Monoclonal Antibodies Defining Functional Sites on the Toxin Superantigen Staphylococcal Enterotoxin B

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Summary

Four monoclonal antibodies (mAbs) were produced binding to four nonoverlapping epitopes on the superantigen staphylococcal enterotoxin B (SEB). The mAbs were tested for their ability to detect SEB bound to major histocompatibility complex (MHC) class II, to inhibit SEB binding to MHC class II, to inhibit SEB stimulation of T cell hybridomas, to bind to various nonfunctional mutants of SEB, and to capture and present SEB and its mutants to T cells in the absence of MHC class II. We concluded that two mAbs, B344 and B327, bound to epitopes not required for superantigen function, one mAb, 2B33, blocked an MHC interaction site on SEB, and the fourth mAb, B87, blocked the T cell recognition site on SEB. Moreover, two mAbs (B344 and 2B33) were capable of presenting SEB, although much less efficiently than APC, to CD4⁻ but not CD4⁺ T cell hybridomas. The results confirm the functional domains on SEB originally defined by mutation and show that MHC class II is not always an essential component of the superantigen ligand.

Staphylococcus aureus produces a set of exotoxin superantigens that cause food poisoning and toxic shock in man and rapid weight loss in mice sometimes leading to death (1–5). As superantigens these proteins bind to MHC class II molecules and activate a large set of T cells in a $V\beta$-specific fashion (6–12). This T cell stimulation and the accompanying massive release of lymphokines appear to play an essential role in the toxicity of these proteins (13, 14).

Although the amino acid sequences of the staphylococcal toxins are related, each has a unique $V\beta$ specificity. For example, in mice staphylococcal enterotoxin B (SEB)[1] stimulates T cells bearing $V\beta7$, 8.1, 8.2, and 8.3 (6, 10, 11), whereas SEA stimulates T cells bearing $V\beta1$, 3, 11, and 17a (10). We have identified mutants of SEB impaired either for binding to MHC or for interaction with $\alpha\beta$ TCR-$V\beta$s (14). These mutations in SEB also eliminated in vivo toxicity in mice. When mapped on the structure of SEB, these mutations indicated different sites on the SEB surface for $\alpha\beta$ TCR versus MHC interaction (15).

In the current study we have confirmed these conclusions using a set of four anti-SEB mAbs specific for four nonover-lapping epitopes on SEB. Two of these antibodies define an MHC and an $\alpha\beta$ TCR–interaction site on SEB, whereas, the other two react with sites not involved in SEB superantigen activity. Moreover, we found that two of these mAbs could substitute for MHC class II in presentation of SEB to T cell hybridomas.

Materials and Methods

Cell Lines. Three T cell hybridomas were used in these studies, all derived from B10.BR mice. KS-6.1 ($V\beta8.2+$) and KS-47.1 ($V\beta8.3+$) both respond strongly to SEB presented by a variety of class II MHC molecules (6, 14). KSEA-1 ($V\beta1+$) responds to SEA presented by a variety of class II MHC molecules (16). The HLA-DR1 homozygous lymphoblastoid line, LG2, was used as the antigen-presenting cell line for SEB and SEA (14, 17).

Toxins. SEA was obtained from Toxin Technology (Madison, WI). Recombinant wild type and mutant SEBs were affinity purified from lysates of *Escherichia coli* as previously described (14). Fluoresceinated SEB (F-SEB) was produced using fluorescein isothiocyanate by standard techniques.

IL-2 Assays. Stimulations of T cell hybridomas to produce IL-2 were done in 96-well microtiter plates as previously described (18) using 5 × 10⁴ hybridoma cells, 5 × 10⁴ LG2 APC cells and various concentrations of SEB. IL-2 production was measured using the MTT assay (19).

Mice. B10.QβBR mice (20) were bred in the animal care fa-

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cility at the National Jewish Center for Immunology and Respiratory Medicine.

B Cell Hybridomas and mAbs. B cell hybridomas secreting mAbs specific for SEB were produced by fusion of the hybridoma cell line SP2/0 (American Type Culture Collection, Rockville, MD) to spleen cells from SEB immunized B10.QβBR mice. The mice were immunized first subcutaneously with 50 μg SEB in CFA, then boosted intraperitoneally at biweekly intervals twice with 50 μg of SEB in IFA. After several months rest a final boost with 100 μg i.p. of SEB in saline was given 3 d before sacrifice. Anti-SEB–secreting hybridomas were identified by ELISA and cloned at limiting dilution. mAbs were typed with isotype-specific reagents (Fisher Scientific, Pittsburgh, PA) and purified from ascites fluid or culture supernatants using protein A (for γ2b) or protein G (for γ1) (Sigma Chemical Co., St. Louis, MO). Purified antibodies were biotinylated using N-hydroxylsuccinylbiotin (Sigma Chemical Co.).

Affinity Measurements. The affinities of anti-SEB mAbs were determined using a BIAcore (Biosensor; Pharmacia, San Diego, CA). Each mAb was coupled to the support of a single channel of the instrument to a level of ∼20,000 RU using carbodiimide and n-hydroxysuccinimide. On and off rates were measured at a flow rate of 5 μl per min using SEB in the range of 25 to 400 nM in a buffer of phosphate saline. 5 μl of glycine buffer (100 nM), pH 2.7, was used to regenerate the antibody between measurements.

ELISA. Wells of microtiter plates were coated with one of the anti-SEB mAbs (10 μg/ml), washed, and blocked with fetal bovine serum (FBS). They were then loaded with excess SEB. The captured SEB was detected using biotinylated forms of other anti-SEB mAb, alkaline phosphatase-coupled avidin (Fisher Scientific), and p-nitrophenylphopshate as substrate. Optical density of the wells was read at 405 nm using an automated plate reader (BIO-TEK Instruments Inc., Winooski, VT).

Toxin Binding to DR1 Class II MHC. Binding of toxin to DR1 class II molecules on LG2 was measured using two fluorescence assays. In the first, 3×10^5 LG2 cells were incubated with different concentrations of F-SEB at 37°C for 2 h in balanced salt solution containing 0.1% Na azide. The cells were washed thoroughly and the extent of F-SEB binding assessed using an Epics C flow cytometer (Coulter Corp., Hialeah, FL). In the second assay, LG2 cells were incubated with various concentrations of nonfluoresceinated SEB, washed, then incubated with excess of each of the anti-SEB mAbs, washed again, then incubated with fluoresceinated goat antibody to the appropriate mouse heavy chain class (Fisher Scientific), and washed again. Finally the cell-bound fluorescence was analyzed as above.

Results

Identification of Four mAbs Specific for Different Epitopes on SEB After preliminary characterization of 10 anti-SEB mAbs produced from B10.QβBR mice, four: B334 (γ1), B327 (γ2b), B87 (γ1), and 2B33 (γ1), were chosen for these experiments. Using a capture ELISA we attempted to detect SEB captured by each of the mAbs with biotinylated forms of the mAbs (Fig. 1). In each case all of the biotinylated mAbs bound well except the one that had been used to capture the SEB indicating that each mAb bound to an independent nonoverlapping epitope on SEB. Using the BIAcore we estimated the affinity of each mAb for SEB (Table 1). The three γ1 mAbs had similar affinities of 5–10 nM, while the γ2b mAb had a somewhat lower affinity of ∼100 nM. Thus in the subsequent experiments differences in affinities played a minor role
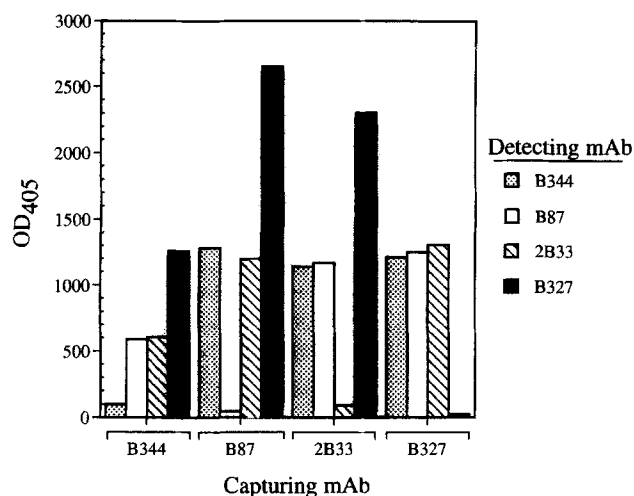
Figure 1. Identification of four mAbs that are specific for independent nonoverlapping epitopes on SEB. The biotinylated version of each mAb was used to detect SEB captured with the unconjugated forms of the four mAbs. Wells of Immulon® three-microtiter plates (Dynatech Labs., Inc., Chantilly, VA) were coated overnight with 100 μl of each mAb (10 μg/ml PBS) and blocked with 25% FBS in PBS. SEB (6 μg/ml PBS) was added at 100 μl/well for 2 h at room temperature, then after extensive washing, 100 μl of the biotinylated form of each mAb (20 μg/ml) was added to a specific set of wells. The bound biotinylated mAbs were detected using Extravidin-alkaline phosphatase and p-nitrophenyl phosphate as substrate. OD of the wells was read at 405 nm using Microplate Autoreader.

in the functional differences among the mAbs, especially the γ1 mAbs.

B87 and 2B33 Inhibit T Cell Hybridoma Response to SEB. The four mAbs were tested for their ability to inhibit the response of the mouse Vβ8.2+ T cell hybridoma, KS-6.1, to a limiting dose of SEB presented by the DR1+ cell line,

Table 1. *Kinetics and Affinity Measurements of the mAbs for SEB*

mAb	k_{ass}	k_{diss}	K_D
	$mol^{-1} s^{-1}$	sec^{-1}	
B344 (γ1)	1.8×10^5	1.0×10^{-3}	6.0×10^{-9}
B87 (γ1)	5.5×10^4	5.2×10^{-4}	9.3×10^{-9}
2B33 (γ1)	3.9×10^5	2.0×10^{-3}	5.2×10^{-9}
B327 (γ2b)	3.9×10^4	3.7×10^{-3}	9.5×10^{-8}

Isotypes of the mAbs were determined using an ELISA. Wells of Immulon® 3 microtiter plates were coated overnight with different mAbs. Isotype-specific secondary reagents coupled to alkaline phosphatase were used to determine the isotype of tested mAbs. Affinity of the mAbs for SEB were measured using the BIAcore machine (Biosensor; Pharmacia). About 20,000 RU of each mAb was immobilized onto a sensor chip, then different concentrations of SEB were used to measure association and dissociation rates for each mAb. Glycine buffer (100 mM), pH 2.7, was used to regenerate mAbs between measurements.
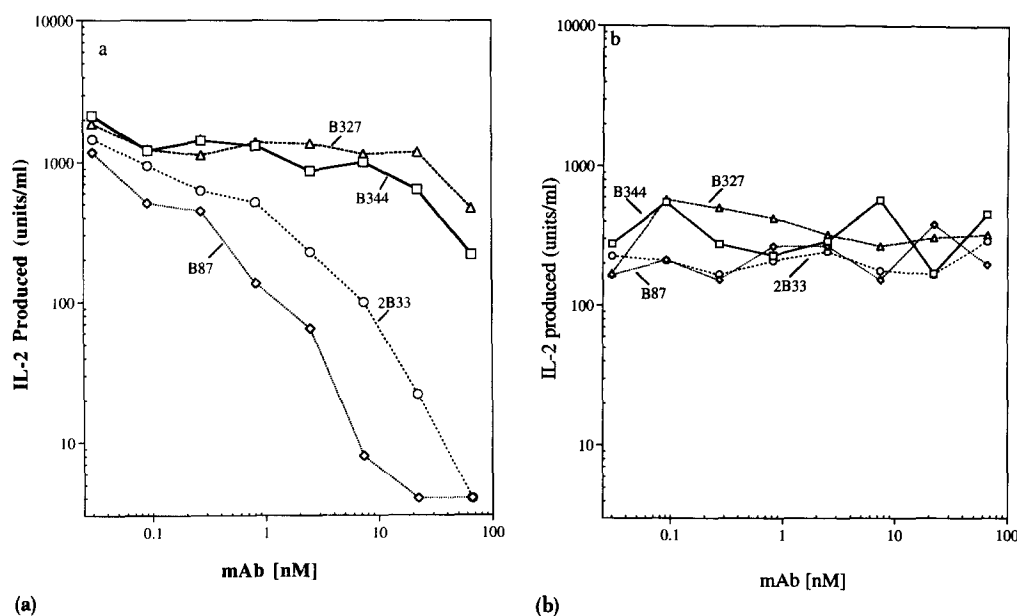
Figure 2. B87 and 2B33 mAbs inhibit the response of KS-6.1 (Vβ8.2) T cell hybridoma to SEB. The mAbs were tested for their capacity to inhibit the response of KS-6.1 (Vβ8.2) T cell hybridoma to SEB (a) or the response of KSEA-1 (Vβ1) T cell hybridoma to SEA (b), in both cases the toxin was presented by (DR1⁺) LG2 cells. Various concentrations of each mAb were incubated overnight at 37°C with 5 × 10⁴ T cells and 5 × 10⁴ LG2 cells in the presence of a limiting dose of toxin (71.4 nM). IL-2 production by was measured as described in the Materials and Methods.

LG2 (Fig. 2 a). The response of the Vβ1⁺ T cell hybridoma, KSEA-1, to SEA was used as a negative control (Fig. 2 b). Dose-dependent inhibition of the response to SEB was seen with both mAb B87 and 2B33. Little inhibition was seen with mAb B344 or B327. None of these antibodies inhibited the response of KSEA-1 to SEA, indicating that the inhibition was specific for SEB.

Correlation of mAb 2B33 and DR1-binding Site on SEB. Since binding of toxin to MHC class II molecules is a prerequisite for presentation by LG2 to the T cell hybridomas (6, 21–23), it is possible that B87 and 2B33 mAbs could have blocked the T cell hybridoma response either by blocking binding of SEB to MHC or by blocking T cell recognition of the toxin/MHC complex. To distinguish these possibilities we tested the ability of the mAbs either to interfere directly with SEB binding to MHC class II or to detect SEB already bound to MHC class II. In the first case we preincubated a limiting amount of F-SEB with various concentrations of each mAb and then tested the ability of the complex to bind to DR1 on LG2 cells. The results (Fig. 3) show that mAb 2B33 was able to block F-SEB binding to LG2, whereas the other three mAbs could not. Interestingly, mAbs B344 and B87, instead of inhibiting, produced enhanced F-SEB binding, whereas B327 had no effect at all. This may have something to do with an increased avidity of the F-SEB for LG2 after dimerization with either B344 or B87. The failure of B327 to show this effect may have been because the location of this epitope on the SEB molecule led to a geometry of the complex which precluded bivalent binding (see below).

In the second experiment the LG2 cells were preincubated with a high concentration of SEB. The unbound SEB was washed away and an attempt was made to detect the bound SEB with each mAb in conjunction with a fluoresceinated secondary reagent (Fig. 4). Both B344 and B87, but neither 2B33 or B327, detected SEB after it was complexed with DR1.

Taken together the results thus far suggested that the epi-

tope detected by 2B33 coincided with an MHC-interaction site on SEB and that the B87 epitope was a good candidate for a TCR-interaction site. The B344 epitope was apparently at some distance from any functional site on the toxin. The B327 epitope was apparently close enough to an MHC interaction site to be blocked after binding of SEB to DR1, but itself did not appear to define a functional site.

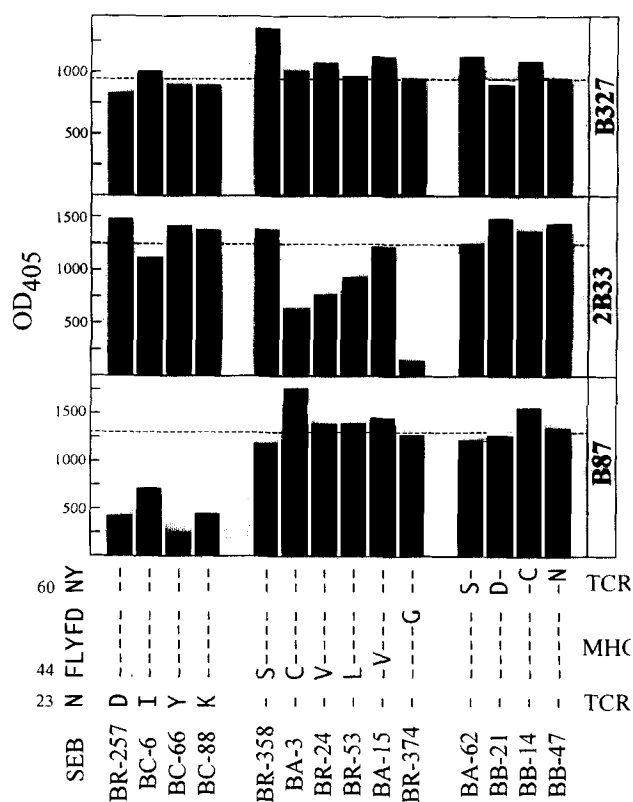*Functional Mutations in SEB Affecting the Binding of mAb 2B33 and B87.* In a previous study we identified amino acids
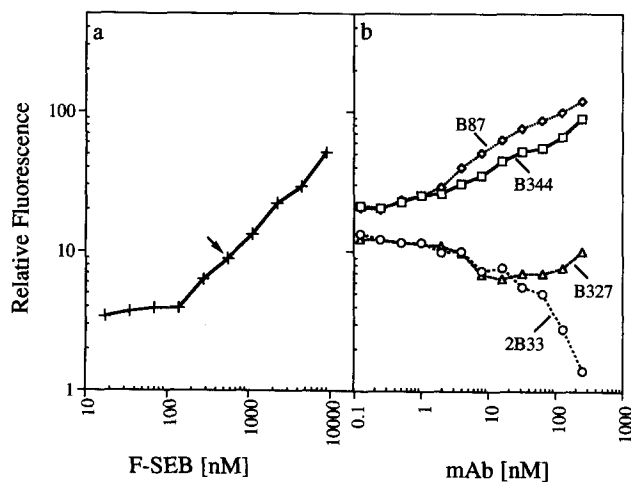
Figure 3. Inhibition of binding of SEB to DR1 expressed on the surface of LG2 cells. (a) Various concentrations of F-SEB were incubated with 3 × 10⁵ LG2 cells for 2 h at 37°C. Excess SEB was washed away from the LG2 cells and the cells were then analyzed for surface fluorescence on an Epics C flow cytometer (Coulter Corp.). Position of the arrow indicates the SEB dose used in the next inhibition experiment. (b) Various concentrations of each mAb were incubated with a limiting dose of F-SEB (as indicated in Fig. 2 a) in complete tissue culture medium (CTM) for 1 h at 37°C. After the first incubation, 3 × 10⁵ LG2 cells were added to each well for 2 h at 37°C. The cells were then washed 4× and analyzed for surface fluorescence.
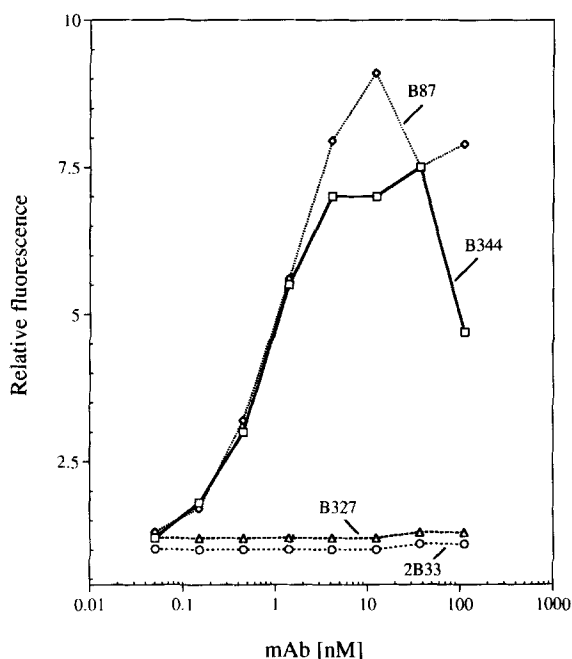
Figure 4. Detection of DR1-bound SEB by B344 and B87 mAbs. SEB (50 pmole) was incubated with 3×10^5 LG2 cells in 100 μl of CTM at 37°C for 2 h. After thorough washing, various concentrations of each mAb were added to the cell suspension for 1 h at 37°C. The cells were washed 4× and incubated again for 15 min at 4°C with fluorescinated goat anti-mouse γ1 or γ2b secondary reagents (Fisher Scientific). The cells were washed thoroughly and then analyzed for surface fluorescence with an Epics C flow cytometer.

Figure 5. Functional mutations in SEB affecting the binding of 2B33 and B87 mAbs. The capacity of the mAbs to bind SEB mutants is tested using a capture ELISA as described in Fig. 1. All mutants were captured with B344 and detected with biotinylated forms of B87, 2B33, and B327 mAbs, respectively. SEB mutants along with their residues that have been changed are illustrated at the bottom of the figure. The functional site affected (MHC or TCR) by specific residue(s) substitution is shown directly below the residue(s) at the bottom right of the figure.

in SEB important in either MHC binding or T cell recognition (14). Mutations in these amino acids diminished either the ability of SEB to bind to MHC class II or the ability of MHC bound SEB to stimulate T cells. To confirm our characterization of the anti-SEB mAbs were tested their ability to bind to SEB carrying either of these types of functional mutations.

Since we had previously shown that the binding of mAb B344 was unaffected by any of the SEB mutations we had produced (14), we used B344 to capture wild-type SEB or any of a number of mutant SEBs. The ability of the captured toxin to bind B327, 2B33, or B87 was tested with biotinylated forms of the mAbs in an ELISA (Fig. 5). None of the mutations tested altered the ability of B327 to bind to SEB. On the other hand the binding of B87 was affected by mutations in N23. Mutations in this amino acid have minimal effect on SEB binding to MHC, but nearly eliminate T cell stimulation (14). Likewise, the binding of 2B33 was reduced by a number of the mutations in amino acids in the region of amino acids 44–48. Most dramatic was the mutation D48>G. Smaller effects were seen with some of the mutations in F44. These mutations all reduce the ability of SEB to bind to MHC by ~1,000-fold (14). Mutations in 60N or 61Y, which also form part of the TCR-interaction site, had no effect on the binding of any of the mAbs. These results mapped the 2B33 epitope as overlapping, but not identical, with the MHC-binding site including amino acids 44–48 and the B87 epitope as including N23.
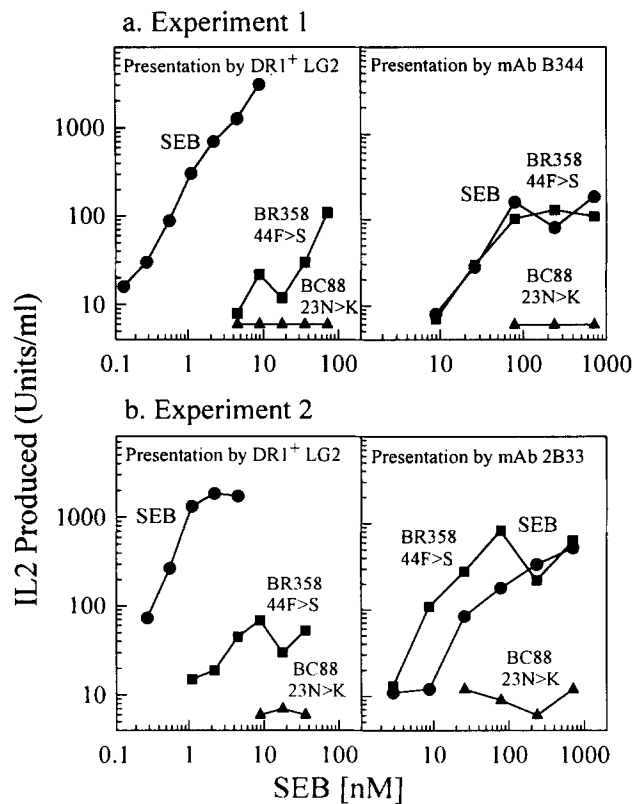
Anti-SEB Substitutes for MHC Class II in Presenting SEB to T Cells. A recent report indicated that toxin superantigens could stimulate T cells in the absence of MHC class II when made polyvalent by capturing with antibody on a solid support (24). Therefore, in a final set of experiments we tested the ability of the mAbs to substitute for MHC class II in presenting SEB. In preliminary experiments, we screened a panel of SEB-reactive T cell hybridomas for response to SEB captured by the immobilized mAbs. All the tested T cell hybridomas that were CD4⁻, but not those which were CD4⁺, responded (data not shown). We have subsequently confirmed that CD4 inhibits recognition of SEB presented by antibody, but not by MHC class II, although the exact mechanism is not yet clear (Hamad, A. R. A., P. Marrack, and J. W. Kappler, manuscript in preparation). Therefore, the CD4⁻ hybridoma, KS47.1, was used in the following experiments. Microtiter plate wells were coated at 100 μg/ml with each of the mAbs in order to saturate the surface. After blocking with FBS, various concentrations of SEB were added along with the T cell hybridoma. As controls in parallel cultures SEB was presented by DR1⁺ LG2 cells. Virtually no response was seen with SEB presented by B87 or B327 (Fig.
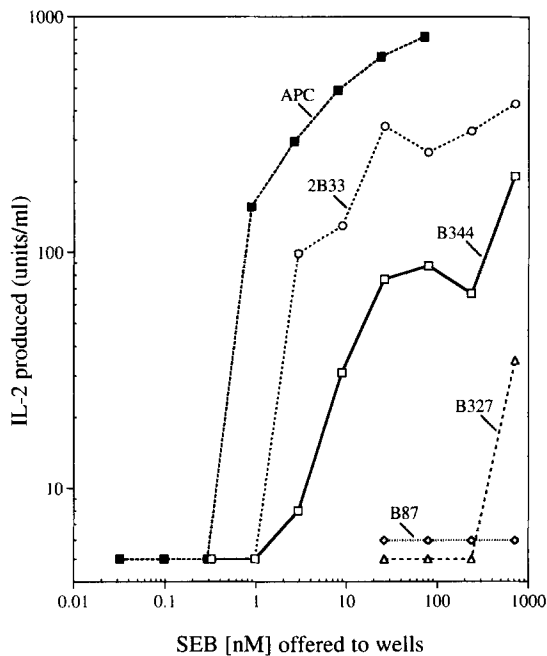
Figure 6. Presentation of SEB to the KS-47.1 (Vβ8.3) T cell hybridoma by B344 and 2B33 mAbs. Wells of Immunolon® 3 microtiter plates were coated with 100 μl of each mAb (666.6 nM) overnight at 4°C. Excess mAbs were washed away and various concentrations of SEB were added at 100 μl/well overnight at 4°C. After a single wash, 5 × 10⁴ KS-47.1 cells were added to each well in CTM. In a parallel set of positive controls wells, LG2 cells were used as an APC. After a 24-h incubation, the amount of IL-2 produced by the T cells was measured.


Figure 7. Wild type and mutants of SEB induce comparable T cell responses when presented by B344 or 2B33 mAbs. SEB and selected mutants were used to stimulate KS-47.1 T cell hybridoma as in Fig. 6. In *a* B344 mAb was used whereas in *b* 2B33 mAb was used to present SEB and its mutants.

6). Strong responses were seen with SEB presented by 2B33 or B344, although ~10–80 times as much SEB was required as with the DR1 bearing antigen-presenting cells. These results indicated that although DR1 was the most efficient in presenting SEB, class II MHC was not absolutely required for presentation provided that SEB was presented in the proper orientation in a multivalent form. The failure of B87 to present SEB was predicted since this antibody blocks the SEB αβ TCR–interaction site. The failure of B327 to present SEB could have been due to its lower affinity or again to the inappropriate geometry of its complex with SEB.

As discussed above, our previous studies established the region around F44-D48 in SEB as important in MHC class II binding, but since this binding was a prerequisite for T cell recognition, we could not formally exclude that these mutations had an effect on the T cell–interaction site of SEB as well. The finding that mAbs could replace MHC in presentation allowed us to test this point in a second experiment. We predicted that we should recover T cell stimulation with these mutants if they are presented with the appropriate mAb rather than with MHC class II. We used the mAbs B344 and 2B33 to present SEB or BR358 (F44>S, which did not effect 2B33 binding [Fig. 5]) to the Vβ8.3⁺ T cell hybridoma, again comparing the responses to those obtained with LG2. The αβ TCR–interaction mutant BC88 (N23>K) was used as a negative control, since its phenotype should be independent of the presenting molecule. Again

wild-type SEB was presented by both LG2 and the mAbs, although the cells were ~10–80 times more efficient than the mAb (Fig. 7, *a* and *b*). As expected, BC88 bearing a mutation in the αβ TCR–interaction amino acid, N23, was not presented by either LG2 or the mAbs. As we previously showed, BR358 was 1,000 times less effective than wild-type SEB when presented by LG2, consistent with its poor MHC-binding properties. However, BR358 was indistinguishable from wild-type SEB when presented by the mAbs (Fig. 7, *a* and *b*). Similar results were obtained with a number of other mutants in the F44-D48 region using B344 as the presenting antibody (data not shown). These results confirm the importance of N23 in T cell recognition and show that mutations in the region around F44, drastically diminish the ability of MHC class II–bearing cell to present SEB but have no effect on αβ TCR interaction.

Discussion

In this study four mAbs were analyzed, each of which recognized a different, nonoverlapping epitope on SEB. Two of these mAbs, B344 and B327, bound to epitopes apparently not involved in the superantigen activity of SEB. We concluded that the epitope bound by the third mAb, B87, included an essential part of the αβ TCR–interaction site on
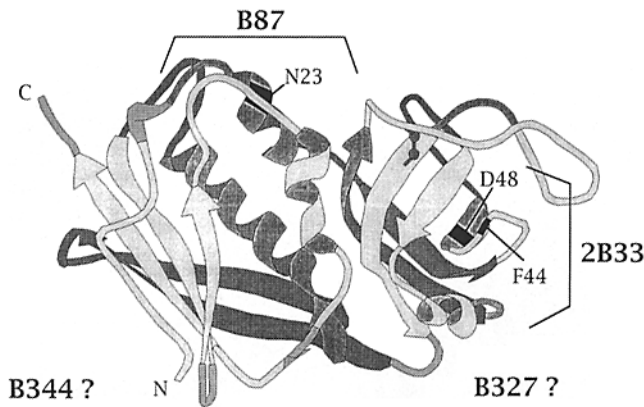
Figure 8. Locations of the epitopes on SEB that are recognized by the mAbs. A schematic diagram of the SEB backbone is shown taken from Swaminathan et al. (15). Amino acids are shown mutations in which effect both mAbs binding and superantigen function. The predicted approximate locations of the epitopes for 2B33 and B87 are shown. The locations of the epitopes for B344 and B327 are not known.

SEB based on the antibody's: (*a*) inhibition of T cell stimulation by SEB; (*b*) failure to inhibit SEB binding to MHC class II; (*c*) ability to detect SEB already bound to MHC class II; (*d*) reduced binding to SEB-bearing mutations in the αβ TCR–interaction amino acid, N23; and (*e*) inability to present SEB or its mutants for T cell recognition. We concluded that the epitope bound by the fourth mAb, 2B33, overlapped an essential MHC-interaction site on SEB based on the antibody's: (*a*) inhibition of T cell stimulation by SEB; (*b*) inhibition of SEB binding to MHC class II; (*c*) failure to detect SEB already bound to MHC class II; (*d*) reduced binding to SEB bearing a number of mutations in the MHC-interacting F44-D48 region; and (*e*) ability to present SEB (or those F44-D48 mutants to which it bound) for T cell recognition. The relevant portions of the SEB molecule are shown schematically in Fig. 8.

The role of MHC class II in superantigen presentation has been a puzzle. Initial experiments appeared to show that MHC class II presentation was essential for αβ TCR recognition

of toxin superantigens suggesting that some portion of the MHC may form an important part of the ligand or that binding to MHC induces a conformational change in the toxin molecule. This view was supported by the inability of nonspecifically immobilized toxins to stimulate T cells. However, thus far no essential αβ TCR/MHC–interaction amino acids have been identified, since most differences in toxin presentation due to allelic, isotypic, or even xenogeneic differences in MHC class II molecules can be attributed to toxin/MHC, rather than αβ TCR/MHC interactions. These findings suggest an alternative view that a large part of the role of MHC class II may be to form a polyvalent ligand by capturing the toxin superantigen in a specific orientation for αβ TCR recognition. This view has received support from several reports of either polyspecific antibodies or other non-MHC class II cellular receptors capable of binding and presenting superantigen toxins to T cells (24, 25).

Our findings here support this latter view. SEB captured by immobilized forms of two of the four anti-SEB mAbs stimulated the T cell hybridoma used in these studies. These results show that an MHC class II molecule is not an absolutely essential part of the SEB ligand for αβ TCRs, but that orientation of the SEB is important, since presentation does not occur with an antibody that interferes with the T cell interaction site on the toxin. However, the efficiency of the presentation was 10–80 times less than that seen with a MHC class II antigen–presenting cell. This could indicate some residual role for αβ TCR/MHC interactions, but could also be due to the differences between a completely immobilized ligand versus one free to move about in a membrane. We have recently produced soluble forms of MHC class II molecules which should allow us to address this question.

Finally antibody titers have been correlated with resistance to the in vivo toxin effects of these bacterial superantigens, although the mechanism is not known (4). This matched set of anti-SEB mAbs with similar affinities should help discriminate among protective mechanisms involving specific blocking of αβ TCR or MHC interaction versus simple antibody-mediated rapid clearance of the toxins.

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