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Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins

(T-cell tolerance/bacterial toxins/T-cell membrane modulation)

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ABSTRACT **The exotoxins produced by certain strains of *Staphylococcus aureus* are able to stimulate powerful polyclonal proliferative responses and to induce nonresponsiveness by clonal deletion of T lymphocytes expressing the appropriate T-cell antigen receptor Vβ gene products. This paper examines the ability of *S. aureus* enterotoxins to modulate the responsiveness of human CD4+ T lymphocytes with defined antigen specificity. It was observed that certain *S. aureus* toxins were able to activate and induce anergy in hemagglutinin-reactive T cells expressing Vβ3+ elements. After exposure to *S. aureus* enterotoxins A, B, and D in the absence of antigen-presenting cells, the T cells failed to respond to their natural ligand presented in an immunogenic form, despite enhanced proliferation to exogenous interleukin 2. The *S. aureus* toxin-induced anergy was associated with modulation of T-cell membrane receptors; down-regulation of the T-cell antigen receptor was concomitant with enhanced expression of CD2 and CD25. Interestingly, CD28 was increased only on stimulation, suggesting this protein may be differentially expressed by activated and anergic T cells. These results indicate that bacterial toxins are able to induce antigen-specific nonresponsiveness in human T cells, the application of which may be relevant in the regulation of T cells expressing a particular family of Vβ gene products.**

The staphylococcal enterotoxins (1, 2) and certain endogenously derived proteins such as Mls (3, 4) are members of a family of antigens termed "superantigens," based on their ability to stimulate powerful polyclonal proliferative responses of murine and human T lymphocytes bearing particular T-cell antigen receptor (TCR) Vβ gene products (4–7). Additionally, superantigens are also able to induce nonresponsiveness in murine T cells either by clonal deletion (5) or functional inactivation (8). With the development of *in vitro* experimental systems, it has been possible to demonstrate that occupancy of the TCR by peptidic fragments of antigen complexed with class II major histocompatibility complex (MHC) molecules, in the absence of additional signals (costimulatory activity), is able to induce antigen-specific anergy (9–12). However, direct evidence to support clonal anergy as an operational mechanism in the development and maintenance of tolerance to either self or extrinsic antigens *in vivo* has been difficult to obtain. The results of recent experiments examining T-cell tolerance to nonlymphoid-expressed MHC molecules (13, 14) or to the self superantigen Mls-1[a] (8) suggest that nonresponsiveness, in certain instances, may be accounted for by functional inactivation. Similarity between the functional characteristics of these *in vivo* experimental models and those of peptide-specific T-cell anergy induced *in vitro* (9–12) prompted us to investigate the ability of *Staphylococcus aureus* enterotoxins to induce antigen-specific nonresponsiveness in cloned human CD4+ T cells specific for the carboxyl terminus of influenza virus hemagglutinin (HA), residues 307–319 [HA-(307–319)] (15, 16). In this report we demonstrate that the *S. aureus* toxins which were able to stimulate proliferation could also render the HA-reactive T cells nonresponsive to an immunogenic challenge of viral antigen and that the mechanism of nonresponsiveness is associated with modulation of T-cell membrane proteins.

MATERIALS AND METHODS

Antigens. Staphylococcal enterotoxins A, B, C1, C2, C3, and D (SEA, SEB, SEC1, SEC2, SEC3, and SED) were purchased from Toxin Technology (Madison, WI) or Serva Fine Biochemicals (New York). The HA peptide (residues 307–319) was synthesized using standard solid-phase methods on an Applied Biosystems model 430A synthesizer, purified by reversed-phase HPLC, and analyzed by amino acid analysis as described (16). This peptide was generously provided by J. Rothbard (ImmuLogic).

Antibodies. For flow cytometric analysis, fluorescein-conjugated murine monoclonal antibodies, anti-Leu5b (CD2), anti-Leu4 (CD3), anti-Leu3a (CD4), anti-interleukin 2 (IL-2) receptor (CD25), and fluorescein isothiocyanate-conjugated mouse IgG1 control were purchased from Becton Dickinson. The murine monoclonal antibodies anti-CD28 (9.3; ref. 17) and anti-CD3 were generously provided by J. Ledbetter (Oncogen, Seattle, WA) and H. Spits (DNAX), respectively.

Cloned Human Antigen-Reactive T Lymphocytes. The isolation and characterization of the cloned human T cells reactive with HA-(307–319) have been reported in detail elsewhere (15). Briefly, T cells activated with immunochemically purified HA were resuspended in RPMI 1640 medium (GIBCO) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 5% (vol/vol) screened human AB+ serum and cloned by limiting dilution in the presence of autologous irradiated peripheral blood mononuclear leukocytes, IL-2 [10% (vol/vol) Lymphocult T; Biotest Folex, Frankfurt, F.R.G.], and antigen. Growing T cells were expanded by cyclic stimulation with antigen and filler cells every 7 days and with IL-2 every 3 or 4 days. Prior to their use in experiments the T cells were allowed to rest for 7 days after the last exposure to antigen and filler cells.

Induction of T-Cell Nonresponsiveness. T cells (10^6 cells per ml) were incubated for 16 hr with the *S. aureus* toxins (0.5 μg/ml) or HA-(307–319) (50 μg/ml; ref. 9). Control cultures

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Abbreviations: APC, antigen-presenting cell; HA, influenza virus hemagglutinin; MHC, major histocompatibility complex; TCR, T-cell antigen receptor; IL-2, interleukin 2; SE, staphylococcal enterotoxin.

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of T cells in medium or of T cells activated with insolubilized anti-CD3 antibody (12 μg/ml) and IL-2 were performed in parallel. The cells were washed extensively after the pretreatment before determining their ability to respond to either an immunogenic challenge of antigen [HA-(307–319)] and antigen-presenting cells (APCs) or IL-2 (10%).

Proliferation Assays. Cloned T cells (10^5 cells per ml) were stimulated with HA-(307–319) (1.0 μg/ml) or the *S. aureus* toxins at the concentrations as indicated in the figures, in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DR1 (10^5 cells per ml; ref. 16) as a source of APCs, or in IL-2 alone. After 60 hr of incubation, [*methyl*-^{3}H]thymidine (1 μCi/ml; 1 Ci = 37 GBq; Amersham) was added and the cultures were harvested onto glass fiber filters 8–16 hr later. Proliferation as correlated with [^{3}H]thymidine incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures. In all cases the standard error of the mean was <20%.

Fluorescence Flow Cytometry. T cells were stained directly with saturating concentrations of fluorescein-conjugated murine monoclonal antibodies, anti-Leu5b (CD2), anti-Leu4 (CD3), anti-Leu3a (CD4), or anti-IL-2 receptor (CD25) using a mouse IgG1 fluorescein isothiocyanate-conjugated control, or indirectly with 9.3 (CD28). Viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry using a FACScan (Becton Dickinson). The cell population was analyzed by gating on the volume and light-scatter characteristics.

RESULTS

The Proliferative Response of Cloned HA-Reactive T Cells (HA1.7) to the *S. aureus* Enterotoxins. Distinct patterns of responsiveness were observed when T cells of clone HA1.7 were cultured alone or with APCs or IL-2 in the presence of the *S. aureus* enterotoxins, over a broad concentration range (Fig. 1). These cloned cells express αβ TCRs bearing Vβ3 gene products (M. J. Owen, personal communication). SEA at 0.5 μg/ml in the presence of APCs induced a weak but reproducible proliferative response (Fig. 1*a*). Although mediated at different concentrations, with SED (Fig. 1*f*) being two orders of magnitude more potent, the effects of this toxin and SEB (Fig. 1*b*) on the T cells were similar. Interestingly, proliferation in response to the natural ligand HA-(307–319), in association with DR1, was always at least 5-fold greater than that induced by any of the *S. aureus* toxins tested. At the appropriate concentration, SEB or SED alone induced T-cell proliferation in the absence of APCs; nevertheless, the response was decreased compared to that observed when APCs were present. In parallel the doses of toxin capable of inducing proliferation decreased responsiveness to exogenous IL-2. The patterns of response to SEC1, -2, and -3 were generally similar in that these toxins failed to induce T-cell proliferation even in the presence of APCs (Fig. 1 *c–e*).

Induction of HA-Specific Nonresponsiveness After Exposure to *S. aureus* Toxins. Preincubation with SEA, SEB, and SED for 16 hr in the absence of APCs induced nonresponsiveness in the T cells such that they were unable to proliferate in response to an immunogenic challenge of HA presented by murine fibroblasts expressing HLA-DR1 (Fig. 2). When peripheral blood mononuclear leukocytes or Epstein–Barr virus-transformed B cells were used as a source of APCs, the enterotoxin-pretreated T cells also failed to respond to specific antigen. This suggests that the nonresponsiveness observed in the presence of the DR1$^+$ transfectants is not the result of a lack of accessory-cell costimulatory activity. In the presence of APCs, the toxins are also able to reduce the

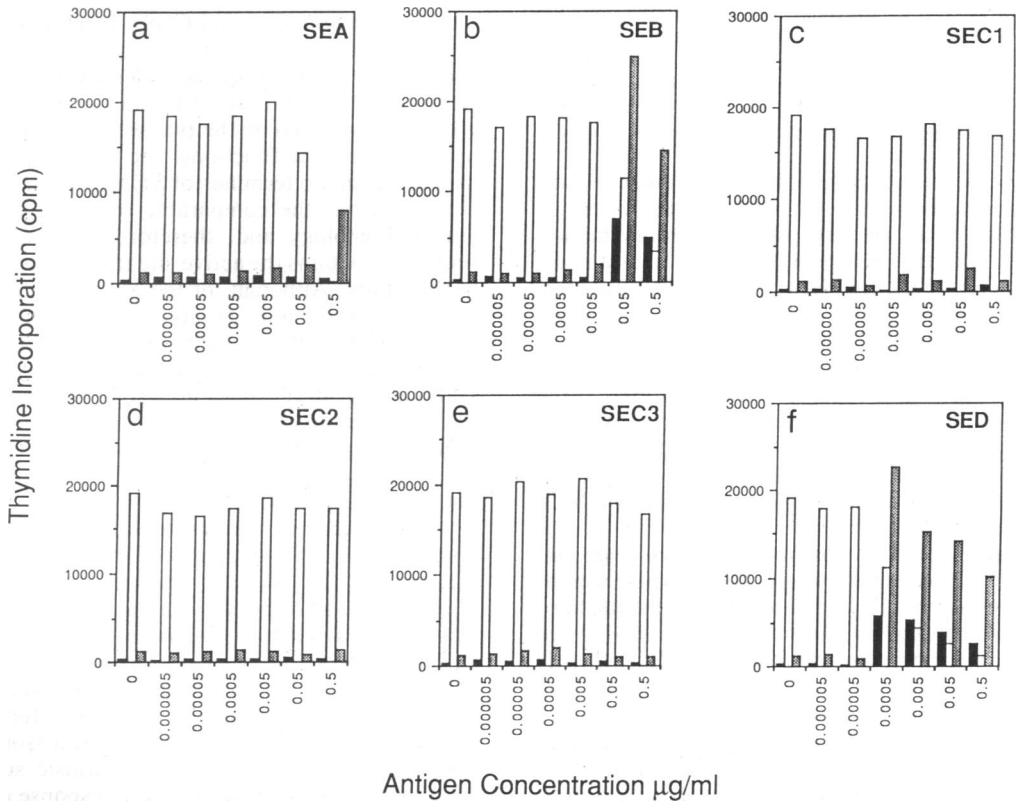

FIG. 1. Effect of *S. aureus* toxins on the proliferative response of HA1.7. Cloned T cells were stimulated with increasing concentrations of staphylococcal enterotoxins, SEA (*a*), SEB (*b*), SEC1 (*c*), SEC2 (*d*), SEC3 (*e*), and SED (*f*) alone (solid bars), with IL-2 (open bars), or with mitomycin C-treated murine fibroblasts expressing HLA-DR1 (stippled bars). The control response of the T cells to HA-(307–319) at an optimum concentration of 1 μg/ml was 96,070 cpm (± 5%) (mean ± SEM).

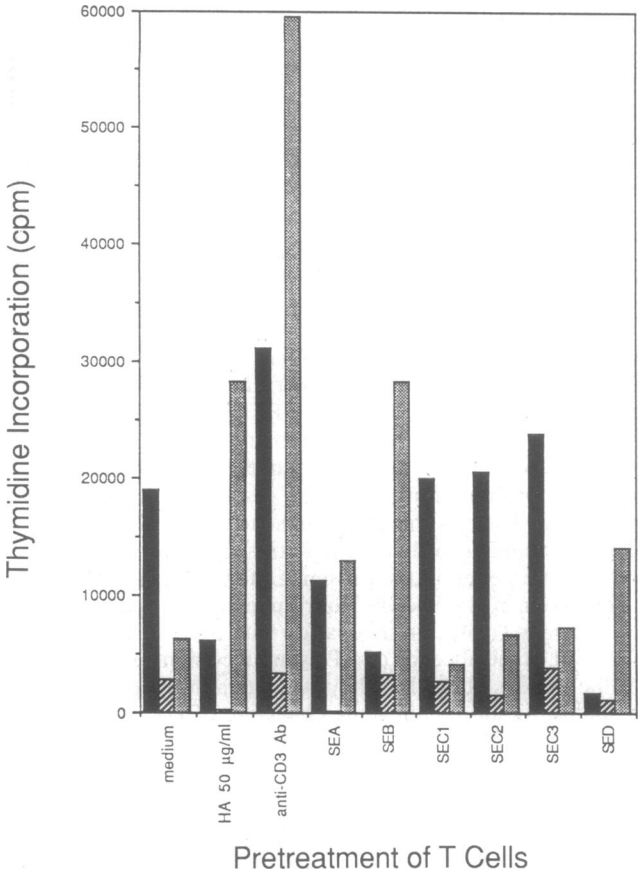

FIG. 2. Functional inactivation of T-cell clone HA1.7 after exposure to *S. aureus* toxins. T cells were exposed to the *S. aureus* toxins under conditions that induce unresponsiveness (as indicated). In the control cultures, T cells were incubated in medium alone or with the HA peptide or anti-CD3 antibody and IL-2. From each group of treatments T cells were assayed for their ability to respond to an immunogenic challenge of HA-(307–319) and accessory cells (mitomycin C-treated murine fibroblasts expressing HLA-DR1; solid bars), accessory cells alone (hatched bars), or IL-2 (stippled bars).

response of the T cells to specific antigen, although higher concentrations are required. A similar state of specific anergy resulted when the T cells were exposed to a supraimmunogenic concentration of free HA peptide but not to a peptide of unrelated sequence (e.g., see Fig. 4*c*). Unlike the activated T cells, both HA-peptide- and toxin-tolerized cells were refractory to an immunogenic challenge for up to 5 days. Concomitant with the loss of antigen-specific nonresponsiveness, a reciprocal enhancement of the proliferative response to IL-2 was demonstrated. As observed in activation, the tolerogenic effects of the toxins could be ranked as SED > SEB > SEA. In contrast, neither antigen- nor IL-2-dependent proliferation was modulated by exposure of the T cells to SEC1, -2, or -3 (Fig. 2).

Phenotypic Modulation Accompanying *S. aureus* Toxin and HA-Peptide-Induced Nonresponsiveness. To determine whether or not nonresponsiveness was due to receptor modulation, the T cells were analyzed by flow cytometry. Changes in phenotype observed after exposure to SEA, SEB, and SED were comparable (Fig. 3). The reduced expression of CD3 (Fig. 3*a*) was accompanied by up-regulation of CD2 (Fig. 3*b*) and CD25 (Fig. 3*c*). The TCR was modulated in parallel with CD3, as determined by staining with the monoclonal antibody WT31, which recognizes the $\alpha\beta$ TCR (data not shown). Activation with insolubilized anti-CD3 antibody and IL-2- or HA-peptide-induced anergy revealed similar changes in the phenotype. Membrane levels of CD4 were unaltered by exposure to HA-(307–319) or most of the *S. aureus* toxins tested, the exception being SEA, which enhanced CD4 expression, although the effect was marginal (Fig. 3*d*). The level of CD28 was marginally, but reproducibly, down-regulated (20–35%, $n = 6$) in toxin- and HA-peptide-induced anergy, whereas activation with anti-CD3 antibody and IL-2 markedly enhanced the expression (Fig. 3*e*). The phenotype of the T cells after pretreatment with SEC1, -2, and -3 were indistinguishable from the medium control.

To determine whether or not functional inactivation paralleled phenotypic modulation, the T cells were exposed to increasing concentrations of SEB and the loss of antigen-dependent proliferation was compared to the expression of CD3 and CD25. At concentrations of SEB >0.05 µg/ml, the down-regulation of CD3 (Fig. 4*a*) correlated with functional inactivation (Fig. 4*c*). Similarly, no changes in the expression of CD25 (Fig. 4*b*) were observed in the presence of SEB at concentrations that failed to induce anergy. Control cultures of T cells tolerized with HA-(307–319) revealed the same phenotypic modulation, whereas an irrelevant peptide derived from the group II allergen of dust mite, residues 36–60, had no effect.

DISCUSSION

The present study demonstrates that human T cells of defined antigen specificity exposed to certain *S. aureus* toxins, in the absence of accessory cells, become anergic to an immunogenic challenge by their natural ligand but retain responsiveness to IL-2. Distinct patterns of proliferation were observed when cloned human $V\beta3^+$ T cells specific for HA were cultured with the *S. aureus* toxins (SEA to -D) under stimulatory conditions. SEA, SEB, and SED induced proliferation in the presence of APCs, albeit with different potencies. Murine and human T cells expressing $V\beta3$ elements are able to interact specifically with SEB (6, 7); therefore, it was not surprising that this toxin is able to stimulate the HA-specific T cells. Human $CD4^+$ and $CD8^+$ T-cell clones activated by SEA and SEB have been identified (18), and since these toxins have ≈30% sequence identity (19, 20), a common sequence may be present that allows binding to the $V\beta$ gene products expressed by the two subsets of T cells. An immunologically related functional site has tentatively been localized at the amino terminus of SEA within residues 1–27 (ref. 21). However, the comparable region in SEB shows only limited homology and, therefore, it is unlikely that this sequence contains the active site that triggers the T cells used in this study. Although, of the *S. aureus* toxins, SEC1 and SEB (22) are the most homologous, the $V\beta3^+$ T cells would appear to bind only the latter. Weak stimulation of the T cells by SEB and SED in the absence of APCs was observed. Although bacterial toxins appear to require no cellular processing to stimulate T cells (1, 18) and activated human T cells express class II MHC molecules, the inability of the T cells to provide adequate accessory signals may account for the suboptimal activation.

Interestingly, in the absence of APCs, those *S. aureus* toxins capable of inducing proliferation were able to modulate antigen recognition by the cloned T cells, such that the T cells failed to respond to an immunogenic challenge of the appropriate ligand. The failure to respond to antigen was not due to cytolysis since IL-2 responsiveness was enhanced. This phenomenon of T-cell nonresponsiveness is similar to that induced by free antigen in peptidic form (9) or antigen presented by chemically modified accessory cells (10–12). This finding demonstrates that extrinsic superantigens are able to functionally inactivate the response of human T cells to their natural ligand, in this case HA. Our observations parallel those reported for specific tolerance to $Mls\text{-}1^a$, the self superantigen in adult $Mls\text{-}1^b$ mice (8). The observation that $V\beta6^+$ peripheral T cells are excluded after stimulation

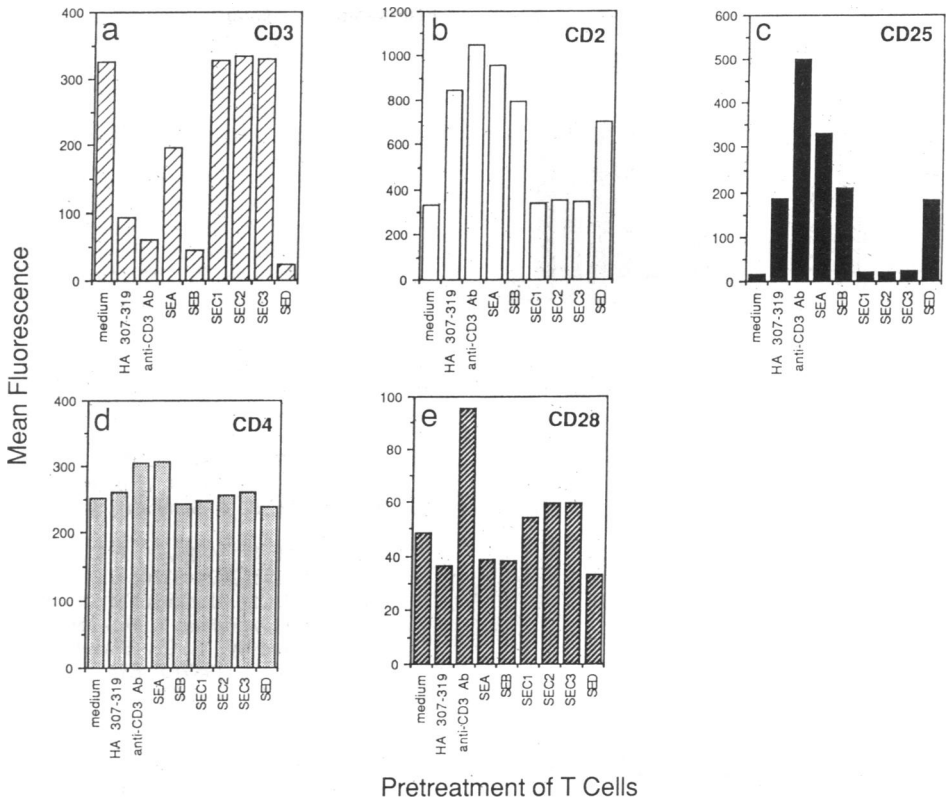

FIG. 3. Phenotypic modulation of the T cells functionally inactivated by *S. aureus* toxins. T cells were exposed to the *S. aureus* toxins under conditions that induce unresponsiveness. Membrane expression of CD3 (*a*), CD2 (*b*), CD25 (*c*), CD4 (*d*), and CD28 (*e*) was examined by flow cytometry and compared to control cultures of T cells in medium alone, tolerized with HA-(307–319), or activated with anti-CD3 antibody and IL-2.

with SED is compatible with the ability of enterotoxins to induce nonresponsiveness of human T cells (6). In experimental animal models, tolerance to SEB may also result from the physical elimination of T cells (5). The direct interaction of SEB with the TCR (4, 18) or through MHC class II molecules on APCs could account for the clonal deletion. From serological inhibition studies, it appears that, in contrast to the HA peptide (23), under conditions that induce anergy, the toxins bind to TCR independently of MHC class II molecules and transduce negative signals directly to the T cells (unpublished observations). However, whether or not bacterial superantigens bind directly to TCR remains controversial (1, 4, 18).

The induction of anergy by the *S. aureus* toxins resulted in changes in the phenotype of the T cells. The Ti–CD3 antigen receptor complex was modulated from the cell surface after exposure to SEA, SEB, or SED and correlated with the failure of the T cells to proliferate in response to specific peptides. However, after overnight activation with anti-CD3 and IL-2, unlike the HA peptide (24) and despite the down-regulation of membrane Ti–CD3, an immunogenic challenge still elicited proliferation. The rapid recovery of Ti–CD3 after activation may account for the antigen-dependent response. The longevity of anergy (9, 10) and the lack of Ti–CD3 on the cells tolerized by chemically modified APCs and antigen (12) indicate that anergy is associated with complex molecular regulation and is not solely the result of TCR modulation. In the nonresponsive T cells, the expression of TCR and CD25 is reciprocal. The up-regulation of CD25 and the subsequent increased IL-2 responsiveness of the anergic T cells may lower the effective IL-2 concentration and account for the apparent suppressor activity of SEA (25) without invoking the generation of an additional regulatory cell type.

There was no comodulation of CD4 with CD3 from the T-cell membrane in the anergic T cells, which suggests that for these cloned T cells CD4 is not structurally part of the antigen-recognition complex (26). However, the interactions between Ti–CD3 and CD2 appear to be considerably more complex. Coprecipitation studies have demonstrated that ≈40% of membrane CD2 is physically associated with Ti–CD3 (27). Interestingly, in the experiments reported here the expression of CD2 and Ti–CD3 are reciprocal in both toxin-mediated anergy and activation. Their relationship is further complicated by the observation that cholera toxin modulates only Ti–CD3 on the human T-cell lymphoma Jurkat (28). Collectively, these findings suggest that two populations of CD2 may exist that are modulated independently and may have different functional roles in the regulation of T-cell activation. The regulation of CD28 expression was intriguing by virtue of its association with an alternative pathway of activation independent of antigen recognition by the Ti–CD3 complex (29). Although, marginally down-regulated in anergy, enhancement occurred in activation. It has been postulated that CD28 may be the receptor for costimulatory activity that determines the outcome of tolerance or activation after occupancy of the TCR, based on the molecular analysis of peptide-induced anergy (12). Our experiments were not designed to address this issue; nevertheless they demonstrate that CD28 is differentially expressed in activated (anti-CD3) and anergic [HA-(307–319), SEA, SEB, and SED] T cells (Fig. 3).

S. aureus toxins react with particular Vβ gene products of TCRs (4–7) and, as is reported here, are also able to inactivate T cells such that they fail to respond to their natural ligand. It has been observed that carboxymethylation, although not altering antigenicity, which remains equal to that of the native molecule, removes the enterotoxic properties of

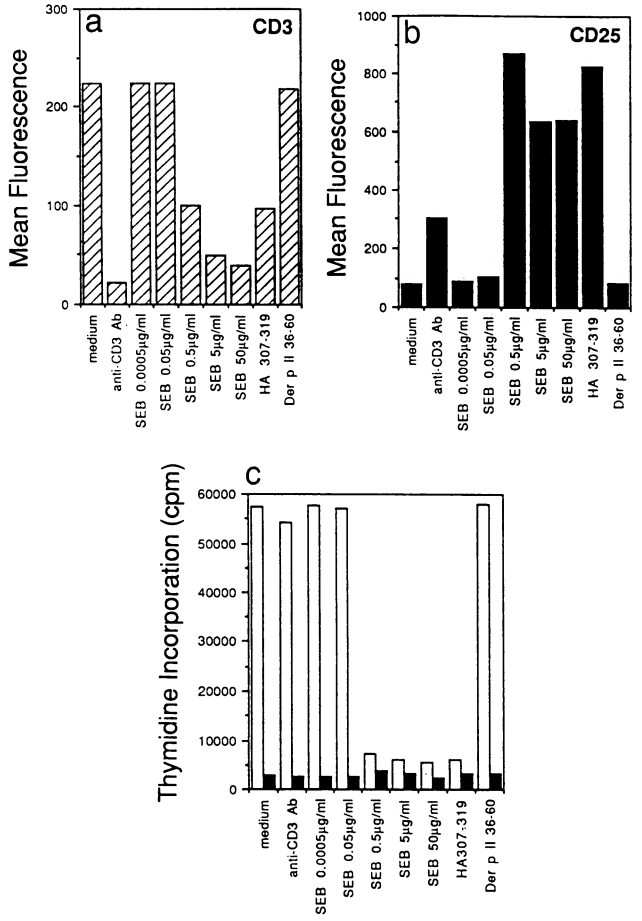

FIG. 4. Dose dependence of functional inactivation and phenotypic modulation. After exposure to SEB at increasing concentrations, T-cell membrane expression of CD3 (*a*) and CD25 (*b*) were compared to their responsiveness to an immunogenic challenge (*c*) of HA-(307–319) and $DR1^+$ APCs (open bars) or $DR1^+$ APCs alone (solid bars). Control cultures of T cells in medium alone, activated with anti-CD3 or pretreated with tolerogenic concentrations of HA-(307–319) or an irrelevant peptide derived from dust mite (Der pII 36–60) were examined.

SEA (32). The ability of the enterotoxins to induce nonresponsiveness of T cells in the presence of APCs, although less efficiently than in their absence, suggests they will retain this property *in vivo* (8). This raises the possibility of using superantigens as tolerogens to inactivate subpopulations of T cells that express TCR with common features. This approach would be of particular relevance in certain autoimmune diseases where the diversity of TCR is limited (30, 31). Furthermore, by genetic manipulation both the tolerogenic activity and affinity of enterotoxins for TCRs may be enhanced and, therefore, have potential as an alternative method of therapeutic intervention.

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