

Cell Surface Molecules Involved in Early Events in T-Cell Mitogenic Stimulation by Staphylococcal Enterotoxins

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We tested the mitogenic response to staphylococcal enterotoxin (SE) type A and SE type B in spleen cells from five strains of mice and found consistent and significant differences among the strains. We chose to study the mitogenic responses of two of these strains, C58BL/6J and BALB/cJ, in greater detail. We investigated the effects of specific monoclonal antibodies to cell surface determinants on SE-induced mitogenesis. Monoclonal antibodies against Ia (class II major histocompatibility complex) determinants blocked SE-induced mitogenesis. Both I-A and I-E molecules can participate in the stimulation, and in BALB/cJ mice which express both types of class II molecules both must be blocked to prevent mitogenesis. Mitogenesis was not inhibited by monoclonal antibodies specific for class I major histocompatibility complex antigens or monoclonal antibodies specific for Mac-1, Lyt-1, or Lyt-2 cell surface proteins. Monoclonal antibodies specific for the T-cell surface antigens L3T4 and T3 also substantially inhibited SE-induced mitogenesis. This implicates participation of the T-cell antigen receptor complex in stimulation induced by the SEs. Elimination of L3T4⁺ helper-inducer T cells abolished the mitogenic response of spleen cells to SE. Reconstitution of L3T4-depleted spleen cells with L3T4⁺ T cells showed that the level of the mitogenic response was directly proportional to the number of L3T4⁺ cells added. Elimination of Lyt-2⁺ cells resulted in a 50% decrease in the response to SEs. These results indicate that L3T4⁺ T cells are required for the mitogenic response to SE, but both L3T4⁺ and Lyt 2⁺ T cells participate in SE-induced mitogenesis. Our results suggest that both Ia and the T-cell antigenic receptor complex are involved in SE-induced mitogenesis.

A family of protein toxins produced by *Staphylococcus aureus* was originally isolated because of its ability to cause food poisoning, and the toxins are called staphylococcal enterotoxins (SEs) because of that biological activity (1). The proteins responsible for these effects were purified and found to be single-chain polypeptides with molecular weights of approximately 28,000 (1, 2). SEs are classified into five types based on their reactivity with specific antisera and are designated SEA, SEB, SEC, SED, and SEE (1, 2). In addition to their ability to cause food poisoning, the toxins stimulate mitogenesis of murine and human lymphocytes (29). SEs were originally classified as T-cell mitogens, since they induce thymocytes to proliferate but cannot induce mitogenesis of spleen cells from nude mice (14). These protein mitogens are not structurally related to plant lectins, do not agglutinate lymphocytes, and do not appear to bind to simple carbohydrate sites on cell surface glycoproteins (37).

Numerous effects of staphylococcal enterotoxins on cells of the immune system have been described. These effects include pronounced enhancement of gamma interferon production in murine and human lymphoid cells (18, 21), induction of mitogenesis (29, 30, 35), suppression of antibody production (19, 35), and suppression of *in vivo* humoral and cellular immune responses (30). SEs are potent inducers of interleukin 2 production and interleukin 2 receptor expression in human lymphocytes (8). More recent reports show that SEs are also active in inhibition of cytotoxic T-cell reactions in both human (31) and murine systems (25). In this report, we present evidence that the SEs mediate their initial action on murine lymphocytes via class II major histocompatibility antigens and T-cell antigen receptors.

Amino acid sequences for some of the SEs have been published (15, 16, 34). A comparison of SEA with SEB

reveals a surprisingly low degree of conserved primary structure between them. A question which arises from this is, do these two structurally different proteins mediate stimulation of T cells in an identical manner? One parameter which may also be relevant in regard to this question is the major histocompatibility complex (MHC) background of the cells used experimentally. To address this issue we used spleen cells from more than one strain of inbred mice with clearly different MHC backgrounds. Two of these strains, C57BL/6J and BALB/cJ, were emphasized since they are the strains of mice used most frequently in studies of SEs.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: Con A, concanavalin A; LPS, lipopolysaccharide; MAb, monoclonal antibody; APC, antigen-presenting cell; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate.

Mice. C57BL/6J (H-2^b), A/J (H-2^a), SJL/J (H-2^s), C3H/HEJ (H-2^k), C3H/OUJ (H-2^k), and BALB/cJ (H-2^d) female mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

Spleen cell preparation. Mice were killed by cervical dislocation, and their spleens were removed aseptically. The spleens were placed in ice-cold, serum-free RPMI 1640 (KC Biologicals, Lenexa, Kans.) containing 50 mg of gentamicin (GIBCO Laboratories, Grand Island, N.Y.) per liter. A single cell suspension was prepared by pushing the cells through a 40-mesh screen. The cells were suspended in 144 mM NH₄Cl with 17 mM Tris (pH 7.4) to lyse the erythrocytes (27). The cells were washed twice in serum-free RPMI 1640, counted, and suspended at 4 × 10⁶ cells per ml in complete RPMI 1640. Complete RPMI 1640 contained 10% fetal bovine serum (GIBCO), 1 mM pyruvate, 2 mM L-glutamine, and 50 mg of gentamicin per ml. Viability of cells

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TABLE 1. MAbs used in these studies

Designation	Relevant specificities
M5/114	I-A ^{b,d} and I-E ^d
14.4.4S	I-E (all)
BP107	I-A (all)
MKD6	I-A ^d
11.5.2	I-A ^k
28.16.8S	I-A ^{b,d}
GK1.5	L3T4
RL172	L3T4
145-2C11	T3 epsilon-chain
53-6.72	Lyt-2
3.168	Lyt-2

was determined with trypan blue (27) and was greater than 90% for cells used in *in vitro* assays.

Mitogenicity assay. Spleen cells (4×10^5) in 100 μ l were added to individual wells of a 96-well tissue culture dish, along with 100 μ l of a 2 \times concentration of SEA or SEB (Toxin Technology; Madison, Wis.), Con A (Sigma Chemical Co., St. Louis, Mo.), PHA (Burroughs-Wellcome Co., Research Triangle Park, N.C.), or LPS (L-4005; Sigma), in complete RPMI 1640. The purity of SEs was 95% or greater as reported by Toxin Technology and confirmed by our using polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Control wells contained cells without mitogen in 200 μ l. The cells were incubated at 37°C in 5% CO₂ for 44 h. At that time, the cells were pulsed for 4 h with 1 or 0.5 μ Ci of [*methyl*-³H]thymidine (TdR) (2.0 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 20 μ l of complete RPMI 1640. Contents of wells were harvested by using a semiautomated microdilution harvester (MA Bioproducts, Walkersville, Md.) onto glass fiber disks and were counted in 4 ml of ACS scintillation fluid (Amersham).

A complication of comparing results from numerous [³H]TdR incorporation experiments was significant day-to-day variation in the absolute amounts of [³H]TdR incorporated. We observed that unstimulated control [³H]TdR incorporation also varied significantly day to day. However, if a stimulation index was calculated by dividing the stimulated counts of [³H]TdR incorporated per minute by the unstimulated counts of [³H]TdR incorporated per minute, the values obtained were much less variable. This is indicative of variation in the background, unstimulated population, and a consistent degree of SE-mediated stimulation. Therefore, where a summary of numerous experiments is presented, the results are shown as a stimulation index. Where results from a single representative experiment are presented, the actual counts incorporated per minute are shown.

MAbs. Hybridomas producing MAbs specific for Lyt-1, Lyt-2, Mac-1, L3T4 (clone GK 1.5), and class I and II antigens were obtained from the American Type Culture Collection (Rockville, Md.). Anti-T3 (kindly provided by J. Bluestone, University of Chicago) and anti-L3T4 (clone RL172)-producing hybridomas have been described previously (9, 24). The specificities of the MAbs used in these studies are summarized in Table 1. We used MAbs in any of three forms: crude supernatants from batch cultures, ascites fluid from pristane-primed and irradiated (600 R) BALB/cJ mice, or highly purified. The form of antibody used in individual experiments is indicated on the relevant figures or in figure captions as supernatant dilution, ascites dilution, or micrograms of antibody (Ab) protein per milliliter, respec-

tively. MAbs were purified from tissue culture supernatant or ascites by DEAE chromatography on a high-pressure liquid chromatography system (Waters Associates, Div. Millipore Corp., Milford, Mass.) by a previously reported method for mouse MAbs (13). Fluorescein-conjugated monoclonal MAR 18.5 (22), anti-Lyt-2 (23), and phycoerythrin-labeled anti-L3T4 (10) were obtained from Becton Dickinson Monoclonal Center, Inc. (Mountain View, Calif.). All MAb preparations were tested for the ability to mediate the staining of cells with appropriate cell surface markers before use in assays testing the capacity of these antibodies to block mitogenesis.

Fluorescent-antibody staining. Spleen cells (2×10^6) in 500 μ l were added to a sterile test tube (12 mm \times 75 mm) (Fisher) containing 500 μ l of 2 \times mitogen. These cells were incubated for 0, 24, or 48 h in the presence of mitogen and pelleted by centrifugation at 200 $\times g$ for 3 min. The cells were washed twice in phosphate-buffered saline (pH 7.4) with 1% bovine serum albumin fraction 5 and 0.02% NaN₃, incubated with 100 μ l of monoclonal supernatant or 10 μ g of purified MAb in 100 μ l for 45 min at 4°C, and washed three times in phosphate-buffered saline (pH 7.4) with 1% bovine serum albumin fraction 5 and 0.02% NaN₃. Cells treated with rat MAb were stained with 1.25 μ g of FITC-MAR 18.5, and cells incubated with hamster or mouse MAb were stained with 2 μ g of FITC-labeled goat anti-hamster immunoglobulin G (IgG) (H + L) or 2 μ g of F(ab)₂-goat anti-mouse IgG Fc fragment (Organon Teknika, West Chester, Pa.), respectively, for 30 min at 4°C. The cells were washed three times, fixed in 1% paraformaldehyde in phosphate-buffered saline (pH 7.4), and stored at 4°C. Controls consisted of cells that were washed and fixed without incubation with antibody (background fluorescence control) and cells that were incubated with FITC-MAR 18.5, FITC-labeled anti-mouse, or FITC-labeled anti-hamster only (nonspecific fluorescence control). Cell suspensions were analyzed for fluorescence by flow cytofluorography (Ortho Diagnostics model 2150) by using an argon laser at 488 nm and 400 mW. For each sample, a minimum of 20,000 cells were counted per histogram.

Cell separation. Spleen cell preparations were separated by panning, as previously described (38). Cells obtained by the panning procedure were compared with the initial population by staining representative samples for immunofluorescence of cell surface markers (e.g., Lyt-2 and L3T4) and viability.

Statistical analysis. To determine the concentration of culture supernatant, ascites fluid, or purified MAb which significantly inhibited the mitogenic response, statistical analyses were performed according to techniques described by Sokal and Rohlf (36). All analyses were performed by using the SAS computer package (SAS Institute, Cary, N.C.). Briefly, the counts-per-minute data from scintillation counting were transformed by using the square root transformation, and analysis of variance was performed on the transformed data. The individual significant mean values were determined by using Scheffe contrasts and are designated by an asterisk (see Fig. 2 through 4). The level of significance reported is $P < 0.01$ unless stated otherwise. This level of significance was used to prevent reporting of marginally significant differences. The cost of using this level of stringency obviously entails some risk of missing small significant values. However, a difference between the $P < 0.01$ and the $P < 0.05$ levels of significance was only rarely observed in these analyses.

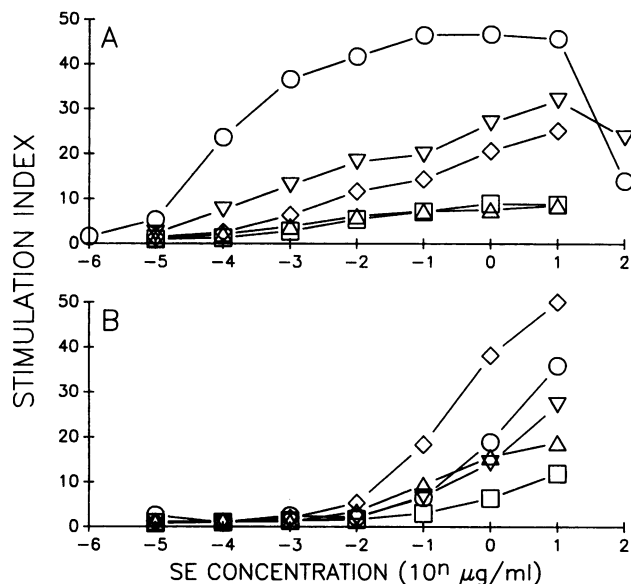


FIG. 1. Mitogenesis of SJL/J (○), C57BL/6J (▽), BALB/cJ (◇), C3H (△), and A/J (□) spleen cells induced by SEA (A) or SEB (B). Mitogenic stimulation was determined as described in Materials and Methods. The number of independent complete dose-response experiments used to calculate the average stimulation indices for SEA and SEB were, respectively, 5 and 5 for SJL/J, 11 and 13 for C57BL/6J, 15 and 17 for BALB/cJ, 3 and 7 for C3H, and 5 and 6 for A/J. Data for C3H spleen cells were pooled from experiments using C3H/HeJ and C3H/OuJ mice. The spleen cell responses for these two substrains of C3H mice did not differ significantly from each other. Stimulation indices were calculated from [³H]TdR incorporation as counts of [³H]TdR incorporated per minute in stimulated spleen cells divided by counts incorporated per minute in unstimulated cultures. Mean counts incorporated per minute into unstimulated cells were 1,348 for SJL, 368 for C57BL/6J, 699 for BALB/cJ, 1,049 for C3H/J, and 558 for A/J.

RESULTS

(i) **Evaluation of the mitogenic potency of SEA and SEB.** We examined the mitogenic response to SEA and SEB of spleen cells from five inbred strains of mice (Fig. 1). The magnitude of mitogenic response to SEA occurred in the following order: SJL > C57BL/6J > BALB/cJ > C3H ≈ A/J. Thus, SJL can be termed high responders, C57BL/6J and BALB/cJ can be termed intermediate responders, and C3H and A/J can be termed low responders to SEA. The same relationship among strains does not apply for SEB stimulation. For SEB stimulation, the relative order for the magnitude of mitogenic response is BALB/cJ > SJL > C57BL/6J > C3H > A/J. For the two inbred strains of mice used most commonly in studies of these toxins, it was evident that C57BL/6J spleen cells exhibited a greater response to SEA than to SEB while BALB/cJ spleen cells were better responders to SEB than to SEA. The surprising result was that the minimum stimulatory concentration was the same for all strains tested. SEA was more potent with respect to stimulating a detectable amount of mitogenesis at a lower concentration (10^{-4} μg/ml) than was SEB (10^{-2} μg/ml), regardless of the inbred strain used.

(ii) **Effects of MAbs against class II MHC antigens on SE-induced mitogenesis.** MAbs specific for cell surface markers were tested for the ability to inhibit SEA- or SEB-induced mitogenesis. The anti-MHC class II MAbs M5/114, 14.4.4S, and BP107 all significantly inhibited mitogenesis of

BALB/cJ spleen cells (Fig. 2). These MAbs interact specifically with I-A^d+I-E^d, I-E^d, and I-A^d, respectively, for BALB/cJ mice. This indicates the participation of both I-E and I-A class II MHC molecules in the response of BALB/cJ spleen cells to SEA and SEB. The antibody 14.4.4S was particularly effective against SEB-stimulated BALB/cJ spleen cells. The I-A^k-specific MAb 11.5.2 was not significantly inhibitory for either SEA- or SEB-stimulated BALB/cJ spleen cells. The I-A^d-specific MAb, MKD6, was not significantly inhibitory to SE-induced mitogenesis. Since M5/114 does not compete for fluorescent staining mediated by MKD6 (S. M. Vroegop and S. E. Buxser, unpublished data), MKD6 and M5/114 apparently recognize different epitopes on the I-A^d molecule.

The abilities of anti-class II MAbs to inhibit SEA- and SEB-stimulated spleen cells from C57BL/6J mice were also tested (results not shown). M5/114, BP107, and 28-16-8S (each recognizes epitopes on I-A^b molecules) all significantly inhibited SEA- and SEB-stimulated C57BL/6J spleen cells. As expected, 14.4.4S, an antibody which recognizes multiple haplotypes of I-E molecules, was not inhibitory, since C57BL/6J spleen cells do not express I-E molecules on their surfaces. Additionally, the 11.5.2 antibody (I-A^k specific) did not inhibit the mitogenic response to SEA or SEB. These experiments with C57BL/6J spleen cells further support the hypothesis that Ia molecules are important for the reaction of SEA and SEB.

Since BALB/cJ mice express both I-A and I-E class II molecules, the relative contributions of both forms of class II molecules can be evaluated in spleen cells from these mice. The antibody 14.4.4S (I-E specific) appears to be a more potent inhibitor than BP107 (I-A specific) (Fig. 2). SEA stimulation was more effectively inhibited by anti-I-A antibody than by anti-I-E antibody. In contrast, anti-I-E-specific antibody (14.4.4S) was more effective than anti-I-A-specific antibody (BP107) in inhibiting SEB-stimulated mitogenesis.

The effect of combined treatment with both BP107 (anti-I-A^d) and 14.4.4S (anti-I-E^d) antibodies was cumulative (Table 2). It was possible to completely inhibit mitogenesis stimulated by either SEA or SEB by treating with both antibodies. Complete inhibition of SEA-induced mitogenesis required relatively high concentrations of both 14.4.4S and BP107 antibody-containing supernatants. In contrast, SEB-induced mitogenesis was nearly completely blocked (83% inhibition) with 10-fold lower concentrations of both antibodies. Thus, BP107 and 14.4.4S alone or in combination were less effective at inhibiting the SEA-stimulated mitogenic response than the SEB-stimulated mitogenic response of BALB/cJ spleen cells. SEB stimulation of BALB/cJ spleen cells was particularly susceptible to neutralization by the anti-I-E-specific antibody 14.4.4S. SEA-stimulated spleen cells were only inhibited 9% by a 1/40 dilution of 14.4.4S. In contrast, SEB stimulation was inhibited 54% by the same dilution of 14.4.4S.

(iii) **Effects of MAbs specific for cell surface molecules in the T-cell receptor complex and relative mitogenic responses by subpopulations of T cells.** The effects of MAbs specific for parts of the T-cell antigen receptor complex were tested in spleen cell cultures from BALB/cJ mice (Fig. 3). Both anti-L3T4 (GK 1.5) and antibody against the epsilon chain of the mouse T3 complex (145-211C) were potent inhibitors of SEA- and SEB-stimulated spleen cells. These results suggest that SE-induced mitogenesis occurs through stimulation of the T-cell antigen receptor complex. In contrast, neither the anti-Lyt-2 MAb, 53-6.72, nor RL172 had a statistically significant effect on SE-stimulated mitogenesis. We also

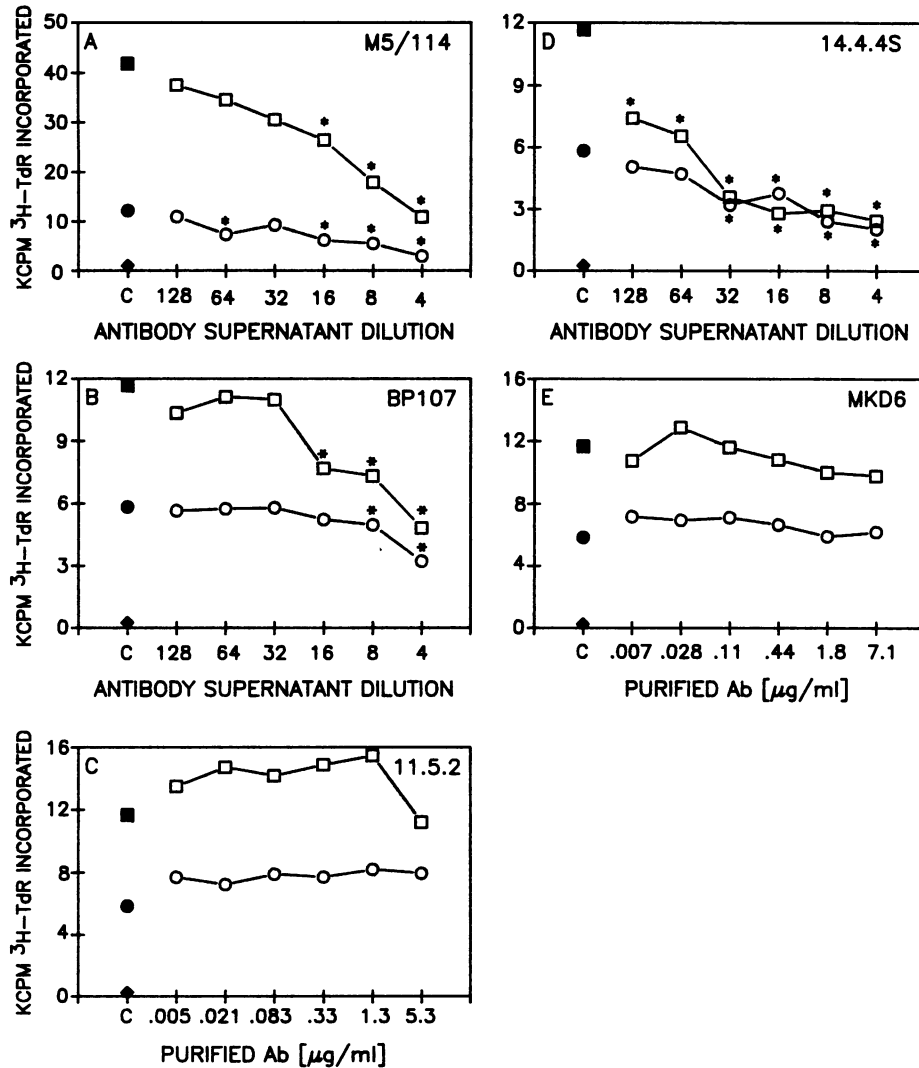


FIG. 2. Inhibition of SEA- or SEB-induced mitogenesis of BALB/cJ spleen cells by MAbs specific for MHC antigens. SEA (1 µg/ml) (○) or SEB (1 µg/ml) (□) was used to stimulate mitogenesis of 4×10^5 BALB/cJ spleen cells. The MAb-containing solution at the concentration indicated was added simultaneously with the SE at time zero. The mean incorporations of [3 H]TdR for four replicate microdilution wells after 48 h of incubation with SE and antibody and a 4-h pulse with 0.5 µCi of [3 H]TdR are shown. Values significantly different ($P < 0.01$) from those for cultures incubated without antibody (solid symbols) are indicated by asterisks. The specific MAb used for each experiment is indicated in the upper right-hand corner of each graph. The results shown are representative of the several experiments, as follows: M5/114 ($n = 10$), 14.4.4S ($n = 3$), BP107 ($n = 3$), MKD6 ($n = 3$), and 11.5.2 ($n = 3$). Kcpm, Thousands of counts per minute.

used a second antibody specific for Lyt-2 (3.168), and no significant inhibition was detected with this antibody.

Both GK 1.5 and 145-211C significantly inhibited SEA- and SEB-stimulated mitogenesis in C57BL/6J spleen cell cultures (data not shown). Anti-Lyt-2 (53-6.72) and RL172 were ineffective at inhibiting mitogenesis of C57BL/6J spleen cells. The effect of these antibodies, specific for conserved molecules in the T-cell antigen receptor complex, was indistinguishable in BALB/cJ compared with the effect on C57BL/6J spleen cell cultures.

We observed the time course of the effect of GK 1.5 on spleen cell mitogenesis. Purified GK 1.5 was only effective at inhibiting mitogenesis when added during the first 4 to 8 h of the 48-h incubation period (Fig. 4). The requirement for early administration of GK 1.5 in order to affect SE-stimulated mitogenesis suggests that L3T4-bearing cells participate in early events in SE-stimulated mitogenesis.

To test the relative participation of spleen T-cell subpopulations in the early stage of SE-induced mitogenesis, we

fractionated the T-cell subpopulations. Spleen cells were depleted of L3T4⁺ cells to greater than 95%, as detected by flow cytometry, and were tested for mitogenesis induced by SEA or SEB. The results of a representative experiment with BALB/cJ spleen cells are shown in Fig. 5. L3T4-depleted spleen cells were inhibited 90 to 100% in their mitogenic response to SE compared with unfractionated spleen cells (Fig. 5A and B). Thus, it appears that the mitogenic response to SEs specifically requires L3T4⁺ cells. Mitogenesis of L3T4-depleted spleen cells stimulated with 2.5 µg of Con A per ml was only partially inhibited, compared with unfractionated spleen cells (Fig. 5C). The mitogenic response to LPS (10 µg/ml) was unaffected by the removal of L3T4⁺ cells (Fig. 5D). This indicates that the effects observed were not due to nonspecific suppression of mitogenesis. L3T4⁺ cells were also depleted from C57BL/6J spleen cells and tested as described above. The results from

TABLE 2. Inhibition of SE-induced mitogenesis of BALB/c spleen cells by combinations of 14.4.4S and BP107 MAb treatments^a

Amt of BP107 added to cells stimulated with SEA or SEB	Mean \pm SE cpm (% inhibition) of cells stimulated with indicated amt of 14.4.4S ^b		
	None	1/40	1/4
Cells with SEA			
None	3,764 \pm 154 (0%)	3,443 \pm 288 (9%)	1,254 \pm 205 (67%) ^c
1/40	6,317 \pm 1,310 (-68%)	2,763 \pm 553 (27%)	1,185 \pm 543 (69%) ^c
1/4	1,847 \pm 855 (51%)	945 \pm 145 (75%)	-259 \pm 48 (107%) ^d
Cells with SEB			
None	4,825 \pm 173 (0%)	2,202 \pm 452 (54%)	653 \pm 173 (86%) ^c
1/40	4,968 \pm 692 (-3%)	812 \pm 154 (83%)	6 \pm 65 (100%) ^d
1/4	1,538 \pm 277 (68%) ^c	80 \pm 76 (98%) ^d	-271 \pm 42 (106%) ^d

^a A total of 4×10^5 BALB/c spleen cells per well in a 96-well microdilution dish were incubated with 1 μ g of SEA (A) or SEB (B) per ml for 48 h in the presence or absence of BP107, 14.4.4S, or both. A total of 0.5 μ Ci of [³H]TdR was added per well during the final 4 h of the incubation before the cells were harvested onto glass fiber filters.

^b The values shown are the mean counts per minute (\pm standard error) of four replicate wells. The values in parentheses are percent inhibitions calculated as: $100 - [(100 \times \text{mean cpm with antibody}) / \text{mean cpm without antibody}]$. Mean incorporation of [³H]TdR for unstimulated cells was 404 ± 140 cpm and was subtracted from each of the values shown.

^c Significantly different from untreated (no antibody) cells.

^d Not significantly different from 100% inhibition.

these assays were indistinguishable from the results obtained with BALB/cJ spleen cells.

Addition of L3T4⁺ cells (enriched by panning with RL172) to L3T4-depleted cells reconstituted the mitogenic response of these cells to both SEA and SEB. Incorporation of [³H]TdR was directly proportional to the number of L3T4⁺ cells added for 0 to 40% L3T4⁺ cells stimulated with SEB and 0 to 60% L3T4⁺ cells stimulated with SEA (Fig. 6). At higher proportions of L3T4⁺ cells, the response leveled off or declined. Use of the highly L3T4 enriched population alone (shown as 100% on Fig. 6) resulted in a relatively

low-level response. This could be because the low number of APCs present in this L3T4-enriched population was capable of supporting only a low-level response. Alternatively, the greater degree of response at lower ratios of L3T4 to other spleen cells may indicate that other lymphocyte subpopulations participate in the mitogenic response to SEs.

The effect of removing Lyt-2⁺ cells is shown in Fig. 7. This resulted in approximately a 50% decrease in the mitogenic response to SE at all concentrations of SEA or SEB tested (Fig. 7A and B). This indicates that although the Lyt-2⁺ cells are not required for the mitogenic response to

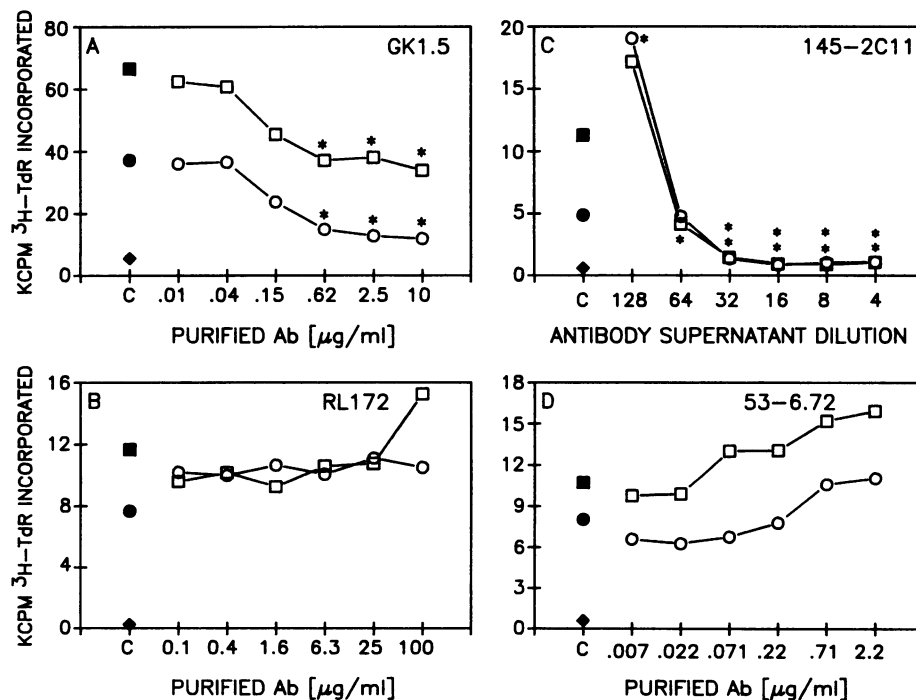


FIG. 3. Inhibition of SE-induced mitogenesis in BALB/cJ spleen cells using MAb specific for the T-cell receptor complex. The procedure used was as described in Materials and Methods and in Fig. 2, except that the antibody specificities were for proteins in the T-cell antigen receptor complex. Values significantly different from the values obtained from cultures stimulated by SEA (O) or SEB (□) in the absence of antibody are indicated by an asterisk. Closed symbols represent cultures not treated with antibody. The results shown are representative of several experiments, as follows: GK1.5 ($n = 16$), 145-211C ($n = 3$), RL172 ($n = 3$), and 53-6.72 ($n = 3$). Kcpm, Thousands of counts per minute.

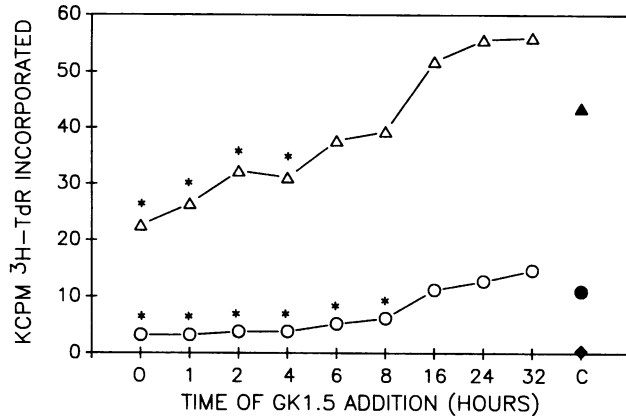


FIG. 4. Time course of inhibition of SE-induced mitogenesis in BALB/cJ spleen cells by the anti-L3T4-specific MAb GK 1.5. BALB/cJ spleen cells (4×10^5) were incubated in 200- μ l cultures in wells of 96-well microdilution culture dishes in the presence of 1 μ g of SEA (O) or SEB (Δ) per ml. At the times indicated, 20 μ l of purified GK 1.5 antibody was added to result in a final antibody concentration of 1.6 μ g/ml. Incubation of the cultures continued for a total of 48 h. [3 H]TdR (0.5 μ Ci) was present during the last 4 h of the incubation period. The incorporation of [3 H]TdR was determined as described in Materials and Methods. Kcpm, Thousands of counts per minute. Values significantly different from the values obtained for cultures stimulated by SEA (●) or SEB (▲) in the absence of antibody are indicated by asterisks.

SEA or SEB, they appear to participate in the response. Con A-induced mitogenesis was decreased by approximately 45% in Lyt-2-depleted spleen cells relative to unfractionated cells (Fig. 7C). Mitogenesis induced by PHA resulted in a 50% decrease in response after depletion of the Lyt-2 cells (Fig. 7D). Lyt-2⁺ cells were also depleted from C57BL/6J

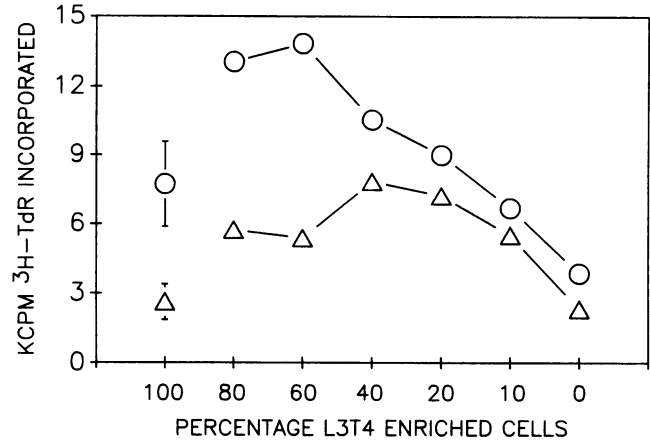


FIG. 6. Reconstitution of L3T4⁺ cells with L3T4-depleted cells. L3T4⁺ cells were prepared from BALB/cJ spleen cells by panning with purified RL172 MAb and obtaining the adherent population. Adherent cells were enriched to greater than 93% L3T4⁺ cells, as measured by flow cytometry. L3T4-depleted cells were the nonadherent cells from the same panning procedure treated with GK 1.5 and complement to deplete any remaining L3T4⁺ cells. L3T4-depleted cells were depleted by greater than 92%, as measured by flow cytometry. Mitogenesis of these reconstituted cells was done in the presence of 1 μ g of SEA (Δ) or SEB (O) per ml. Mean [3 H]TdR incorporations of unfractionated cells were $29,333 \pm 2,867$ for 1 μ g of SEA per ml and $29,737 \pm 1,075$ for 1 μ g of SEB per ml. Mean incorporation of [3 H]TdR for unstimulated cells was $2,884 \pm 625$ cpm. Kcpm, Thousands of counts per minute.

spleen cells and tested for response to SEA, SEB, Con A, and LPS. The results from this assay were indistinguishable from the results described for Lyt-2-depleted BALB/cJ spleen cells.

MAbs with specificity for Lyt-1, Mac-1, or the class I MHC did not inhibit SE-induced mitogenesis significantly.

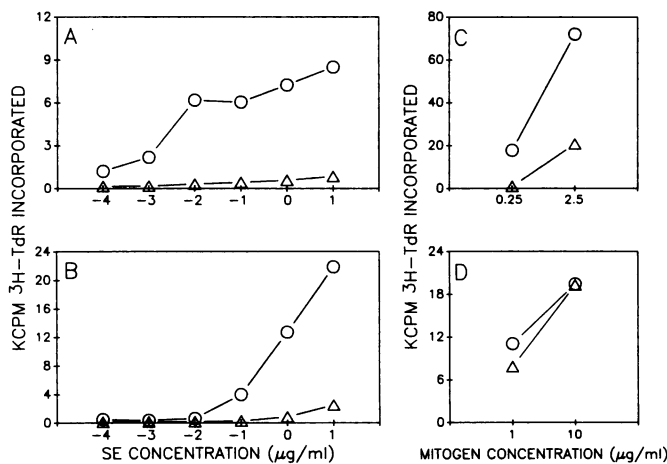


FIG. 5. The effect of L3T4 depletion on SE and lectin mitogenesis. L3T4⁺ cells were depleted from BALB/cJ spleen cells to greater than 90% (as measured by flow cytometry) by panning with the GK 1.5 MAb. Mitogenesis in the presence of a range of concentrations of SEA (A), SEB (B), Con A (C), and LPS (D) was determined by using untreated (O) or L3T4-depleted (Δ) populations. Mean incorporation of [3 H]TdR for unstimulated cells was 408 ± 84 cpm for untreated cells and 217 ± 52 cpm for L3T4-depleted cells. The results shown are representative of the results from two experiments. Two experiments performed with C57BL/6J spleen cells yielded results indistinguishable from those shown here for BALB/cJ cells. Kcpm, Thousands of counts per minute.

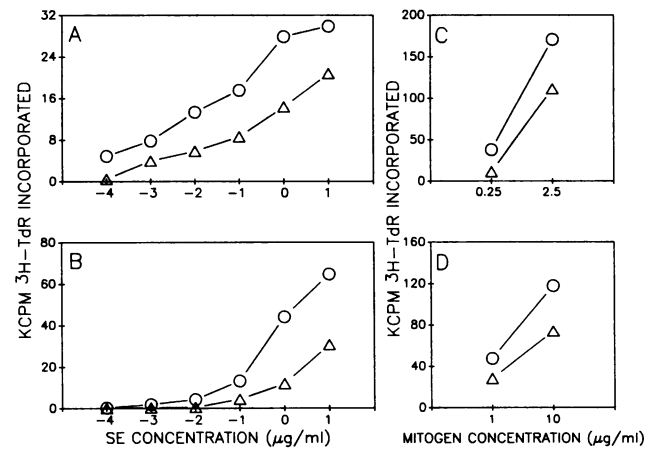


FIG. 7. The effect of Lyt-2 depletion on SE and lectin mitogenesis. Lyt-2⁺ cells were depleted from BALB/cJ spleen cells by panning with the 3.168 MAb. Depletion of Lyt-2⁺ cells was greater than 90% as measured by flow cytometry. Mitogenesis in the presence of a range of concentrations of SEA (A), SEB (B), Con A (C), or PHA (D) was done with untreated (O) or Lyt-2-depleted (Δ) populations. Mean incorporations of [3 H]TdR for unstimulated cells were 453 ± 75 cpm for untreated cells and 458 ± 124 cpm for Lyt-2-depleted cells. The results shown are representative of two experiments. Two experiments performed with C57BL/6J spleen cells yielded results indistinguishable from those shown here for BALB/cJ cells. Kcpm, Thousands of counts per minute.

These results emphasize the specificity of the inhibitions noted above. The only antibodies which reproducibly inhibited the 48-h mitogenic response were the specific class II MHC molecules expressed by the murine strain being tested or antibodies specific for the T-cell antigen receptor complex.

DISCUSSION

Earlier studies showed that SEs are polyclonal T-cell mitogens (14, 21, 29, 37). The pattern of mitogenesis and lymphocyte activation generated by plant lectins such as PHA and Con A are clearly distinguishable from activation induced by the SEs. For example, simple sugars such as alpha-methyl mannoside and a number of others did not inhibit SE-stimulated mitogenesis (37). This is consistent with the failure of SEs to agglutinate cells (37, 39) in contrast to the plant lectins. Additionally, antibody inhibition of PHA-stimulated cloned T cells was different from antibody inhibition of SE stimulation of the same cloned T cells (17).

We surveyed the mitogenic responses of spleen cells from five commonly available inbred strains of mice. We wanted to choose the two most sensitive strains of mice differing in degree of responsiveness to SEA and SEB for use in studying the cell surface molecules involved in the mitogenic response to the SEs. The magnitude of the mitogenic response was highly strain dependent. The strain maximally responsive to SEA (SJL) was different from the strain maximally responsive to SEB (BALB/cJ) (Fig. 1). Clearly, the magnitude of mitogenic response by SJL spleen cells to SEA was greater than the magnitude of response by any other inbred strain tested. However, there are more antibodies specific for the cell surface markers expressed on C57BL/6J spleen cells than for those on SJL spleen cells. Also, since C57BL/6J mice have been used extensively for studies of these toxins (6, 18, 21, 25, 37), we chose to use the C57BL/6J strain as a major responder to SEA. Fortuitously, BALB/cJ spleen cells were the highest responders to SEB, and since they have been used previously to study the immunological effects of these toxins (11, 25, 30, 39), we chose to study cells from this strain as high responders to SEB.

SEA was able to stimulate significant mitogenesis at 100-fold lower concentrations (10^{-4} $\mu\text{g/ml}$) than SEB (10^{-2} $\mu\text{g/ml}$), regardless of the haplotype of the spleen cells tested (Fig. 1). Also, it was more difficult to neutralize the activity of SEA than that of SEB (Table 2). A study published earlier (6) showed more effective competition by SEA than SEB in direct binding studies on spleen cells. This evidence is consistent with a higher affinity of SEA than SEB for its binding site on spleen cells. It is interesting to note that the earlier direct binding studies for SEA (6) indicated a binding affinity in a mixed spleen cell population equivalent to the affinities reported recently for antigenic protein fragments to Ia molecules (5). This hypothesis predicts that the stimulatory concentrations of SEA and SEB are directly dependent on their respective affinities for binding sites on spleen cells (e.g., Ia molecules).

This hypothesis does not explain the strain-dependent differences in the magnitude of the mitogenic responses observed. Although it will require further experiments to demonstrate, one hypothesis consistent with the data presented here is that the magnitude of the response is dependent upon the number of clones stimulated by the Ia-SE complex.

MAbs against MHC antigens were examined for their effects on mitogenesis induced by SE. MAbs against class I

molecules or class II molecules of an irrelevant haplotype did not inhibit SE-stimulated mitogenesis during the 48-h cultures used in this work. Most MAbs against class II MHC molecules of the relevant haplotype were effective inhibitors of SE-induced mitogenesis (Fig. 2). However, MKD6, a MAb specific for I-A^d, bound to BALB/cJ (H-2^d) spleen cells (determined by indirect immunofluorescence; data not shown) but did not significantly inhibit SE-induced mitogenesis. This result can be explained by the difference in epitopes recognized by the inhibitory M5/114 compared with the noninhibitory MAb MKD6 (3).

Our evidence is also consistent with the participation of both I-A^d and I-E^d molecules in the mitogenic response to SEA and SEB (Table 2). Addition of antibody to both types of Ia molecules was necessary to completely block SE-induced mitogenesis. Additionally, SEB-stimulated mitogenesis was more effectively inhibited by an anti-I-E-specific MAb, while SEA-stimulated mitogenesis was inhibited more by an anti-I-A-specific MAb (Table 2). This result suggests a preference of association (SEA to I-A and SEB to I-E) in the H-2^d haplotype. It is also interesting to note that the highest responders to SEA were the SJL and C57BL/6J strains, both of which express I-A exclusively. C57BL/6J does not express I-E on the cell surface because of a deletion in the gene coding for the E alpha-chain (26). SJL fails to express I-E because of a similar genetic defect. The proposed preferential association of SEA for I-A is consistent with the results observed with these strains. Likewise, the lower stimulation by SEB in SJL and C57BL/6J relative to the I-E^d-expressing BALB/cJ strain is consistent with a preferential association of SEB with I-E.

Antigenic activation of T cells involves the T-cell antigen receptor. The specific antigen recognition is mediated through the isotypic disulfide-linked heterodimer antigen-specific T-cell receptor (TcR) composed of alpha-beta (20) or gamma-delta-chains (4). As demonstrated by immunoprecipitation (28, 32), the antigen-specific TcR is linked noncovalently to a protein complex termed T3. Evidence presented in this report indicates that SEs may activate T cells through the TcR-T3 complex since the mitogenic stimulation was effectively blocked by an anti-T3 MAb (145-2C11). Likewise, with the recent demonstration of the association of L3T4 with the TcR complex (33), blocking the mitogenic activity of the SEs with a MAb specific for L3T4 (GK 1.5) supports the hypothesis for the direct participation of the TcR in SE-mediated mitogenesis. This inhibition of SE-induced mitogenesis by GK 1.5 is an early event, since this MAb was significantly inhibitory only if added within 8 h of SE addition (Fig. 4). Recently, Janeway et al. (17) showed that the MAbs 3D3 and F23.1 (antibodies specific for variable regions on the TcR) were able to block SEB-induced stimulation of D10 or AK8 cloned T cells, respectively. These are much more difficult experiments to perform definitively in spleen cell cultures since the SEs are polyclonal stimulators. An antibody specific for a single variable region on the TcR complex may not block mitogenesis in a sufficiently large subpopulation of the stimulated spleen cell clones to yield a detectable inhibition. However, the data presented above, especially in combination with the data presented by Janeway et al. (17), strongly implicate a direct role for the T-cell receptor in SE-stimulated lymphocytes. In addition to antibody inhibition data, the direct role of L3T4-bearing cells in SE stimulation was shown (Fig. 5 and 6). These results show that class II-restricted T cells are required for the mitogenic response to SE.

Lyt-2⁺ cells also appear to be activated by SE, since

depletion of Lyt-2⁺ cells reduced the response to SE by 50%. However, class I-restricted T cells (Lyt-2⁺) are not required for SE-induced mitogenesis since Lyt-2 depleted spleen cells, in contrast to L3T4-depleted spleen cells, still exhibited 50% of the mitogenesis of unfractionated cultures (Fig. 7). When tested in C57BL/6J cultures, the remaining mitogenic response was ablated after treatment of the Lyt-2-depleted cells with anti-L3T4 MAb GK 1.5 (unpublished observations). Further investigation of the role of Lyt-2⁺ cells in response to SE stimulation is of interest. The participation of Lyt-2⁺ cells in SE-stimulated suppression of antibody generation has been documented (11). However, suppression of antibody generation only occurred 3 to 5 days after treatment with SE (11, 35). In this context, our results may indicate an early, required participation by L3T4⁺ cells. Participation by Lyt-2⁺ cells may be a later event in SE-stimulated immunomodulation mediated by L3T4⁺ cells or a product released by L3T4⁺ cells.

Although the data described above indicate a requirement for class II MHC molecules for the mitogenic response of murine spleen cells to SEs, the cellular source of these molecules was not characterized. Recently, several laboratories have described the relationship between antigen-presenting cells and response to the SEs. Carlsson et al. (7) observed that SEA activation of human T cells was absolutely dependent on accessory cells. By using cloned human cytotoxic and proliferative T lymphocytes, Fleischer and Schrezenmeier (12) found that the presence of autologous or allogeneic MHC class II molecules was required on accessory or target cells in order to get a proliferative or cytotoxic response. Other experiments with cloned T-cell lines indicated a requirement for APCs in order to get interleukin 4 production in response to an SE stimulus (17). All of these observations indicate that class II MHC molecules as presented by APCs are required in order to get a response to the SEs. Preliminary experiments (to be further described elsewhere) also indicate that paraformaldehyde-fixed APCs treated with SEs can still lead to T-cell stimulation. We have not yet determined whether Ia molecules on the surface of B cells, macrophages, or both act as APCs for SEs within the spleen cell mixtures.

An interesting consideration for future studies of the SEs and related proteins is their apparent specificity for molecules on the surface of cells in the immune system. Particularly considering the previously described capacity of these toxins to modulate the immune response (11, 18, 30, 31, 35, 39), these toxins need to be examined for participation in infectious processes of *S. aureus* in addition to their well-documented activity in mediating food poisoning.

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