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High Incidence of Spontaneous Autoimmune Encephalomyelitis in Immunodeficient Anti-Myelin Basic Protein T Cell Receptor Transgenic Mice

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Summary

We have generated TCR transgenic mice (T/R$^+$) specific for myelin basic protein (MBP) and crossed them to *RAG-1*-deficient mice to obtain mice (T/R$^-$) that have T cells expressing the transgenic TCR but no other lymphocytes. Both T/R$^+$ and T/R$^-$ mice carry, in the lymph nodes and spleen, large numbers of the potentially encephalitogenic CD4$^+$ anti-MBP T cells. These cells respond to MBP in vitro but show no signs of activation in vivo. Nevertheless, ~14% of H-2^u T/R$^+$ and 100% of H-2^u T/R$^-$ mice developed spontaneous experimental autoimmune encephalomyelitis (EAE) within 12 months. These data indicate that EAE can be mediated by CD4$^+$ anti-MBP T cells in the absence of any other lymphocytes and that nontransgenic lymphocytes that are present in T/R$^+$ but absent in T/R$^-$ mice have a protective effect. The data also suggest that spontaneous EAE may be triggered by an in situ activation of CD4$^+$ anti-MBP cells in the nervous system.

Introduction

Chronic inflammatory diseases such as rheumatoid arthritis (Harris, 1990), insulin-dependent diabetes mellitus (Eisenbarth, 1986), or multiple sclerosis (MS) (ffrench-Constant, 1994) are referred to as autoimmune diseases because they are assumed to be caused by CD4 T cell responses to antigens of the joints, the pancreatic islets, and the white matter of the brain, respectively. This assumption is based on three lines of evidence. First, CD4 T cells and major histocompatibility complex (MHC) class II–expressing and putative antigen-presenting cells are found in the inflamed tissues (Traugott, 1987; Burmester et al., 1987). Second, each of these diseases is associated with certain MHC class II alleles, suggesting that MHC class II–restricted T cells play a crucial role in the pathogenesis (Todd et al., 1988; Olerup and Hillert, 1990). Third, CD4 T cells from healthy human donors and from patients can respond in vitro to antigens of the affected tissues (Ota et al., 1990; Honeyman et al., 1993), and immunization of animals with such antigens can lead to tissue-specific lesions and clinical symptoms that are similar to those observed in patients (Stuart et al., 1988; Zamvil and Steinman, 1990). For example, immunization of H-2^u mice with myelin basic protein (MBP) or with an MBP-derived peptide in complete Freund's adjuvant (CFA) induces an MS-like disease that is called experimental autoimmune encephalomyelitis (EAE) (Zamvil and Steinman, 1990). The disease is also inducible in naive animals by injecting individual MBP-specific CD4 T cell clones that can be generated in tissue culture from lymph node cells of immunized mice (Zamvil and Steinman, 1990).

Recently, two groups generated T cell receptor (TCR) transgenic mice to study the development and the pathological potential of CD4 T cells expressing TCRs for MBP(1–11) (Goverman et al., 1993) and for an unknown islet cell antigen (Katz et al., 1993), respectively. The analyses of these mice confirmed the lack of negative selection of the self-antigen-specific T cells and their potential pathogenic role, which was previously observed in normal mice. However, these studies did not exclude the participation of other CD4 T cells and of other classes of lymphocytes in the development of the lesions in the brain and the pancreas, respectively. Other studies do point to a role of lymphocytes of different classes and specificities in the pathogenesis of autoimmune diseases. Recently, a cascade of CD4 T cell responses to various self-antigens has been observed in nonobese diabetic (NOD) mice that spontaneously develop diabetes (Kaufman et al., 1993; Tisch et al., 1993) and in H-2^u mice that develop EAE after immunization with an MBP-derived peptide (Lehmann et al., 1993). In both models the initial response to one peptide was followed by responses to other peptides derived from the same protein and to peptides from other proteins expressed in the same tissue. Besides CD4 T cells, other classes of lymphocytes (such as B cells, CD8 T cells, and γδ T cells) have been found in EAE and MS lesions (Raine, 1991; Hvas et al., 1993; Shimenkovitz et al., 1993). However, the mere presence of these cells in inflamed tissues does not necessarily imply that they have a pathological function. Indeed, lymphocyte infiltrates in the white matter of the brain (Sedgwick and Mason, 1986; Day et al., 1992; Linington et al., 1993) and in the islets of the pancreas (Podolin et al., 1993; Singer et al., 1993) have been observed that were not associated with tissue destruction nor with any clinical symptoms. While some of the infiltrating cells or their products may contribute to the generation of lesions, others may have protective functions. The course of chronic inflammatory diseases appears to depend on complex interactions of disease-causing and protective cells with each other and with other host cells. With the aim to reduce this complexity to a minimum, we generated mice that have CD4 T cells expressing a transgenic TCR specific for MBP(1–9) but no other lymphocytes. Such mice were obtained by crossing TCR transgenic mice with *RAG-1* gene–deficient mice (Mombaerts et al., 1992). The protein RAG-1 is required for the assembly of functional antibody and TCR genes. Thus, in *RAG-1*-deficient mice, lymphocytes can mature only if they express rearranged antigen receptor transgenes. To our sur-

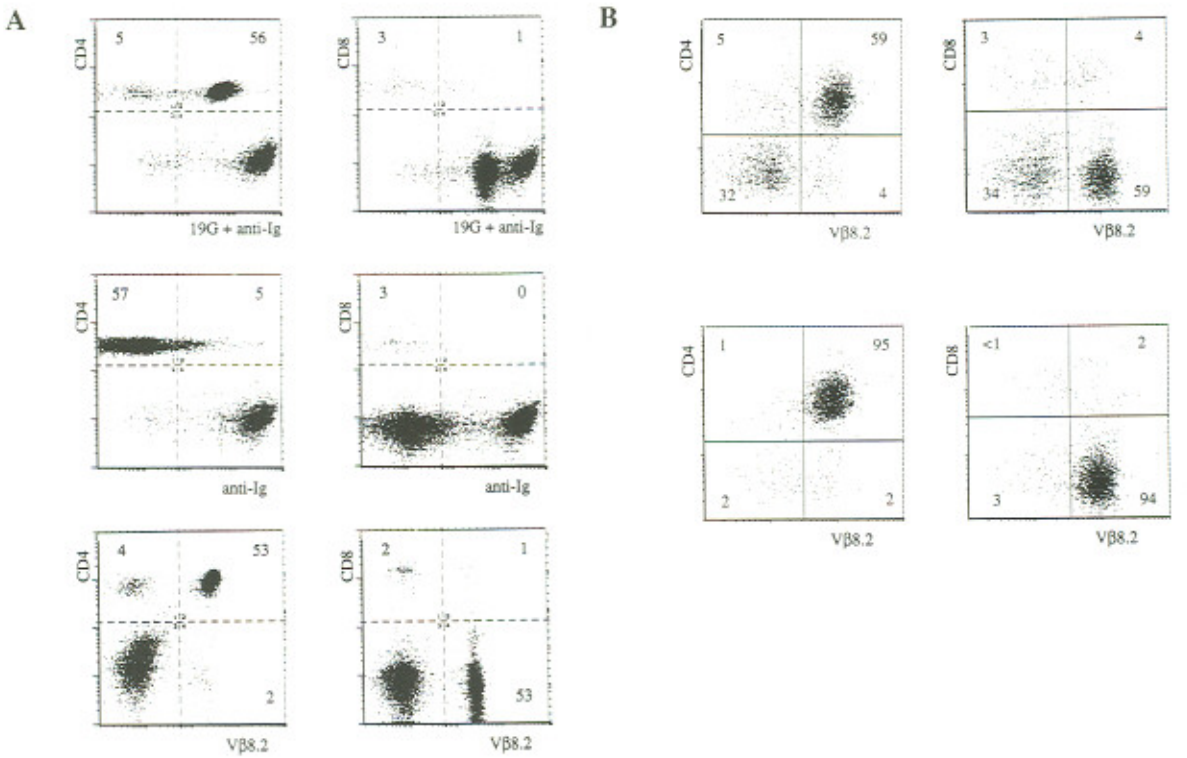

Figure 1. Stainings of Peripheral Lymphocytes from T/R$^+$ and T/R$^-$ Mice

(A) Cells from the mesenteric lymph nodes of a 3-month-old H-2$^{u/u}$ T/R$^+$ mouse. Cells were stained with anti-TCR antibodies (horizontal axis) and either CD8 or CD4 (vertical axis). The top panels are stainings of the TCR with unlabeled anti-clonotypic antibody 19G (Baron et al., 1993) followed by PE-labeled anti-mouse immunoglobulin. The middle panels show a control staining with no anti-clonotypic antibody. The bottom panels are stainings of the TCR with anti-Vβ8.1/8.2 FITC-labeled antibody. The population in the far right-hand side of the fourth quadrants of the top four panels are B cells stained with anti-immunoglobulin PE.

(B) Cells from the mesenteric lymph nodes of a 6-month-old H-2$^{u/u}$ T/R$^+$ mouse and a T/R$^-$ littermate. Cells were stained with anti-CD8 FITC or anti-CD4 PE in combination with biotinylated anti-Vβ8.1/8.2 followed by streptavidin allophycocyanin. The horizontal axis corresponds to the Vβ8.1/8.2 staining. The vertical axis corresponds to either CD8 (right panels) or CD4 (left panels) staining. The top panels show T/R$^+$ and the bottom panels show T/R$^-$.

prise, EAE developed spontaneously in all TCR transgenic *RAG-1*-deficient (T/R$^-$) mice but only in some TCR transgenic mice with a functional *RAG-1* gene (T/R$^+$ mice).

Results

All Lymphocytes in T/R$^-$ Mice Express the Transgenic TCR

Clone 19 is an I-A^u-restricted, encephalomyelitogenic CD4 T cell clone that recognizes MBP(1–9), an acetylated peptide corresponding to the N-terminal 9 amino acids of MBP (Baron et al., 1993). The rearranged TCR α and β genes of this clone were used to generate TCR transgenic mice expressing the H-2^u haplotype. By crossing these TCR transgenic mice with *RAG-1*$^-$ mice (Mombaerts et al., 1992), we obtained TCR transgenic H-2^u mice that were homozygous for the *RAG-1* mutation (T/R$^-$ mice) as well as littermates that carried a normal *RAG-1* gene (T/R$^+$ mice). T cells expressing the transgenic TCR could be identified by staining with a clonotypic antibody (Baron et al., 1993) or with a Vβ8.1/8.2-specific antibody. The two antibodies stained virtually the same number of cells in either T/R$^+$ (Figure 1A) or T/R$^-$ (data not shown) mice, indicating that these mice have few, if any, T cells expressing endogenous Vβ8.1 or Vβ8.2 genes. In all other experiments described in the present study, we used Vβ8.1/8.2-specific antibodies to detect cells expressing the transgenic TCR. Figure 1B shows the fluorescence-activated cell sorting (FACS) analysis of lymph node cells from T/R$^+$ and T/R$^-$ littermates that were double stained with antibodies specific for Vβ8.1/8.2 and antibodies specific for CD4 or CD8. The total numbers of mesenteric lymph node cells were ~10 × 10^6 and 5 × 10^6 in 6-month-old T/R$^+$ and T/R$^-$ littermates, respectively. The majority of these cells were TCR transgenic CD4 T cells, 59% in the T/R$^+$ mice and 95% in the T/R$^-$ mice. Thus, the absolute numbers of TCR transgenic CD4 T cells were similar in the two types of mice. The cells of T/R$^+$ mice that do not express the transgenic TCR include CD4$^-$CD8$^-$ B cells (30% of all lymphocytes), CD4$^+$ T cells (5% of all T cells), and CD8$^+$ T cells (3% of all T cells). None of these cell types are found in the lymph nodes of T/R$^-$ mice. Since the transgenic TCR was obtained from an MHC class II–restricted T cell clone, it was expected that the vast majority of the transgenic TCR-expressing cells were CD4 T cells. A few CD8$^+$Vβ8.2$^+$ cells were found in T/R$^+$ (4%) and T/R$^-$ (2%) lymph node

Table 1. Lymphocyte Populations in Spleen and CNS of T/R$^+$ (n = 8) and T/R$^-$ (n = 7) Mice

	Healthy T/R$^+$		EAE-Bearing T/R$^-$	
	Spleen	CNS	Spleen	CNS
Transgenic TCR$^+$ T cells	31.5×10^6	7×10^4	24.4×10^6	1.2×10^6
Transgenic TCR$^-$ T cells	2.8×10^6	1×10^4	–	–
B cells	57.0×10^6	9×10^4	–	–

cells. In T/R$^-$ mice, these cells are CD8 T cells expressing the transgenic TCR. In T/R$^+$ mice, these cells are composed of two subpopulations, one expressing the transgenic TCR α and β chains and one expressing the transgenic TCR β chain paired with endogenous α chains.

As shown in Table 1, the spleens of T/R$^+$ mice also contain a large number of B cells and T cells expressing the transgenic TCR as well as smaller numbers (~9% of total T cells) of T cells expressing nontransgenic TCRs. By contrast, the lymphocytes from the spleens of T/R$^-$ mice are entirely composed of T cells expressing the transgenic TCR. As expected, nontransgenic TCRs expressed by peripheral T cells from T/R$^+$ mice are diverse. For instance, these nontransgenic T cells proliferate in vitro as vigorously as splenic T cells derived from normal mice in response to several different allogeneic stimulators (data not shown).

EAE Develops Spontaneously in All T/R$^-$ Mice but Only in Some T/R$^+$ Mice

We monitored large numbers of T/R$^+$ and T/R$^-$ mice for the spontaneous development of EAE. Although both types of mice have similar numbers of potentially encephalitogenic CD4 T cells and were kept in the same room under specific pathogen-free conditions, the incidence of spontaneous EAE was very different in the two types of mice (Figure 2). In the first 5 weeks, no EAE symptoms were observed in any mice. Thereafter, all T/R$^-$ H-2^u mice developed spontaneous EAE. The disease begins with weakness or paralysis of the hind legs and then extends to the front legs. Once the hind legs were completely paralyzed (level 3), the disease always progressed very rapidly. All mice were sacrificed when they reached a moribund state (level 5). We have not observed remission of the disease in any T/R$^-$ H-2^u mice. The onset and the rate of progression of the disease varied. In some mice, symptoms developed by the age of 2 months and progressed to a moribund state within 3 months. In other mice, the first symptoms were noted at the age of 4 months or later, and the disease often remained stable for several months at level 1/2 before it progressed to a moribund state. In one mouse, symptoms did not develop until 11 months of age. The average age of the onset of disease was 13 weeks. We also kept a smaller number of T/R$^-$ mice with the H-2^b haplotype in the same room. These mice had no H-2^u-restricted CD4 T cells (a detailed analysis of T cell development in these mice will be published elsewhere), and no signs of EAE were observed in any of these mice within the test period of 12 months. Symptoms of EAE were also never observed in any normal H-2^u mice nor in any nontransgenic *RAG-1*$^-$ mice.

In contrast with the high incidence of EAE in T/R$^-$ H-2^u mice, spontaneous development of EAE was observed only in 11% of T/R$^+$ H-2^u littermates by 6 months of age and in 14% by 12 months of age. In those mice that developed EAE, the course of the disease was indistinguishable from that seen in the T/R$^-$ littermates. The disease was stable in some mice for a few months, but again no signs of remissions were noted in any of these mice. Immunization with MBP and CFA led to a rapidly progressing form of EAE in all T/R$^+$ H-2^u mice. In T/R$^+$ H-2^b mice, the disease never developed, even after immunization with MBP(1–17) and CFA.

To summarize, these findings show that EAE spontaneously develops in TCR transgenic H-2^u mice that have large numbers of MBP(1–11)-specific, H-2^u-restricted CD4 T cells. The incidence of the disease is 100% in mice that have only the potentially encephalitogenic CD4 T cells expressing the transgenic TCR but no other lymphocytes. Interestingly, the incidence of the disease is only 14% in T/R$^+$ mice, which have, besides large numbers of transgenic TCR-expressing CD4 T cells, other lymphocytes (such as

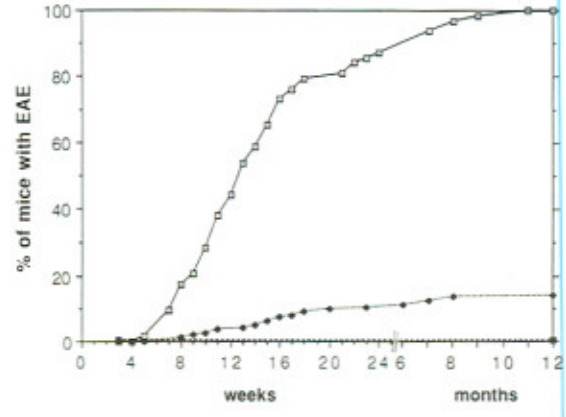

Figure 2. Spontaneous Incidence of EAE in T/R$^-$ and T/R$^+$ Mice

The cumulative percentages of mice with clinical manifestations of EAE (for details see Experimental Procedures) are plotted. Solid line, disease incidence among 63 T/R$^-$ H-2^u mice; dotted line, disease incidence among 162 T/R$^+$ H-2^u mice; broken line, disease incidence among 16 T/R$^-$ H-2$^{b/b}$ mice. Mice were scored positive upon initial detection of levels 1 or 2 of disease (often level 1 symptoms were not observed). The vast majority of mice with EAE never showed any improvement in severity of the disease, and in the few cases in which some improvement was observed, it never encompassed more than one clinical level.

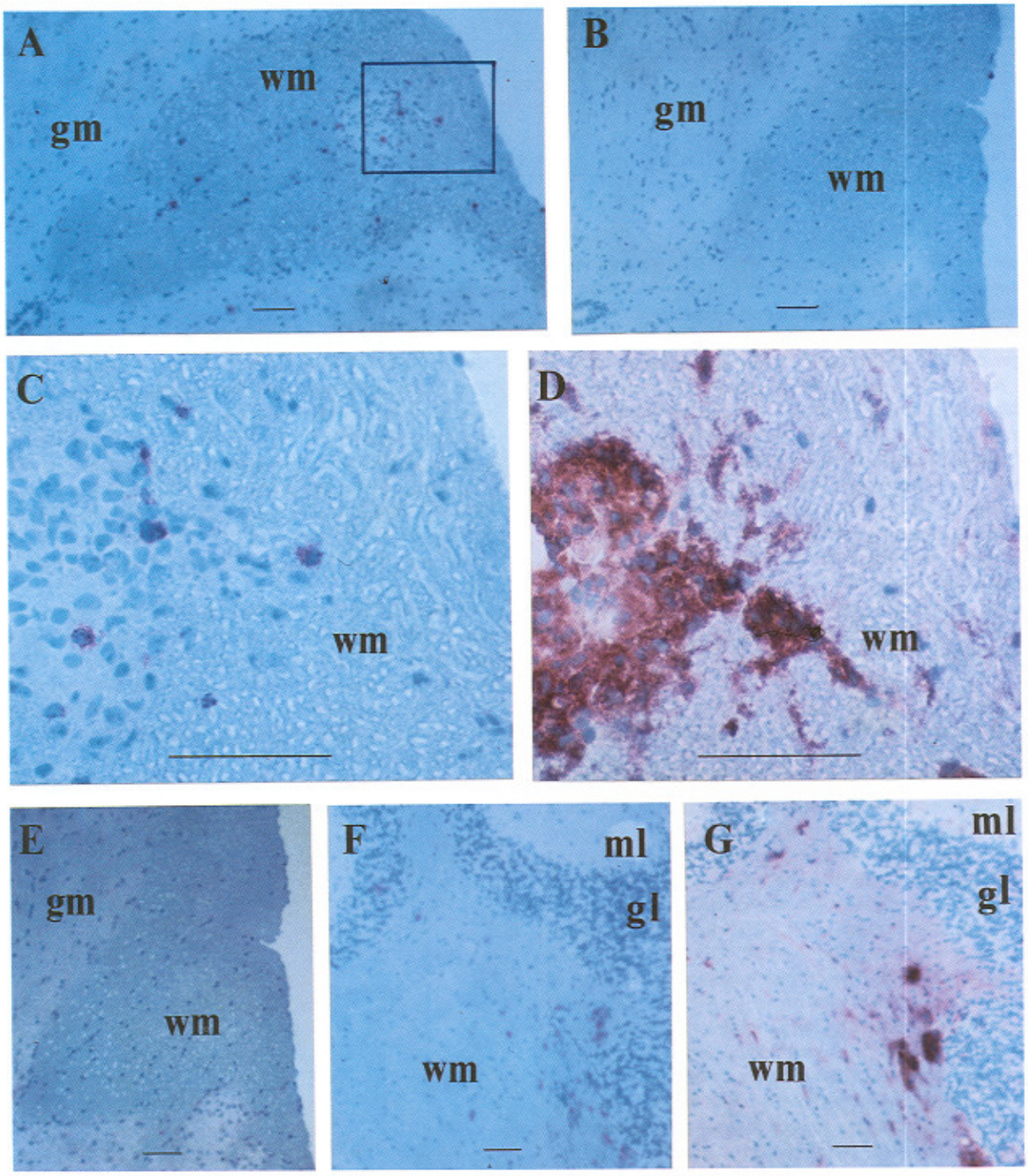

Figure 3. Immunohistological Analysis of CNS Tissue from Mice with and without Clinical EAE

A 4-month-old T/R$^-$ H-2$^{b/u}$ mouse with level 3 progression of spontaneous disease and a T/R$^+$ littermate H-2$^{b/u}$ without clinical signs of EAE were analyzed.

(A) CD3 staining of spinal cord from diseased mouse at low magnification.

(B) CD3 staining of spinal cord from healthy littermate at low magnification.

(C) Magnified view of rectangular area from (A).

(D) I-A$^{b/u}$ staining of spinal cord from diseased mouse at high magnification.

(E) I-A$^{b/u}$ staining of spinal cord from healthy littermate at low magnification.

(F) CD4 staining of cerebellum from diseased mouse at low magnification.

(G) Anti-I-A$^{b/u}$ staining of cerebellum from diseased mouse at low magnification.

Abbreviations: wm, white matter; gm, gray matter; gl, granular layer; ml, molecular layer. Scale bar, 100 μm.

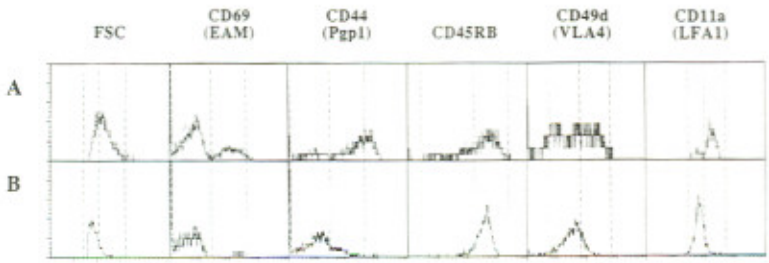

Figure 4. Flow Cytometric Analysis of Cells from Popliteal Lymph Nodes Draining the Site of MBP or PBS Injection

(A) Cells from mice injected with MBP(1–17) in CFA. (B) Cells from mice injected with PBS without CFA. H-$2^{u/u}$ T/R$^+$ mice at 3 months of age were injected in the left hind footpad. Mice were sacrificed at different timepoints after injection, and their lymphoid and CNS tissues were analyzed. Histograms represent surface staining of cells from draining lymph nodes at 5 days (CD69) or 8 days (remaining data) after injection. Samples were gated for Vβ8.1/8.2$^+$ cells for analysis with forward light scatter (FSC), CD69, CD44, CD49d, and CD11a and for CD4$^+$ cells for CD45RB analysis.

B cells and T cells) expressing endogenously rearranged TCR genes.

Spontaneous EAE in TCR Transgenic Mice Is Associated with Typical EAE Lesions in the Central and Peripheral Nervous Systems

Histological examination of sections of the cerebellum and the spinal cord from T/R$^-$ mice with EAE revealed patchy lesions with mononuclear cell infiltrates. To characterize the infiltrating cells, we stained frozen sections of the cerebellum and the spinal cord with anti-CD3, anti-CD4, and anti-MHC class II antibodies. Staining with anti-CD3 antibodies (Figures 3A and 3C) and with anti-CD4 antibodies (Figure 3F) revealed only a few T cells. They were predominantly localized at the edges of the lesions. These T cells must be specific for the MBP(1–11) peptide since no other T cells are generated in T/R$^-$ mice. The vast majority of the infiltrating cells stained with anti-MHC class II antibodies (Figures 3D and 3G). These cells are probably macrophages, which are thought to be responsible for the demyelination.

Most T/R$^+$ mice do not spontaneously develop EAE, although they have as many transgenic TCR-expressing CD4 T cells as the T/R$^-$ mice, which all develop EAE (see above). To examine possible reasons for this difference, we examined sections of the brain and spinal cord of several T/R$^+$ mice that had not shown any signs of EAE. Representative examples of stained frozen sections are shown in Figures 3B and 3E. Very few cells were stained with the anti-CD3 antibody. They were scattered throughout the sections of the spinal cord (Figure 3B) and brain (data not shown). Likewise, MHC class II–positive cells were also very rare in the spinal cord of healthy T/R$^+$ mice (Figure 3E).

Activated MBP-Specific T Cells Are Found in the Brain but Not in the Peripheral Lymphoid Tissues of Mice That Suffer from EAE

To understand the pathogenic mechanisms leading to spontaneous EAE in our TCR transgenic mice, we wished to find out whether the MBP-specific T cells are activated before or after they emigrate from blood vessels in the brain. T cell activation is associated with an increase in cell size and with alterations in the expression of cell surface markers. The adhesion molecules CD44, CD49d, and CD11a are up-regulated. CD69 and CD25, which are not expressed by resting mature T cells, appear at the surface of activated cells, while the surface expression of the exon 5–containing form of CD45 (CD45RB) is diminished after activation. Using the FACS and fluorescence-labeled antibodies against these markers, we found many activated T cells in lymph nodes of mice that were immunized with the MBP(1–17) peptide in CFA. Using double staining, we determined the expression of the activation markers by Vβ8.2$^+$ (MBP-specific) T cells in the draining lymph nodes at various times after immunization. Lymph nodes from mice mock immunized with phosphate-buffered saline (PBS) served as negative controls. Almost all of the MBP-specific T cells were enlarged 1 day after immunization and stained brightly with antibodies against CD44 and CD11a. An increase in expression of CD49d occurred more gradually, reaching a peak level of 80% of the MBP-specific T cells 1 week after injection. CD69$^+$ cells did not exceed 25% of the T cells at any given point; however, we do not know whether the expression of this marker is quickly down-regulated or whether it is restricted to a small subset of T cells. A similar expression pattern was observed for CD25 (data not shown). Staining with antibodies against CD45 exon 5 (CD45RB) revealed only a modest decrease in intensity after immunization with MBP. Figure 4 shows the expression of activation markers by MBP-specific T cells in the lymph nodes of immunized mice and mock-immunized control mice 5 or 8 days after immunization.

We then searched for activated MBP-specific T cells in nonimmunized T/R$^-$ mice. Figure 5 shows the analysis of transgenic T cells in the spleens of T/R$^-$ mice with EAE and of healthy T/R$^+$ mice. The vast majority of MBP-specific T cells from both types of mice were in a resting state as judged by cell size and by staining with antibodies against CD25, CD69, CD44, and CD49d. We also analyzed T cells from several young T/R$^-$ mice that had not yet shown any signs of paralysis. Again, no activated MBP-specific T cells were found in these mice (data not shown). Finally, we analyzed MBP-specific T cells that could be isolated from saline-perfused brains of mice with and without EAE. As shown in Figure 5, significant proportions of transgenic T cells from mice with EAE were CD69$^+$ and expressed the

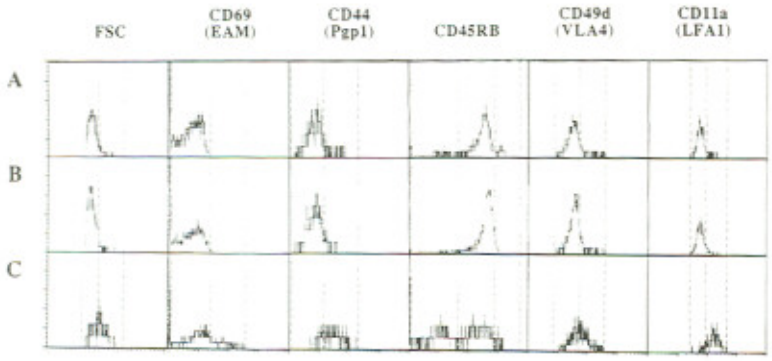

Figure 5. Flow Cytometric Analysis of Splenic Lymphocytes and CNS-Infiltrating Lymphocytes

(A) Spleen cells from mice without EAE. (B) Spleen cells from mice with EAE. (C) CNS-infiltrating lymphocytes from mice with EAE. Spleen cells from a 3-month-old T/R$^-$ H-2$^{a/u}$ animal with level 3 progression of EAE and a T/R$^+$ H-2$^{a/u}$ littermate with no clinical signs of EAE were analyzed for size (forward light scatter [FSC]) and surface expression of CD44, CD49d, and CD11a. A similar pair of mice at 7 months of age was used to analyze surface expression of CD69 and CD45RB. Data were gated for Vβ8.1/8.2$^+$ cells for analysis with forward light scatter, CD69, CD44, CD45RB, and CD11a. Data were gated for CD4$^+$ cells for analysis with VLA4.

adhesion molecules CD44, CD49d, and CD11a at high levels. The few T cells that were obtained from the brains of healthy T/R$^+$ mice were in a resting state (data not shown), although we cannot exclude the possibility that these cells originated from blood rather than from the parenchyma of the brain.

Table 1 summarizes numbers of T and B cells isolated from the saline-perfused central nervous system (CNS) and spleen of healthy T/R$^+$ and EAE-bearing T/R$^-$ mice. Transgenic T cells infiltrating the CNS are almost 20-fold more abundant in T/R$^-$ mice than in T/R$^+$ mice. The ratio of T cells expressing the transgenic TCR and those expressing nontransgenic TCRs is similar in the spleen and the CNS of T/R$^+$ mice. This suggests that there is no preferential extravasation of one type of T cell over the other.

T Cells from the Spleens of TCR Transgenic Mice with EAE Respond to MBP

We were surprised to find activated MBP-specific T cells only in the brains of mice with EAE but not in the peripheral lymphoid tissues of T/R$^-$ mice, neither before nor after they develop the disease. One possible reason for this finding was that the activated T cells were recruited to the brain while those remaining in the peripheral lymphoid organs were refractory to stimulation (i.e., anergic) because of previous encounters with tolerogenic forms of MBP. To test this possibility, we cultured spleen cells from T/R$^-$ mice with EAE and from T/R$^+$ mice without EAE in the presence and absence of MBP(1–17) and mitomycin C–treated syngeneic spleen cells as antigen-presenting cells. Both spleen cell populations proliferated vigorously in the presence but not in the absence of MBP(1–17) (Figure 6), indicating that these cells are not anergized.

Discussion

The T/R$^-$ mice described here represent an animal model with a high incidence of a spontaneous autoimmune disease that is caused by lymphocytes of known specificity. The only mature lymphocytes that are generated in these immunodeficient mice are CD4 T cells expressing a transgenic TCR that is specific for the peptide MBP(1–11) in association with I-A^u. All T/R$^-$ mice spontaneously develop EAE. The time of onset and the rate of progression of EAE varied in these mice. Two groups may be distinguished. The first group develops EAE between 8 and 16 weeks after birth. In the second, smaller group, the onset of the disease is delayed for up to 11 months of age and tends to progress more slowly. Since all T/R$^-$ mice were kept in the same pathogen-free colony, their variable fate is likely to be due at least in part to the fact that these mice have genes from three different inbred strains that occur in variable combinations in individual mice (see Experimental Procedures).

EAE Can Be Mediated by MBP-Specific CD4 T Cells in the Absence of Other Lymphocytes

Beside MBP-specific CD4 T cells, lymphocytes of other classes and specificities have been implicated in the pathogenesis of EAE. Immunization of (SJL × B10/PL)F1 mice with MBP(1–11) in CFA has been shown to trigger responses to other peptides, such as MBP(35–47),

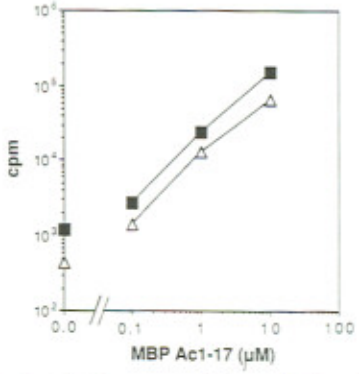

Figure 6. Proliferative Response to Antigen of Spleen Cells from Animals with and without EAE

Mean values of counts per minute for triplicate cultures are plotted versus peptide concentration. Spleen responders from transgenic mice were stimulated to proliferate with increasing amounts of the MBP(1–17) peptide as indicated. After 60 hr of stimulation, cultures were pulsed with tritiated thymidine. Closed squares, cells from T/R$^-$ animals with EAE; open triangles, cells from T/R$^+$ animals without EAE.

MBP(81–100), and MBP(121–140), that must be derived from MBP molecules of the host (Lehmann et al., 1993). Similarly, immunization with MBP provoked T cell responses to proteolipid protein, another abundant myelin protein (Perry et al., 1991). The initial insult by MBP-specific CD4 T cells may not only initiate responses of other CD4 T cells but may also recruit self-antigen-specific B cells, CD8 T cells, and γδ T cells. These cell types, as well as antibodies, have been found in the demyelinating lesions of animals with EAE and of patients suffering from MS (Raine, 1991; Bernard and de Rosbo, 1992). Antibodies against the myelin oligodendrocyte glycoprotein cause demyelination (Bernard and de Rosbo, 1992), and oligodendrocytes may be killed by CD8 T cells (Jewtoukoff et al., 1989). However, it is not known whether these effects are essential for the development of EAE in animals or of MS in humans. The finding that MBP-specific T cell clones could transfer EAE to nude mice (Tabira and Sakai, 1987) argues against an essential role of multiple types of T cells in the development of the disease, and more recent studies with $CD8^{-/-}$ mice (Koh et al., 1992) and with anti-CD8 antibody-treated mice (Jiang et al., 1992) indicate that CD8 T cells are not essential for the pathogenesis of EAE. The present study shows that neither diverse sets of CD4 T cells, nor CD8 T cells, nor γδ T cells are required for the generation of EAE lesions and clinical symptoms. It also shows that the disease may occur in the absence of B cells and antibodies.

How Are the Disease-Causing Cells Activated?

Many previous studies (for review see Sinha et al., 1990) and the present study show that mature T cell populations include cells that recognize and respond to self-antigens. These cells are normally not harmful because they are not activated. T cells responsive to autologous MBP, for example, are found in healthy humans (Ota et al., 1990) and in mice (Fritz et al., 1983). Their pathogenic potential can be revealed in animals by immunization with MBP in CFA (Paterson, 1976). The important unresolved question is how self-reactive cells are "spontaneously" activated. A widely held view is that they are activated by infections. Recently, support for this view has come from the study of Goverman et al. (1993), who found that some TCR transgenic mice similar to our T/R^+ mice developed spontaneous EAE when kept in normal (infectious) conditions but not when kept in a specific pathogen-free facility. In contrast with these findings, the spontaneous EAE in our TCR transgenic mice developed under specific pathogen-free conditions. We have not found any evidence of infections by known encephalitogenic viruses such as mouse hepatitis virus or Theiler's murine encephalitis virus nor by any other common mouse pathogens. However, since our mice were not kept under germ-free conditions, we cannot exclude a contribution of infectious organisms to the disease induction.

There are several possible reasons why self-antigen-specific T cells may be activated by infectious organisms. First, the T cells may express two TCRs, one for a self-antigen and one for a microbial antigen (Padovan et al., 1993). Second, the same TCR may recognize a peptide derived from a microbial protein as well as a peptide derived from a self-protein (Oldstone, 1987). Third, a self-antigen-specific TCR may interact with a bacterial superantigen (Marrack and Kappler, 1990; Brocke et al., 1993). Fourth, the infection may lead to the aberrant presentation of self-antigens by professional antigen-presenting cells (Bottazzo et al., 1983; Sinha et al., 1990). Since all T cells of T/R^- mice express only a single TCR, we can exclude the first possibility as an explanation for the spontaneous occurrence of EAE in these mice. While we cannot exclude any of the other possibilities, there is also no evidence in their favor. The absence of significant numbers of activated T cells in the peripheral lymphoid organs of our TCR transgenic mice argues against so-called microbial activation of MBP-specific T cells in our transgenic mice. Indeed, since all T cells in the T/R^- mice have the same TCR, the chance of activation by a cross-reactive microbial antigen is minimal.

The only other murine autoimmune disease that is known to occur spontaneously with high frequency is the diabetes of NOD mice. Recently, Katz et al. (1993) generated TCR transgenic NOD mice using the rearranged TCR α and β genes from a diabetogenic CD4 T cell clone. Diabetes developed spontaneously in 85% of the TCR transgenic mice and in 40% of non-TCR transgenic littermates. The potentially diabetogenic T cells that are found in large numbers in the lymph nodes of these mice were neither anergic nor did they express any activation markers (CD69, CD25, CD44, CD45RB, CD45RC, and Mel-142). This was true for lymph node cells from 7-week-old prediabetic mice as well as for lymph node cells from 6-month-old diabetic mice. These findings by Katz et al. (1993) are very similar to ours in TCR transgenic mice with and without EAE. In both models, only very small fractions of the large numbers of potential disease-causing cells are spontaneously activated. Because of the absence of activated T cells in the peripheral lymph nodes of the TCR transgenic mice, we suspect that the disease-causing responses are initiated in the target organs. Resting T cells may leave small blood vessels in the CNS at a very low rate. While the exit rate is presumably the same in normal and TCR transgenic mice, the chance that an MBP-specific CD4 T cell reaches the parenchyma of the brain is much higher in the TCR transgenic mice. This may be the reason why EAE occurs spontaneously in our TCR transgenic mice but rarely (if ever) in normal $H\text{-}2^u$ mice. However, the clear difference in the incidence of EAE in T/R^- and TR^+ mice cannot be attributed to differences in the numbers of MBP-specific CD4 T cells.

Lymphocytes Can Protect against Spontaneous EAE

Spontaneous EAE occurs in all T/R^- mice but only in 14% of the T/R^+ mice. Since the only difference between these two strains of mice is the presence of nontransgenic lymphocytes in the former mice, our finding indicates a protective role of some of these cells. At the present time, we only know that the protection must involve lymphocytes. Many different mechanisms of protection of animals against EAE have been proposed that involve CD8 T cells

(Sun et al., 1988; Jiang et al., 1992; Koh et al., 1992; Miller et al., 1992; Zhang et al., 1993; Gaur et al., 1993), CD4 T cells (Ellerman et al., 1988; Aharoni et al., 1993), or CD4 T cells and CD8 T cells (Kumar and Sercarz, 1993). Recently, Kumar and Sercarz (1993) generated protective CD4 T cell clones from lymph node cells of PL/J mice that had recovered from MBP-induced EAE. These clones recognized a peptide corresponding to the framework region III of Vβ8.2 chains. Intraperitoneal injection of as few as 5×10^5 cells protected PL/J mice against the subsequent induction of EAE by immunization with MBP and CFA. The TCR–peptide-specific cells might interfere with the response of the disease-causing cells by secreting anti-inflammatory factors such as transforming growth factor β (Racke et al., 1991; Miller et al., 1992), interleukin-10 (Kennedy et al., 1992), or interleukin-4 (Karpus et al., 1992) or more indirectly by recruiting protective CD8 T cells (Kumar and Sercarz, 1993; Gaur et al., 1993). If T/R^+ mice were protected by TCR αβ–specific regulatory T cells, protection should be abrogated if the expression of endogenous TCR α genes was prevented. An alternative possibility is that the transgenic TCR-expressing cells acquire a protective function after activation by MBP(1–11)-presenting B cells. Support for this possibility comes from work by Day et al. (1992). These investigators found that rats that were immunized with an encephalitogenic MBP peptide did not develop EAE when simultaneously injected with the same peptide conjugated to anti–immunoglobulin D antibodies. The MBP peptide presentation by B cells was shown to induce protective T cells, presumably Th2 cells. In our T/R^+ mice, even rare MBP(1–11) peptide-presenting B cells are likely to encounter the transgenic MBP(1–11)-specific CD4 T cells because of the high frequency of the latter cells. Protective Th2 cells might prevent the induction of disease-causing Th1 cells or might counteract their pathological effects. Depending on the conditions of priming, the TCR transgenic CD4 T cells might develop into disease-causing Th1 cells or into protective Th2 cells. A result consistent with this notion was recently reported for an animal model of autoimmune diabetes (Scott et al., 1994). The TCR transgenic mice described here are well suited to study this and other potential models of lymphocyte-mediated protection against autoimmune diseases.

Experimental Procedures

Mice

Anti-MBP transgenic mice were generated by injection of α and β TCR constructs into pronuclei of fertilized C57BL/6 eggs. The α and β TCR clones were derived from an encephalomyelitogenic T cell clone (clone 19; Baron et al., 1993). The β construct consists of a cloned 20 kb genomic KpnI fragment, and the α construct consists of an EcoRV fragment derived from a genomic clone containing the rearranged α gene and subcloned into the cosmid CαBS2 (Sha et al., 1988). The $H\text{-}2^u$ haplotype was introduced into the transgenic background by crosses with either PL/J or B10/PL mice (Jackson Laboratories).

RAG-1-deficient mice were originally generated in the 129/Sv genetic background and have been previously described (Mombaerts et al., 1992).

Mice were housed in the Massachusetts Institute of Technology (MIT) Center for Cancer Research Specific Pathogen-Free Facility. All mice (T/R^- and T/R^+ littermates) were maintained in autoclaved cages, with autoclaved bedding, food, and water.

Antibodies

The anti-TCR clonotypic antibody 19G has been described (Baron et al., 1993) and was a gift from the laboratory of C. A. Janeway, Jr. All other antibodies were purchased from Pharmingen: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11a (2D7), anti-CD25 (7D4), anti-CD44 (1M7), anti-CD45RB (23G2), anti-CD49d (R1-2), anti-CD69 (H1.2F3), anti-Vβ8.1/8.2 (MR5-2), and anti-$I\text{-}A^{b/u}$ (AF6-120.1).

Peptide

The 17-mer N-acetylated peptide derived from mouse MBP was synthesized at the MIT Center for Cancer Research Biopolymers Facility. The amino acid sequence of this peptide is ASQKRPSQRSKYLATAS.

Immunohistology

Mice were anesthetized with methoxyflurane (Metofane, Pitman-Moore Incorporated) and perfused with PBS for ~10 min via the left ventricle of the heart. Brain and spinal cord tissue were dissected in one piece, cut into smaller pieces with a razor blade, and immediately placed in Tissue-Tek OCT (Miles 4583), frozen in liquid nitrogen, and stored at −70°C.

Sections (2–6 μm) were cut and stored at −20°C.

Slides were briefly fixed in acetone and blocked with normal serum and avidin, and biotinylated antibodies were added at dilutions ranging from 10 to 100 (depending on the antibody) in PBS. After 1 hr at room temperature, slides were washed three times in PBS, incubated for 30 min with 0.3% H_2O_2 to block endogenous peroxidase activity, and washed three times in PBS. Biotin was developed using the Vectastain ABC kit (Vector Laboratories). Aminoethylcarbazole color development was monitored by microscopy, and slides were then rinsed in water, counter stained with hematoxilin, and mounted with DAKO Glycergel.

Flow Cytometry

CNS tissue was prepared as described above. Single-cell suspensions were prepared by forcing the tissue through a metal sieve (Cellector, Bellco Glass Incorporated) with a syringe plunger. The single-cell suspension was brought to a 38% Percoll (Pharmacia) concentration and spun at 1800 rpm for 20 min at room temperature, and the pellet was washed three times. Before staining, cells were incubated with 100 μl of normal mouse serum per 10^6 cells for 1 hr. Spleen and lymph node single-cell suspensions were prepared by disrupting the organs between two glass slides.

For stainings with directly labeled antibodies, $\sim 5 \times 10^5$ cells were incubated with antibodies at 4°C for 45 min. Cells were washed twice and analyzed with either a FACScan (Beckton Dickinson) for fluorescein isothiocyanate (FITC) and phycoerythrin (PE) stainings or a FACStar Plus for FITC, PE, and allophycocyanin stainings. In all cases, dead cells were gated out using propidium iodide.

Stainings with the anti-TCR clonotypic antibody 19G were done in four steps. Cells were incubated with 1 μg of unlabeled antibody (19G; Baron et al., 1993) per 10^6 cells at 4°C for 45 min. After washings, PE-labeled goat anti-mouse serum was added and the cells left at 4°C for 45 min. After the secondary antibody was washed, the cells were incubated with 100 μl of normal mouse serum per 10^6 cells for 10 min. Anti-CD4 FITC or anti-CD8 FITC was added to the cells without removing the normal mouse serum, and the procedure was continued as described above for directly labeled antibodies.

T Cell Proliferation

$V\beta8.2\ CD4^+$ spleen cells (10^5 per well) were incubated in 96-well plates in the absence or presence of the peptide MBP(1–17). Culture medium consisted of RPMI supplemented with 10% fetal calf serum, glutamine, 2-mercaptoethanol, and antibiotics. Mitomycin C (Sigma)–treated total spleen cells (2×10^5 per well) were added as antigen-presenting cells. After 48–60 hr, the plates were pulsed for 6–12 hr, cells were harvested, and the radioactivity was counted. Every experiment was done in triplicate.

Induction and Evaluation of EAE

Mice were anesthetized with Metofane and injected in the left hind footpad with 20 μl of MBP(1–17) (20 mg/ml) emulsified in CFA (GIBCO 660-5721AD). At the same time, 200 μl of 0.5 ng/μl Pertussis toxin (List Biological Laboratory) in PBS was injected intravenously.

Disease was scored as described by Baron et al. (1993): level 1, limp tail; level 2, partial hind leg paralysis; level 3, total hind leg paralysis; level 4, hind and front limb paralysis; level 5, moribund. Animals were observed daily and sacrificed when they reached level 5.

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