Acquired Tolerance to Experimental Autoimmune Encephalomyelitis by Intrathymic Injection of Myelin Basic Protein or Its Major Encephalitogenic Peptide

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Summary

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system that can be induced in a number of species by immunization with myelin basic protein (MBP) in adjuvant, and serves as an experimental model for the study of multiple sclerosis. The role of the thymus in acquired tolerance in autoimmune models has not been thoroughly investigated. In this study, we examined the effects of intrathymic injection of MBP or its major encephalitogenic peptide on the course of EAE in Lewis rats. A single intrathymic injection of MBP 48 h pre- but not postimmunization protects animals from actively induced EAE. An intact MBP-primed thymus was required up to 10 d postimmunization, as thymectomy on days 1, 2, and 7 postimmunization abrogated the protective effect, whereas thymectomy on day 10 did not. The proliferative response of primed lymphocytes was significantly reduced in animals that were intrathymically injected with MBP. Protection against clinical EAE was induced by thymic injection of the major encephalitogenic region (residues 71–90) but not a nonencephalitogenic (21–40) MBP epitope. Immunohistologic examination of the brain from rats intrathymically injected with encephalitogenic peptide showed markedly reduced cellular infiltrate and virtual absence of activation and inflammatory cytokines as compared with rats intrathymically injected with the nonencephalitogenic peptide. These results indicate that the thymus may play an active role in acquired systemic immunologic tolerance in T cell–mediated experimental autoimmune diseases. This effect may be mediated by a process of clonal inactivation of autoreactive T cell clones circulating through the thymus.

Experimental autoimmune encephalomyelitis (EAE)[1] is an inflammatory disease of the central nervous system that can be induced in a number of species by injection of myelin basic protein (MBP) with adjuvant (1). In the Lewis rat, the major encephalitogenic determinant is peptide 71–90 of guinea pig MBP (2, 3). Nonencephalitogenic epitopes of MBP, residues 21–40 for example, are immunogenic and can generate a cell-mediated immune response without inducing disease (4, 5).

The thymus plays the major role in development of self-tolerance. Tolerance to autoantigens is mediated by clonal deletion or clonal anergy (6, 7), and recent evidence suggests that different types of APC in the thymus mediate different responses (8, 9). However, it is unclear how tolerance to self-antigens not constitutively expressed in the thymus is mediated, and the role of the thymus in acquired systemic tolerance has not been thoroughly investigated. There is evidence that a subset of peripheral T cells, when activated, circulate to the thymus (10), providing a potential mechanism for peripheral activated cells to be exposed to thymic APC and become inactivated.

More than 20 yr ago Ellison and Waksman (11) induced partial tolerance to EAE by injecting MBP into the shielded thymus but not into the shielded spleen of irradiated Lewis rats. Recently there has been renewed interest in studying the role of the thymus in acquired systemic tolerance. Thymic injection of pancreatic islet cells prevents autoimmune diabetes in the BB rat (12, 13) and the NOD mouse (14). In addition, intrathymic injection of donor cells (15, 16), or synthetic class II MHC allopeptides (17), induce specific unresponsiveness to skin (15) and vascularized allografts (16, 17). There are no data on the systemic effects of thymic recognition of

[1] *Abbreviations used in this paper:* EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; p, peptide.

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soluble antigen or peptides in an induced T cell–mediated experimental autoimmune model such as EAE.

Using intrathymic injection of guinea pig MBP or synthetic MBP peptides in adult Lewis rats that were not irradiated or subjected to other immunological manipulations, we found that we can induce systemic unresponsiveness and suppress clinical and pathological features of EAE. Intrathymic injection of MBP or MBP peptides provides a novel approach for studying antigenic epitopes and investigating the role of the thymus in acquired systemic tolerance.

Materials and Methods

Induction of EAE. Female Lewis rats 6–8 wk of age were obtained from Charles River Inc. (Wilmington, MA). EAE was induced by immunizing the animals in the foot pad with 50 μg of MBP in CFA. Scoring of clinical disease was performed as previously described (18). Duration of disease was calculated by counting the total number of days from clinical onset to recovery for each animal; mean duration was calculated as the average for the whole group. Maximal grade is the highest clinical grade achieved for each animal; mean maximal grade is calculated as the average for each group. Statistical analysis was performed using a one-tailed student's *t* test. Disease index is a composite score and was calculated as follows: mean duration of disease × mean highest disease grade × incidence (18).

Antigens. Preparation of guinea pig MBP was performed as previously described (18). Peptide sequences were obtained from published data (19), and MBP peptides were prepared at the Biopolymer Center at the Center for Neurologic Diseases of the Brigham and Women's Hospital (Boston, MA).

Intrathymic Injections. Intrathymic injections were performed under ether anesthesia by exposing the thymus through a small incision above the sternum. Volumes up to 50 μl can be injected into each lobe, without evidence of leakage, using a 27-gauge needle. Thymic injections were performed 48 h before immunization unless specified otherwise. Experimental animals were injected with MBP or MBP peptides. Control animals were injected with PBS or OVA.

Thymectomy. Under ether anesthesia, the sternum was opened as for thymic injection and the thymus was removed using forceps. The chest wall was sutured after ensuring that no bleeding occurred.

Proliferation Assays. Draining lymph nodes were collected from Lewis rats immunized with MBP/CFA on day 10 postimmunization. The lymph nodes were passed through 60-gauge sterile stainless steel sieves to make single-cell suspensions. The cells were washed in HBSS (Sigma Chemical Co., St. Louis, MO) then resuspended in RPMI 1640 (Whitaker Bioproducts, Walkersville, MD) containing 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 20 μM 2-ME, and 5 mM Hepes. The cells were plated in flat-bottomed 96-well plates at a concentration of 10^6/ml. Antigen was added at 10–50 μg/ml. The plates were incubated at 37°C with 5% CO_2 for 4 d, then pulsed with [^{3}H]thymidine (1 μCi/well), harvested, and placed in a beta counter. Experiments were performed in quadruplicate and results expressed as experimental counts per minute. Relative response is calculated as: Δcpm of experimental group/Δcpm of control group.

Antibodies. Murine mAbs were obtained, unless otherwise specified, from Sera-Lab (Accurate Chem. & Sci. Corp., Westbury, NY). This panel included mAbs to all rat leukocytes (CD45, OX-1); all T cells (CD5, OX-19); TCR α/β chains (R73); T cell subsets (CD4, BWH-4; CD8, OX-8); and mononuclear phagocytes (ED-1, ED-2). Activation of mononuclear or endothelial cells was assessed using antibodies to class II antigens (OX-3); p-55 chain of the IL-2R (CD25, ART-18; courtesy of Dr. T. Diamantstein, Berlin, Germany); intercellular adhesion molecule 1 (ICAM-1; CD54, 1A29); and by labeling for the cytokines IL-1β (Olympus Corp. Precision Instr. Div. Lake Success, NY); IL-2 (15); IL-4 (Genzyme, Boston, MA); IL-6 (R&D, Minneapolis, MN); IL-8 (ICN, Costa Mesa, CA); IFN-γ (courtesy of Dr. P. van der Meide, Rijswijk, Holland); TNF-α (courtesy of Dr. I. McKenzie, Melbourne, Australia); TGF-β (R&D); and PGE_2 (Sigma Chemical Co.). Rat Ig-absorbed goat anti–mouse Ig (Sigma Chemical Co.), rabbit anti–goat Ig and rabbit peroxidase-antiperoxidase (Dako Corp., Santa Barbara, CA) were obtained commercially. Details of the use of these antibodies, isotype-matched control mAb, and purified rabbit Ig in immunohistologic studies were recently described (20, 21).

Immunohistology. Brain samples (cerebrum and cerebellum) were harvested from each rat at day 12 after disease induction (three samples/group). Tissues were frozen in liquid nitrogen and stored at −80°C in preparation for immunohistologic studies, or fixed in neutral-buffered formalin, embedded in paraffin, and sectioned for light microscopy. Cryostat sections were fixed in paraformaldehyde-lysine-periodate for demonstration of leukocytes and activation antigens, or in acetone for the labeling of cytokines, and stained by a three-layer (for polyclonal antibodies) or four-layer (for mAbs) peroxidase-antiperoxidase method as previously described (20, 21). Data quantitation and statistics were done as previously described (21). Evaluation of the slides was performed blindly.

Results

Intrathymic Injection of MBP before Immunization Protects against EAE. We investigated whether intrathymic injection of MBP before immunization protected animals against the development of clinical EAE. In these experiments, 1 mg of guinea pig MBP was injected intrathymically into Lewis rats 24 or 48 h before immunization with MBP/CFA. The animals received no other treatment. Control unmodified animals had an incidence of 5/5, a mean disease duration of 3.8 ± 0.2 d, and a mean maximal score of 2.6 ± 0.4. The MBP-injected animals had an incidence of 1/6, a mean disease duration of 0.5 ± 0.5 and 0.33 ± 0.33 d for 48 and 24 h, respectively, and a mean maximal score of 0.33 ± 0.33 and 0.17 ± 0.17 for 48 and 24 h, respectively (Fig. 1). These data indicate that intrathymic injection of MBP 24 or 48 h before immunization will protect against the development of clinical EAE.

To establish the uniqueness of the intrathymic approach, and to confirm that the tolerogenic effect of intrathymic injection of MBP was not due to systemic leakage of the antigen, animals were injected in the perithymic area with 1 mg of MBP, and were immunized with MBP/CFA 48 h later. Perithymic injection of MBP was not protective against development of clinical EAE (mean duration = 5.6 ± 0.6 and mean grade = 3.0 ± 0, p = NS, compared with OVA-injected animals), indicating that the tolerogenic effect observed with intrathymic injection is due to thymic recognition of the antigen, and not due to systemic leakage of antigen into the periphery.

We then investigated the time course of intrathymic MBP-induced hyporesponsiveness. Intrathymic injection of 1 mg

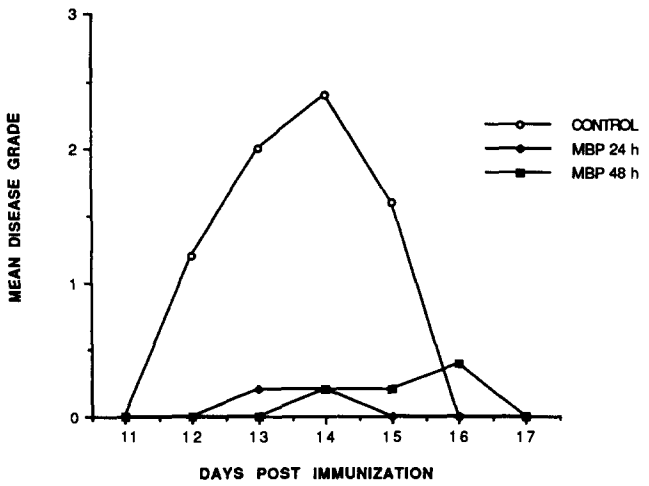

Figure 1. Disease course in animals injected intrathymically with 1 mg MBP 24 and 48 h before immunization compared with control immunized animals. Each data point represents the mean disease grade for five animals per group.

of MBP was performed at various time intervals after immunization including days 1, 3, and 7, and the clinical scores were compared with those animals that were intrathymically injected with 1 mg MBP 48 h before immunization. In addition, to establish the specificity of the intrathymic tolerogenic effect, control animals were injected intrathymically with 1 mg OVA. As seen in Table 1, the protective effect of intrathymic injection of MBP is lost when performed after immunization. These data indicate that thymic recognition of MBP has to occur before immunization if systemic tolerance is to be effected.

Effect of Thymectomy. To investigate the potential mechanisms mediating the tolerogenic effect of intrathymic injection of MBP, we performed thymectomies at various time intervals after immunization. The animals were intrathymically injected either with 1 mg MBP or 1 mg OVA 48 h before immunization with MBP/CFA. As seen in Fig. 2, thymectomy done 1, 2, and 7 d after immunization abrogated the tolerogenic effect of intrathymic injection of MBP. Thymectomy done on day 10 postimmunization, however, did not abrogate the tolerogenic effect, suggesting that the injected thymus is no longer required after 7 d postimmunization. It is interesting to note that the onset of clinical disease in this model is approximately day 10 postimmunization, and pathological changes can be seen 2–3 d before the onset of clinical disease (22). Thymectomy did not prevent the induction of EAE in the control animals, although a slight decrease in severity and a delayed onset of disease were seen especially in animals thymectomized on day 10 postimmunization.

Proliferative Response of Draining Lymph Node Cells. We then studied the proliferative responses of primed lymphocytes from intrathymically tolerized animals. Fig. 3 shows the proliferation of draining lymph node cells from intrathymically tolerized and control animals, as measured by [^{3}H]thymidine incorporation. Lymphocytes from animals that were injected intrathymically with 1 mg of MBP 48 h before immunization with MBP/CFA exhibited markedly reduced proliferation to MBP (relative response = 0.2, $p = 0.001$, $n = 3$), but not to *Mycobacterium tuberculosis* (MT) (relative response = 2.6, $n = 3$), as compared with lymphocytes from control rats injected with PBS.

Intrathymic Injection of the Major Encephalitogenic Epitope of MBP Is Tolerogenic. We compared the systemic tolerogenicity of intrathymic injection of the major encephalitogenic epitope of MBP (peptide [p] 71–90), with that of a nonencephalitogenic epitope (p 21–40) (Fig. 4). Intrathymic injection of 100 μg of p 71–90 48 h before immunization with

Table 1. *Effect of Intrathymic Injection of MBP Pre- and Postimmunization*

Day of intrathymic injection*	Thymic injection	Incidence	Duration‡	Maximal grade‡	Disease index
			d		
−2	MBP	6/13	1.77 ± 0.63§	1.15 ± 0.39§	0.94
	OVA	12/15	3.8 ± 0.59	2.2 ± 0.31	6.69
+1	MBP	4/5	3.6 ± 1.0	2.4 ± 0.6§	6.9
	OVA	5/5	5.6 ± 0.24	4.0 ± 0.0	22.4
+3	MBP	5/5	4.2 ± 0.86	2.6 ± 0.4	10.9
	OVA	5/5	4.2 ± 0.2	3.2 ± 0.4	13.4
+7	MBP	5/5	4.2 ± 0.37	3.0 ± 0.55	12.6
	OVA	5/5	4.8 ± 0.2	3.2 ± 0.2	15.4

*Day of intrathymic injection is relative to the day of immunization with MBP/CFA.
‡Duration and maximal grade are expressed as mean ± SE.
§$0.0125 < p < 0.025$.

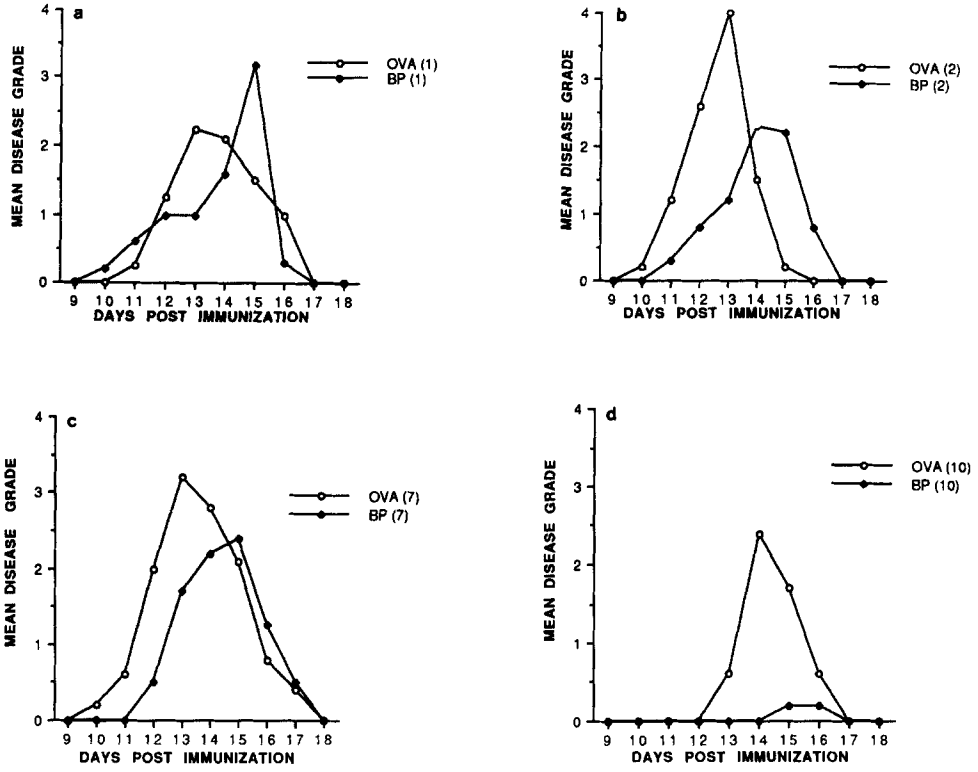

Figure 2. Disease course in animals intrathymically injected with 1 mg MBP 48 h before immunization and thymectomized on days 1 (*a*), 2 (*b*), 7 (*c*), and 10 (*d*) postimmunization. Each data point represents the mean disease grade of five animals per group.

MBP/CFA was protective against development of clinical EAE (mean grade = 1.2 ± 0.2, mean duration = 1.8 ± 0.6) indicating that thymic recognition of the immunodominant epitope of MBP downregulates the systemic immune response to MBP. Intrathymic injection of 21–40 was not protective (mean grade = 3.0 ± 0, mean duration = 4.6 ± 0.4).

Intrathymic Injection of the Encephalitogenic Epitope of MBP Suppresses CNS Inflammation. Immunohistologic evaluation of brains from rats injected with the MBP encephalitogenic epitope showed almost complete absence of an inflammatory response, which was in marked contrast to specimens from rats injected intrathymically with the nonencephalitogenic epitope (Table 2). Thus, sections from the latter group showed large perivascular and submeningeal infiltrates, consisting of T cells and macrophages, many of which were seen to be in contact with the cell bodies of neurons (Fig. 5 *a*). About 20–30% of such cells expressed IL-2R (Fig. 5 *c*) and were associated with expression of IL-2 (*e*), IFN-γ (*g*), and TNF-α (*i*), whereas minimal or no cell infiltration (*b*) or cytokine expression was seen in sections from animals receiving in-

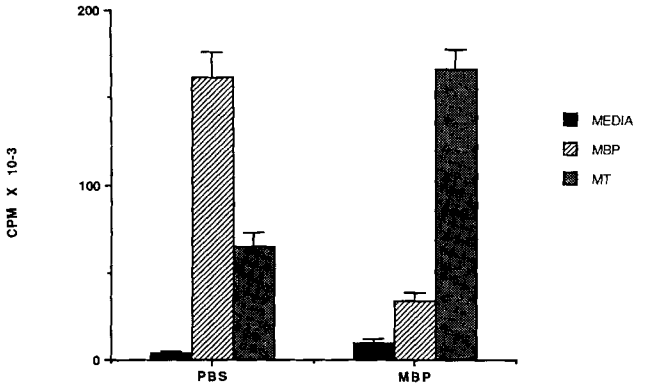

Figure 3. Proliferative response of draining lymph node (LN) cells taken on day 9 postimmunization. The results shown are from pooled LN of three animals per group. The two groups are represented on the *x*-axis as PBS and MBP for the animals receiving intrathymic injection of PBS and MBP, respectively. MBP and Mt antigens were used at 10 μg/ml.

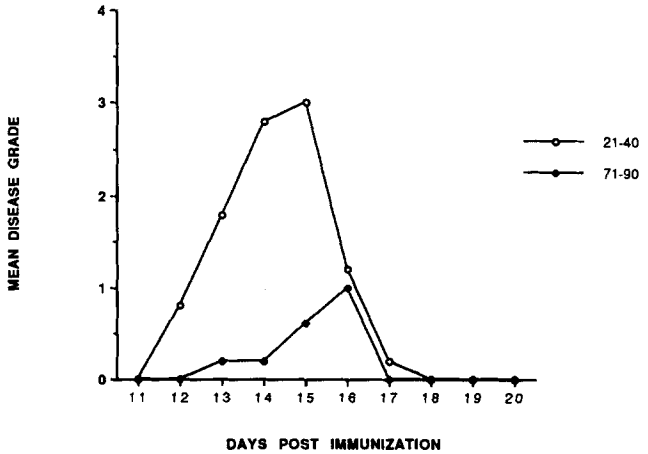

Figure 4. Disease course in animals injected intrathymically with the encephalitogenic MBP p 71–90 or the nonencephalitogenic p 21–40 and immunized 48 h later with MBP/CFA. Each data point represents the mean disease grade of five animals per group.

Table 2. *Perivascular and Submeningeal Mononuclear Cells, Activation Markers, and Cytokines in Brains of Rats Immunized with MBP after Intrathymic Injection of Encephalitogenic or Nonencephalitogenic MBP Peptide*

	Nonencephalitogenic (p 21–40)	Encephalitogenic (p 71–90)
Leukocytes	96.2 ± 33.5	3.2 ± 1.1*
TCR α/β	58.4 ± 26.1	0.9 ± 0.8*
Macrophages	33.7 ± 18.7	3.7 ± 2.1*
IL-2R	18.6 ± 7.3	0.0*
Ia	78.3 ± 25.3	1.5 ± 0.9*
ICAM-1	85.4 ± 35.32	2.8 ± 1.5*
TNF-α	2+	±
IL-1β	2+	0
IL-2	2+	0
IL-4	1+	0
IL-6	1+	0
IL-8	2+	±
IFN-γ	2+	0
TGF-β	±	0
PGE	±	0

Based on examination of 20 high-power fields/rat and 3 rats/group. Numbers indicate: mean labeled cells ± SD/100 nucleated cells in or adjacent to cerebral and cerebellar small vessels. Cytokine data were graded semiquantitatively as: (0) absence of labeling, (±) trace labeling, (1+) few small foci, and (2+) multiple foci.
*p <0.001 compared with nonencephalitogenic peptide–injected group.

trathymic injection of the encephalitogenic MBP peptide (*d*, *f*, *h*, and *j*). Samples from the latter group also lacked expression of IL-4, TGF-β, or PGE, each of which was previously associated with suppression of development of EAE in orally tolerized animals (21), and sections incubated with control antibodies were all unstained.

Discussion

We have shown that intrathymic injection of MBP induces systemic immune hyporesponsiveness as evidenced by protection against actively induced EAE in vivo and decreased proliferative lymphocyte responses in vitro. The protective effect of intrathymic injection is not the result of systemic leakage of antigen, suggesting that MBP is processed and presented by thymic APC. A recent report indicates that a portion of an activated T cell population circulates to the thymus, and may reside there for a prolonged period of time (10). Thus, peripheral T cells generated by immunization with MBP could potentially circulate through the thymus where they become inactivated. This hypothesis would explain abrogation of the protective effect by thymectomy until day 10 postimmunization, when pathological and clinical disease has already been initiated. Alternatively, regulatory thymocytes could migrate to the periphery (23) to inactivate or suppress MBP-reactive T cells.

There are two types of thymic APC, bone marrow–derived macrophages/dendritic cells and epithelial cells. Which cell type processes and presents MBP or MBP peptides is unclear. There is evidence that each type of thymic APC has a different function in the induction of self-tolerance, and in T cell repertoire selection (8, 9). Positive selection may be mediated by thymic epithelial cells, and negative selection (clonal anergy or deletion) may be mediated by the bone marrow–derived cells (7). T cells usually require two distinct signals for optimal stimulation: an antigen presented in the context of self-MHC, and a costimulatory signal, usually provided by APC (24). Thymic macrophages have been shown to have a selective antigen presentation defect to Th1 clones that was attributed to lack of a costimulatory signal (25). In addition, thymic epithelial cells have also been shown to induce in vivo tolerance to class I–incompatible skin grafts mediated by peripheral T cell anergy (26, 27). Studies are in progress to investigate which thymic APC is responsible for MBP presentation in our model, and to better understand the exact mechanisms of acquired intrathymic tolerance.

Protection from EAE is achieved by intrathymic injection of the encephalitogenic (p 71–90), but not by injecting the nonencephalitogenic (p 21–40), peptide of MBP. Immunohistologic studies also showed that intrathymic injection of the encephalitogenic MBP peptide abrogated T cell and macrophage infiltration, and prevented upregulation of Ia and ICAM-1 expression. These effects were associated with lack of T cell activation, as evidenced by complete absence of IL-2R, IL-2, or IFN-γ expression in the central nervous system, and provide in vivo morphologic correlates of the clinical and the in vitro proliferation data.

Systemic tolerance has been induced in the EAE model by oral administration of MBP (28), or oral administration of either the encephalitogenic p 71–90 or the nonencephalitogenic p 21–40 (29). We have previously shown that oral tolerance in EAE is associated with secretion of the suppressive cytokine TGF-β in the target organ (21). This phenomenon is enhanced by oral administration of LPS, which results in IL-4 expression in the brain and is probably due to selective inhibition of Th1 and stimulation of Th2 cells in vivo (21). Intrathymic injection of MBP or MBP peptides causes suppression of disease clinically and pathologically with evidence of downregulation of activation and inflammatory cytokines in the target organ. However, in contrast to the oral tolerance model, there is no upregulation of TGF-β and no evidence of immune deviation from Th1 to Th2 cell function as evidenced by the absence of IL-4 expression. Immunization with MBP/CFA leads to the generation of cells that are mainly reactive to p 71–90 (30). This would suggest that thymic recognition of the immunodominant MBP epitope leads to clonal inactivation of the encephalitogenic cells via functional inactivation or anergy, or conversely, it may lead to peripheralization of regulatory cells that act on the afferent limb of the immune system, i.e., by active suppression of the encephalitogenic cells in the lymph nodes.

Intrathymic injection of an autoantigen or its immuno-

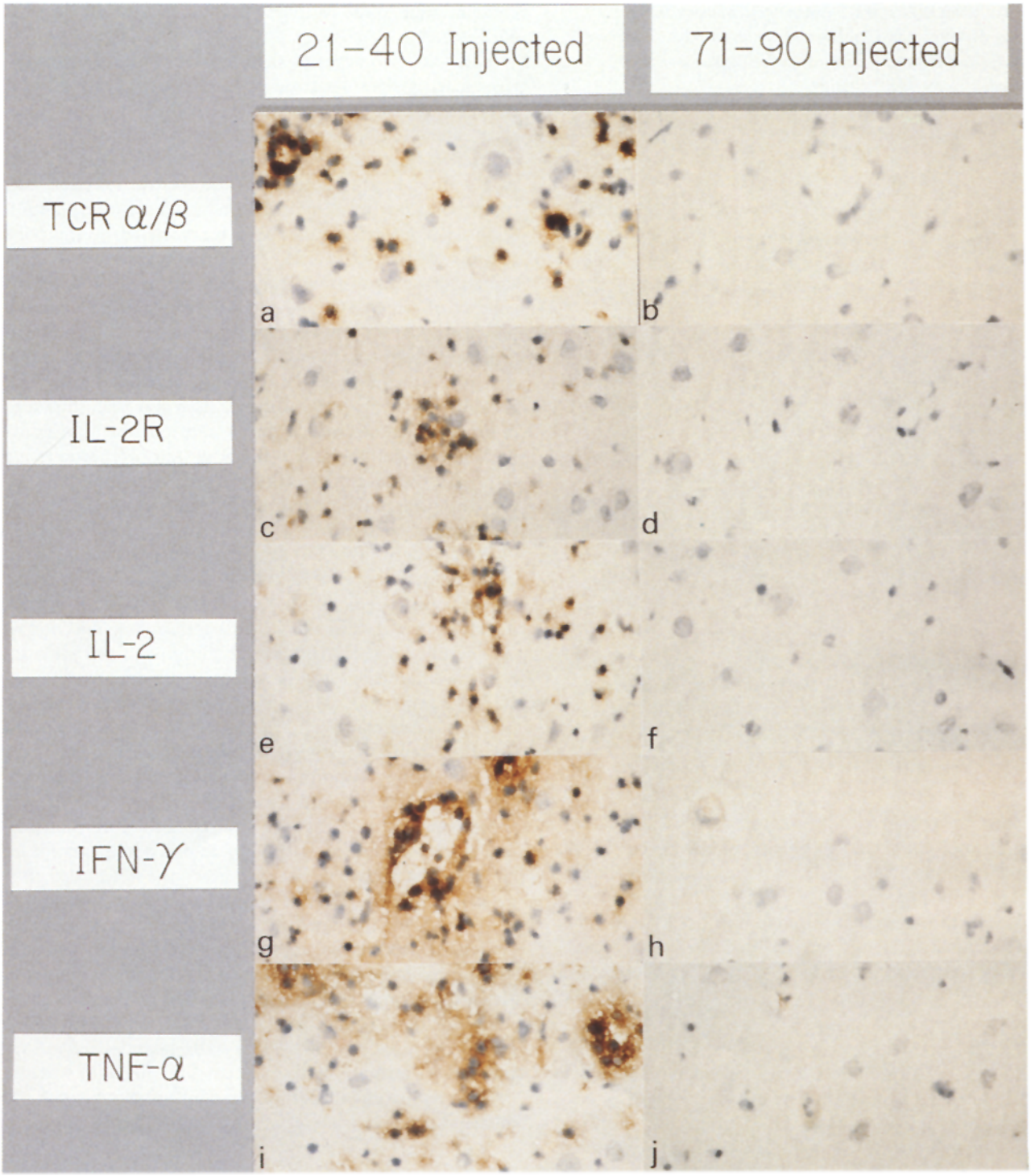

Figure 5. Paired photomicrographs of immunoperoxidase staining of cryostat sections of cerebri from rats on day 12 (peak of disease) after immunization with MBP/CFA. (*Left*) Rats injected intrathymically with nonencephalitogenic MBP peptide; (*right*) rats receiving intrathymic injection of encephalitogenic MBP peptide (hematoxylin counterstain; ×400). Intrathymic injection of encephalitogenic MBP peptide essentially abolished CNS inflammation, compared with injection of the control, nonencephalitogenic peptide, as shown by markedly decreased perivascular infiltrates of: (*a* and *b*) TCR α/β^+ cells, which, in the control group, were often (*c* and *d*) IL-2R$^+$ (CD25), and associated with expression of (*e* and *f*) IL-2, (*g* and *h*) IFN-γ, and (*i* and *j*) TNF-α.

dominant peptide provides a novel approach for the study of the induction and regulation of T cell–mediated autoimmune disease and an opportunity to define the contribution of the thymus in modulating T cell–mediated immune responses in adult animals.

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