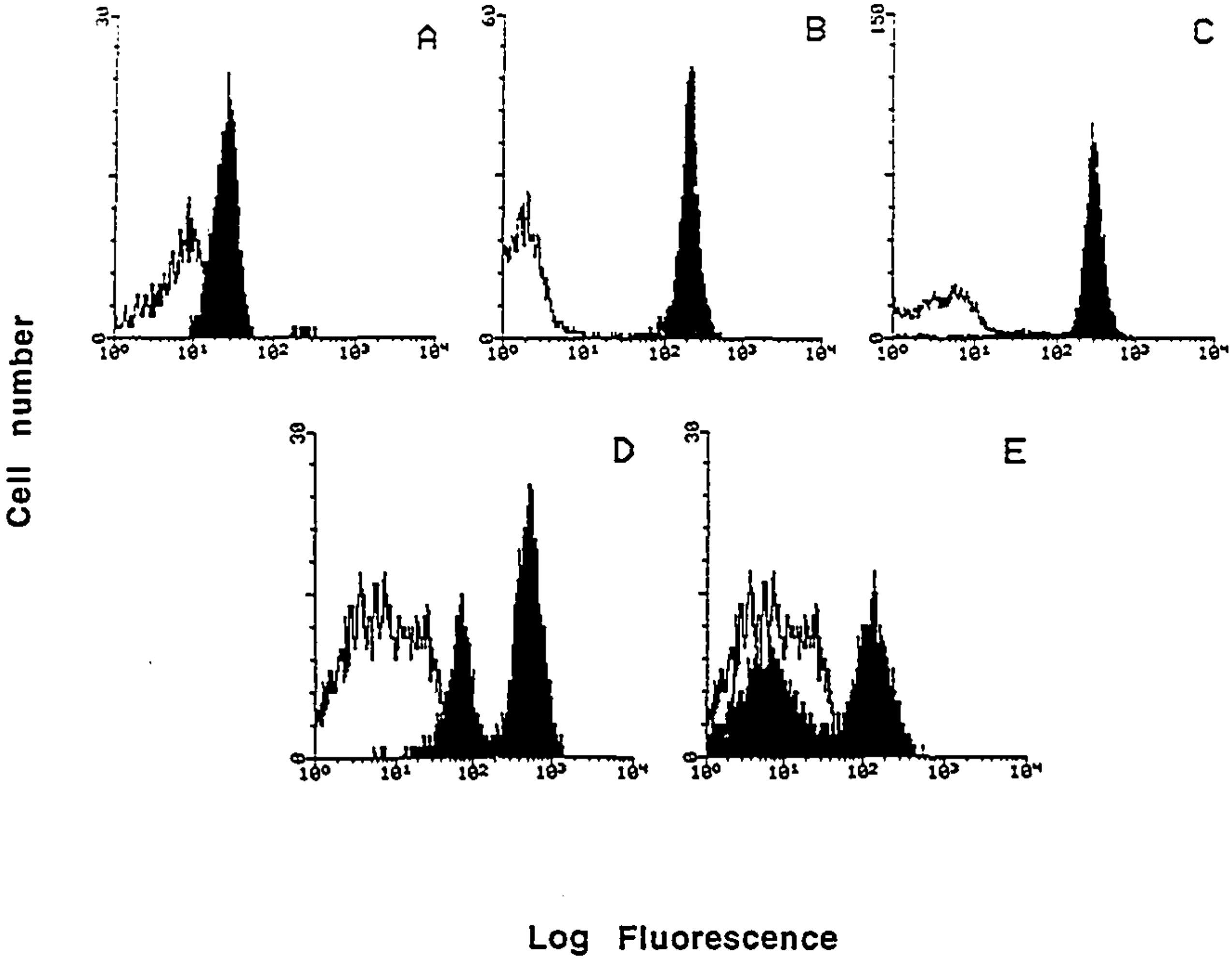
**Microglia can be induced to express TNFα by Th1 cytokines**

The fact that the kinetics of TNFα expression correlated with those of T cell infiltration, but TNFα was expressed by a non-T cell population that included microglia, suggested that TNFα transcription was regulated by infiltrating T cells. Previous studies have suggested soluble T-derived cytokines as likely mediators of such regulation ( ). To investigate this, we asked whether

Figure 6.

**Phenotyopic characterization of mononuclear cells derived from CNS.**

Mononuclear cells were isolated from normal CNS (A and C) and spleen (B), and from the CNS of mice with EAE (D and E). Cells were then stained with FITC-coupled anti-CD45 (A, B, and D) or anti-Mac-1 (C and E), and analysed on a Becton Dickinson FACScan. Open curves: irrelevant isotype-matched FITC-coupled MAb.


A
B
C
D
E
30
60
150
30
30
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Cell number
Log Fluorescence

freshly-derived microglia could be induced to express TNFα by cytokines known to be produced by activated T cells in EAE. Microglia from normal, uninfiltrated CNS (characterized in Figs. 6A, B) were incubated overnight with supernatant from an activated Th1 clone (E9.D4). A Th1 clone was selected since we have previously shown the infiltrating $CD4^+$ T cells in EAE to be overwhelmingly of the Th1 phenotype (3). RNA was then extracted, and a TNFα-specific RT-PCR was performed. Fig. 8 shows that TNFα mRNA was induced in cells that were incubated with Th1 supernatant, compared to cells incubated with medium alone.

Figure 7.

TNFα production by CD45$^{low}$ and CD45$^{high}$ cells from EAE CNS.

Mononuclear cells were isolated from mice with grade 3 EAE and stained with PE-coupled anti-CD4 and anti-CD8, and with FITC-coupled anti-CD45. Cells were sorted using sort gates that excluded PE-labelled events, and separated the remaining cells into two populations based on levels of CD45 expression (Panel A). RNA was isolated from these populations and TNFα and actin mRNA determined by 25-cycle RT-PCR with specific primers. The autoradiograms in Panel B are of Southern blots of PCR amplimers, and show equivalent mRNA production by both CD45$^{low}$ and CD45$^{high}$ cells.

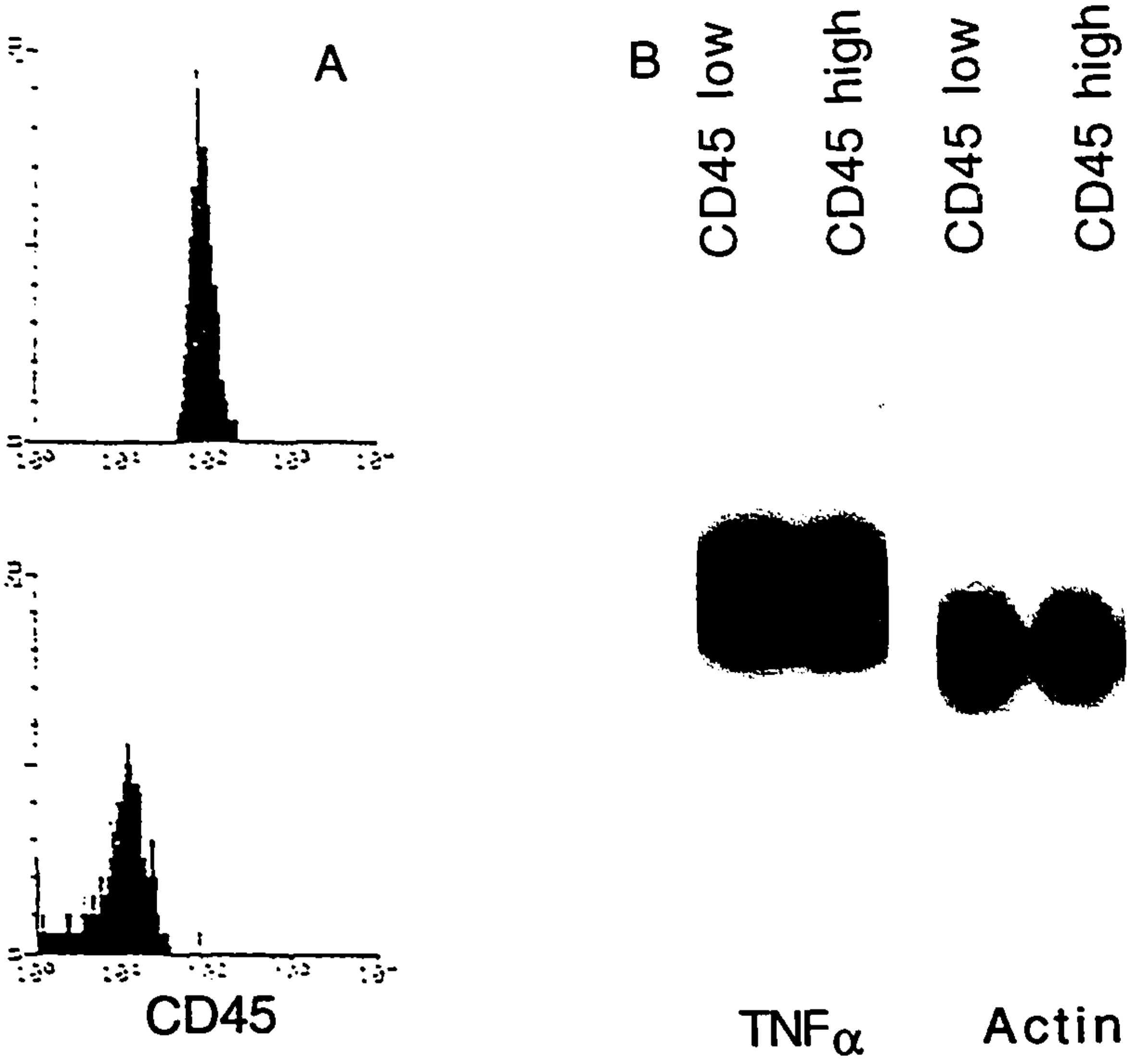
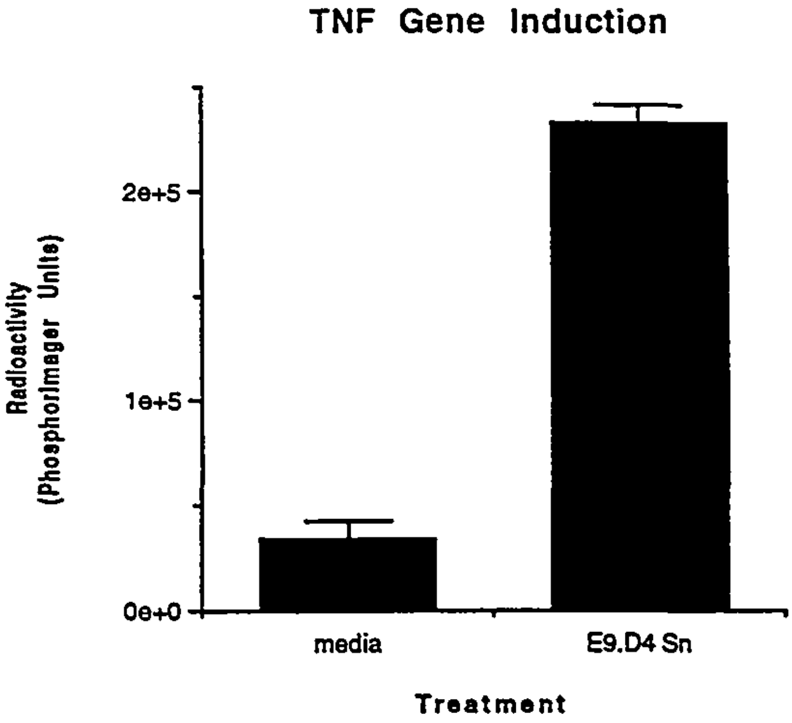

A
CD45
B
CD45 low
CD45 high
CD45 low
CD45 high
TNFα
Actin

Figure 8.

TNFα induction in microglia by Th1 supernatant.

Mononuclear cells were isolated from normal uninfiltrated CNS, as described above. Microtiter wells containing $10^5$ cells were incubated overnight in duplicate in the presence or absence of supernatant from an activated Th1 clone (E9.D4) at a final concentration of 40 U/mL IFN-γ and 0.006 U/mL IL-2. Cells from each treatment group were pooled, mRNA was extracted, and RT-PCR was carried out using TNF and actin oligonucleotide primers. Southern blotting and PhosphorImager analysis were then performed. TNF signals were normalized to their respective actin signals. Results shown represent the mean radioactivity from triplicate RT-PCR amplifications.


TNF Gene Induction
Radioactivity
(PhosphorImager Units)
2e+5
1e+5
0e+0
media
E9.D4 Sn
Treatment

## DISCUSSION

In this study, we show a strong correlation between T cell infiltration and IL-2, IFN-$\gamma$, and TNF$\alpha$ gene expression during EAE in SJL/J mice. This study shows for the first time the cell source of these cytokines in inflamed CNS, and directly demonstrates that whereas IL-2 and IFN-$\gamma$ are produced by infiltrating CD4$^+$ T cells, TNF$\alpha$ is expressed by macrophages and microglia. This, and the ability of microglia to produce TNF in response to T cell cytokines suggest tight regulation of TNF$\alpha$ expression in macrophages/microglia by infiltrating T cells.

Consistent with previous reports, we find IL-2, IFN-$\gamma$, and TNF$\alpha$ in the CNS during peak disease, and we show their loss in remission (4, 6). By contrast, Khoury et al. (5) found TNF$\alpha$ to be expressed during remission. This may be due to the fact that they studied early remission (day 18 in Lewis rat, with peak disease on day 14), when infiltrates were still present, as opposed to our studies, which were performed relatively late (one week into remission), when infiltration had greatly subsided.

We also show that the expression of these cytokines correlates with T cell infiltration, as defined by detection of CD3 mRNA. Coordinate upregulation of CD3, IL-2, IFN-$\gamma$, and TNF$\alpha$ in peak EAE, and their concerted decrease in remission, could in principle be due to the expression of all the above cytokines by infiltrating T cells. Their downregulation in remission would then be the direct result of the loss of T cells at this stage of the disease (28). Alternatively, both T and non-T cells could contribute to the expression of these cytokines in EAE. In this case, their coordinate expression with T cell infiltration would be the result of cross-regulation between different cytokines and/or the cells producing them. Our results point to the latter alternative as the more likely scenario.

In characterising TNFα-producing cells in the CNS, we found that the majority (65-75%), but not the totality of these cells expressed Mac-1. This marker is not expressed on all CNS-derived microglia/macrophages (30), so the Mac-1⁻ cells may include macrophages, as well as other infiltrating CD45⁺, Mac-1⁻infiltrating leukocytes, such as B cells and neutrophils. That macrophages produce TNFα is not surprising (10, 13). Our data show for the first time that freshly-derived, adult microglia produce TNFa, so extending studies showing the ability of *in vitro* propagated, fetal microglia (14) to produce TNFα in responce to T cell cytokines. Our study does not however rule out the possibility that other glial cells such as astrocytes contribute to TNFα production in vivo, since our cell isolation method excludes these CD45⁻ Mac-1⁻ cells (Fig. 6). Cultured rodent astrocytes are inducible by LPS, IFN-γ, and IL-1 to produce TNFα (31), and astrocytes in MS lesions were colocalized immunohistochemically with TNFα (10).

The paucity of TNFα expression by infiltrating T cells raises another point Others have shown encephalitogenic T cell clones to express this cytokine (9), and we have found activated $CD45RB^{low}$ $CD4^+$ T cells in LN to produce TNFα (TR, unpublished). One explanation for this apparent discrepancy is that the requirements for encephalitogenic T cells in other studies (9) to produce detectable TNFα are fulfilled through the extensive stimulation inherent in the cloning process. Such requirements may not be met through in vivo activation. The T cells in our study had not been restimulated in vitro, and this might account for their lack of detectable TNFα expression. Alternatively, TNFα-producing encephalitogenic T cells may indeed infiltrate the CNS early in EAE, but represent such a small percentage of the T cells we isolate during peak disease that their TNFα message is undetectable in our 25-cycle PCR amplification.

Given that T cells infiltrate the CNS early in inflammation (32), we propose that the cytokines they produce (eg. IFN-γ) induce TNFα expression by microglia, before infiltration by macrophages and other leukocytes. This may be important for the recruitment of inflammatory cells since TNFα was shown to be directly chemotactic to monocytes and PMNs (33). The ability of TNFα to enhance macrophage activation in vitro (34) suggests that once macrophages infiltrate the CNS, their activation could be potentiated by the microglia-produced TNFα. Moreover, TNFα can synergize with IFN-γ in the induction of MHC class II on the surface of glial cells (35), which can act as APC for pre-activated T cells (14, 36, 37). Eventually, macrophage- and microglia-derived TNFα may contribute to pathology since this cytokine was shown to be cytotoxic to oligodendrocytes (7).

Whether T cell cytokine-activated microglia are sufficient to mediate EAE pathology remains to be addressed. It is probable that macrophages are essential in the inductive phase of EAE, as activators of autoantigen-specific T cells in the periphery. It is also clear that they participate in the pathology of EAE by stripping myelin from axons and secreting toxic mediators (2). Huitinga et al. addressed the requirement for infiltrating macrophages in EAE by treating rats with mannosylated toxin-loaded liposomes that were shown to cross the blood brain barrier (38). The fact that treated animals had milder EAE symptoms was interpreted as demonstrating an essential role for macrophages in the effector arm of the inflammatory reaction. Given that the liposomes crossed the blood brain barrier and that they targeted phagocytic cells (which includes microglia), it is conceivable that alleviation of EAE was also due to effects on microglia in the CNS. It is likely that microglia can effect many of the functions of infiltrating macrophages and that both cell types play complementary roles in the pathology of EAE.

If TNFα expression is indeed regulated by T cell cytokines, then all that would be required for remission to occur is the removal of the T cell-generated signal(s). For this to be true, one must postulate that for a sustained TNFα expression, continuous presence of the inducing cytokine(s) is required. Swoboda et al. (39) showed that in T cells, continued presence of the activating signal was required for sustained expression of IL-2 and IL-4, and that transcription of mRNA for these cytokines, but not that for IL-2R, stopped as soon as that signal was removed. In EAE, a similar effect can be achieved through T cell-regulating cytokines such as TGFβ (40, 41) and IL-10 (6, 42).

Therefore, if infiltrating T cells in MS similarly regulate cytokine production by other CNS-infitrating or -resident cells, then interventions targeting T cells would remove not only the cytokines they produce, but also those cytokines whose production they regulate, without the need to directly target the cells producing them.

Finally, what is the relevance of having resident CNS cells capable of producing TNFα? The TNFα gene is inducible on hematopoietic cells by a variety of viruses (43), and TNFα amplifies virus-induced Ia induction on astrocytes (44). It is therefore likely that microglia evolved into TNF-producing cells under the pressure of neurotropic pathogens. Given the antiviral properties of TNFα, its expression by microglia during the initial stages of a viral infection would constitute a first line of defense against the pathogen. Subsequently, TNFα would synergize with the viruses in the induction of MHC on brain cells, a crucial step for the development of a local virus-specific immune response. In this context, TNFα production by microglia in EAE represents an aberrant response wherein this host-protective mechanism has been subverted by autoimmune T cells.

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# CHAPTER 4

## Exacerbation of experimental allergic encephalomyelitic in transgenic mice expressing interferon-gamma in the central nervous system

# Preface

In previous chapters, I have shown a correlation between the expression of IFN-γ in the CNS and the activity and severity of EAE. I also showed data suggesting that IFN-γ is an important player in the cytokine cascades leading to pathology in the CNS. Here, I describe the generation and preliminary characterization of transgenic mice that express IFN-γ in the CNS.

## ABSTRACT

Interferon-gamma (IFN-γ) is a T cell- derived cytokine that has been implicated in delayed-type hypersensitivity reactions. To assess the role of this cytokine in inflammation of the central nervous system (CNS), we generated transgenic mice that express IFN-γ specifically in that compartment. IFN-γ expression, placed under the control of the myelin basic protein promoter, was higher in the spinal cord than in the brain. MHC antigen expression was induced in the CNS of transgenic animals, and was most pronounced in the hippocampus and the cerebellum, coincident with areas of myelination in young animals. When experimental allergic encephalomyelitis was induced in transgenic mice by immunization with MBP in Freund's adjuvant, the penetrance of the disease was greater, the symptoms were more severe, and remission was delayed, in comparison to non-transgenic littermates. This suggests that IFN-γ plays a role in the amplification and perpetuation of EAE.

# INTRODUCTION

IFN-γ is a T cell derived cytokine that has been implicated in a variety of inflammatory conditions. In previous chapters, I have extensively reviewed the existing literature showing IFN-γ expression and regulation in EAE. I also presented data correlating IFN-γ expression in the CNS with disease severity and activity (v/s remission), and presented evidence suggesting that this cytokine may play a role in the cytokine cascade that eventually leads to tissue pathology. None of these studies, however, directly demonstrates a role for IFN-γ in EAE.

Early efforts in that direction used the approach of injecting INF-γ into animals with EAE. This yielded conflicting results, ranging from protection to exacerbation of the disease (Simmons and Willenborg, 1990; Voorthuis et al., 1990). The fact that injections of IFN-γ involved breaching the blood brain barrier and were performed in different anatomical compartments in the CNS makes interpretation of these data difficult. To make things more complicated, systemic administration of anti-IFN-γ Abs was found to exacerbate the disease (Billiau et al., 1988).

We decided to address the question by producing transgenic mice that express IFN-γ in the CNS. The advantage of the transgenic approach is that, unlike other modes of administration, it does not require breaching the blood-brain barrier to deliver the cytokine. We found that IFN-γ expression in the CNS induces class I MHC production, and makes mice more susceptible to actively-induced EAE and relatively refractory to remission.

## MATERIALS AND METHODS

### DNA construct

A plasmid containing mouse MBP gene segments and an ampicillin resistance gene (PM2) was obtained from Dr Arthur Roach (Fig. 1). The MBP portion consisted of 3 Kb of upstream sequences including 35 bp of 5' UT sequences from exon 1, fused to 3 Kb downstream regions including 1.1 Kb of 3' UT sequences from exon 7. PM2 was linearized using the restriction enzyme BspMII, which cuts between the 5' and 3' UT regions (Fig. 1). The DNA overhang thus generated was filled with the Klenow fragment of DNA polymerase I, to generate blunt ends, as described (Wartell and Reznikoff, 1980).

A 1.4 Kb full-length cDNA encoding the murine IFN-γ was excised from pCD-γIFNmu (kindly provided by DNAX) with BamHI. Loose ends were filled with Klenow to generate blunt ends.

The blunt-ended PM2 and IFN-γ cDNA were ligated using DNA ligase and a buffer containing 10 mM ATP, as described (Maniatis et al., 1982). The ligation products (PM2/IFN-γ) were used to transform E. coli (strain DH5α), which were selected by overnight propagation on ampicillin-containing growth medium. Bacteria from colonies that survived the selection process were further grown in ampicillin-containing media, and minipreps were performed to isolate plasmid DNA, using the boiling method (Holmes and Quigley, 1981).

Sequencing was carried out using the dideoxy method (Sanger et al., 1977) and oligonucleotide primers from MBP sequences present in PM2. These were a sense primer from the 5' UT region of exon 1 (5'-TTCAAAGACAGGCCCTCAGA-3'), and an antisense primer from the 3' UT region of exon 7 (5'-TAGGGGTGAACTTGAAAGGG-3').

Large quantities of the PM2/IFN-γ construct were obtained by maxi-prep and CsCl purification (Maniatis et al. 1982).

### Transfection of PM2/IFN-γ into GA-5 cell line

Transfection was carried out by cationic liposome-mediated transfer using Lipofectin (Gibco BRL, Montreal, Qc.), as described (Felgner et al., 1987). GA-5 is an oligodendrocyte precursor cell line containing the SV40 large T Ag (LTA) carrying a heat-sensitive mutation, kindly provided by DR G. Almazan (Almazan and McKay, 1992). At 33°C, the LTA is active, and the cells proliferate without differentiating. It is at this stage that PM2/IFN-γ (10μg), in addition to 1 μg of a resistance gene to hygromycin (152.2 Hygro), were used to co-transfect the cell line. The transfected cells were then incubated in DMEM (ICN, Mississauga, Ont) containing 10% FCS in the presence of 60 μg/ml hygromycin (Sigma, St. Louis, MO) for a week, after which the hygromycin was removed. The surviving cells were incubated at 37°C for 2 weeks. At this temperature, the oncogene is inactive and the cells stop dividing and differentiate morphologically and express MBP and galactocerebroside (Almazan and McKay, 1992).

### RT/PCR

Transfected GA-5 cells were pelleted and their RNA was extracted using the acidic guanidinium method (Chomczynski and Sacchi, 1987). RNA from equivalent cell numbers was subjected to a one-step reverse transcription and amplification protocol. Briefly, RNA was added to a tube containing 10 U avian myoblastosis virus reverse transcriptase (Pharmacia, Montreal, Qc.), 2.5 U Taq DNA polymerase (Gibco

BRL), 20 U RNA Guard$^{TM}$ RNAse inhibitor (Pharmacia), 10 mM of each dNTP, 50 pmoles of each primer, and a buffer mixture consisting of 50 mM KCl, 100 mM Tris (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin. Primers used were IFN-γ sense primer 5'-ACACTGCATCTTGGCTTTGC-3', IFN-γ antisense primer 5'-CGACTCCTTTTCCGCTTCCT-3'. These primers span 450 bp. Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler (Norwalk, CT) for 15 minutes at 50°C then amplified for 25 cycles (denaturation 1 min 94°C, annealing 2 min 60°C, extension 2.5 min 72°C). Amplification products were resolved on a 1% agarose gel in TAE (0.04 M Tris-acetate, 0.002 M EDTA) to detect the expected 450 bp product.

### IFN-γ bioassay

An IFN-γ-specific bioassay was performed as described (Kelso, 1990). WEHI-279 is a B lymphoma line whose proliferation is inhibited by IFN-γ. Briefly, two-fold dilutions of supernatant from transfected GA-5 cells were incubated with $10^4$ WEHI-279 cells/microtiter well in the presence or absence of 10 µg/ml neutralizing Ab to IFN-γ (XMG1.2). 3 days later, 10 µl MTT [3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma) at 50 µg/ml was added to each well, and incubated for 6 hours at 37°C. The resulting formazan precipitate was solubilized by addition of 100 µl 10% SDS-0.01N HCL and incubation at 37°C overnight. Optical density was measured at 560 nm with a reference wavelenght of 690 on a multiwell plate photometer (SLT EAR 400 AT) (Fisher scientific, Montreal, Qc), and compared to a standard curve of recombinant IFN-γ (Genzyme, Cambrige, MA).

### Generation of transgenic mice

HindIII was used to excise a linear DNA fragment from PM2/IFN-γ that included the IFN-γ cDNA, flanked by 1.3 Kb upstream, and 2.2 downstream MBP sequences. Microinjection and reimplantation were carried out as described (Hogan et al., 1986).

### DNA screening

Integration of the transgene was evaluated 3 weeks after birth by PCR analysis of tail DNA. DNA was isolated as follows: tail tissue was incubated overnight at 50°C in a solution containing 400 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.25% SDS, 10 mM EDTA, and 400 µg/ml Proteinase K (BRL, Montreal, Qc). After microcentrifugation, the supernatant was extracted twice with phenol/chloroform, the aqueous phase precipitated in 2 volumes ethanol at -20°C, and the resultant DNA aggregate resuspended in distilled $H_2O$.

PCR amplification was then carried out for 30 cycles using the conditions described above and a mixed pair of oligonucleotide primers consisting of either MBP sense and IFN-γ antisense primers, or MBP antisense and IFN-γ
sense primers (primer sequences were described above).

### RNA expression

RNA was extracted from various organs and subjected to RT/PCR using the IFN-γ sense and antisense primers, as described above. Equal volumes from amplification reactions were run in 1% agarose gels in TAE buffer. PCR amplimers were transferred to Nytran membranes (Schleicher and Schull Inc., Keene, NH) by vacuum blotting (Pharmacia), and hybridized by incubation with $^{32}P$-labelled cDNA probes for 18 hours at 42°C in 50% formamide, 1 M NaCl, with 1.0% SDS and 10% Dextran Sulphate (Maniatis et al., 1982), then washed for 5 minutes at room temperature in 1 X SSC, 0.1% SDS, 10 minutes 1 X SSC 0.1 % SDS at 65°C, 10-15 minutes in 0.1 X SSC, 0.1% SDS at 65°C and 20 minutes in 0.1 X SSC, 0.1% SDS at 65°C. Probes were labelled by random priming 25 ng DNA with $[a^{32}P]$dCTP (5 µCi) (ICN), using a kit

(Gibco/BRL). The IFN-γ probe was a 643 base pair PstI fragment purified from a plasmid described by Kelso and Gough (1988).

### Screening for homozygosity

Screening for homozygous mice was performed by Southern blotting (Southern, 1975). Briefly, 10 µg tail DNA was digested with SstI, electrophoresed in agarose, blotted onto a membrane, and hybridized with a $^{32}$P-labelled IFN-γ cDNA probe, as described above. After washing, radioactive bands on the blot were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### EAE induction

EAE was induced by two subcutaneous injections 7 days apart, in the base of the tail and flanks, of 400 µg purified myelin basic protein (MBP) (Sigma) in CFA (Difco, Detroit, MI), containing 50 µg H37RA *M. tuberculosis* (Difco). Symptoms were first observed 14 days after the initial injection. Before removal of brains for histology, animals were anesthetized with chloral hydrate (3.5 g/Kg) (Fisher), then perfused through the heart with PBS.

### Histology

10-20 µm sections were cut from frozen tissue, layed onto gelatin (Sigma)-coated slides, and blocked in 3% ovalbumin (Calbiochem, San Diego, CA) for 1 hour at room temperature (RT). Sections were then incubated overnight with primary (rat-derived) mAbs at 4°C. After washing with PBS, a mouse-preabsorbed biotinylated rabbit anti-rat Ig secondary Ab (Dimension Laboratories, Mississauga, Ont) was added for 1 hour at RT, washed, after which the slides were dipped in 0.3% $H_2O_2$ in methanol, to quench endogenous peroxidase. Sections were then incubated twice in Avidin-Biotin Complex (ABC) solution (Dimension Laboratories) for 1 hour at RT, separated by an amplification step with biotinyl tyromide (DuPont, Mississauga, Ont). Slides were washed, and incubated in 0.3 mg/ml diaminobenzidine (DAB) solution (Sigma) containing 0.03% $H_2O_2$ and 2% nickelammonium sulfate (Sigma) for 8-10 minutes. The reaction was then stopped by washing with double deionized water. When desired, counterstaining with hematoxylin (BDH, Ville Saint Laurent, Qc) was performed, as described (Wordinger et al., 87). Finally, slides were dehydrated in ethanol, cleared in xylene (Fisher), and mounted in Permount (Fisher)

Hematoxylin and eosin staining (Fisher), and a myelin stain were normally performed on sections from the same animals. Myelin stain was performed as follows: tissue sections were fixed 1-2 hours in formalin (Fisher), then hydrated to 95% ethanol. Slides were incubated overnight in a luxol fast blue solution (Fisher) in ethanol and glacial acetic acid (Fisher) at RT, then differentiated by successive incubations in 0.05% lithium carbonate (Fisher), 70% ethanol, and distilled $H_2O$. Sections were then counterstained in cresyl violet (Fisher) for 6 minutes, washed in 95% ethanol for 2 minutes, then in terpineol (Anachemia, Lachine, Qc)-xylol for 2 minutes, cleared in xylene (Fisher), and mounted in permount.

MAbs used were: XMG1.2 (Cherwinski et al., 1987), R4-6A2 (Spitalny and Havell, 1984), AN.18 (anti-IFN-γ); GK1.5 (anti-CD4) (Dialynas et al., 1983); P7/7 (anti-class II MHC) (Momburg et al., 1986); M1/42 (anti-class I MHC) (Springer, 1980); 53.6.72 (anti-CD8α) (Ledbetter and Herzenberg, 1979).

## RESULTS

### Generation of MBP/IFN-γ construct

An MBP/IFN-γ construct, PM2/IFN-γ, was generated by blunt-ended cloning of a full-length mouse IFN-γ cDNA into a plasmid, PM2, (Fig. 1) containing containing 3 Kb upstream and 2 Kb downstream mouse MBP DNA.

To determine the orientation of integration of the IFN-γ cDNA insert into the MBP sequences, the plasmid was digested with combinations of restriction enzymes and run on agarose gels. Double digestion with XbaI and EcoRV was one of the combinations used. XbaI cuts PM2 (see Fig. 1), but not the insert, whereas EcoRV cuts the insert at bp 225, but not PM2. Digestion with either of these REs alone would therefore linearize the construct, yielding a band of 9260 bp. Digestion with both REs, on the other hand, would yield 2 bands, 5940 and 3320 bp, if the insert is in the right orientation, and 5335 and 3925 bp, if it is in the wrong orientation. Fig. 2 shows an agarose gel with a PM2/IFN-γ clone that was determined to be in the right orientation using this method. Other RE combinations were used to confirm the insertion and orientation in this clone (not shown). Sequencing confirmed that the cDNA inserted in the plasmid in the right orientation and that no new reading frames were created (not shown).

### Transfected MBP/IFN-γ construct yields IFN-γ mRNA and protein

To ascertain that the MBP regulatory sequences in PM2/IFN-γ are capable of driving transcription of IFN-γ mRNA and that this message can yield a functional IFN-γ protein, PM2/IFN-γ was transfected into GA-5, an

Figure 1.

**The PM2 vector.**

5' end of the mouse MBP gene (from cos 138) fused with its 3' end by ligating the BspMII site in the 5'UT region of exon 1 with the BspMII site in the 3'UT region of exon 7. Italics indicate restriction sites which are not unique (letters), or positions which are approximate.

# pM2

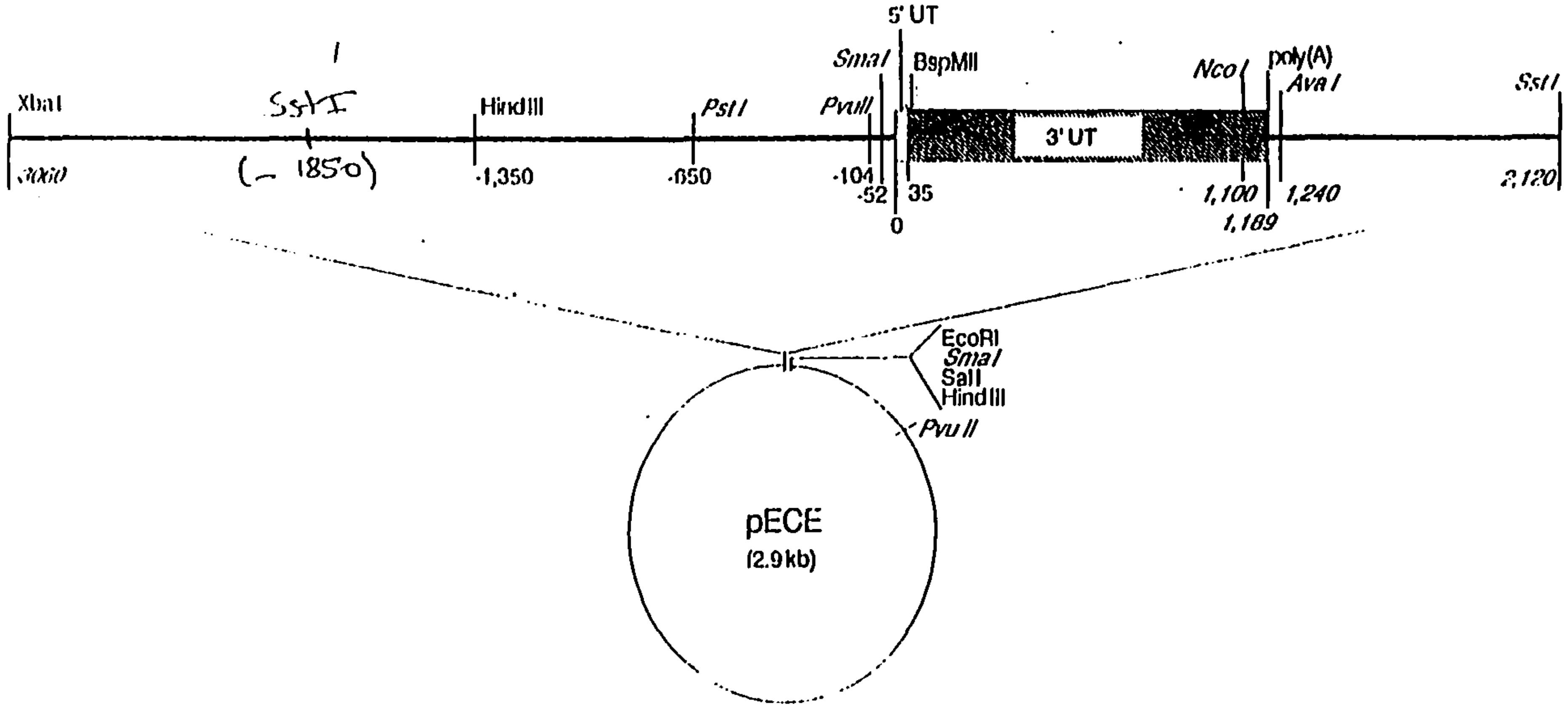

5' UT
SmaI
BspMII
NcoI
poly(A)
AvaI
XbaI
SstI
HindIII
PstI
PvuII
SstI
3' UT
-3000
(~ 1850)
-1,350
-650
-104
-52
0
35
1,100
1,189
1,240
2,120
EcoRI
SmaI
SalI
HindIII
PvuII
pECE
(2.9 kb)

Figure 2.

## Restriction mapping of PM2/IFN-γ

1 μg miniprep DNA from PM2/IFN-γ clone 28 was subjected to digestion with the restriction endonucleases EcoRV and/or XbaI. Digested DNA, as well as undigested DNA from the same clone, were electrophoresed on a 1% agarose gel and visualized with ethidium bromide. Size markers: lambda DNA digested with either HindIII or with HindIII and EcoRI.

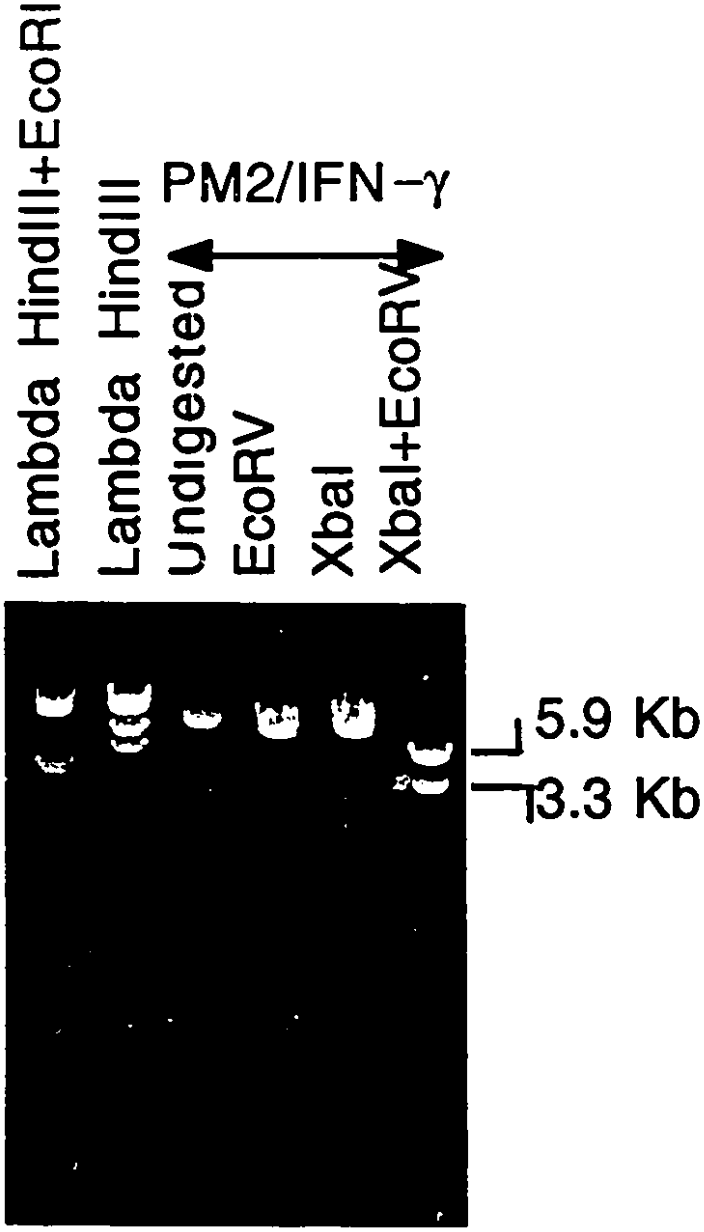
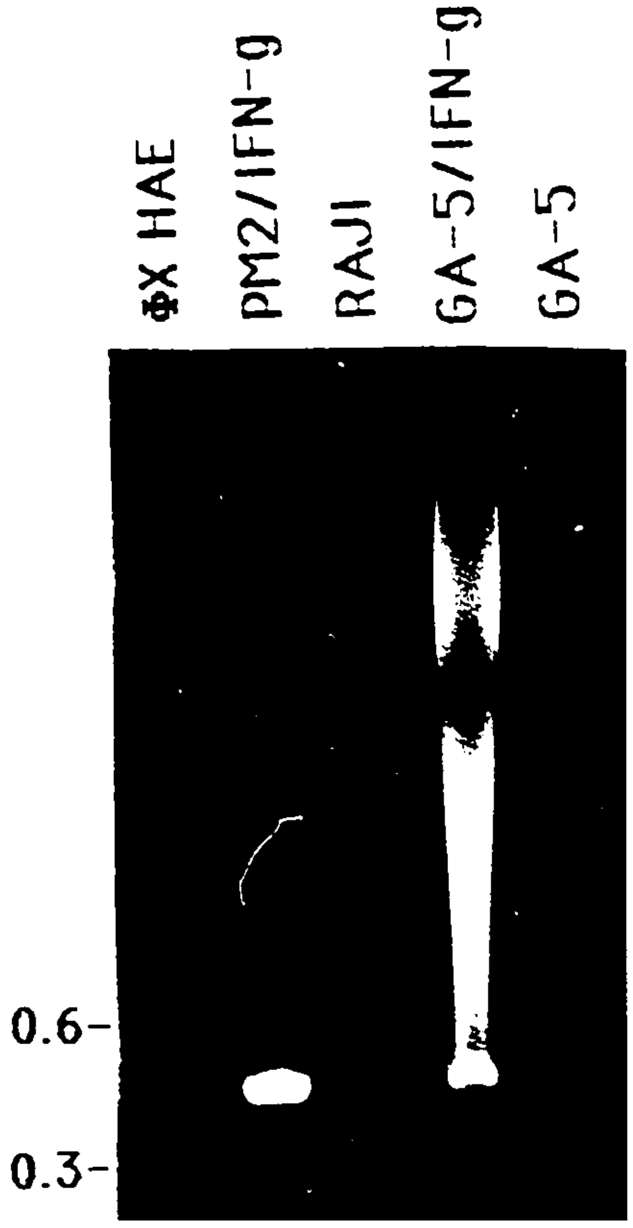

Lambda HindIII+EcoRI
Lambda HindIII
Undigested
EcoRV
XbaI
XbaI+EcoRV
PM2/IFN-γ
5.9 Kb
3.3 Kb

Figure 3.

## RT/PCR amplification of IFN-γ in transfected GA-5 cells

Plasmid containing PM2/IFN-γ sequences was transfected into GA-5 cells, as described in materials and methods. RT/PCR amplification was performed for 30 cycles using IFN-γ-specific primers, and amplification products were run on agarose gel and visualized with ethidium bromide. GA-5: mock transfected GA-5 cells; GA-5/IFN-g: PM2/IFN-γ- transfected GA-5 cells; RAJI: human lymphoblast line; PM2/IFN-g: PM2/IFN-γ plasmid used for transfection.


ΦX HAE
PM2/IFN-g
RAJI
GA-5/IFN-g
GA-5
0.6-
0.3-

oligodendrocyte precursor cell line transformed with SV40 large T antigen (LTA) carrying a heat-sensitive mutation.

The transfected cells were pelleted and their supernatant (SN) was collected. RT/PCR was performed on RNA extracted from the cell pellet. Fig.3 shows that a 450 bp IFN-γ band could be amplified from GA-5 cells transfected with PM2/IFN-γ (GA-5/IFN), but not from mock-transfected GA-5 cells (GA-5), or from a human B cell line (RAJI). PM2/IFN-γ plasmid was included as a positive control.

To determine whether biologically-active IFN-γ protein was produced by the transfected cells, the SN was subjected to a IFN-γ-specific bioassay. As shown in Fig. 4, SN from PM2/IFN-γ-transfected GA-5 cells was able to inhibit proliferation of WEHI-279 cells in a dose-dependent manner, and this inhibition was blocked by anti-IFN-γ mAb, indicating that biologically-active IFN-γ was produced by the transfected cells.

### Transgenic founders are identified

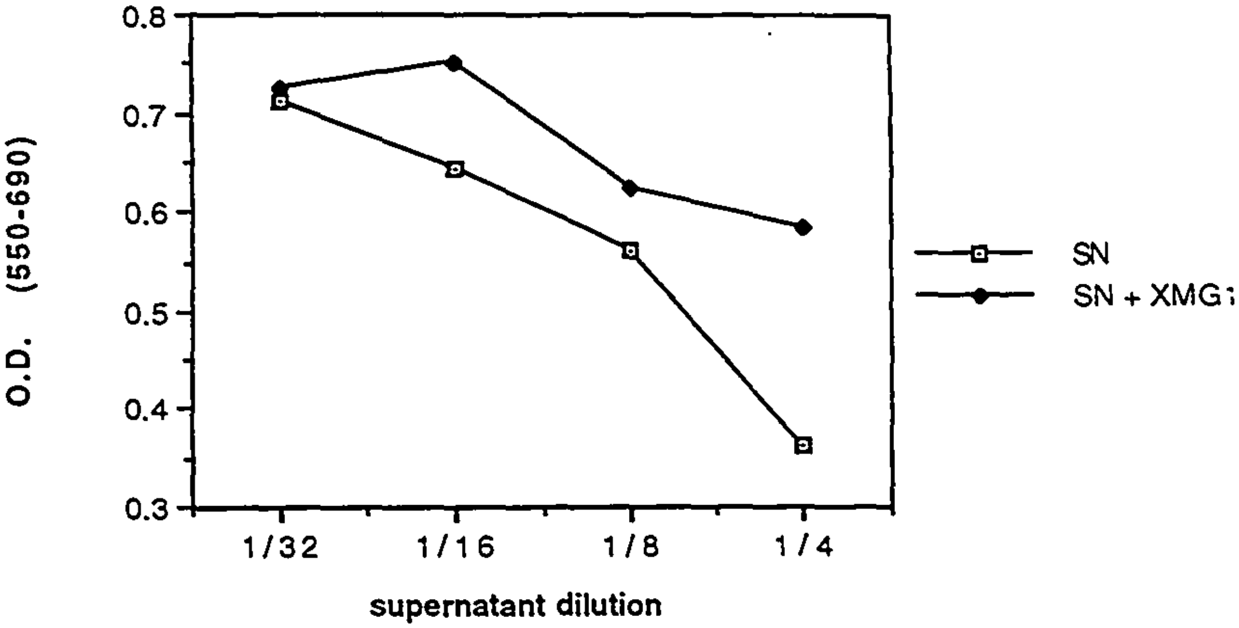
Having confirmed that the PM2/IFN-γ construct was able to drive the expression of IFN-γ mRNA in the appropriate cells and that biologically-active IFN-γ was translated from that message and secreted, the construct was linearized prior to microinjection. A linear HindIII fragment was obtained (Fig. 5) and microinjected into male pronuclei of fertilized (C3H X C57BL/6) F2 oocytes, which were then implanted into pseudopregnant females.

Out of 40 pups that were born from surrogate mothers, 8 were found to have integrated the transgene using RT/PCR and mixed MBP/IFN-γ primers (Fig. 5), a combination that insures that only PM2/IFN-γ-containing DNA, but not the

Figure 4.

IFN-γ bioassy of supernatant from transfected GA-5 cells

Plasmid containing IFN-γ/MBP sequences was used to transfect the oligodendrocyte precursor cell line, GA-5. IFN-γ titers were determined by inhibition of proliferation of the WEHI-279 cell line. Briefly, $10^4$ cells were added to titrations of supernatants or standards, with or without 25% anti-IFN-g mAb for confirmation of specificity of response. Cell proliferation was read out colorimetrically after 3 days, using MTT and an SLT-EAT400 multiwell photometer.

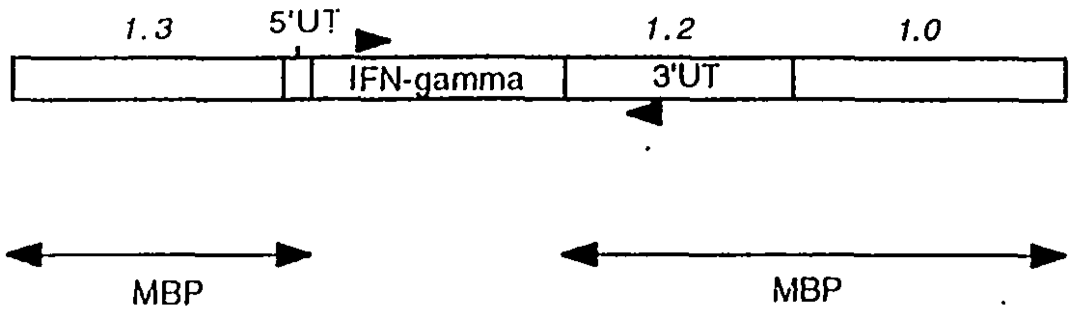


O.D. (550-690)
0.8
0.7
0.6
0.5
0.4
0.3
1/32
1/16
1/8
1/4
supernatant dilution
SN
SN + XMG1

**Figure 5.**

**IFN-γ/MBP promoter construct**

A mouse IFN-γ cDNA was subcloned into the BspMII site of PM2. The DNA construct was linearized by digestion with HindIII and microinjected into the pronuclei of fertilized (C57BL/6 X C3H) F1 fertilized oocytes. Arrowheads indicate the location of the oligonucleotide primers that were used to screen for transgene integration.


1.3
5'UT
1.2
1.0
IFN-gamma
3'UT
MBP
MBP

endogenous MBP or IFN-γ genes, will be amplified. This is illustrated in Fig. 6, where amplification of 1 μg DNA from mice that had integrated the transgene yielded a band that co-migrated with the amplification product of PM2/IFN-γ plasmid on agarose gel. DNA from unmanipulated mice or mice that were microinjected but did not integrate the transgene showed no such bands.

**IFN-γ is expressed in the CNS of MBP/IFN-γ mice**

To study the expression of the transgene, the founders were crossed to C3H mice, and RNA was obtained from the brains and spinal cords of their offspring. Transgenic offspring from one founder (A519) were found to express readily detectable levels of IFN-γ mRNA, by RT/PCR, in the spinal cord and brain, with consistently higher expression levels in the former than in the latter. This is shown in Fig. 7, where RNA from B201 and C302, two transgenic offspring of A519, yielded an appropriate RT/PCR band. This was abrogated upon treatment of the PCR mix with DNAse-free RNAse prior to amplification, ruling out amplification of contaminating transgene-containing genomic DNA. CNS tissue from B409, a non-transgenic littermate, was used as a negative control. IFN-γ mRNA in these mice was undetectable by Northern blot analysis.

To ascertain tissue specificity of expression of the transgene, RNA was extracted from various tissues and subjected to RT/PCR with IFN-γ primers and Southern blotting. As shown in Fig. 8, RNA from heart, testes, lungs (in addition to kidney and liver, not shown) of a transgenic animal did not contain IFN-γ message, whereas brain, spinal cord, spleen and thymus RNA did. The presence of IFN-γ mRNA in spleen and thymus could be due to "leakiness" of

the promoter. Alternatively, given the extreme sensitivity of our detection

Figure 6.

## PCR screening of tail DNA for transgene integration.

Tail tissue from 3-week old mice was digested with Proteinase K and DNA was obtained using phenol/chloroform extraction. 1 µg DNA was amplified in a PCR reaction using an upstream primer specific to the mouse IFN-γ cDNA and a downstream primer complementary to the 3'UT sequences of the murine MBP gene. Amplification products were run in an agarose gel and visualized with ethidium bromide. Plasmid DNA: PCR-amplified PM2/IFN-γ plasmid (100 ng).

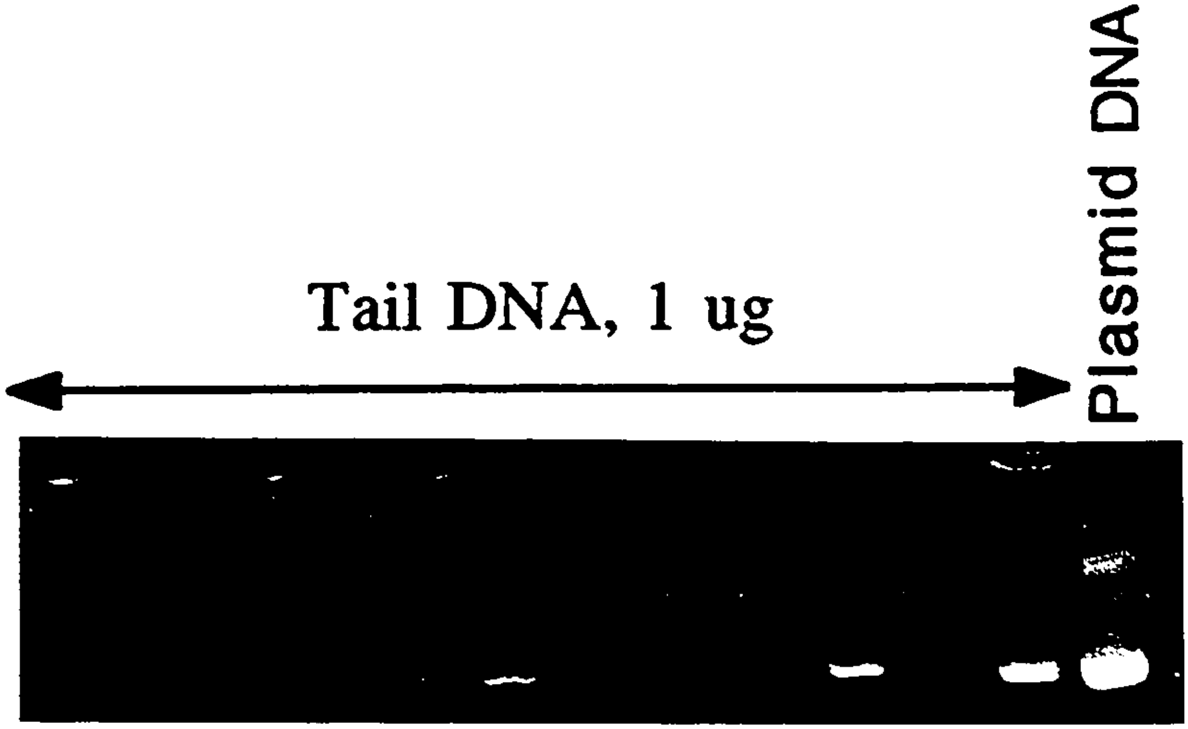
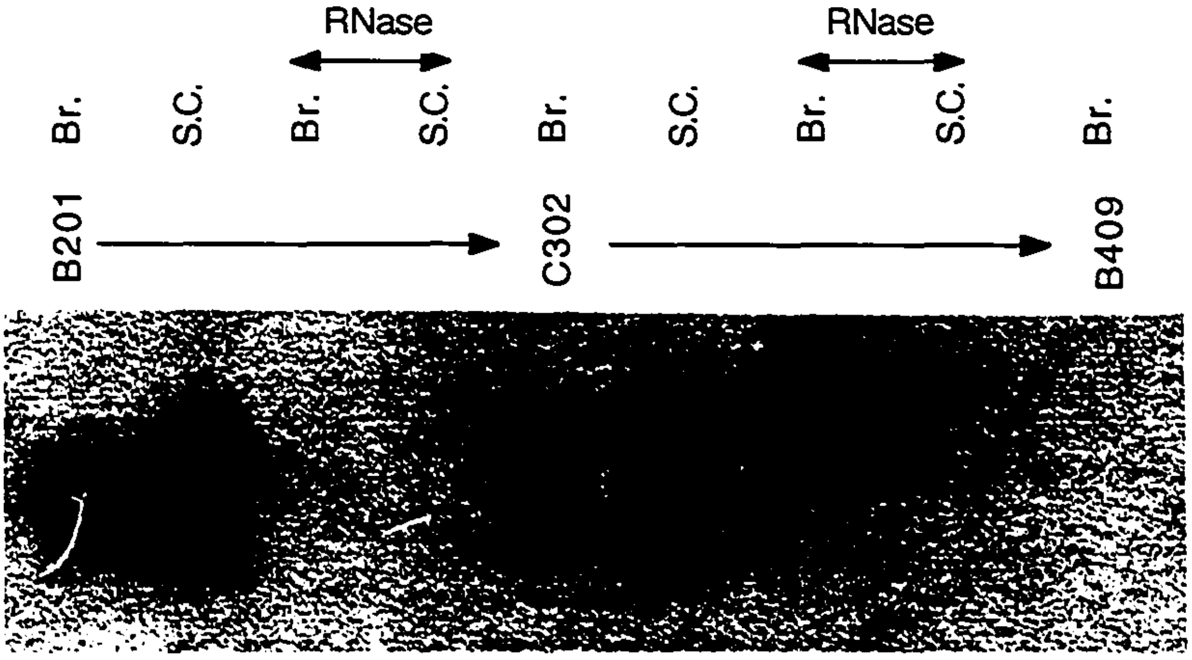

Tail DNA, 1 ug
Plasmid DNA

Figure 7.

## IFN-γ expression in CNS of MBP/IFN-γ transgenic mice

RNA was extracted from brain and spinal cord, reverse transcribed, and subjected to a 25-cycle PCR amplification using IFN-γ-specific primers in the presence of absence of DNAse-free RNAse. Amplimers were visualized by Southern blotting and hybridization with a $^{32}$P-labelled IFN-γ cDNA. B201 and C302 are transgenic mice from different crosses. B409 is a non-transgenic littermate of B201. Br.: brain; S.C.: spinal cord.

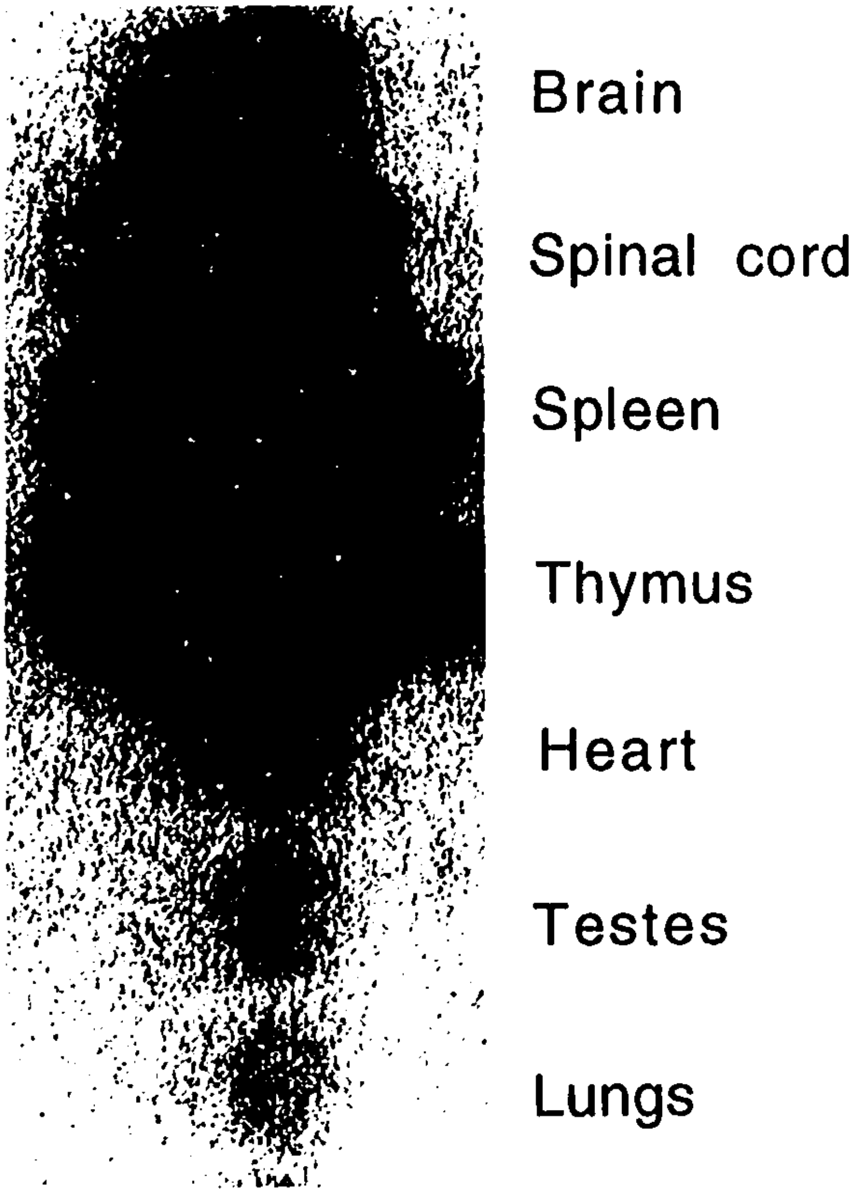


B201
C302
B409
Br.
S.C.
Br.
S.C.
Br.
S.C.
Br.
S.C.
Br.
RNase
RNase

Figure 8.

Tissue specificity of the IFN-γ/MBP construct.

RNA was extracted from different tissues, reverse transcribed, and subjected to a 25-cycle PCR amplification using IFN-γ-specific primers, and visualized by Southern blotting and hybridization with a $^{32}$P-labelled IFN-γ cDNA. In addition to brain and spinal cord, IFN-γ message was also detected in spleen and thymus.


Brain
Spinal cord
Spleen
Thymus
Heart
Testes
Lungs

system, and given the lymphoid nature of these organs, the IFN-γ message in these tissues could come from endogenous sources. Fig. 9 supports this latter hypothesis, since it shows that IFN-γ mRNA can be detected in the spleen and thymus, but not in the spinal cord of a normal, unmanipulated C3H mouse.

### Derivation of homozygous MBP/IFN-γ lines

Transgenic animals were then backcrossed 5 generations onto the EAE-susceptible SJL background, after which homozygous lines were generated by intercrossing heterozygous mice. For example, in Fig. 10, mice #1 to 6 are the offspring of a cross between a homozygous and a heterozygous mouse, whose DNA was used as controls. DNA from a non-transgenic littermate was used as a negative control. As expected, all the mice in the litter were transgenic, and the intensities of the bands identified mice #2, 3, 4, and 6, as homozygous, and mice #1 and 5 as heterozygous.

### Class I MHC is induced in the CNS of MBP/IFN-γ mice

Histologic examination of hematoxylin and eosin (H&E)- stained brain and spinal cord sections from transgenic animals did not reveal any gross morphological abnormalities in comparison to those from littermates. No infiltrating cells were detected, and myelin staining failed to reveal differences between transgenic and non-transgenic mice.

For technical reasons, we were unable to demonstrate expression of IFN-γ protein in the CNS by immunohistochemical staining, using a technique that has identified IFN-γ in a Th1 clone and in in vitro-activated LN T cells. We were, however, unable to immunoblot IFN-γ protein from total lysates of the same Th1 clone, despite being able to immunoblot CD45, using the same

Figure 9.

PCR screening of spleen and thymus of transgenic and control aminals for IFN-γ mRNA.

RNA was extracted, reverse transcribed, and amplified using IFN-γ- specific primers, as described. Agarose gel and Southern blotting were performed as described above. C3H: normal C3H/J mouse; Tg.: a transgenic offspring from a cross between a normal C3H and a transgenic (C3H X C56BL/6)F1. S.C.: spinal cord; Spl.: spleen; Thy.: thymus.

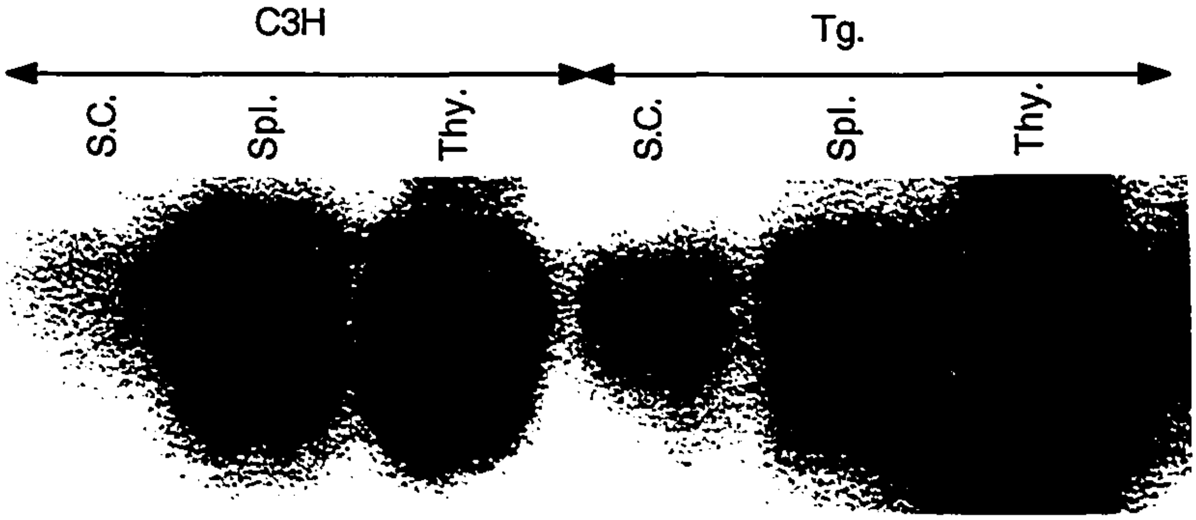
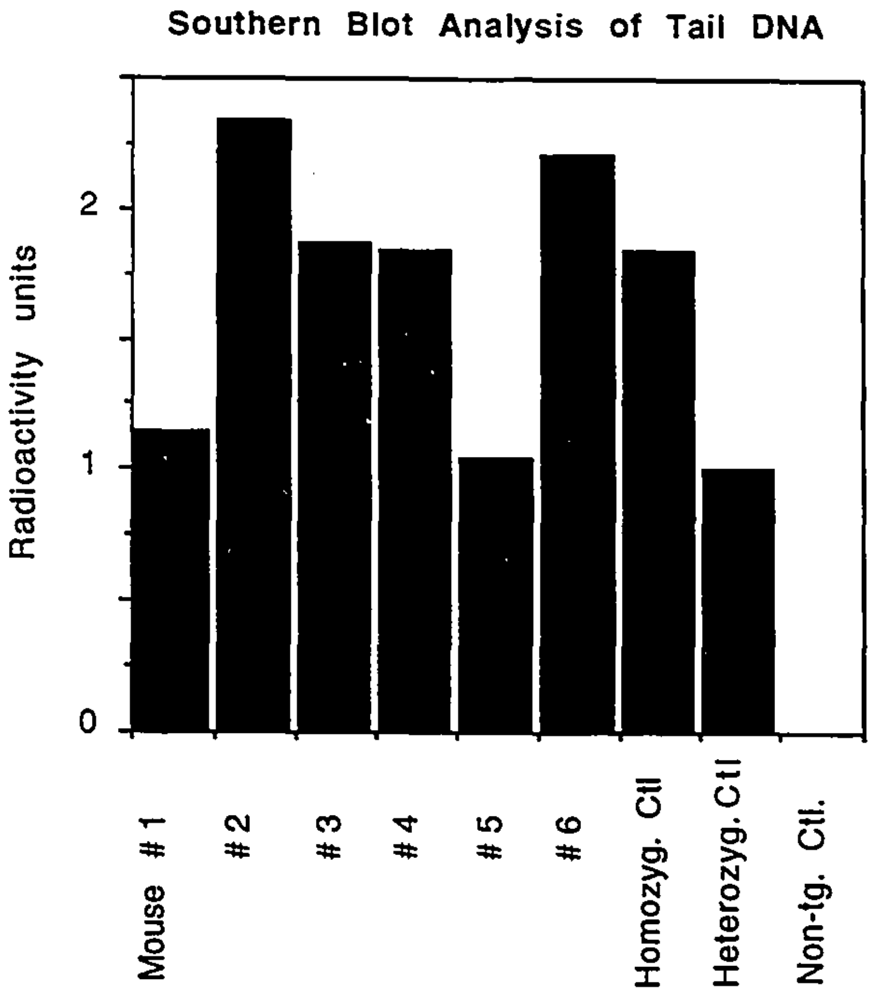

C3H
Tg.
S.C.
Spl.
Thy.
S.C.
Spl.
Thy.

Figure 10.

Southern blot screening for homozygous transgenic mice.

Genomic DNA was extracted and Southern blot anlysis carried out, using an IFN-γ-specific cDNA probe. Radioactive bands were quantitated using PhosphorImager, and plotted as arbitrary radioactivity units. Mice #1 to #6 are the offspring of a homozygous and heterozygous transgenic mice (included as controls). A non-transgenic mice was also used a control.


Southern Blot Analysis of Tail DNA
Radioactivity units
0
1
2
Mouse # 1
# 2
# 3
# 4
# 5
# 6
Homozyg. Ctl
Heterozyg. Ctl
Non-tg. Ctl.

technique. We used two monoclonal and 1 polyclonal anti-IFN-γ Abs for blotting. None of these had previously been used successfully in a western blot. It is possible that these Abs cannot recognize epitopes on the incompletely renatured protein bound to nitrocellulose.

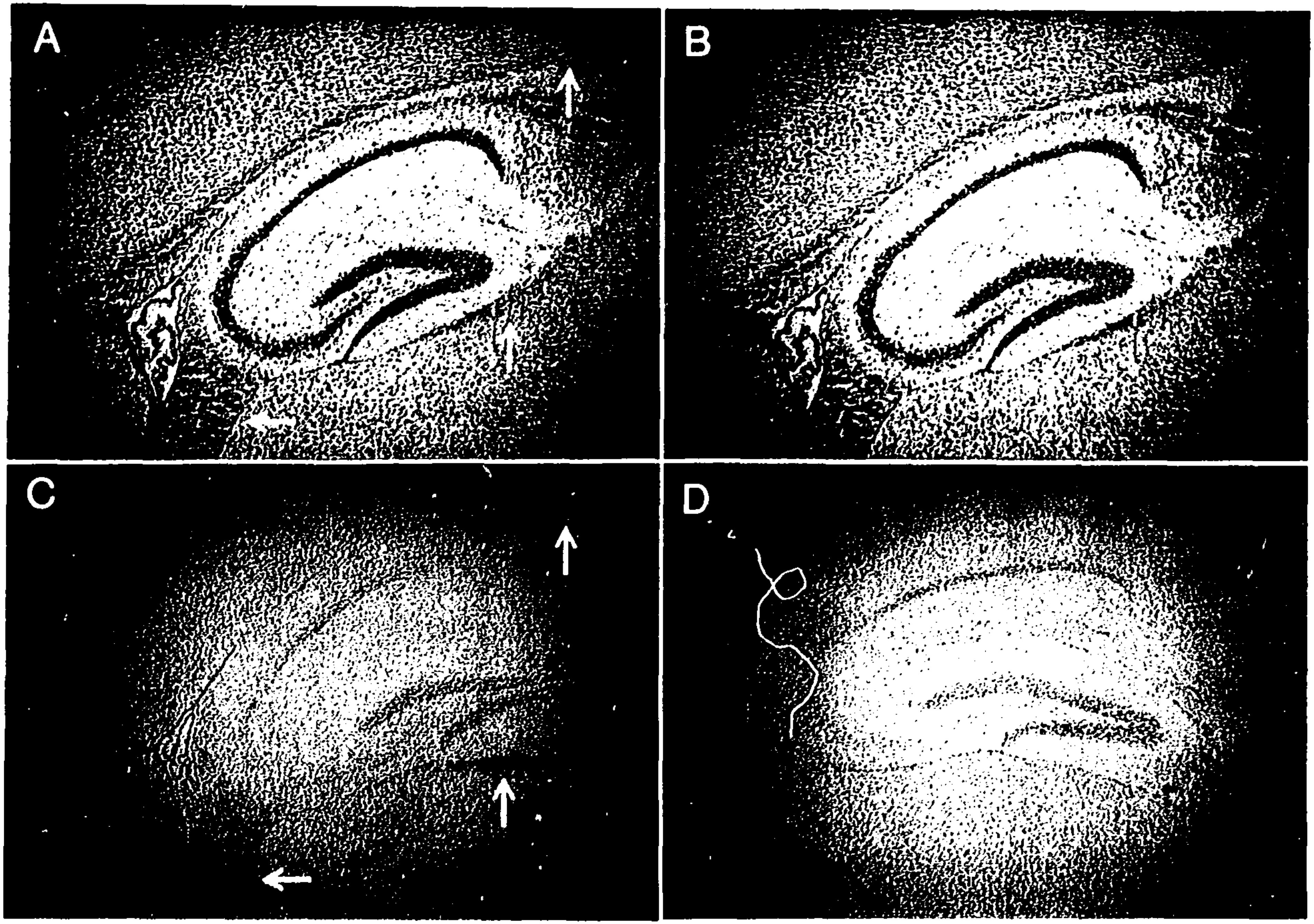
In the absence of direct demonstration of IFN-γ protein in the CNS, we stained for IFN-γ-inducible genes. Class I MHC antigens were chosen because they are expressed at low to undetectable levels in normal CNS, but are readily detectable in inflamed CNS, presumably as a result of induction with IFN-γ. Fig. 11 shows sagittal sections through the hippocampus of a 3 week old transgenic mouse (A,C) and a non-transgenic littermate (B,D) stained with with luxol fast blue (myelin stain) (A,B) or anti-Class I MHC mAb (M1/42, rat IgG2a) (C,D). Class I MHC staining was observed in sections from 3-week-old, but not adult, transgenic animals, or from non-transgenic littermates. Class 1 MHC reactivity was detected predominantly in the hippocampus, cerebellum, and spinal cord, and coincided with areas of myelination. An isotype-matched anti-CD8α Ab (53.6.72) showed no staining (not shown). The coincident expression of class I MHC and myelin is consistent with IFN-γ being driven by a transgenic MBP promoter, whose peak activity was shown to occur at age 3 weeks (Foran and Peterson, 1992), during active myelination. It is likely that class I MHC expression is induced in regions where myelination was occuring or had recently occured.

**MBP/IFN-γ mice develop severe, non-remitting MBP-induced EAE.** MBP/IFN-γ transgenic mice were observed for over a year. No neurological signs were detected, even in mice that had been backcrossed 5 generations onto SJL. This was consistent with the lack of CNS infiltration and the normal

Figure 11.

Myelin and class I MHC staining in the CNS

Sagittal sections through the hippocampus of a 3 week old transgenic mouse (A,C) and a non-transgenic littermate (B,D) were stained with with luxol fast blue (myelin stain) (A,B) or anti-Class I MHC mAb (M1/42, rat IgG2a) (C,D). Arrows show areas of myelination (A) or class I MHC staining (C).


A
B
C
D

myelin patterns in these mice. In the absence of spontaneous autoimmune disease, we asked whether IFN-γ expression in the CNS affected the suceptibility of mice to actively-induced EAE, or altered the course of the disease. EAE was induced by s.c injections of MBP in CFA. Table 1 shows that a large proportion of transgenic animals developed a severe, non-remitting, EAE. By contrast, a lower frequency of non-transgenic littermates developed EAE, severity was lower, and duration was similar to that seen in normal SJL mice. The lower incidence of EAE in non-transgenic littermates v/s normal SJL mice may reflect the differences in genetic background, and as such the littermates are the appropriate controls for the transgenic mice.

Table 1.

Exacerbated EAE in PM2/IFN-γ transgenic mice.

Expt. I

| | | |
|---|---|---|
| Transgenic | (+/-) | Severe, unremitting, EAE. Moribund at 4 weeks |
| Transgenic | (+/-) | Severe, unremitting, EAE. Moribund at 4 weeks |
| Transgenic | (+/-) | No symptoms |
| Littermate | (-/-) | No symptoms |
| Littermate | (-/-) | No symptoms |

Expt. II

| | | |
|---|---|---|
| Transgenic | (+/-) | Severe EAE. Delayed remission (3 weeks) |
| Littermate | (-/-) | Mild EAE. Normal remission |
| Littermate | (-/-) | No symptoms |
| Littermate | (-/-) | No symptoms |

EAE was induced by two immunizations with MBP in CFA. All mice were (C3H X C57BL/6) F1 females, backcrossed twice onto SJL/J. (+/-) indicates heterozygous transgenic mice; (-/-) non-transgenic littermates. Remission in non-transgenic mice usually occurs within one week after appearance of symptoms.

## DISCUSSION

We have generated transgenic mice that constitutively express IFN-γ in the CNS. The CNS of these mice was morphologically normal and free of infiltrating cells. Mice did not develop spontaneous autoimmune disease, even after extensive backcrossing into the SJL background. This lack of spontaneous pathology contrasts with a previous study where mice developed inflammatory diabetes as a consequence of transgenic IFN-γ expression in the pancreas (Sarvetnick et al., 1988). This apparent discrepancy might reflect substantially lower levels of IFN-γ mRNA in our mice. Indeed, unlike the studies of Sarvetnick et al. where IFN-γ mRNA was detectable by Northern blotting in the pancreas, we had to use the more sensitive RT/PCR with Southern blotting to detect IFN-γ transcripts in the CNS. The level of expression of the IFN-γ transgene in the CNS of our mice were comparable to those found in the CNS of mice with EAE (not shown), and therefore possibly more physiologically relevant than the pancreatic model. Another difference may simply relate to differences inherent to the tissues where the transgene is expressed. It is noteworthy that transgenic expression of IFN-α, or IL-10 (an immunosupressive cytokine) in the pancreas also produced inflammation in that organ (Stewart et al., 1993, Wogensen et al., 1993), suggesting an enhanced susceptibility of the pancreas to cytokine-mediated inflammation. Also, whereas both class I and class II MHC are induced by transgenic IFN-γ in the pancreas, only class I MHC could be detected in the CNS of our transgenic mice. Interestingly, the pattern of class I expression (strong expression in the hippocampus and cerebellum, colocalizing with active myelination at age 3 weeks) in the MBP/IFN-γ transgenic mice closely resembled that of β-Gal in MBP/LacZ transgenic mice using the same promotor sequences (Foran and Peterson, 1992).

Although MBP/IFN-γ mice did not develop spontaneous disease, they were more susceptible to MBP-induced EAE. Indeed, a more severe and unremitting form of EAE was observed in transgenic animals, in comparison to the mild, rapidly-remitting EAE seen in non-transgenic littermates. This suggests that IFN-γ alone is not sufficient to initiate of an inflammatory response in the CNS. Rather, the continuous expression of this cytokine in the CNS seems to amplify a peripherally-induced, CNS-directed immune response and to prevent remission. The lack of remission in MBP/IFN-γ mice and our demonstration (in chapter 3) that IFN-γ mRNA is lost in remission from actively-induced EAE in non-transgenic mice suggest that this loss is required for remission to occur. It is noteworthy that IFN-γ, a Th1-type cytokine, inhibits the proliferation of Th2 T cells (Gajewski and Fitch, 1988). These cells produce IL-10, a cytokine that has been implicated in remission from EAE (Kennedy et al., 1992). IFN-γ also negatively suppresses the action of prostaglandin E2, an inhibitor of macrophage-mediated cytoxicity (Russell and Pace, 1984).

Studies in which IFN-γ was injected into rodent CNS yielded conflicting results. Intraventricular administration of IFN-γ in rats protected from the EAE (Voorthuis et al., 1990). By contrast, injection of IFN-γ into rodent spinal cord induced EAE-like symptoms (Simmons and Willenborg, 1990). Finally, intrathecal injection of IFN-γ in rats induced MHC expression in the CNS, but did not lead to overt pathology (Vass and Lassmann, 1990). The discrepancies between these studies may relate to the differences in the site of injection and the extent of vascularization in those sites. This is an especially important consideration given the critical role the blood-brain barrier plays in the pathology of EAE (Brosnan et al., 1993). The transgenic approach has allowed us circumvent that problem by expressing IFN-γ without breaching the blood-brain barrier or perturbing peripheral IFN-γ status. Our results are not consistent with a protective role for IFN-γ in EAE. Rather, they are in line with the observation of Vass and Lassmann (1990). More importantly, our data are reminescent of those obtained in humans, where administration of IFN-γ to MS patients shortened remission periods and exacerbated the disease (Panitch et al., 1987).

We are currently using the recently available PM2/IFN-γ homozygous lines to dissect the cellular and molecular events leading to the exacerbated pathology and lack of remission in MBP/IFN-γ transgenic mice. We are also in the process of generating another MBP/IFN-γ construct using a 9 Kb MBP promoter that has been shown to direct a more abundant and widespread expression of transgenes in the CNS (A. Peterson, unpublished observations), to study the effects of IFN-γ hyperexpression in that compartment.

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# CHAPTER 5

## Cytokine production by cells in cerebrospinal fluid during Experimental Allergic Encephalomyelitis in SJL/J mice.

## Preface

In chapter 3, I showed that the expression of IL-2 and IFN-γ in total CNS tissue from mice with EAE correlated with the active phase, and with the severity of the disease. The next question was whether this information can be used in the evaluation of inflammation of the human disease, MS, for which EAE is a model. The only compartment avilable for study in MS is the CSF. To study the validity of using CSF to evaluate the immune activity in the CNS, I here compare the levels of expression of IL-2 and IFN-γ in these two compartments, and the severity of the disease, in mice with EAE.

## ABSTRACT

Cytokine production by T cells in the cerebrospinal fluid (CSF) and central nervous system (CNS) of SJL/J mice during myelin basic protein (MBP)-induced experimental allergic encephalomyelitis (EAE) was examined. Reverse transcriptase/polymerase chain reaction (RT/PCR) was used to measure interleukin-2 (IL-2) and interferon-gamma (IFN-γ) mRNA levels from perfused CNS tissue (brain and spinal cord) and from cells isolated from CSF. Animals were grouped according to EAE severity, ranging from asymptomatic (adjuvant only) to severe disease (paralysis or severe paresis). Cytokine signals, normalized to actin, were almost undetectable in control tissues, and only slightly elevated in whole CNS tissue from animals with mild EAE. Both cytokine messages were strongly upregulated in CNS tissues derived from severely affected animals, consistent with previous observations correlating disease progression with infiltration by memory/effector CD4$^+$ T cells, the major source of these cytokines. This cytokine upregulation was specific to the CNS, since other organs from the same animals did not express significant levels of IL-2 and IFN-γ. CSF was obtained from the cisterna magna of unperfused mice and verified as such by absence of red blood cells (RBCs) and by immunoglobulin concentration orders of magnitude lower than in serum. Cytokine message was measured in RNA isolated from cells in CSF. Levels of IL-2 and IFN-γ mRNA in CSF cells were significantly elevated in mild EAE and strongly upregulated in severe disease, correlating with those in total CNS tissue. These results confirm the CSF as representative of the immune status of the CNS and indicate a role for IL-2 and IFN-γ in inflammatory CNS disease.

## Introduction

Immune-mediated mechanisms are postulated to underlie the development of multiple sclerosis (MS), a disorder characterized by multifocal areas of inflammation, demyelination and gliosis within the CNS (Prineas, 1985). Immune-mediated mechanisms have been shown to mediate the tissue injury in animal models of CNS inflammatory-demyelinating diseases induced either by auto-antigen sensitization (EAE) (Raine, 1985) or by a viral infection such as Theiler's murine encephalitis virus (Miller et al., 1990). In both the human and animal disorders cytokines produced by immune cells infiltrating the CNS are implicated as important determinants of disease development and progression (Selmaj, 1992). We have shown that $CD4^+$ T cells which infiltrate the CNS in EAE are predominantly of the memory/effector phenotype (Zeine and Owens, 1992), which is associated with active cytokine production (Bottomly et al., 1989), and we have shown these T cells to produce IL-2 and IFN-γ (Renno et al, submitted).

In humans, analysis of cytokine production in concert with disease development is limited by lack of direct access to the CNS *in vivo*. The systemic circulation is accessible to analysis, but studies of this immune compartment in affected individuals have at best shown a weak correlation of cytokine profiles and disease activity (Olsson et al., 1990). Analysis of cytokines present in the fluid phase of the CSF have suggested correlation with disease activity in some studies (Sharief et al., 1991; Franciotta et al., 1989) but comparisons among these studies are complicated by methodologic differences, and limits of detectability of cytokine proteins. Cytokine production by T cell clones generated from CSF in response to antigen has been reported (Benvenuto et al., 1991), as has cytokine production by individual CSF-derived T cells following short-term exposure to antigen in vitro, using an immunospot assay (Olsson et al., 1990). In the latter studies, cytokine production by

MS patient-derived T cells exceeded that of control donor cells. The above studies involved restimulation of antigen-reactive CSF-derived T cells *in vitro* prior to assay, rather than analysis of the total T cell population immediately *ex-vivo*, raising questions about the relevance of these findings to the *in vivo* situation.

The aims of the present study were to determine whether cytokine production by immediately ex-vivo cells derived from the CSF parallels that in total CNS tissue, eg. reflects the immune status of the CNS of EAE animals, and whether such cytokine production correlates with the severity of clinical symptoms. The advent of molecular techniques that permit amplification of cDNA signals has allowed analysis of gene transcription in small numbers of cells such as are found in CSF, whereas previous studies have required in vitro expansion of CSF cells prior to analysis. In the present study we describe a RT/PCR analysis of mRNA from total CNS tissue and of mRNA in T cells from the CSF of mice with varying grades of EAE. We show correlation of IL-2 and IFN-γ expression in CNS with severity of EAE. We also demonstrate that changes in the level of production of IL-2 and IFN-γ by CSF T cells parallel those seen in total CNS tissue. This demonstrates that the CSF is representative of infiltrating lymphocytes in a CNS autoimmune disease, and validates study of this compartment in analysis of human disease.

## Materials and Methods

### Mice and induction of EAE

Female SJL/J mice (5-8 weeks) were obtained from Harlan Sprague Dawley (Indianapolis, IN). EAE was induced by two subcutaneous injections, 7 days apart, of myelin basic protein (MBP) (Sigma, St. Louis, MO) in CFA (Difco, Detroit, MI), containing 50 µg H37RA *M. tuberculosis* (Difco). Symptoms were first observed 14 days after the initial injection. The animals used for this study either had mild EAE (grade 1, eg. limp tail) (n=8), or severe EAE (grade 3-4, eg. severe paresis/paralysis of one or both hind limbs) (n=8) (Kennedy et al., 1988). Before collection of brains and spinal cords for PCR analysis, animals were perfused through the heart with 150 ml PBS.

### CSF extraction

CSF was extracted using a slight modification of the method of Griffin (1981). Briefly, mice were anesthetized with chloral hydrate (3.5 g/Kg); the skin and musculature over the head and the upper back was reflected and a 30-gauge Butterfly needle (Abbot, Montreal, Quebec) fitted with a mouth tube was used to pierce the dura mater and aspirate fluid from the medial cisterna. The fluid was examined visually and microscopically to confirm absence of red cells and the white cells were counted on a hemocytometer. The cells were then pelleted and snap-frozen in liquid nitrogen until needed. The supernatant was assayed for immunoglobulin content by ELISA. Serum was separated from clotted peripheral blood by centrifugation and immunoglobulin measured by ELISA (Owens, 1988).

### RNA extraction and PCR.

Cellular RNA from total CNS, heart, lung, and kidney was isolated by acidic guanidine thiocyanate (Terochem Scientific Inc., Markham, Ontario) and phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Messenger RNA was isolated from CSF cells using Quick Prep™ Micro (Pharmacia, Montreal, Quebec).

RNA was subjected to a one-step reverse transcription and amplification protocol. Briefly, 0.5 µg total CNS RNA and aliquots of CSF mRNA were added to a tube containing 10 U avian myoblastosis virus reverse transcriptase (Pharmacia), 2.5 U Taq DNA polymerase (Gibco BRL), 20 U RNA Guard™ RNAse inhibitor (Pharmacia), 10 mM of each dNTP, 50 pmoles of each primer, and a buffer mixture consisting of 50 mM KCl, 100 mM Tris (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin. Primers used were as follows: CD3γ sense primer 5'-ATGGAGCAGAGGAAGGGTCTG-3', CD3γ antisense primer 5'-TCACTTCTTCCTCAGTTGGTT-3', IL-2 sense primer 5'-TGCAGCTCGCATCCTGTCTCA-3', IL-2 antisense primer 5'-AGAAGGCTATCCATCTCCTCA-3', IFN-γ sense primer 5'-ACACTGCATCTTGGCTTTGC-3', IFN-γ antisense primer 5'-CGACTCCTTTTCCGCTTCCT-3', actin sense primer 5'-TGGGTCAGAAGGACTCCTATG-3', Actin antisense primer 5'-CAGGCAGCTCATAGCTCTTCT-3'. Reactions were incubated in a Perkin-Elmer/Cetus DNA Thermal cycler for 25 cycles (denaturation 1 min 94°C, annealing 2 min 60°C, extension 2.5 min 72°C). 40 µl per sample were run in 1% agarose gels in TAE buffer. Gels were transferred onto membranes and hybridized with the respective $^{32}$P-labelled cDNA probes: a 700 bp HindIII + EcoRI CD3 fragment purified from clone pB10.AT3γ-1 (Krissanssen et al., 1987), kindly provided by Dr R.P. Sekaly (IRCM, Montreal); an N-terminal 337 base pair PstI-HindIII fragment of the IL-2 cDNA, and a 643 base pair PstI fragment from IFN-γ (Kelso and Gough, 1988), provided by N. Gough (WEHI, Melbourne). The actin probe was a 1.1 Kb PstI fragment from the coding sequences of an a-actin cDNA (Minty et al., 1981). The radioactive signal was visualized by autoradiography, and the signal intensity was quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

## Results

### T cell and cytokine gene expression in CNS of mice with EAE.

Infiltration by T cells, and production of IL-2 and IFN-γ in the brains of mice with EAE was demonstrated by mRNA analysis. RNA was isolated from brain and spinal cord, cDNA was generated by reverse transcription was amplified by PCR. Whereas actin PCR products were detectable by ethidium bromide staining, cytokine and CD3 amplification products, while readily detectable by ethidium bromide staining at 40 cycles of amplification, required the more sensitive Southern blotting with specific cDNA's for detection in the linear range (25 amplification cycles). Figure 1 shows that IL-2-, IFN-γ-, and CD3-specific message was readily detectable from the CNS of mice with EAE, whereas analogous PCR products were barely detectable in unprimed controls. The intensity of actin cDNA amplimers from both groups was equivalent (Fig. 1).

The radioactive signal obtained in Southern blot analysis of PCR products was quantitated by phosphorimager analysis and the CD3 and cytokine signals were normalized to the actin signal. Figure 2 shows actin-normalized quantitations of IL-2 and IFN-γ signals obtained from CNS tissue of groups of 8 animals with varying grades of EAE. EAE severity was assessed on the basis of symptoms and animals were pooled according to grade. A strong correlation between cytokine levels and disease severity was observed. The signals obtained from the CNS of mice with mild EAE were too weak to accurately quantify the difference between them and control animals. Previous work has shown that IL-2 and IFN-γ are produced by infiltrating $CD4^+$ T cells that infiltrate the CNS ( Renno et al. submitted).

In order to confirm that this upregulation of IL-2 and IFN-g in EAE was specific to the CNS, we compared the expression of these cytokines in the brain, lung, kidney,

Table 1.

Characterization of CSF.

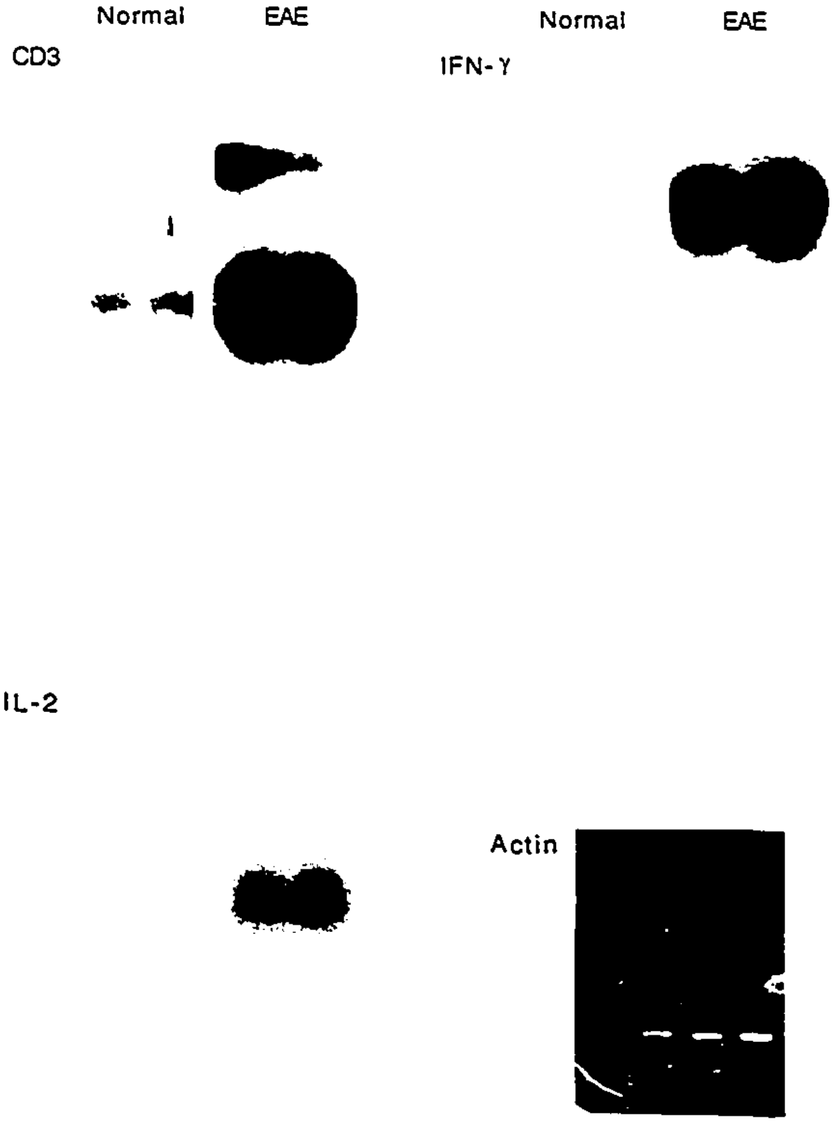
| | Number of mice | Number of CSF cells/mouse | CSF [Ig] /serum [Ig] |
|---|---|---|---|
| Normal | 8 | 250 | 1/100 |
| PBS+CFA | 6 | 300 | 1/122 |
| G1 EAE | 8 | 1000 | 1/350 |
| G3 EAE | 8 | 2300 | 1/77 |

CSF was obtained as described (Griffin, 1981), and pooled according to treatment or to disease grade. Cells were counted on a hemocytometer. Immunoglobulin ELISA was performed as described (Owens, 1988). Normal: unmanipulated mice. G1: mild EAE; G3: severe EAE.

Figure 1

PCR analysis of IL-2, IFN-γ, CD3 and actin mRNA in control and EAE CNS.

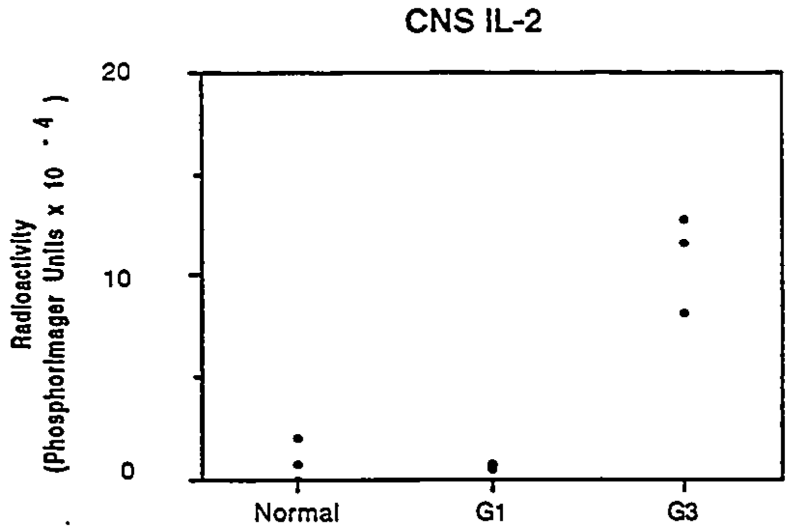
RNA was isolated from the brain and spinal cord of SJL/J mice that were unprimed (Control) or had been immunized with (RSCH + CFA) to induce Grade 3 EAE. Tissues were collected from PBS-perfused animals during the first episode (15 days after immunization). cDNA was transcribed from RNA and amplified by PCR using primers specific for IL-2, IFN-γ, CD3γ , or actin, then separated by agarose gel electrophoresis. Cytokine and CD3 products were transferred to a membrane and visualized by hybridization with specific cDNA probes. Actin PCR amplimers were visualized by ethidium bromide staining of the agarose gel.


Normal
EAE
CD3
Normal
EAE
IFN-γ
IL-2
Actin

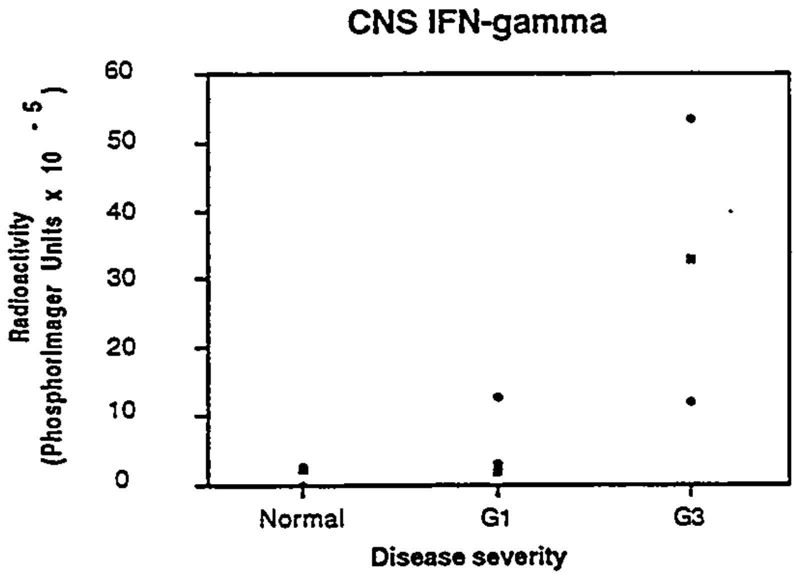
## Figure 2

**Quantitative analysis of PCR amplification of IL-2 and IFN-γ message from total CNS RNA.**

CNS sample collection, EAE induction, and RT/PCR were performed as described in the legend to Fig. 1 and in Materials and Methods. Radioactive bands on Southern blots of PCR amplification products were quantitated by PhosphorImager analysis. Each point represents a separate mouse and was normalized to the actin signal from the same experiment. Normal: unimmunized. G1: mild EAE. G3: severe EAE.



CNS IL-2



CNS IFN-gamma

and heart of mice with severe EAE. As shown in Figure 3. IL-2 and IFN-g messages were strongly upregulated in brain, whereas their expression in the lung, kidney, and heart was low (Fig. 3), comparable to that in the CNS of unimmunized mice (data not shown).

### Isolation of cells from CSF.

To assess cytokine production by CSF cells, CSF was obtained from animals with varying grades of EAE, and cells isolated for PCR analysis. The fluid obtained from the cisterna magna was identified as CSF by the following criteria. Firstly, although mice were not perfused, there were no or very few red blood cells in CSF, arguing against significant contamination with blood. Secondly, the immunoglobulin content of CSF ranged between 5.0 and 14 µg/mL, orders of magnitude lower than detectable in serum from the same animals (600-2700 µg/mL) (Table 1). Thirdly, the number of cells counted in CSF varied with CNS inflammation as predicted for a compartment draining an inflamed tissue. Animals immunized with CFA without neuroantigens yielded about the same number of CSF cells as obtained from normal, unmanipulated animals (Table 1). Too few cells were obtained in most experiments to allow surface phenotype analysis. However, preliminary data from selected samples confirmed the presence of $CD4^+$ T cells, as has been described (Griffin et al., 1987).

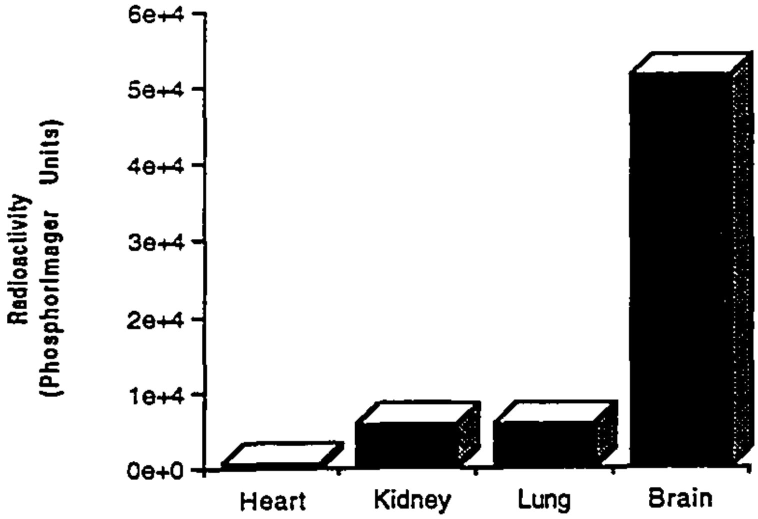
### Cytokine gene expression in CSF cells of mice with EAE.

RT-PCR analysis of cells from pooled CSF samples showed that IL-2 and IFN-$\gamma$ gene expression correlated with severity of disease (Figure 4). Message levels for these cytokines were essentially undetectable in CSF from control animals, and increased significantly with disease severity. Differences between CSF cytokine gene expression in CSF from control animals and from mice with mild EAE were more distinct than those noted when using total CNS tissue (Fig. 2).

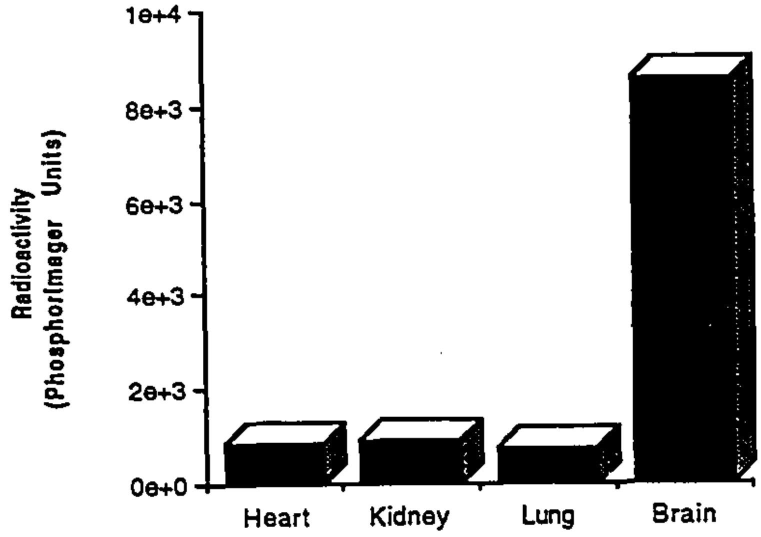
Figure 3.

Organ specificity of IL-2 and IFN-γ expression in mice with EAE.

Sample collection, EAE induction, and RT/PCR were performed as described in the legend to Fig. 1 and in Materials and Methods. Radioactive bands on Southern blots of PCR amplification products were quantitated by PhosphorImager analysis after normalization to actin.



IL-2



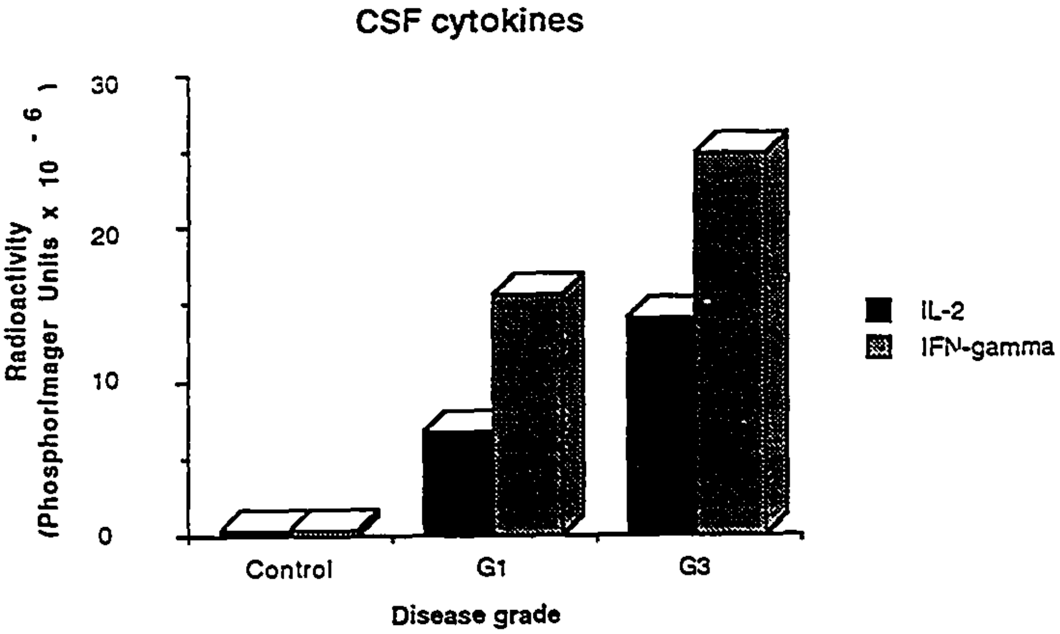
IFN-gamma

Tissue

**Figure 4.**

**Quantitative analysis of PCR amplification of IL-2 and IFN-γ mRNA from CSF.**

CSF was obtained as described in Materials and Methods. EAE induction, RT/PCR, and signal quantitation were carried out as described above. Each bar represents the signal obtained from pooled CSF cells of 6-8 mice and was normalized to the actin signal from the same experiment. Control: mice immunized with PBS/CFA. G1: mild EAE. G3: severe EAE.


CSF cytokines
Radioactivity
(PhosphorImager Units x 10 - 6 )
30
20
10
0
Control
G1
G3
Disease grade
■ IL-2
▨ IFN-gamma

## Discussion

We undertook to examine the variation in expression of two cytokines, IL-2 and IFN-γ, in total CNS tissue and in CSF cells, with respect to severity of EAE. Histological studies have shown cytokine-producing cells around the lesion both in EAE (Merrill et al., 1992) and MS (Hofman et al., 1986). Our results indicate a correlation between IL-2 and IFN-γ gene expression and disease severity in the CNS of animals with EAE. Cytokine gene expression increased with increasing severity of the disease. The cytokines IL-2 and IFN-γ are mediators of inflammation. IL-2 is a cytokine implicated in the proliferation of T cells, the cell type responsible for the induction of EAE (Sriram et al., 1987) and presumably MS (Selmaj, 1992). Antibodies directed against IL-2 blocked the development of EAE in mice (Duong et al., 1991). IFN-γ is a cytokine involved in macrophage activation and in the upregulation of of MHC expression on the surface of antigen presenting and other cells (Schreiber et al., 1985). The intracerebral administration of IFN-γ to rat was shown to induce an influx of inflammatory cells into the CNS (Sethna and Lampson, 1991). Moreover, injection of MS patients with recombinant IFN-γ resulted in exacerbation of the disease (Panitesh et al., 1987), consistent with a role for this cytokine in inflammatory disease of the CNS. Surprisingly, when anti-IFN-γ antibodies were administered to mice with EAE, the disease was exacerbated (Billiau et al., 1988, Duong et al., 1991).

Although the leukocytes isolated from inflamed CNS tissue are heterogenous and contain a high proportion of non-T non-B cells, cell sorting experiments have shown that only $CD4^+$ T cells- all displaying the $CD45RB^{low}$ phenotype of activated cells- produce IL-2 and IFN-γ (Renno et al., submitted). In this study, we also show an increase in cellularity and in cytokine expression in the CSF of mice with EAE. The predominant leukocytic component of inflamed CSF, as for the CNS, is $CD4^+$ T cells (Griffin et al., 1987), although the low number of cells makes routine verification of this (by flow cytometry) impractical. The increased cellularity in the CSF (presumably due to increased numbers of $CD4^+$ cells) correlates with the increase in $CD4^+$ T cells in CNS tissue of mice with severe disease (Zeine and Owens, 1992; TR, unpublished observation). Because we have normalized cytokine measurement to actin, a cellular message whose expression is relatively invariant, it is unlikely that the increase in cytokine expression in the CSF resulted solely from the increased cellularity, but instead reflects the activation status of T cells in the CSF. The cytokine signals detected in CSF from control mice and mice with mild EAE were easily resolved, whereas such signals were almost indistinguishable in total CNS. This is possibly a consequence of the enormous dilution of T cell mRNA with irrelevant mRNA in total CNS, which might interfere with efficient binding of specific primers to their template.

Activation of T cells to cytokine production requires antigen recognition or TCR/CD3 crosslinking. The fact that T cells in the CSF actively transcribed cytokine mRNA indicates that these cells were so activated. In our experiments, the immunizing antigen was MBP. Although it is possible that CSF cells were activated through recognition of PPD or other non-encephalitogenic proteins from Freund's adjuvant, this is unlikely since the same number of cells was recovered from the CSF of normal mice and from mice immunized with adjuvant (Table 1). Moreover, transfer experiments using radiolabeled autoantigen-activated T cells have shown that these T cells home to the CNS and remain there, whereas activated T cells of an irrelevant specificity (OVA) could be found in the CNS for only 2-3 days (Wekerle, 1986). Furthermore, we have shown that OVA-reactive T cells in the CNS do not express the memory/effector phenotype that is associated with active cytokine production (Zeine and Owens, 1992). It is therefore likely that the cytokine-producing CSF cells were autoantigen-specific and were activated by recognition of CNS antigens such as MBP.

It remains to be determined whether their activation state was due to encounter with antigen locally in the CSF or whether they were activated by the autoantigen in the parenchyma, and then drained into the CSF.

Given the high sensitivity of the techniques used in this study, and given that activated T cells are likely to migrate stochastically through the body, it was important to ascertain that the elevation of cytokine message observed in EAE was indeed specific to the CNS and therefore related to neuroautoantigen recognition. As expected, IL-2 and IFN-γ expression was readily detectable in the brain of animals with severe EAE. By contrast, mRNA levels for these cytokines in the heart, kidney, and lung of the same animals were indistinguishable from the background levels detected in the CNS of unimmunized mice.

This study in the EAE animal model supports the validity of CSF as a marker for CNS inflammation. In a recent report, CSF cells from MS patients with active disease, but not from those in remission, were shown to induce inflammation and demyelination when injected intracerebrally into mice with severe combined immune deficiency (scid) (Seaki et al., 1992). Our experiments provide a mechanistic framework for the induction of CNS inflammation, whereby cytokine production by CNS T cells, including those in the CSF, can initiate a cascade of events leading to the observed pathology. An approach similar to that used in this study can be applied to human CSF where, as in the mouse, limited cell numbers pose technical problems for the analysis of cytokine proteins produced by immediately ex-vivo cells, and might provide a clue to disease mechanism. More importantly, the analysis of CSF cytokines would provide a useful marker of inflammatory activity in the CNS.

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# CHAPTER 6

# SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

EAE is a prototype T cell-mediated autoimmune disease. The disease can be transferred into naive recipient with activated, neuroantigen-specific CD4$^+$ T cells. Activated CD4$^+$ T cells effect their function through the release of cytokines. In this thesis, I have studied the expression of cytokines in EAE with the hope of better understanding their contribution to the induction of this organ-specific autoimmune disease, and eventually, that of the human disease, multiple sclerosis.

I approached the question from two complementary angles. First, I assessed the expression of cytokines in total CNS tissue and in cells isolated from the CNS and LN of animals with EAE. I analysed those cells immediately after their isolation, without in vitro manipulation. This allowed determination of which cytokines these cells actually expressed, as opposed to their potential cytokine expression. The second approach was to express one of these cytokines (IFN-γ) transgenically in the CNS of mice. This allowed direct assessment of the role of this cytokine in CNS inflammation.

Previous studies from our laboratory had shown an accumulation of CD4$^+$ T cells exhibiting the CD45RB$^{low}$ "effector" phenotype in the CNS of animals with EAE (Zeine and Owens, 1992). An obvious question was whether this phenotype actually reflected the ability of CD4$^+$ T cells to produce cytokines in an in vivo inflammatory situation. I found that CD45RB$^{low}$ CD4$^+$ T cells from the CNS and LN of animals with EAE expressed IL-2 and IFN-γ, whereas CD4$^+$ T cells expressing high levels of CD45RB did not. This directly demonstrated the functional relevance of CD45RB as a marker of effector T cells. The enrichment of CD45RB$^{low}$ CD4$^+$ T cells in inflamed CNS raised the issue of whether MBP-specific T cells in mice with EAE are CD45RB$^{high}$ in the LN, then are converted into CD45RB$^{low}$ following their entry to the CNS, or whether they acquire the CD45RB$^{low}$ phenotype in the LN before migration to the CNS. In either case, the CD45RB$^{low}$ T cells are retained in the CNS. In the absence of data on the Ag specificity of the CD4$^+$ T cells isolated from our autoimmune mice, we cannot distinguish between these two possibilities. Our laboratory, however, is currently investigating this issue, by analyzing the surface phenotype and cytokine expression of CD45Rb-sorted, passively transferred, lipophilic dye-labelled, OVA-specific T cells isolated from the CNS and LN of mice with MBP-induced EAE.

Differential production of IFN-γ or IL-4 by CD4$^+$ T cells has been used to subdivide these cells into the Th1 (DTH type) and Th2 (B cell helper type) subsets, respectively. The relevance of these subsets in vivo has been shown in immune responses to certain parasitic and bacterial infections (Locksley et al., 1987; Heinzel et al., 1989; Yamamura et al., 1991). I found that sorted CD45RB$^{low}$ cells from the LN of animals with EAE made abundant IL-4, as well as IL-2 and IFN-γ, whereas T cells (mostly CD45RB$^{low}$) isolated from the CNS of the same animals expressed IL-2, IFN-γ, but effectively no IL-4 (at least 250-fold less than in LN). This enrichment of Th1 cytokines in inflamed CNS is consistent with the observation that Th1, but not Th2 clones, are encephalitogenic, and raises the question of what determines the Th1 profile of a CD4$^+$ T cell. Recent data have suggested that the APC, rather than the TCR, determines whether a CD4$^+$ T cell makes Th1 or Th2 cytokines (Seder al., 1992). It is likely that in EAE, CD4$^+$ T cells are primed to autoantigen in the LN by dendritic cells. These have been shown to be able to induce IL-4 production by T cells (Seder et al., 1992). Activated T cells then migrate to the CNS, where they again recognize the priming Ag, this time presented by CNS APC, eg. macrophages/microglia. If these CNS APCs are unable to reactivate IL-4-producing cells, then IFN-γ and IL-2-producing cells will predominate in the CNS. An experiment to test this possibility would involve isolating microglia (sorting CD45$^{low}$) from MBP-primed autoimmune mice. The ability of these microglia to induce proliferation of MBP-specific T cell lines in the absence of exogenously added Ag would indicate that microglia have processed MBP in vivo and present it on their surface in the context of their MHC molecules. IFN-γ and IL-4 gene expression by responding T cells could then be investigated, to assess whether microglia activated Th1, Th2, or both. Such experiments are underway in this laboratory.

Autoimmune disease is not the only instance where a DTH-like response arises in the CNS. Viral and bacterial infections also elicit inflammatory reactions and IFN-γ production in that compartment. TNFα is a cytokine implicated inflammatory reactions. I found that TNFα expression correlated with T cell infiltration and that it was produced by macrophages and microglia, but not by T cells in the CNS of animals with EAE. The possibility that T cells were regulating the expression of TNFα by macrophages/microglia was supported by showing that T cell cytokines induced the expression of TNFα in freshly-derived microglia.

Why did CNS-resident cells evolve to produce TNFα, a potential cytophatic mediator? It is possible that the production of this cytokine by microglia evolved to fight infection by neurotropic pathogens. TNFα is directly inducible by viral infection and is cytotoxic to infected cells (Wong and Goedell, 1986). Moreover, it has been shown to recruit inflammatory cells (Ming et al., 1987) and to upregulate MHC expression (Massa et al.,1987), which leads to amplification of the immune response. The coordinate regulation of TNFα production and T cell infiltration suggest the possibility of a positive feedback loop resulting from viral infection of the CNS: Virus -> TNFα production -> recruitment of inflammatory (T) cells -> IFNγ production -> TNF production. Given the toxicity of TNFα to oligogendrocytes in vitro, it would be interesting to investigate why many viral infections of the CNS do not lead to demyelination.

Having shown that the expression of a variety of cyokines correlated with the clinical course of EAE, and having identified their cellular sources, I directly investigated the role of one of these cytokines, IFN-γ, in the pathogenesis of the disease. I used the non-invasive approach of transgenesis to express this cytokine in the CNS, under the control of a CNS-specific MBP promoter. Transgenic expression of IFN-γ induced class I, but not class II, MHC expression in the CNS. This finding is noteworthy, since others have shown upregulation of Class II MHC upon injection of IFN-γ in the CNS (Vass and Lassmann, 1990, Sethna and Lampson, 1991). The lack of class II MHC induction in the CNS of our mice might explain in part why these mice do not develop spontaneous autoimmune disease. Indeed, in the transgenic model of IFN-γ-mediated diabetes, class II MHC was upregulated in the islets of Langerhans. It is impossible to discriminate between this expression being required for diabetes to occur, or it being a secondary consequence of inflammation.

So IFN-γ is not sufficient to cause spontaneous inflammation in the CNS.
In this case, can any other cytokine by itself induce CNS inflammation? For instance, if early expression of TNFα in the CNS is indeed required for the generation of a DTH response in that compartment, is it sufficient to cause inflammation? To address this issue, our lab is currently in the process of generating transgenic mice expressing TNFα in the CNS. These mice should provide some insight on the cytokines or cytokine combinations required for the induction of CNS autoimmunnity.

That the continuous expression of IFN-γ in our transgenic mice exacerbated MBP-induced EAE, and blocked or delayed remission represents an interesting finding. Together with the finding that IFN-γ falls to background levels during remission from MBP-induced EAE, this raises the possibility that loss of IFN-γ from the CNS is required for remission from EAE to occur. The mechanisms responsible for that loss are not entirely clear. It has been postulated that cytokines which negatively regulate the inflammatory response, such as TGFβ and IL-10, play a role in the induction of remission. Although IL-10 is considered to be a Th2 cytokine, and Th2 cells are largely absent from the CNS, other cells, such as monocytes have been shown to produce this cytokine. Since IFN-γ has been shown to downregulate Th2-type cytokine production (Gajewski and Fitch, 1988), its constitutive production in the CNS may inhibit the expression or action of these cytokines, therefore interfering with the downregulation of the immune response and the induction of remission.

Can we use the knowledge acquired by studying EAE to improve our understanding of MS? As discussed in the Chapter 1, the study of MS has been hampered by many difficulties, in particular the scarcity of human tissue available for experimental analysis. Taking advantage of the availability of both CNS and CSF from animals with EAE, I compared the expression of cytokines in these two compartments in relation to severity of the disease. I showed that IL-2 and IFN-γ expression in the CNS and CSF increased with increased severity of EAE, suggesting that as far as these cytokines are concerned, the immune status of the CSF is an accurate reflection of that of the CNS parenchyma. This finding reinforces the usefulness of the study of CSF in MS.

Finally, it is likely that the elucidation of the central role that cytokines might play in autoimmune diseases will not only further the development of cytokine-based strategies in the treatment of these diseases (eg. IFNβ in MS), but is also likely to significantly increase our understanding of the intricate cellular interactions that characterize immune responses in general.

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