Mechanisms of Acquired Thymic Tolerance in Experimental Autoimmune Encephalomyelitis: Thymic Dendritic-enriched Cells Induce Specific Peripheral T Cell Unresponsiveness In Vivo

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Summary

Experimental autoimmune encephalomyelitis (EAE), an experimental model for the study of multiple sclerosis, is an autoimmune disease of the central nervous system that can be induced in a number of species by immunization with myelin basic protein (MBP). MBP-reactive $CD4^+$ T cells, predominantly expressing the $V\beta8.2$ T cell receptor (TCR), migrate from the peripheral lymphoid organs and initiate the inflammatory response in the brain. We have previously shown that a single intrathymic injection of MBP or its major encephalitogenic peptide (p71-90), but not a nonencephalitogenic peptide (p21-40), induces antigen-specific systemic tolerance and inhibits the induction of EAE in Lewis rats. In this study, we investigated the mechanisms of induction and maintenance of acquired thymic tolerance in this model. First, we investigated which thymic cell is responsible for "induction" of systemic tolerance. Thymic dendritic-enriched cells, isolated by plastic adherence, when incubated in vitro with p71-90 and injected intravenously into Lewis rats, were capable of preventing the development of EAE, but his protection was lost in thymectomized recipients. In addition, intravenous injection of thymic dendritic cells isolated from animals that had been previously injected intrathymically with p71-90 but not p21-40 also prevented the development of EAE. Second, to determine the "effector" mechanisms involved in acquired thymic tolerance, we compared TCR expression in the brains of animals with actively induced EAE with TCR expression in animals that received intrathymic injection of p71-90 or p21-40. Using a semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) technique, we found increased expression of CD4 and $V\beta8.2$ message in brains of immunized animals compared with those of naive animals. In animals intrathymically injected with p71-90 but not p21-40, CD4 and $V\beta8.2$ transcript levels were significantly reduced compared with immunized controls. Immunohistologic studies of brain tissue and spleens with specific $V\beta8.2$ and control $V\beta10$ monoclonal antibodies confirmed these observations in vivo. These findings, taken together with recent data demonstrating that activated T cells circulate through the thymus, suggest that interaction of thymic dendritic cells with specific TCR of activated peripheral T cells can lead to inactivation of these antigen-specific cells and confirm the role of $V\beta8.2$-expressing T cells in EAE.

Experimental autoimmune encephalomyelitis (EAE)[1] 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) and adjuvant (1). In Lewis rats, the major encephalitogenic epitope of guinea pig MBP (GP-MBP) is peptide 71-90 (2, 3). Nonencephalitogentic 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 appears to play a major role in the develop-

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[1] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; GP-MBP, guinea pig myelin basic protein; MBP, myelin basic protein; RT, reverse transcription; TxA_2, thromboxane A_2.

ment of self-tolerance and may also play a role in acquired tolerance in experimental autoimmune (6–9) and transplantation (10–14) models. We have recently shown that injection of MBP or its encephalitogenic peptide p71-90 into the thymus induces systemic tolerance and prevents development of EAE in the Lewis rat model. Immunohistologically, there was marked reduction of mononuclear cell infiltrates and absent activation and inflammatory cytokines in the brains of intrathymically tolerized animals (15). The cellular interactions in the thymus between specific T cells and thymic APCs that ultimately lead to induction of systemic tolerance and the effector mechanisms of acquired thymic tolerance are not well understood. Agus et al. reported that activated but not resting T cells circulate through the thymus and may stay there for a prolonged period of time (16). Furthermore, dendritic cells are known for their ability to migrate to T cell–dependent areas of lymphoid organs (17). These observations provide potential mechanisms whereby presentation of immunogenic peptides by specific thymic APCs to activated peripheral T cells circulating through the thymus might lead to specific T cell inactivation (18) and specific systemic tolerance. We now report results of studies directed at defining the thymic APCs that induce specific peripheral T cell unresponsiveness to EAE in vivo.

Materials and Methods

Induction of EAE. Lewis rats, 6–8 wk old, were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) or were bred in our own animal facility. EAE was induced by immunizing the animals in the foot pad with 50 μg MBP in CFA, which was prepared by mixing IFA (Sigma Chemical Co., St. Louis, MO) with 4 mg/ml mycobacterium (GIBCO BRL, Gaithersburg, MD). Scoring of clinical disease was performed as previously described (15, 19, 20). Briefly, duration of disease was calculated by counting the total number of days from clinical onset to recovery for individual animals; mean duration of disease was calculated as the average for the whole group. Maximal grade was defined as the highest clinical grade achieved by individual animals; mean maximal grade was calculated as the average for each group. Statistical analysis was performed using a one-tailed Student's t test (n = 4–5 per group). Disease index is a composite score and was calculated as follows: mean duration of disease × mean highest disease grade × incidence (19).

Antigens. Preparation of guinea pig MBP was performed as previously described (19). Peptide sequences 71-90 and 21-40 (p71-90 and p21-40) were obtained from published data (21) and synthesized at the Biopolymer Center, Center for Neurologic Diseases, Brigham and Women's Hospital (Boston, MA).

Intrathymic Injections. Animals were injected intrathymically with synthetic peptides dissolved in sterile PBS as previously described (14, 15). Intrathymic injections were performed with the animals under ether anesthesia by exposing the thymus through a small suprasternal incision. Volumes of up to 50 μl can be injected into each lobe, without evidence of leakage, using a 27–30 gauge needle. Thymic injections were performed 48–72 h before immunization.

Isolation of Nylon Wool–adherent Cells. Thymus was isolated from naive Lewis animals and mashed through a metal sieve to obtain a single-cell suspension. The cells were washed and resuspended at 2–3 × 10⁶/ml and then incubated for 1 h at 37°C over 20-ml

nylon wool columns containing 10% FCS in RPMI. The columns were washed 5–10 times with RPMI to remove nonadherent T cells, and then the columns were tapped for several minutes to mechanically dissociate the adherent cells. These cells were then collected and washed before use.

Isolation of Nylon Wool–nonadherent Primed T Cells. Draining lymph nodes were isolated on day 9 after immunization with GP-MBP/CFA and prepared into a single-cell suspension. The preparation was enriched for T cells by depletion of nylon wool–adherent cells as described above. The cells were washed and placed in flat-bottomed 96-well plates (Fisher Scientific Co., Pittsburgh, PA) at 2 × 10⁵ per well and used as responder cells. The ratio of APCs to responders was 1:2, 1:5, 1:10, and 1:100 in quadruplicates. APCs were preincubated with 50 μg/ml GP-MBP before being added to the responders.

Enrichment of Dendritic Cells. A dendritic cell–enriched population was prepared from thymus or spleen according to the method reported by Banuls et al. (22) with minor modifications. Briefly, the isolated thymus was cut into small fragments and digested in RPMI 1640 (Sigma Chemical Co.) containing 2 mg/ml collagenase type IV (Boehringer Mannheim GmbH, Mannheim, Germany) for 15 min at 37°C. Digested fragments were filtered through a stainless steel screen. The cells were then washed in RPMI 1640 and resuspended in the same medium supplemented with 10% FCS at 5 × 10⁷ cells/ml and cultured in 10-ml plates (Costar Corp., Cambridge, MA) for 60 min at 37°C in a 5% CO_2 incubator. The nonadherent cells were removed by gentle washing with warm RPMI medium, and the adherent cells, i.e., dendritic cells and macrophages, were cultured for 12–16 h in 10% FCS-RPMI. Most macrophages remained adherent after this culture period, whereas dendritic cells became nonadherent (23). After isolation, the dendritic cell–enriched population consisted mostly of large cells, with some small mononuclear cell contamination when viewed under phase-contrast microscopy. Staining with the following monoclonal antibodies was performed to identify the populations by flow cytometry: TCR α/β (R 73), ED1 (MCA 341), ED2 (MCA 342), ED3 (MCA 343), OX-33 (MCA 340), OX-22 (MCA 53), OX-3 (MCA-45), and OX-18 (MCA 51). All antibodies and isotype controls were purchased from Bioproducts for Science Inc. (Indianapolis, IN). Flow cytometry was performed on a FACSort® (Becton Dickinson & Co., Mountain View, CA) and showed that the isolated cells consisted of two distinct populations: a large-cell population that was ED1⁺, ED2⁺, ED3⁻, OX-33⁻ (B cell), CD45R⁻, class I MHC⁺, and class II MHC⁺, consistent with the expected profile of dendritic cells (22), and a small-cell population that consisted mostly of thymocytes (TCR⁺, ED1⁻, ED2⁻, ED3⁻, OX-33⁻, class I⁺, and class II⁻). These thymocytes constituted 10–20% of the total population. Notably, B cell contamination (OX-33) was negligible (<1%). The dendritic-enriched cells had the typical dendritic structure when viewed on cytospin slides and strongly expressed class-II MHC antigen; some of these cells also expressed OX-62, which stains rat veiled cell–enriched populations (24). Functionally, the dendritic-enriched cells were potent APCs and were capable of inducing significant proliferation (SI = 700) of a peptide T cell clone at ratios of dendritic/responder as low as 1:30 (data not shown).

In Vitro Pulsing of APCs. Nylon wool–adherent cells or dendritic-enriched cells were incubated with antigen at a concentration of 50 μg/ml for 3 h at 37°C, and then washed twice to remove excess antigen.

Reverse Transcription PCR. Total cellular RNA was extracted from brain sections using RNAzol B (Cinna/Biotecx, Friendswood, TX), and cDNA was prepared as previously described (25). We

used a published (RT)-PCR technique developed to measure relative transcript levels (25, 26) and modified as follows for use with CD4 and Vβ8.2. Oligonucleotide primers were synthesized by the Protein/Nucleic Acid Laboratory, Brigham and Women's Hospital. For each primer combination, conditions were optimized to generate a single specific band, and sequence analysis confirmed amplification of the predicted fragment. Logarithmic ranges of PCR amplification were established as a function of cycle number and cDNA dilution. Reaction conditions included 1.25 μl cDNA (diluted 1:3 parts in 1 × PCR buffer), 1 μM (each) 5' and 3' primers, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% (wt/vol) gelatin, 800 μM dNTPs, and 0.625 U AmpliTaq DNA polymerase (Perkin Elmer Corp., Foster City, CA) in a total volume of 25 μl. [^{32}P]dCTP (150,000 cpm) was included for quantitative PCR studies. The thermal cycling variables were denaturation at 94°C for 15 s, annealing temperature as listed for 20 s, and extension for 60 s (with a final extension of 7 min at the end of all cycles). Primer sequences, annealing temperature, and number of cycles were as follows:

CD4   5' TGC GAG CTG GAG AAC AAF AAA GAG G
3' TCA CAG GTC AAA GTG TTG CTG TCG G
(reference 27)
(57°C, 24 cycles)

Vβ8.2   5' AAA GGT GAC ATT GAG TTG TAA G
3' AAG GGC CCA CCA GCT CAG CTC CAC
ATG GTC AGG (reference 28)
(62°C, 32 cycles)

G3PDH 5' TGA AGG TCG GTG TCA ACG GAT TTG
GC;
3' CAT GTA GGC CAT GAG GTC CAC CAC
(reference 29)
(58°C, 22 cycles)

[^{32}P]dCTP incorporated into PCR product bands was measured from dried agarose gels on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) as described (25, 26). PCR amplification with the G3PDH housekeeping gene was performed to assess variations in total RNA or cDNA loading between samples. The mean obtained from at least three analyses was used to normalize other transcript level measurements from the same sample. Corrected values were derived by dividing the measured ^{32}P value for the transcript of interest by the mean G3PDH value for the sample. Relative transcript levels were then determined from cDNA sets that included negative control samples (for which reverse transcriptase had been omitted during cDNA synthesis or water had been used instead of cDNA) performed in the same evaluation. PCR analysis was performed in duplicate on all cDNA samples in an experimental set. The experimental set included cDNAs from the brains of naive rats (n = 3), rats immunized with MBP without treatment to induce EAE (n = 5), rats immunized with MBP after intrathymic injection of p21-40 as control (n = 6), and after intrathymic injection of p71-90 to inhibit EAE (n = 6). Each bar in Fig. 2 represents the mean corrected levels obtained by pooling the levels obtained from all animals per group that had been analyzed in duplicate. Results were subjected to multivariate analysis of variance (MANOVA) without replication.

Immunohistology. The extent of correlation of Vβ usage in situ with mRNA data was sought by immunohistologic analysis of sections of rat brain and spleen sections at day 14 (n = 3 per group) using mAbs to Vβ8.2 and control Vβ10 (PharMingen, San Diego, CA). Immunoperoxidase labeling was performed using a sensitive four-layer peroxidase-antiperoxidase technique, as previously described (15, 20).

Results

Intrathymic Injection of p71-90 of GP-MBP Protects Animals from EAE Even After 30-d Delay of Immunization. In our initial studies, we showed that intrathymic injection of p71-90 but not p21-40 24 h, 48 h, or 1 wk before immunization prevented development of clinical EAE. In the current study, animals injected intrathymically with p71-90 or p21-40 were immunized 30 d after intrathymic injection and were observed for disease. As seen in Fig. 1, animals injected with p71-90 were completely protected from development of EAE compared with control animals injected intrathymically with p21-40. The incidence in the p21-40 injected group was 5/5 with a mean duration of 5.4 ± 0.6 d and a mean grade of 3.0 ± 0, whereas none of the p71-90–injected animals were sick. These observations indicated to us that the thymic APCs, which are presenting p71-90 to primed T cells had to have the unique characteristics of holding on to antigen for a prolonged period of time.

Thymic Nylon Wool-adherent Cells Pulsed In Vitro with p71-90 Inhibit EAE. We tested the hypothesis that thymic nylon wool-adherent cells recognize and present antigen to primed T cells. Thymic nylon wool-adherent cells from a naive animal were pulsed in vitro with GP-MBP for 3 h at 37°C and then added to responder T cells at various ratios. Splenic nylon wool-adherent cells served as control APCs. Thymic nylon wool-adherent cells were able to present antigen to primed T cells (SI = 24 and ∂CPM = 31,611 at a ratio of 1:2, SI = 30 and ∂CPM = 39,117 at a ratio of 1:5, SI = 11 and ∂CPM = 14,681 at a ratio of 1:10, and SI = 2.3 and ∂CPM = 1,780 at a ratio of 1:100). We then tested the hypothesis that nylon wool-adherent cells pulsed with the encephalitogenic peptide p71-90 and injected intravenously lead to systemic tolerance in vivo. We isolated nylon wool-adherent cells from naive thymus and pulsed them in vitro for 3 h at 37°C with 50 μg/ml p71-90 or p21-40. The cells were then washed twice to get rid of excess peptides and injected intravenously into naive Lewis animals. The recipients were immunized 48–72 h later with MBP/CFA. Significant pro-
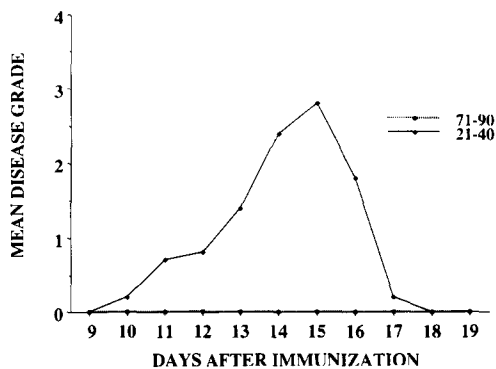

Figure 1. Protection from EAE even after delay of immunization after intrathymic injection. Lewis rats were injected intrathymically with 100 μg of p71-90 or p21-40 of MBP. 30 d later they were immunized and scored for disease. There were five animals per group. Each point represents the mean score for the group.

tection from EAE was seen in animals injected with p71-90–pulsed cells but not with p21-40 pulsed cells, compared with unmodified controls (Table 1 a). A dose–response effect was observed such that injection of >30 × 10⁶ cells was protective, while injection of <30 × 10⁶ cells was not (two experiments with 10⁷ and 17 × 10⁶; data not shown).

Thymic but not Splenic Dendritic Cell–enriched Populations Pulsed In Vitro with p71-90 Induce Tolerance to EAE. To define which thymic cells are responsible for induction of unresponsiveness, a thymic dendritic cell–enriched population was prepared from naive animals. The cells were pulsed in vitro with p71-90 as described above, washed, then injected intravenously into naive animals 48–72 h before immunization with MBP/CFA. Injection of 3–4 × 10⁶ cells of this dendritic cell–enriched population significantly diminished the incidence, severity, and duration of EAE (Table 1 b). Again, a dose–response effect was observed such that injection of 1.5 × 10⁶ cells caused a decrease in severity of disease without affecting incidence (data not shown). Furthermore, injection of 4 × 10⁶ thymic non–plastic-adherent cells (after 2 h incubation) pulsed in vitro with p71-90 was not protective (Table 1 c), providing evidence that the plastic-adherent, dendritic cell–enriched population (that become nonadherent after overnight incubation), and not contaminating thymocytes or thymic B cells, is responsible for induction of tolerance. Depletion of Fc receptor-positive cells from the dendritic-enriched cell population by incubation over immunoglobulin-coated plates did not result in loss of disease protection, suggesting that potentially contaminating macrophages do not contribute to this effect (data not shown).

To address the question of whether thymic dendritic cells were unique in their capability to induce tolerance, we injected dendritic-enriched splenic cells pulsed in vitro with p71-90 intravenously into naive recipients, which were then immunized. Table 2 shows that a splenic dendritic-enriched population pulsed in vitro with p71-90 did not protect the recipients from EAE. Furthermore, parallel experiments showed that injection of p71-90 at a dose of 100 μg intravenously did not protect animals from EAE (Table 2). This provides evidence against the carry-over of peptide by nonspecific adherence to cells as a mechanism for protection.

Thymectomy before Injection of Dendritic-enriched Cells Abrogates Protection from EAE. Animals were thymectomized and then injected intravenously with thymic dendritic-enriched cells pulsed in vitro with p71-90 and immunized 48 h later. Control animals were thymectomized and then immunized. Table 3 shows that thymic dendritic-enriched cells were not protective when injected into thymectomized recipients. As expected, splenic dendritic-enriched cells pulsed in vitro and injected into thymectomized recipients also were not protective. These data suggest that the native thymus is required for induction of tolerance by intravenous injection of antigen-pulsed dendritic cells.

Transfer of Dendritic-enriched Cells from Intrathymically Injected Animals Inhibits EAE. The observations described above, although suggestive, do not provide direct evidence that thymic dendritic-enriched cells are responsible for induction of acquired thymic tolerance. To study this further, thymic dendritic-enriched cells were isolated from the thymus of Lewis rats 2 d after intrathymic injection of p71-90 or p21-40. After

Table 1. *Disease Characteristics in Animals Receiving Thymic Nylon Wool–adherent, Dendritic-enriched, or Non–plastic-adherent Cells*

Type of cells	Number of cells injected	Incidence	Mean duration ± SEM	Mean maximal score ± SEM	Index
a.					
Thymic nylon wool					
adherent 71-90 pulsed	35 × 10⁶	2/4	1.25 ± 0.7*	0.3 ± 0.3*	0.18
Thymic nylon wool					
adherent 21-40 pulsed	35 × 10⁶	4/4	6.0 ± 0	2.75 ± 0.2	16.5
Control	0	4/4	5.25 ± 0.7	3.5 ± 0.5	18.4
b.					
Thymic dendritic enriched					
71-90 pulsed	4 × 10⁶	2/5*	1.8 ± 1.1*	0.8 ± 0.5*	0.6
Control noninjected	0	5/5	6.4 ± 0.4	3.0 ± 0	19.2
c.					
Nonplastic adherent					
71-90 pulsed	4 × 10⁶	5/5	6.2 ± 0.4	2.1 ± 0.4	13.0
Control noninjected	0	5/5	6.5 ± 0.6	2.5 ± 0.5	16.2

Data presented are representative of two to four experiments. Each group of animals consists of four to five animals.
* P <0.05.

Table 2. *Disease Characteristics in Animals Receiving Splenic Dendritic-enriched Cells or Injected Intravenously with 100 µg of Peptide*

Type of cells	Number of cells injected	Incidence	Mean duration ± SEM	Mean maximal score ± SEM	Index
Splenic dendritic-enriched					
71-90 pulsed	4 × 10⁶	4/4	5.50 ± 0.6	2.38 ± 0.9	13.09
Injected p71-90					
100 µg IV	0	5/5	4.40 ± 0.8	2.40 ± 0.4	10.60
Control noninjected	0	5/5	4.80 ± 0.8	2.60 ± 0.4	12.48

Each group consisted of five animals. There were no significant differences in incidence, duration, or severity of disease between the groups.

repeated washing, 3–4 × 10⁶ cells were injected intravenously into naive animals without in vitro pulsing. The recipients were immunized with MBP/CFA 48–72 h later and observed for disease. Fig. 2 shows that recipients of dendritic-enriched cells from animals intrathymically injected with p71-90 were protected against EAE compared with recipients of dendritic-enriched cells from animals intrathymically injected with p21-40. The incidence was two out of four in the p71-90 group vs four out of four in the p21-40 group; the mean maximal grade was 0.5 ± 0.3 (p71-90 vs 2.12 ± 0.52 (p21-40), $P = 0.016$; and the mean duration was 2.0 ± 1.2 d (p71-90) vs 7.5 ± 0.6 d (p21-40), $P = 0.004$.

Specific Downregulation of TCR Vβ8.2 Infiltrating T Cells in the Brain. There is evidence that the encephalitogenic CD4⁺ T cells that initiate the immune response in the rat EAE preferentially use the TCR Vβ8.2 (30). We studied the expression of this TCR in the brains of naive animals, animals immunized with MBP/CFA, and animals intrathymically tolerized with the immunodominant peptide p71-90 or the

control peptide p21-40. Brain RNA was extracted and the samples analyzed by RT-PCR for expression of Vβ8.2, and CD4 messages. G3PDH was used as a reference gene, and quantitation of PCR products was expressed as a ratio of the relevant gene to that of G3PDH. Fig. 3 shows that on day 14 (peak of disease), immunized animals had significantly increased expression of CD4 and Vβ8.2 transcripts ($P = 0.001$) compared with naive animals. Animals injected intrathymically with p21-40 had no significant change in expression of these molecules compared with immunized controls, whereas animals injected with p71-90 showed significantly decreased expression of CD4 and of Vβ8.2 transcripts ($P < 0.001$).

Immunohistologic studies with specific mAbs against Vβ8.2 and a specificity control TCR Vβ10 confirmed that Vβ8.2⁺ T cells were selectively decreased by intrathymic injection of p71-90 compared with p21-40. As shown in Fig. 4, brains from animals injected with p21-40 showed 10–20 Vβ8.2⁺ T cells per high power field, whereas samples from rats injected with p71-90 showed <1 Vβ8.2⁺ T cell per high

Table 3. *Disease Characteristics in Animals Receiving Thymic or Splenic Dendritic-enriched Cells after Thymectomy*

Type of cells	Number of cells injected	Incidence	Mean duration ± SEM	Mean maximal score ± SEM	Index
Thymic dendritic-enriched					
71-90 pulsed					
injected after thymectomy	5 × 10⁶	5/5	4.80 ± 0.4	2.0 ± 0.5	9.6
Splenic dendritic-enriched					
71-90 pulsed					
injected after thymectomy	5 × 10⁶	5/5	5.20 ± 0.5	1.8 ± 0.4	9.4
Control noninjected					
thymectomized	0	5/5	5.40 ± 0.5	2.4 ± 0.4	12.9

Each group consisted of five animals. There were no significant differences in incidence, duration, or severity of disease between the groups.
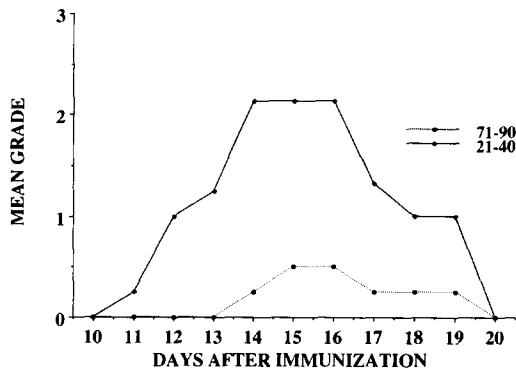
Figure 2. Injection of dendritic-enriched cells from animals intrathymically injected with MBP peptides. Dendritic-enriched cells were isolated from animals injected intrathymically 48 h earlier with p71-90 or p21-40. The cells were washed and injected i.v. into naive recipients at 4×10^6 per animal (four animals per group). The recipients were immunized 48 h later and observed for disease.

power field. mAb against Vβ10 was used as a positive control antibody and showed comparable infiltration by Vβ10$^+$ T cells in the brains of animals injected intrathymically with p21-40 or p71-90. Intrathymic injection of p71-90 had no effect on TCR Vβ expression in corresponding spleen specimens, in which the numbers of Vβ8.2$^+$ T cells were consistently 50% or less that of Vβ10$^+$ T cells (Fig. 4). These data indicate that intrathymic injection of p71-90 results in specific inactivation of Vβ8.2 before they home to the brain.

Discussion

In our previous studies, we determined that thymectomy after intrathymic injection and up to day 7 after immuniza-
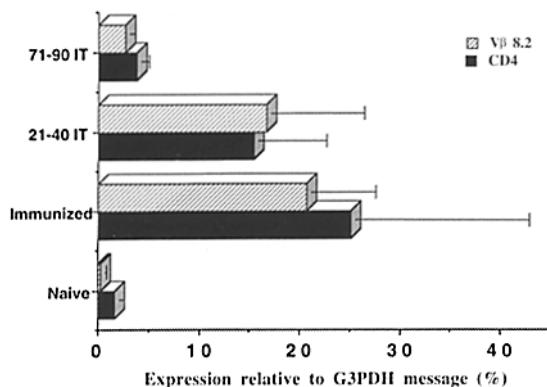

Figure 3. Vβ8.2 and CD4 message in the brain on day 14 postimmunization. Total cellular mRNA was extracted from the brains of animals in the groups listed on the y axis. The numbers of animals per group were naive, 3; immunized, 5; p21-40 intrathymically then immunized, 6; and p71-90 intrathymically then immunized, 6. RT-PCR was performed in duplicates on RNA samples from each individual animal, and the results were normalized to the mean transcript levels of the housekeeping gene G3PDH. The results shown represent the mean of normalized message for each group with SEM.

tion would abrogate acquired thymic tolerance (15). This suggested that a thymic cell population was actively involved in the induction phase of acquired thymic tolerance. Some APCs are capable of presenting antigen in a way that favors tolerogenesis (31, 32). Dendritic cells were shown to take up antigen from the anterior chamber of the eye and transport it systemically to be presented in the periphery, causing immune deviation (33). They are known to express high levels of class II MHC (22, 34) and to capture antigen in an immunogenic form in situ (35). They are extremely efficient APCs in vitro (36, 37) and in vivo (38). Our data indicate that a population enriched with thymic dendritic cells incubated in vitro with the encephalitogenic MBP peptide p71-90 and injected systemically into naive animals mimics the effects of intrathymic injection of p71-90 (15).

The dendritic cell–enriched population may be contaminated with other professional APCs (B cells or macrophages), which potentially could be mediating the tolerogenic effects. We show that intrathymic injection of peptide continues to be effective at preventing disease even after a 30-d delay in immunization. Because the antigen is injected in soluble form, it is not expected to be retained as free antigen for a prolonged period in the thymus; it is more likely to be retained on the surface of APCs. Dendritic cells retain antigen more efficiently than macrophages (38, 39), which have a rapid turnover of antigen-presenting activity (40), suggesting that 30 d after intrathymic injection, the peptide is more likely to be on the surface of thymic dendritic cells than on the surface of macrophages. Thymic B cells were shown to mediate neonatal tolerance by causing clonal deletion (23), but our preparation contained <1% B cells, as assessed by flow cytometry. Dendritic cells adhere to plastic initially but become nonadherent after overnight culture (34), so washing non-plastic-adherent cells after 2 h of incubation removes contaminating thymocytes and B cells. This nonadherent cell population was incapable of inducing tolerance after in vitro incubation with p71-90. Furthermore, the small size of the dendritic cell–enriched population required in our model strongly argues in favor of the dendritic population rather than macrophages or B cells because dendritic cells are very efficient APCs (38, 39). Carry-over of antigen as a mechanism is unlikely because the cells are washed repeatedly before injection, the control nonadherent population failed to induce tolerance after the same in vitro incubation with antigen, and injection of 100 μg of peptide intravenously was not protective.

In vitro incubation of dendritic cells allowed us to control the conditions of the experiment and ensured that the dendritic cells were exposed to the antigen before injection. To establish the link between our in vitro system and the in vivo intrathymic injection, we isolated dendritic cells from animals injected intrathymically with p71-90 or p21-40 and transferred them intravenously into naive recipients without in vitro pulsing with peptides. Protection of the recipients suggests that dendritic cells were exposed to antigen in vivo and that thymic dendritic cells are mediating acquired thymic tolerance. Dendritic cells have been shown to present antigen and prime naive T cells in vivo (38). Interestingly, in our model,

MBP 21-40   **MBP 71-90**
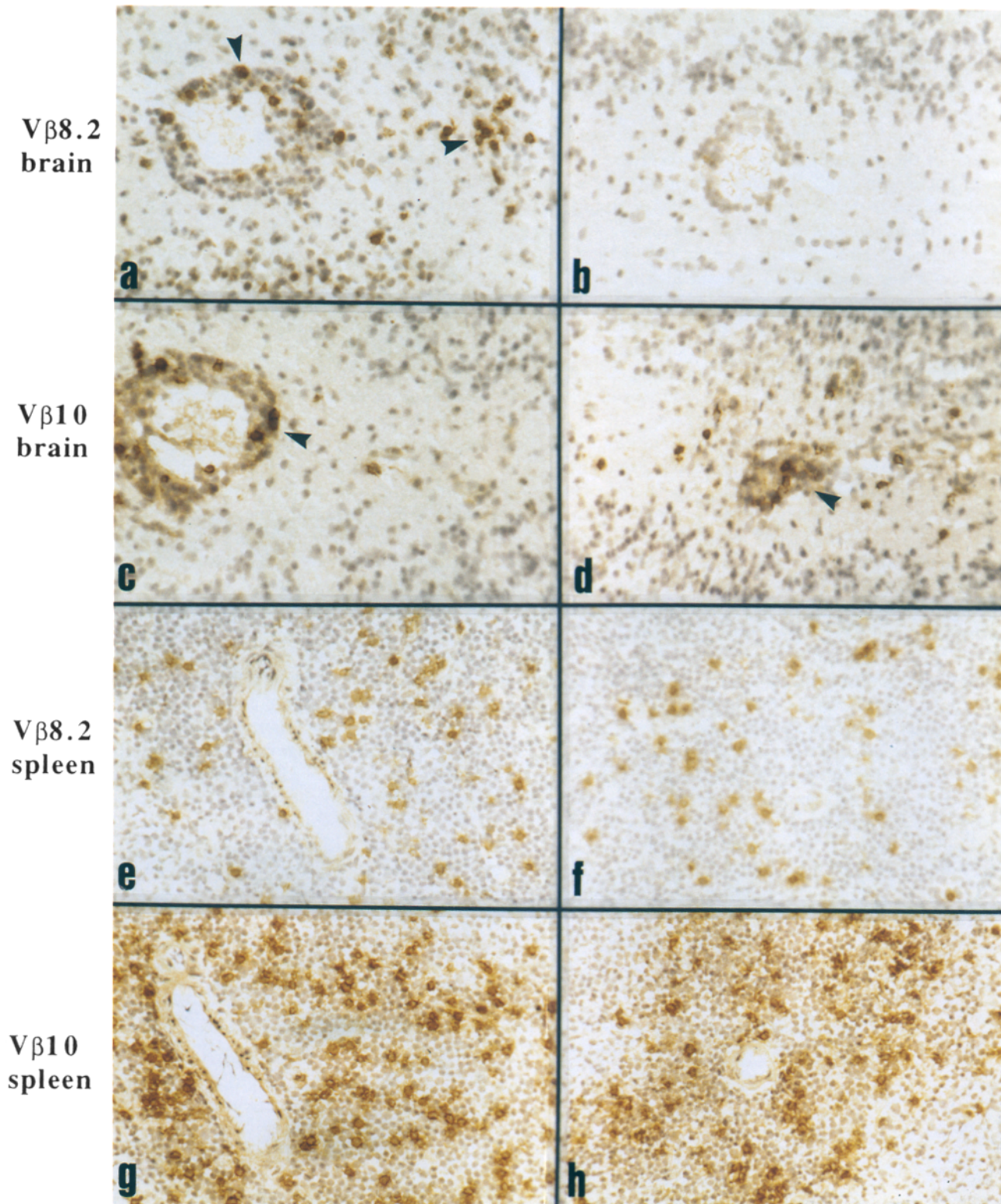
Vβ8.2
brain

Vβ10
brain

Vβ8.2
spleen

Vβ10
spleen

Figure 4. Immunohistologic analysis, in serial sections, of Vβ usage by T cells infiltrating brains of rats immunized with MBP and injected intrathymically with peptides 21-40 or 71-90. Shown are sections from the brain and spleen of corresponding rats. (*a*) Considerable numbers of Vβ8.2$^+$ T cells were present adjacent to cerebellar blood vessels of rats injected with p21-40. (*b*) Intrathymic injection of p71-90 was associated with essentially complete absence of Vβ8.2$^+$ cells within rat brains. (*c*) Lesser numbers of Vβ10$^+$ T cells than Vβ8.2$^+$ cells were seen in cerebellar areas of rats of injected intrathymically with P21-40. (*d*) Intrathymic injection of p71-90 did not diminish cerebellar infiltration by Vβ10$^+$ T cells. (*e*) Vβ8.2$^+$ T cells contributed ∼5–10% of T cells within periarteriolar T cell areas of rat spleen (rat was injected with p21-40). (*f*) Intrathymic injection of p71-90 had no effect on the numbers of Vβ8.2$^+$ cells in rat spleen. (*g*) Considerably more Vβ10$^+$ than Vβ8.2$^+$ T cells were seen in the spleens of rats injected with p21-40. (*h*) Intrathymic injection of p71-90 did not diminish spleen cell labeling for Vβ10$^+$ T cells. Hematoxylin counterstain; ×400.

the outcome of antigen being presented by dendritic cells is suppression of disease and suppression of proliferation of primed lymph node cells (15). This may result from anergizing/deletion of antigen-specific T cells (41, 42) or, alternatively, from the generation of an intermediate regulatory cell that suppresses antigen-specific T cells in vitro and in vivo. Trapping of antigen-reactive cells in the spleen or lymph nodes, thus preventing them from reaching the target organ, is ruled out by the finding that thymectomy before administration of pulsed dendritic cells abrogates protection. This observation suggests that the interaction between dendritic cells and antigen-specific T cells may be occurring in the thymus, where activated cells can circulate (16). Although the relative number of activated T cells circulating through the thymus at any time point is small (43, 44), this process may be a dynamic one, and, theoretically, most activated T cells may become inactivated or deleted in the thymus over a period of time. This is consistent with our previous observation that the thymus is required for the induction of tolerance for up to 7 d after intrathymic injection (15).

The findings that splenic dendritic cells do not duplicate the tolerogenic effect of thymic dendritic cells and that thymectomy abrogates protection from EAE raise the possibility that thymic dendritic cells home to the thymus, whereas peripheral dendritic cells are unable to do so. In their work on anterior chamber–associated immune deviation, Wilbanks et al. showed that peripheral APCs were unable to duplicate the effect of anterior chamber cells in inducing immune deviation unless the peripheral APCs were preincubated with TGF-β (31, 45).

How could the interaction between thymic dendritic cells and activated T cells result in a tolerogenic signal? The observation that maturing thymocytes have very high cell-surface expression of receptors for thromboxane A$_2$ (TxA$_2$) (46), a potent aggregating and vasoconstrictor agent (47), suggested that TxA$_2$ elaborated by thymic stromal cells might act as the key signal for cellular events related to T cell development. A TxA$_2$ agonist, but not an inactive metabolite, induced concentration-dependent DNA fragmentation in CD4$^+$ CD8$^+$ cells (46), which was inhibited by a specific an-

tagonist. Work from Remuzzi et al. in a rat transplantation model showed that blocking TxA$_2$ with a specific receptor antagonist abrogates acquired thymic tolerance to renal allografts induced by intrathymic injection of MHC allopeptides (18). Thymic dendritic cells abundantly express the enzymatic machinery to synthesize TxA$_2$ (48). We suggest that the interaction between thymic dendritic cells and activated T cells leads to a series of events that involve the TxA$_2$/TxA$_2$ receptor, thus triggering intracellular events that increase cytoplasmic calcium (49, 50) and resulting in T cell anergy or deletion by apoptosis (46).

In a murine model of EAE, Cross et al. showed that antigen-specific encephalitogenic cells initiate the inflammatory response in the brain, which is perpetuated by nonspecific cells recruited from the periphery (51). In Lewis rats, the encephalitogenic T cells preferentially express Vβ8.2 (30). We show by immunohistology that the frequency of Vβ8.2-expressing cells in the spleens of naive or immunized animals is less than that of Vβ10 expressing cells, whereas in the brains, Vβ8.2$^+$ cells are equal or more numerous than Vβ10$^+$ cells at the peak of disease, which is consistent with the hypothesis that Vβ8.2$^+$ antigen-specific encephalitogenic cells initiate the inflammatory response in the brain. We also show that animals intrathymically injected with p71-90 expressed significantly lower levels of Vβ8.2 and CD4 message than animals injected with p21-40. This was confirmed at the protein level by decreased staining of Vβ8.2$^+$ cells in the brains of intrathymically tolerized animals. Interestingly, there was an equivalent number of Vβ10$^+$ T cells in the brains of animals intrathymically injected with p21-40 or p71-90. Whether the encephalitogenic cells are anergized or actually deleted before homing to the brain is unknown; however, we found that in our model administration of IL-2 in vivo did not abrogate protection from EAE (data not shown). Since one form of anergy is reversible by exposure to IL-2 (52, 53) and, in some models, in vivo administration of IL-2 abrogates tolerance (41, 54), our observation suggests that IL-2-responsive anergy is not responsible for the protection that we observed, and studies with specific TCR transgenic mice are under way to further delineate these mechanisms.

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