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Accelerated induction of experimental allergic encephalomyelitis in PL/J mice by a non-$V_\beta 8$-specific superantigen

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ABSTRACT **Superantigens such as the staphylococcal enterotoxins can play an important role in exacerbation of autoimmune disorders such as experimental allergic encephalomyelitis (EAE) in mice. In fact, superantigens can reactivate EAE in PL/J mice that have been sensitized to rat myelin basic protein (MBP). The T-cell subset predominantly responsible for disease in PL/J mice bears the $V_\beta 8^+$ T-cell antigen receptor (TCR). The question arises as to whether T cells bearing other V_β specificities are involved in induction or reactivation of EAE with superantigen. Thus, we have investigated the ability of a non-$V_\beta 8$-specific superantigen, staphylococcal enterotoxin A (SEA) (V_β specificities 1, 3, 10, 11, and 17), to induce EAE in PL/J mice that have been previously protected from disease by anergy and deletion of $V_\beta 8^+$ T cells. PL/J mice were first pretreated with the $V_\beta 8$-specific superantigen staphylococcal enterotoxin B (SEB) and then immunized with MBP. These mice exhibited $V_\beta 8$-specific anergy and depletion and did not develop EAE, even when further treated with SEB. However, administration of SEA to these same mice induced an initial episode of EAE which was characterized by severe hindleg paralysis and accelerated onset of disease. In contrast to SEB pretreatment, PL/J mice pretreated with SEA did develop EAE when immunized with MBP, and after resolution of clinical signs of disease these mice were susceptible to relapse of EAE induced by SEB but not by SEA. Thus, superantigens can activate encephalitogenic MBP-specific non-$V_\beta 8^+$ T cells to cause EAE in PL/J mice. These data suggest that superantigens can play a central role in autoimmune disorders and that they introduce a profound complexity to autoimmune diseases such as EAE, akin to the complexity seen in multiple sclerosis.**

The staphylococcal enterotoxin superantigens are the most powerful T-cell-stimulatory molecules known (1–3). Among them, staphylococcal enterotoxin A (SEA) is the most potent, with the ability to stimulate DNA synthesis at concentrations as low as 10^{-16} M in the human system (3). The staphylococcal enterotoxins have been shown to be ubiquitous in the environment and are responsible for a number of maladies, including food poisoning and toxic shock syndrome (4, 5). Such powerful stimulation by these superantigens is based on their ability to engage major histocompatibility complex class II molecules and together as a complex bind to the T-cell antigen receptor (TCR) in a β-chain variable region (V_β)-specific manner (6–10). In addition, staphylococcal enterotoxin B (SEB) can anergize and delete V_β-specific T-cell subsets in naive mice (11–13). As powerful V_β-specific T-cell-stimulatory molecules, superantigens have been suggested to play a role as environmental factors that can influence the course of autoimmune disease (14, 15).

An animal model useful for the study of the inflammatory demyelinating disease multiple sclerosis (MS) is experimental allergic encephalomyelitis (EAE) (16). In the EAE model, myelin basic protein (MBP) has been shown to be one of the primary central nervous system antigens responsible for induction of autoimmunity. Upon immunization with rat MBP, PL/J mice develop clinically observable tail and limb paralysis due to lymphocyte infiltration into the central nervous system accompanied by acute demyelination. In the case of the PL/J strain, acute episodes of disease will usually resolve and clinical relapses do not occur (17).

The predominant T-cell population responsible for initiating disease in PL/J mice possesses the $V_\beta 8^+$ TCR (18–20). Pretreatment with certain staphylococcal enterotoxin superantigens prior to immunization for induction of EAE can prevent development of disease in PL/J mice and Lewis rats (21–23). Such prevention appears to be dependent on the V_β specificity of the superantigen. For example, SEB-induced anergy and deletion of $V_\beta 8^+$ T cells appear to be the mechanism for protection from EAE in PL/J mice (21, 22). In contrast, administration of either SEB or SEA can reactivate disease in PL/J mice that have been immunized with rat MBP and resolved an initial episode of disease (24). SEB can also reactivate disease when an acetylated amino-terminal peptide of MBP is employed as the immunogen in PL/J mice (25). In the case of reactivation of EAE by superantigen, it appears that immunization prior to exposure to superantigen results in stimulation without induction of anergy (24).

In this study, the differential effects of SEB and SEA pretreatment on the induction or reactivation of EAE by superantigen were evaluated. Administration of SEA to PL/J mice previously protected from development of EAE by SEB pretreatment induced an accelerated induction of an initial episode of EAE. Flow cytometric analysis of V_β-specific T-cell subsets and *in vitro* responsiveness for detection of superantigen-induced anergy were also examined.

MATERIALS AND METHODS

Reagents. MBP was purified from rat spinal cords (Pel-Freeze Biologicals) and was shown to be homogeneous by SDS/PAGE. The acetylated amino-terminal peptide encompassing aa 1–17 of MBP [Ac-MBP-(1–17)] was synthesized on a Milligen 9050 peptide synthesizer (PerSeptive Biosystems, Framingham, MA) using fluorenylmethoxycarbonyl chemistry. Reverse-phase HPLC revealed one major peak, and amino acid analysis gave a composition that corresponded closely to the theoretical composition. The superantigens SEA and SEB were obtained from Toxin Technology (Sarasota, FL). Anti-V_β antibodies and streptavidin-phycoerythrin were from PharMingen.

Induction of EAE. PL/J mice (4–6 weeks old; The Jackson Laboratory) were immunized s.c. with 300 μg of whole rat MBP emulsified in complete Freund's adjuvant containing

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Abbreviations: EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; TCR, T-cell antigen receptor; V_β, β-chain variable region.

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Mycobacterium tuberculosis H37Ra (8 mg/ml); pertussis toxin (List Biological Laboratories, Campbell, CA; 400 ng) was injected i.p. at the same time. Pertussis toxin was reinjected 48 hr later. Mice were observed daily for development of disease and assessment of disease severity. Clinical severity score was as follows: 0, normal; 1, loss of tail tone; 2, hind leg paralysis; 3, paraparesis; 4, paraplegia; 5, moribundity/death.

Superantigen Injections. For superantigen pretreatment, 50 μg of either SEB or SEA was injected i.p. in 0.2 ml of phosphate-buffered saline (PBS). A period of 4 days was allowed before mice were further manipulated. In the case of superantigen administration after immunization with rat MBP, 40 μg of the respective superantigen and 400 ng of pertussis toxin in 0.2 ml of PBS were injected i.p. at least 1 month after immunization with MBP or after complete resolution of all clinical symptoms of EAE.

Flow Cytometry. For analysis of V_β-specific T-cell populations, spleen cells were obtained from mice in each of the treatment groups and subjected to ammonium chloride treatment for removal of erythrocytes. Cells (5×10^5) were washed with FACS buffer (PBS containing 0.5% bovine serum albumin and 10 mM sodium azide) and then incubated with anti-V_β antibodies for 45 min at 37°C. Cells were washed and incubated with streptavidin-phycoerythrin for 15 min at room temperature. Cells were then washed twice and incubated with fluorescein isothiocyanate-labeled anti-CD4 antibodies (PharMingen) for 45 min at 37°C. Cells were washed again and analyzed on a FACSort (Becton Dickinson) as 10,000 events per sample.

Proliferation Assays. Spleen cells were obtained and subjected to ammonium chloride treatment for removal of erythrocytes. For $V_\beta 8$-specific stimulation, microtiter plates were coated with purified anti-$V_\beta 8$ antibody F23.1 (10 μg/ml in PBS, 30 μl per microtiter well). Plates were incubated at 37°C for 2 hr and washed with PBS before addition of spleen cells (5×10^5 per well). For stimulation with whole MBP and Ac-MBP-(1–17) peptide, spleen cells (5×10^5 per well) were incubated with whole protein or peptide at 300 μg/ml. After 3 days, cultured cells were incubated with 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine for 18 hr and harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA).

RESULTS

Evaluation of Differential Superantigen Pretreatment on the Induction or Reactivation of EAE in PL/J Mice. We first demonstrated that SEA can induce an initial episode of EAE in PL/J mice whose $V_\beta 8^+$ T cells were anergized and depleted by pretreatment with SEB. As presented in Table 1, Exp. 1, PL/J mice were pretreated with SEB and 4 days later were immunized with rat MBP. Pretreatment of PL/J mice with SEB prior to immunization with rat MBP as outlined in *Materials and Methods* has been shown to provide protection from development of EAE (21, 22). No clinical signs of EAE were observed in these mice for >1 month. At this point, mice were separated into two groups which were administered either SEB or the non-$V_\beta 8$-specific superantigen SEA. Those mice administered SEA exhibited an accelerated induction of EAE as evidenced by a mean time of onset of 4.0 ± 2.0 days. This onset was quite rapid when compared with onset of EAE after immunization with rat MBP, which ranges between 10 and 14 days (16). Only one mouse from the group which was administered SEB developed signs of EAE, with onset at day 9. A small number of $V_\beta 8^+$ T cells may have escaped anergy and/or deletion during the initial SEB pretreatment, which may explain why disease occurred in this mouse.

Another group of PL/J mice were pretreated with SEA prior to immunization with rat MBP (Table 1, Exp. 2). SEA did not provide protection from development of EAE, as these mice exhibited a disease incidence of 8/10. This observation is consistent with the non-$V_\beta 8$ specificity of SEA. Two weeks after resolution of all clinical signs of EAE, mice were separated into two groups which were administered either SEA or SEB. The SEA recipients did not develop EAE. However, the SEB recipients exhibited a reactivation of EAE similar to that which was previously reported (24). From these data, it appears that PL/J mice protected from induction of EAE by SEB pretreatment remain vulnerable to induction of an initial episode of EAE when exposed to a non-$V_\beta 8$-specific superantigen such as SEA. Conversely, pretreatment with SEA does not protect mice from induction of disease by rat MBP, which involves $V_\beta 8^+$ T cells. Thus, although MBP induces EAE via activation of $V_\beta 8^+$ T cells, there are non-$V_\beta 8^+$ T cells that are sensitized to MBP that are apparently not involved in the initial episode of EAE. These cells are driven to cause EAE by non-$V_\beta 8$-specific superantigens such as SEA.

$V_\beta 8^+$ T-Cell Profiles of Superantigen-Treated PL/J Mice. $V_\beta 8$ profiles were determined for the mice which were pretreated with either SEB or SEA in Exps. 1 and 2 of Table 1. SEB-pretreated mice, regardless of which superantigen was administered after immunization with rat MBP, exhibited SEB-induced deletion of the $V_\beta 8^+CD4^+$ T-cell subset when compared with naive PL/J mice (Fig. 1). This suggests that $V_\beta 8^+CD4^+$ T cells may not play a major role in the accelerated induction of EAE caused by the administration of SEA.

PL/J mice which were pretreated with SEA, immunized with rat MBP, and administered SEA exhibited levels of $V_\beta 8^+CD4^+$ T cells similar to their naive counterparts (Fig. 1). In contrast, mice which were pretreated with SEA, immunized with rat MBP, and administered SEB exhibited an expansion of their $V_\beta 8^+CD4^+$ T-cell subset. All superantigen-pretreated

Table 1. The non-$V_\beta 8$-specific superantigen SEA can induce an initial episode of disease in PL/J mice previously protected from development of EAE

					Day 3			Day 9		
Exp.	Superantigen pretreatment*	Immunization with rat MBP	Disease incidence	Superantigen administration†	Disease incidence	Mean severity	Mean day of onset	Disease incidence‡	Mean severity	Mean day of onset
1	SEB	+	0/8	SEA	2/4	3.0 ± 0	2.5 ± 0.7	3/4	2.7 ± 0.6	4.0 ± 2.0
				SEB	0/4	—	—	1/4	2	9
2	SEA	+	8/10	SEA	0/5	—	—	0/5	—	—
				SEB	3/5	1.2 ± 0.3	3.7 ± 0.6	3/5	1.8 ± 0.7	3.7 ± 0.6
3	None	−	0/10	SEA	0/5	—	—	0/5	—	—
				SEB	0/5	—	—	0/5	—	—

*PL/J mice received either SEB (50 μg in 0.2 ml of PBS) or SEA (50 μg in 0.2 ml of PBS) 4 days before immunization with rat MBP.

†At least 1 month after MBP immunization or after resolution of all clinical signs of EAE, mice were separated into two groups and received either SEB (40 μg in 0.2 ml of PBS) or SEA (40 μg in 0.2 ml of PBS) and pertussis toxin (400 ng).

‡We hypothesized that mice that received the sequence SEB → MBP → SEA (Exp. 1) or SEA → MBP → SEB (Exp. 2) would develop EAE, whereas mice that received SEB → MBP → SEB (Exp. 1) or SEA → MBP → SEA (Exp. 2) would not develop EAE. Results showed that 6 of 9 mice developed EAE where expected while 1 of 9 mice developed EAE where EAE was not expected. These two groups were significantly different by χ^2 analysis ($P = 0.0156$).

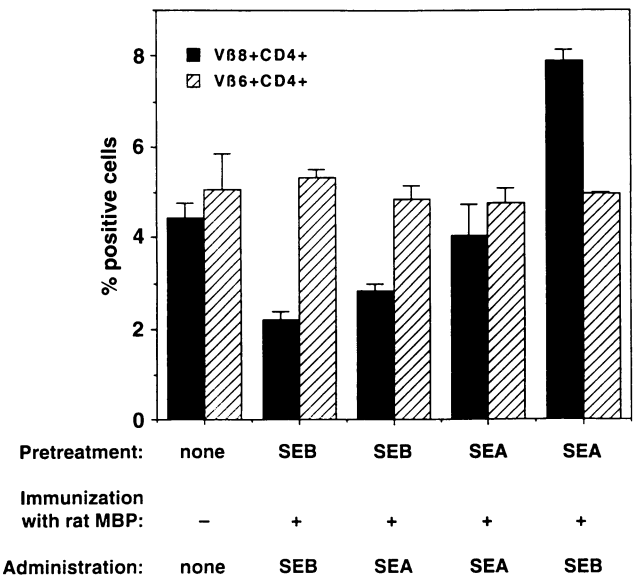

FIG. 1. Two-color flow cytometric analysis of the $V_\beta8^+CD4^+$ T-cell subset in SEB- or SEA-pretreated PL/J mice. Solid bars, $V_\beta8^+CD4^+$; hatched bars, $V_\beta6^+CD4^+$. Samples were run in duplicate for individual mice from each group ($n = 3$ mice per group) and values are presented as percentage of positively stained cells (mean and standard error). Significance as measured by Student's t test was shown for the decrease in the percentage of $V_\beta8^+CD4^+$ T cells in both groups of mice pretreated with SEB as compared with naive counterparts ($P < 0.02$) and for the increase in the percentage of $V_\beta8^+CD4^+$ T cells in SEA-pretreated mice immunized with MBP and administered SEB as compared with naive counterparts ($P < 0.002$).

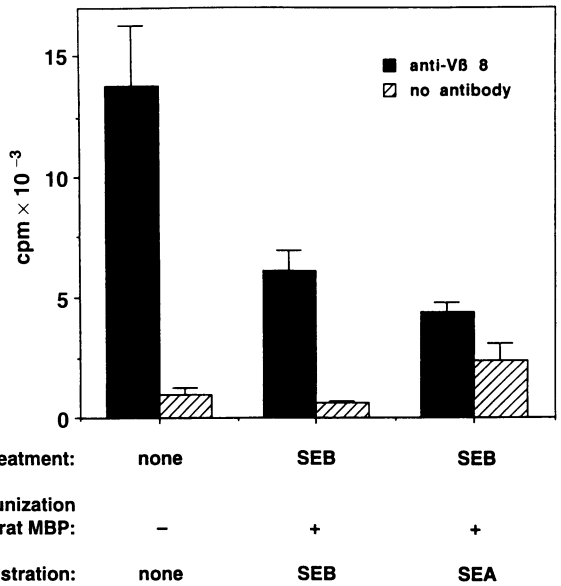

FIG. 2. $V_\beta8$-specific stimulation of T cells from naive PL/J mice and from PL/J mice which were pretreated with SEB, immunized with rat MBP, and administered either SEB or SEA. Solid bars, stimulation with anti-$V_\beta8$ antibody (F23.1); hatched bars, medium alone. Spleen cells were plated at 5×10^5 cells per well. Data are indicated as the mean and standard error of [^{3}H]thymidine incorporation for samples for individual mice from each group ($n = 3$ mice per group). Responsiveness to anti-$V_\beta8$ antibody stimulation between naive and SEB-pretreated mice immunized with MBP and administered SEB was significant ($P < 0.028$), and comparison of naive and SEB-pretreated mice immunized with MBP and administered SEA was also significant ($P < 0.009$), as measured by Student's t test.

mice exhibited levels of $V_\beta6^+CD4^+$ T cells similar to those observed in naive PL/J mice. Thus, expansion of $V_\beta8^+CD4^+$ T cells coincided with reactivation of EAE in mice which were pretreated with SEA, immunized with rat MBP, and then administered SEB for reactivation of EAE.

The SEB-pretreated PL/J mice in Table 1, which were administered either SEB or SEA after immunization with rat MBP, were also examined for their ability to respond to $V_\beta8$-specific stimulation *in vitro*. Spleen cells from naive and both groups of SEB-pretreated mice were stimulated *in vitro* with an immobilized anti-$V_\beta8^+$ antibody (Fig. 2). When compared with cells from naive PL/J mice, cells from SEB-pretreated mice administered SEB after immunization with rat MBP had a proliferative response which was reduced by a factor of 2.3. Cells from PL/J mice which were pretreated with SEB but, upon administration of SEA, developed accelerated onset of EAE exhibited a proliferative response that was reduced by a factor of 3.1. Thus, SEB pretreatment induced anergy and deletion of the $V_\beta8^+$ T-cell subset in mice which developed accelerated onset of EAE after SEA administration.

Induction of EAE by SEA Correlates with Expansion of the $V_\beta10^+CD4^+$ T-Cell Subset. It was next determined whether expansion of a SEA-specific V_β^+ T-cell subset occurred in accordance with accelerated onset of SEA-induced EAE. PL/J mice positive for SEA-induced EAE showed a significant expansion of $V_\beta10^+CD4^+$ T cells (Fig. 3). Expansion of the $V_\beta10^+CD4^+$ T-cell subset was the only detected V_β-specific expansion in SEA-induced EAE mice. SEB pretreatment of these mice resulted in anergy of the $V_\beta11^+$ T-cell subset (data not shown). In the case of $V_\beta3$ and $V_\beta17$, these T-cell subsets exist at nearly undetectable levels in the PL/J strain (27). It is possible that $V_\beta1^+$ T cells may also play a role in SEA-induced EAE; however, we were unable to test this possibility. Thus, it appears that $V_\beta10^+CD4^+$ T cells may be important for induction of EAE by SEA.

Cells from SEB-pretreated mice which were immunized with rat MBP and administered SEB were also analyzed,

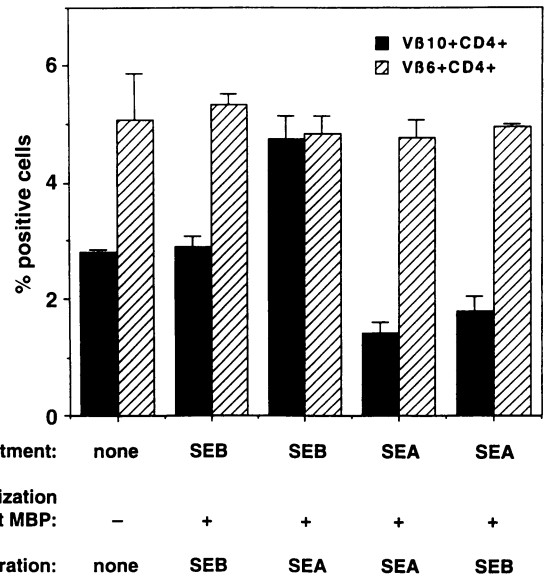

FIG. 3. Two-color flow cytometric analysis of the $V_\beta10^+CD4^+$ T-cell subset in SEB- or SEA-pretreated PL/J mice. Solid bars, $V_\beta10^+CD4^+$; hatched bars, $V_\beta6^+CD4^+$. Samples were run in duplicate for individual mice from each group ($n = 3$ mice per group) and values are presented as percentage of positively stained cells (mean and standard error). Significance as measured by Student's t test was shown for the decrease in the percentage of $V_\beta10^+CD4^+$ T cells in both groups of mice pretreated with SEA as compared with naive counterparts ($P < 0.029$) and for the increase in the percentage of $V_\beta10^+CD4^+$ T cells in SEB-pretreated mice immunized with MBP and administered SEA as compared with naive counterparts ($P < 0.011$).

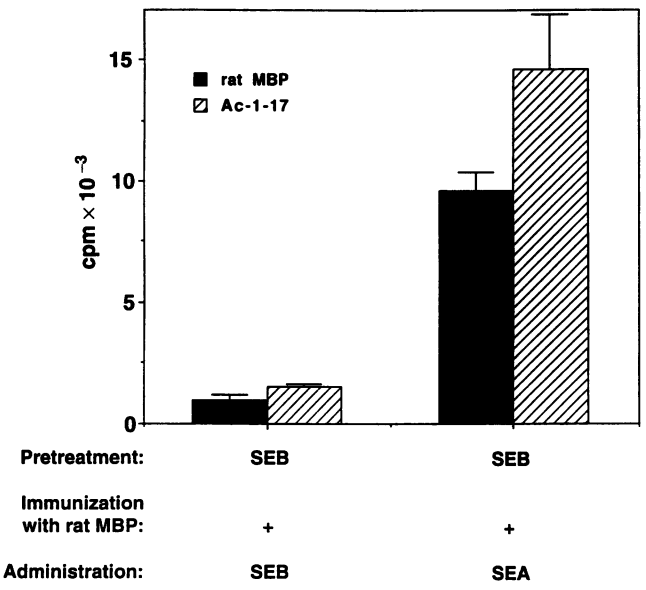

FIG. 4. MBP-stimulated proliferative responses of T cells from SEB-pretreated mice administered either SEB or SEA. Whole MBP and Ac-MBP-(1–17) were used at a concentration of 300 μg/ml [i.e., 16 μM MBP and 150 μM Ac-MBP-(1–17)]. Spleen cells were plated at 5×10^5 cells per well. Background cpm values have been subtracted. Data are indicated as mean and standard error of quadruplicate samples of individual mice from each group ($n = 3$ mice per group). Differences in responsiveness to MBP and Ac-MBP-(1–17) peptide between SEB-pretreated mice immunized with MBP and administered SEB as compared with SEB-pretreated mice immunized with MBP and administered SEA were significant by Student's t test ($P < 0.01$).

revealing levels of $V_\beta10^+CD4^+$ T cells similar to those of their naive counterparts (Fig. 3). In contrast, SEA-pretreated mice which were immunized with rat MBP and administered either SEA or SEB showed a deletion of their $V_\beta10^+CD4^+$ T-cell subset.

PL/J Mice with SEA-Induced EAE Recognize the Amino-Terminal Region of MBP. One of the primary epitopes of MBP recognized by the PL/J strain is the amino terminus (16). The ability of SEB-pretreated mice to proliferate in response to whole MBP or Ac-MBP-(1–17) was examined. SEB-pretreated mice which were immunized with rat MBP and administered SEB displayed a low but significant response to both whole MBP and Ac-MBP-(1–17). Mice exhibiting SEA-induced EAE displayed a vigorous response to both whole MBP and Ac-MBP-(1–17) (Fig. 4). Thus, non-$V_\beta8^+$ T cells specific for the amino-terminal epitope of MBP do exist in SEB-pretreated mice, and the responsiveness to this epitope is amplified after administration of SEA.

DISCUSSION

The effects of superantigen treatment on induction or reactivation of EAE as presented in this study are depicted in Fig. 5. SEB pretreatment prevented induction of EAE. Administration of a second dose of SEB did not induce EAE in SEB-pretreated mice. However, mice protected from EAE by SEB pretreatment remained susceptible to induction of an initial episode of EAE when administered SEA. SEA-induced EAE can be characterized by accelerated onset of disease accompanied by a severity of grade 2–3 within 48 hr. T-cell profiles of these mice revealed the $V_\beta8^+$ T-cell subset to be anergized and deleted. In contrast, the percentage of $V_\beta10^+CD4^+$ T cells was expanded after SEA administration. In addition, T cells from SEA-induced EAE mice responded to *in vitro* stimulation with whole rat MBP and/or Ac-MBP-(1–17) peptide. In common with $V_\beta8^+$ EAE T cells, at least some of the $V_\beta10^+$ T cells were specific for the Ac-MBP-(1–17) encephalitogenic peptide. Thus, the non-$V_\beta8$-specific superantigen SEA can drive MBP-sensitized T cells to induce an initial episode of EAE.

Unlike SEB pretreatment, SEA pretreatment did not prevent development of EAE. However, SEA-pretreated mice could not be reactivated when administered a second dose of

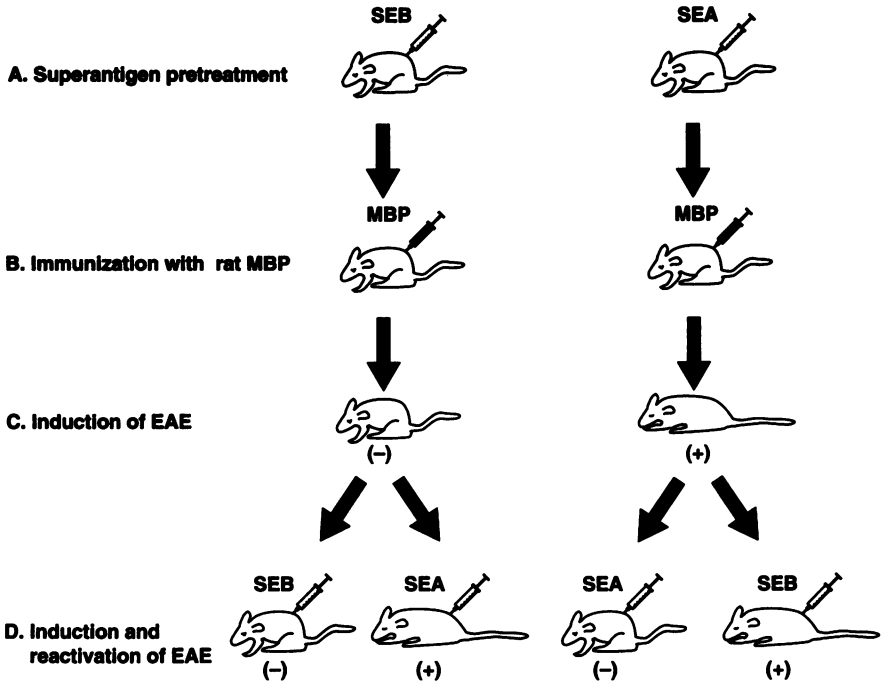

FIG. 5. Modulation of EAE by superantigens with different V_β specificities. Plus (+) and minus (−) signs beneath certain mice denote our original hypothesis regarding development of EAE after administration of either SEA or SEB. In the first group, SEB pretreatment prevented development of EAE and mice administered a second dose of SEB were refractory to development of disease. SEB-pretreated mice administered SEA exhibited accelerated onset of EAE. In the second group, SEA pretreatment did not prevent EAE. After resolution of clinical symptoms, administration of a second dose of SEA did not reactivate disease. SEB administration did, however, reactivate EAE in the SEA-pretreated mice.

SEA. Administration of SEB to SEA-pretreated mice did reactivate EAE. SEA-pretreated mice exhibited a depletion of the $V_\beta10^+CD4^+$ T-cell subset, whereas mice which were administered SEB and underwent reactivation of EAE showed an expansion of the $V_\beta8^+CD4^+$ T-cell subset. Stimulation of $V_\beta8^+$ T cells is thus the cause of reactivation of EAE in mice administered SEB.

This study demonstrates that environmental factors such as the staphylococcal enterotoxin superantigens introduce a level of complexity to disease induction in EAE that may be akin to that observed in MS. Other studies have shown that primary induction of EAE is mediated by $V_\beta8^+$ T cells in naive PL/J mice immunized with either MBP or an amino-terminal peptide of MBP (16). While $V_\beta8^+$ T cells are important for induction of EAE in naive PL/J mice, adding the variable of superantigen treatment suggests that T cells with different V_β specificities also have the ability to mediate disease induction. This is a credible argument since $V_\beta8$ restriction in the PL/J strain is dominant but not exclusive. An expansion of $V_\beta10^+CD4^+$ T cells was observed to correlate with the onset of SEA-induced EAE. Such non-$V_\beta8^+$ T cells that mediate SEA-induced EAE also appear to be specific for the amino terminus of MBP, since T cells from SEA-induced EAE mice proliferated in response to either whole MBP or Ac-MBP-(1–17) peptide. It is not known whether MBP-(1–17) is the specific epitope of MBP that T cells from SEA-induced EAE mice respond to or whether determinant spreading occurs after administration of SEA. Other epitopes of MBP such as aa 35–47, which also have encephalitogenic potential in the PL/J strain, may be of particular importance if such spreading occurs.

A number of studies have attempted to determine whether an oligoclonal T cell population is relevant to the pathogenesis of MS. Production of MBP-specific T cell clones from MS patients revealed biased TCR usage of $V_\beta5.2$ and, to a lesser extent, $V_\beta6.1$ (28). Another study identified a limited oligoclonality among T-cell clones from MS patients using the $V_\beta12$ gene segment (29). In addition, analysis of TCR gene rearrangements from MS patient brain plaques implicated T cells with the TCR $V_\beta5.2$ rearrangement as potentially important in MS (30). Based on information derived from the EAE model and from the studies described above, it has been suggested that a useful avenue of immunotherapy for MS is the targeting and regulation of T cells bearing specific V_β TCRs. Data presented here show that targeting of a V_β-specific T-cell subset that is normally responsible for induction of EAE does not provide absolute protection from further disease induction. It is possible for MBP-sensitized T cells with a different V_β specificity (non-$V_\beta8$) to be responsible for induction of EAE after exposure to the bacterial superantigen SEA. If such a scenario of superantigen-induced autoimmunity does occur in MS pathogenesis, it may be important to consider superantigen effects in the design and development of immunotherapies for MS. This may also explain in part the success of another non-V_β-specific immunotherapy, interferon, as shown by the use of interferon-β for MS (26) and the ability of interferon-τ to block superantigen reactivation of EAE (J.M.S. and H.M.J., unpublished data).

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