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Staphylococcal enterotoxins can reactivate experimental allergic encephalomyelitis

(autoimmunity/superantigen/autoreactive T cells)

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ABSTRACT **Staphylococcal enterotoxins (SEs) are one member of a unique group of molecules known as superantigens. They are potent T-cell activators and stimulate a large number of T cells bearing specific T-cell-receptor β-chain variable regions. It has been proposed that superantigens may trigger autoimmune disorders by stimulation of autoreactive T cells with restricted β-chain variable-chain usage. We investigated the effects of SEs B and A (SEB and SEA) on the reactivation of experimental allergic encephalomyelitis, an animal model for multiple sclerosis. We report that SEB can reinduce encephalitis multiple times in PL/J mice that had previously recovered from an acute episode. SEB was also able to induce encephalitis in mice previously immunized with myelin basic protein but did not show clinical signs of disease. In addition, it was observed that T cells from PL/J mice that had been previously activated by myelin basic protein in complete Freund's adjuvant or in complete Freund's adjuvant alone were resistant to the induction of anergy by SEB. To determine whether reactivation of experimental allergic encephalomyelitis was specific for SEB, another superantigen, SEA, was employed. It was found that SEA was also able to reinduce experimental allergic encephalomyelitis in mice previously recovered from an acute episode and those that had been previously immunized with myelin basic protein but did not show clinical signs of disease. These results indicate that SEs are capable of reactivating autoreactive T cells and inducing autoimmune disease.**

Superantigens such as the staphylococcal enterotoxins (SEs) are among the most powerful T-cell activators known (1–3). Stimulation of T cells by the superantigens occurs first by engaging the class II major histocompatibility complex and then the complex binds to the T-cell receptor (TCR) in a β-chain variable-region (Vβ)-specific manner (1, 4). The SEs B and A (SEB and SEA, respectively), employed in this study, have been shown to be specific for murine T cells bearing Vβ3, -7, and -8.1–8.3 and Vβ1, -3, -10, -11, and -12, respectively (5). The observation that these enterotoxins can activate T cells based on TCR V gene usage has led to the concept that these antigens may trigger autoimmune disorders (6–9).

Experimental allergic encephalomyelitis (EAE) is an animal model of antigen-induced autoimmunity and has been widely studied to gain insight into the inflammatory demyelinating disease multiple sclerosis (MS) (10). PL/J mice immunized with rat myelin basic protein (MBP) develop acute demyelination manifested clinically as tail or hind limb weakness and paralysis. Those mice that survive the acute episode will usually resolve these clinical signs and do not develop clinical relapses (11). It has been shown that in the PL/J mouse, the predominant initiating T cells are an oligoclonal population bearing the Vβ8$^+$ TCR (12–14). Recently, analysis of TCR gene rearrangements directly from MS patient brain plaques suggests that cells with the TCR Vβ5.2 rearrangement may be critical in MS (15).

We wished to evaluate whether SEs could reinduce disease in those mice that had resolved the acute episode of EAE. We report that SEB and SEA can reactivate EAE and that previously activated T cells are resistant to the induction of anergy by superantigen.

MATERIALS AND METHODS

Induction of EAE. PL/J mice (6–8 weeks old; The Jackson Laboratory) were immunized subcutaneously with rat MBP (300 μg) in complete Freund's adjuvant (CFA) plus H37 Ra (4 mg/ml) along with pertussis toxin (List Biological Laboratories, Campbell, CA; 500 ng i.p.; day 0). Two days later pertussis toxin was readministered i.p. Mice developed signs of clinical EAE starting at ≈14 days after immunization. Clinical severity score was based on the following disease severity index: 0, normal; 1, decrease tail tone; 2, tail paralysis; 3, paraparesis; 4, paraplegia; 5, moribund/death.

Injection Schedule for Reactivation of EAE by SEs. For injection of SE for initial reactivation of disease, either SEB (40 μg; Sigma) or SEA (40 μg; Toxin Technology, Sarasota, FL) in 0.2 ml of phosphate-buffered saline (PBS) was administered i.p. with pertussis toxin (500 ng) on the same day 1 month after resolution of clinical symptoms. For subsequent reactivations of EAE by SEB, 40 μg of SEB in 0.2 ml of PBS was administered i.p. with or without pertussis toxin 7–9 days after resolution of clinical symptoms.

T-Cell Proliferation Assay. Spleen cells were obtained from PL/J mice 7 days after the last injection or immunization and the erythrocytes were lysed with 0.84% ammonium chloride. Spleen cells (3×10^5 cells per well) were incubated for 3 days in round-bottom microtiter wells that had been coated with an anti-Vβ8 antibody. The purified anti-Vβ8$^+$ antibody F23.1 was diluted to 10 μg/ml with PBS and 30 μl was added per microtiter well. Plates were incubated at 37°C for 2 h and washed with PBS before adding lymphocytes. After 3 days, cultured cells were pulse-labeled with 0.5 μCi of [^{3}H]thymidine (specific activity, 21 mCi/mg; 1 Ci = 37 GBq) and harvested on a cell harvester (PHD, Cambridge, MA) 18 h later.

Statistical Analysis. Statistical analysis of data presented in Fig. 2 was performed using a nested design with mice nested in treatment groups. Mice were considered a random effect. Statistical significance (at α level 0.05) was assessed by an

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Abbreviations: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; SE, staphylococcal enterotoxin; Vβ, β-chain variable region; CFA, complete Freund's adjuvant; TCR, T-cell receptor; CNS, central nervous system.

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analysis of variance (ANOVA) followed by Student's *t* test. All statistical analysis was done under consultation with the University of Florida Statistics Consulting Division.

RESULTS

Administration of SEB to PL/J Mice Reactivates EAE. Six PL/J mice were initially immunized with rat MBP in CFA followed by pertussis toxin. As can be seen in Fig. 1, 5 of 6 mice developed signs of EAE with a mean severity index of 1.8, which resolved within a week. One month after the development of EAE, when the mice were clinically normal and no other relapse of EAE was noted, 4 mice were injected with SEB and pertussis toxin only (rat MBP was not repeated). Three weeks after this injection, 2 of 4 mice developed a second episode of clinical EAE. This episode resolved and the mice were again clinically normal. Two weeks after resolution of all clinical signs the same 4 mice were again injected with SEB and pertussis, with a relapse of the clinical signs of EAE seen in 3 of 4 mice (onset, 1–2 weeks after injection) (Fig. 1 and Table 1, experiment 1). A similar result was seen in an additional experiment (Table 1, experiment 2). No relapses occurred when mice were injected with pertussis only. Another group of 15 PL/J mice were administered SEB (40 μg i.p.) (mice had not been previously immunized with rat MBP), and none of them developed evidence of encephalitis (data not shown).

Next, we wished to determine whether SEB could reinduce EAE in the absence of pertussis toxin. In a total of four mice that had previously been immunized with MBP and received SEB plus pertussis toxin, two developed clinical signs of EAE (Table 1, experiment 1) with SEB only. However, the severity and duration of clinical signs were less than previous episodes in mice reinduced with SEB only (compared to SEB and pertussis). Nevertheless, SEB alone was capable of reinducing EAE in some mice that had previously received SEB and pertussis toxin.

Finally, we evaluated whether EAE could be induced in those mice that were immunized with rat MBP in CFA and pertussis toxin but who never developed clinical evidence of EAE. A total of three of seven mice developed EAE after injection with SEB and pertussis toxin (Table 1, experiment 3), and of those three, one animal developed EAE after reinjection with SEB only. Thus, SEB is able to induce EAE in mice immunized with rat MBP but who did not develop clinical signs of EAE.

Previously Activated T Cells Are Resistant to the Induction of Anergy by SE. The finding that mice injected repeatedly with SEB developed clinical EAE after each injection was unexpected. Although SEB can activate Vβ8$^+$ cells, it has been demonstrated that after this period of activation the Vβ8$^+$ T cells become unresponsive to further stimulation with SEB (or to stimulation with anti-Vβ8$^+$ antibodies) (16–18). We reasoned that either Vβ8$^+$ T cells that were previously activated must be resistant to the development of anergy upon stimulation with SEB or that the pertussis toxin injected along with the SEB prevented the induction of anergy by SEB. Fig. 2 demonstrates that T cells activated *in vivo* with rat MBP in CFA are indeed resistant to the induction of anergy. In Fig. 2*A*, mice were administered SEB 1 week before immunization with rat MBP in CFA or administered SEB 1 week after immunization with rat MBP in CFA. Vβ8$^+$ proliferation was evaluated by stimulation of T cells for 3 days in microtiter wells coated with an anti-Vβ8 antibody followed by [^{3}H]thymidine incorporation. Vβ8$^+$ T cells obtained from animals administered SEB 1 week before immunization with rat MBP in CFA exhibited a reduced response, whereas Vβ8$^+$ T cells from mice administered SEB 1 week after immunization with rat MBP in CFA were not anergized. Interestingly, mice that were immunized with only CFA and received SEB 1 week later were also not anergized (data not shown). Controls consisted of PL/J mice immunized with only rat MBP in CFA. T cells from all three groups proliferated equally well when stimulated with an anti-Vβ9$^+$ antibody *in vitro* (data not shown), suggesting that Vβ8 T cells had been specifically anergized. In addition, in Fig. 2*B* we evaluated Vβ8$^+$ T-cell proliferation in one group of mice (EAE → SEB) that had previously developed a clinical episode of EAE and who were administered SEB 1 month after the episode of EAE. Vβ8$^+$ T cells from these mice also failed to be anergized after *in vivo* exposure to SEB. These results show that the timing of administration of SEB determines whether cells are anergized or activated. Thus, it appears that Vβ8$^+$ T cells stimulated *in vivo* by rat MBP and CFA or CFA alone are resistant to the induction of anergy by SEB.

Pertussis Toxin Cannot Overcome Anergy Induced by SE. Fig. 2*B* demonstrates that pertussis toxin cannot overcome the anergizing effects of SEB when injected simultaneously with SEB in previously unimmunized animals although pertussis toxin itself is not a superantigen (19). It had previously been demonstrated that pertussis toxin could prevent the induction of T-cell anergy to an encephalitogenic peptide of MBP injected i.v. and that pertussis toxin has strong mitogenic effects (19, 20). However, pertussis toxin, when injected simultaneously with SEB, was not able to overcome SEB induced T-cell anergy.

Administration of SEA to PL/J Mice Also Reactivates EAE. While SEB can activate Vβ8$^+$ T cells, SEA cannot. To determine whether reactivation of EAE was specific for SEB, we immunized a separate group of PL/J mice with rat MBP and pertussis toxin. Six out of 10 mice developed EAE. After remission of clinical EAE, mice were then injected with SEA and pertussis toxin. The six EAE mice developed a clinical relapse of EAE (Table 2). The four mice that were immunized with MBP but did not develop clinical symptoms were also injected with SEA (40 μg i.p.) and pertussis toxin 1 month after resolution of clinical symptoms. A total of two of four

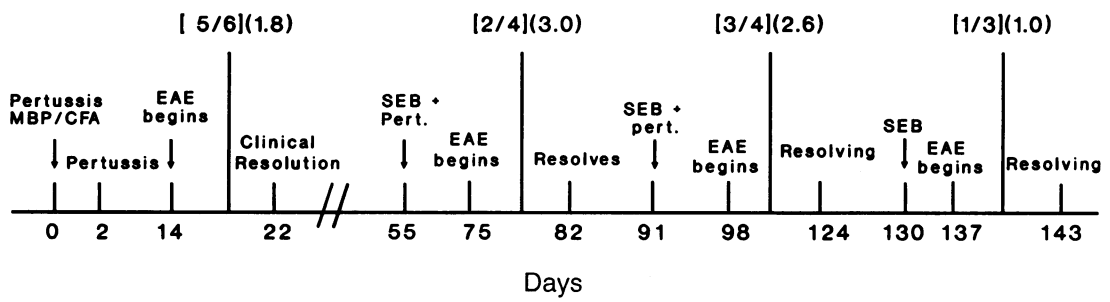

FIG. 1. Time course of reinduction of EAE in PL/J mice initially immunized with rat MBP in CFA and pertussis (Pert.) and given multiple injections of SEB. A relapse of EAE occurred each time after immunization with either SEB and pertussis or SEB alone. Numbers in brackets represent number of mice with EAE/total number injected. Numbers in parentheses represent mean severity index.

Table 1. Reactivation of EAE with SEB

Exp.	Incidence of disease, no. mice with EAE/total no. injected			
	Immunization with rat MBP	First injection SEB plus pertussis	Second injection SEB plus pertussis	Third injection SEB only
1	5/6 (1.8)	2/4 (3.0)*	3/4 (2.6)*	1/3 (1.0)
2	3/3 (2.0)	1/3 (3.0)	—	1/1 (2.0)
3	0/7†	3/7 (3.7)	—	1/3 (2.0)

All mice in all experiments were initially immunized with MBP in CFA and pertussis to induce EAE. Some mice, however, never developed clinical signs of EAE. Whether or not mice developed EAE, they were subsequently administered SEB (40 μg i.p.) and evaluated for clinical signs of EAE. Numbers in parentheses are mean severity index.

*One mouse that did not develop EAE after the first injection of SEB and pertussis developed EAE after the second injection.

†These mice were pooled from several previous experiments in which mice were immunized with MBP and pertussis. Although some mice developed clinical EAE, the seven mice used in this experiment never developed clinical signs of EAE.

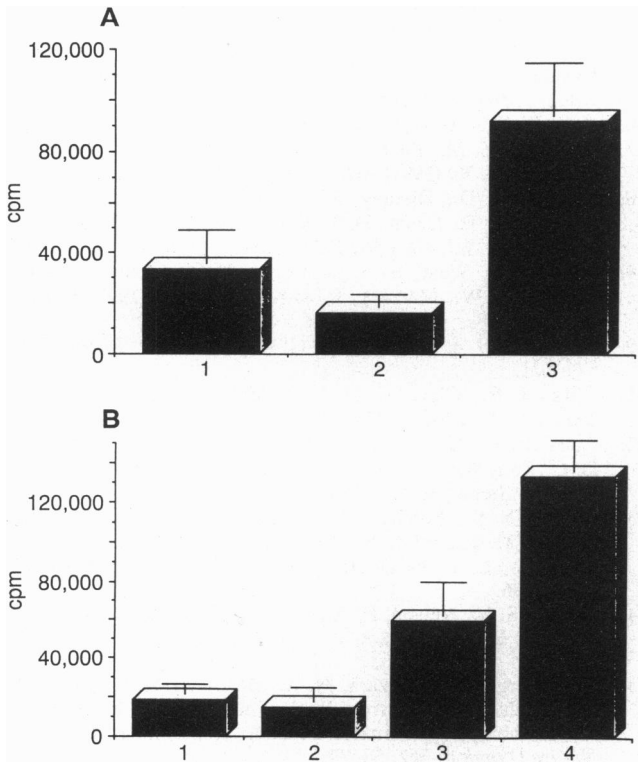

FIG. 2. Induction of T-cell anergy by superantigen is prevented by previous activation. (*A*) Bars: 1, group was immunized with MBP only; 2, group received SEB (40 μg i.p.) and was immunized 1 week later with MBP; 3, group was immunized with MBP and received SEB (40 μg i.p.) 1 week later. All groups were sacrificed 1 week after the last immunization or injection. Procedures were timed for sacrifice of all groups on the same day. Proliferation was induced with anti-Vβ8$^+$ antibodies and measured by [^{3}H]thymidine incorporation (16). Group means differed significantly by an ANOVA ($P >$ 0.0031). (*B*) Bars: 1, group received SEB (40 μg i.p.) only; 2, group received both toxins (SEB at 40 μg and pertussis toxin at 500 ng i.p.) simultaneously; 3, group was immunized with MBP and 1 week later received SEB (40 μg i.p.); 4, group was immunized for induction of EAE and developed acute symptoms, and symptoms were resolved. One week after resolution mice received SEB (i.p.). All groups were sacrificed 1 week after the last immunization or injection. Again, procedures were timed for sacrifice of all groups on the same day. Two to three mice were used per group per experiment and proliferation was induced with anti-Vβ8$^+$ antibodies (16). Significance between groups 1 and 2 versus groups 3 and 4 was determined by an ANOVA ($P < 0.0001$). Controls were performed for all experiments by measuring proliferation induced with anti-Vβ9$^+$ antibodies or in the absence of antibody. Cells from all groups proliferated equally well when stimulated with anti-Vβ9$^+$ antibodies.

of these mice exhibited clinical evidence of EAE (Table 2). Thus, like SEB, SEA can induce EAE in mice that developed clinical signs of EAE and in those mice that were immunized with MBP but did not develop clinical symptoms.

DISCUSSION

There are several interesting features of superantigen-induced EAE. First, we were able to induce relapses of EAE with SEB, SEB plus pertussis toxin, or SEA plus pertussis toxin. We have previously attempted to induce relapses of EAE in PL/J with MBP without success. One possible mechanism for this resistance to reinduction is an active suppression preventing reactivation of rat MBP-reactive T cells. However, with SEB alone or SEB/SEA plus pertussis toxin (but not pertussis toxin alone), we were able to overcome this effect. Furthermore, we were able to demonstrate that EAE could be reinduced multiple times, each incident occurring after an SEB injection. However, at the present time we have not observed any evidence of a spontaneous relapsing EAE in mice treated with SEB but no MBP. We suggest, therefore, that the mechanisms for suppressing the acute autoimmune illness remain intact in mice immunized with MBP but can be overcome with SEB alone. We have also shown that reactivation of EAE is not specific for SEB. We believe that a likely explanation for the finding that SEA can reactivate EAE is that once EAE is initiated by Vβ8$^+$ T cells, T cells with multiple specificities can be found within the inflammatory lesions in the central nervous system (CNS). Enterotoxins of various specificities can then activate these T cells with resultant cytokine release such as interleukin 2, tumor necrosis factor, or interferon γ. This may lead to reactivation of EAE secondary to the direct effects of these cytokines on neuronal function or to the indirect activation of T cells. Such autoreactive T cells (*i*) may be specific for other CNS antigens (e.g., proteolipid protein), (*ii*) may be specific for subdominant epitopes of MBP (21), or (*iii*) may enter the lesion due to breakdown of the blood–brain barrier or be

Table 2. Reactivation of EAE by SEA

Initial development of EAE	Incidence of disease, no. of mice with EAE/total no. injected	
	Immunization with rat MBP	Injection SEA plus pertussis
Yes	6/6 (2.3)	6/6 (2.9)
No	0/4	2/4 (3.0)

PL/J mice were injected with SEA (40 μg i.p.) 1 month after resolution of clinical signs of EAE. Numbers in parentheses are mean severity index.

attracted to the area nonspecifically by inflammatory mediators.

Injection of SEB does not induce anergy in previously activated T cells. We have shown that mice previously immunized with MBP in CFA or CFA alone are resistant to induction of anergy by superantigen and that such resistance is not MBP-specific. Previous *in vivo* studies demonstrating the induction of anergy and apoptosis by superantigen were performed in naive previously unimmunized animals (16–18). In fact we have demonstrated (22) that PL/J mice injected with SEB 5 days before immunization with rat MBP in CFA and pertussis are protected from the development of EAE. The mechanism for the induction of anergy with SEs is not known. In any event, changes in the characteristics of the T cells from a naive to activated cell induced by immunization seem to prevent the induction of anergy in these cells. Therefore, the timing of the administration of SE determines whether cells are anergized or activated.

Clinical signs of EAE could be induced in mice that previously showed no clinical signs of disease. There are several possible explanations for this: The initial immunization was ineffective in producing a critical threshold of MBP-reactive T cells in the CNS, the T cells never reached the CNS to produce clinical disease, or the mice developed subclinical disease that was not evident on examination. Another group has attempted to induce EAE in mice previously immunized with MBP but who did not show clinical evidence of EAE, by using repeated injections of pertussis toxin, MBP, or irradiation without success (23). By using SEB or SEA we were able to do so. Thus, superantigen was able to initiate disease symptoms in immunized but asymptomatic animals harboring autoreactive T cells.

We believe that superantigens such as the SEs are capable of reactivating autoreactive T cells and inducing autoimmune diseases such as MS. It has been suggested (7) that superantigens may contribute to the development of the relapsing and remitting cycles characteristic of various autoimmune diseases. One possibility is that individuals with a previous history of an autoimmune illness, such as MS, systemic lupus erythematosus, or rheumatoid arthritis may be induced to develop acute flares of their illness after a clinical or subclinical staphylococcal infection with a superantigen-producing organism. Therefore, we propose a "two-hit" hypothesis in the induction of some cases of autoimmune disease. First, autoreactive T cells may be stimulated after an infection through, for example, a mechanism of molecular mimicry. No autoimmune disease may develop as either insufficient numbers of autoreactive cells are stimulated or mechanisms to suppress the proliferation of these cells develop. A second infection with a superantigen-producing organism develops (which may also be subclinical) with reactivation of the autoreactive cells leading to clinical manifestations. It is also possible that a single organism may produce factors that activate autoreactive T cells, which are then further stimulated by a superantigen produced by the same organism. At the very least, superantigens appear capable of reactivating autoreactive T cells that may lead to clinical disease.

Finally, the data presented here and by others (24) using superantigens to reactivate disease in EAE or bacterial-cell-wall-induced arthritis suggest that this model of MS is more complex than previously thought. For example, specific $V\beta8^+$ T cells are thought to be the "culprits" in induction of EAE in the PL/J mouse, but reactivation of EAE with a non-$V\beta8$-specific superantigen would suggest that other as yet unknown factors and/or mechanisms may also be involved in the exacerbation of MS.

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