

T CELL STIMULATION BY STAPHYLOCOCCAL ENTEROTOXINS

Clonally Variable Response and Requirement for Major Histocompatibility Complex Class II Molecules on Accessory or Target Cells

BY BERNHARD FLEISCHER AND HUBERT SCHREZENMEIER

*From the Department of Medical Microbiology and Immunology, University of Ulm, Ulm, Federal
Republic of Germany*

Grampositive bacteria produce a variety of exoproteins, many of which are toxins or otherwise involved in pathogenicity or virulence. The Staphylococcal enterotoxins (SE)¹ comprise a group of structurally related but serologically distinct proteins produced by certain strains of *Staphylococcus aureus* (1, 2). Five serological groups (A, B, C, D, and E) of SE have been identified, based on their reactivity with monospecific antisera. SE are the most frequent cause of food poisoning in humans (1, 2). In addition, SE are the most potent mitogens known, stimulating human or murine lymphocytes at concentrations of $<10^{-9}$ M efficiently (3). The only known mitogen active at similarly low concentrations are mAbs against CD3. SE belong to the most widely used mitogens, since SEA and SEB contaminations are the mitogenic principle in Staphylococcal protein A (4). In vivo application of SE leads to polyclonal T cell activation (5) and has significant suppressive effects on cellular and humoral immune responses in vivo (6).

The mechanism of T cell activation by SE is unknown. The extremely low concentration of mitogen required for T cell activation suggests a highly selective mechanism in contrast to mitogenic lectins that bind to many structures on T lymphocytes and accessory cells (AC) and require at least 1,000 times higher concentrations.

In this report, we have analyzed the molecular mechanism of T cell activation by SE. The experimental data show that reactivity to SE is clonally expressed and that MHC class II molecules are required for T cell triggering by SE. A possible explanation of these findings is that SE are functionally bivalent molecules leading to a selective crosslinking of the T cell receptor/CD3 complex with MHC class II molecules.

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¹ *Abbreviations used in this paper:* AC, accessory cell; B-LCL, EBV-transformed B cell line; E⁻, T cell-depleted PBMC; SE, Staphylococcal enterotoxin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Materials and Methods

Reagents. Purified Staphylococcal enterotoxins A, B, and D, as well as type-specific antisera, were purchased from Serva Chemicals, Heidelberg, Federal Republic of Germany. SDS-PAGE of iodinated enterotoxin revealed only a single band in the 30-kD region. PHA was purchased from Difco Laboratories, Inc. (Nordwald KG, Hamburg, Federal Republic of Germany). mAbs against HLA class I (BBM.1), class II antigens (DA6.231, L243, L227) (7), CD3 (OKT3), or CD2 (CB.219) were purified from the supernatant of hybridomas, most of them obtained from the American Type Culture Collection (Rockville, MD).

Cells. PBMC were obtained by Ficoll Hypaque centrifugation. T cell-depleted mononuclear cells (E^-) were obtained by rosetting with sheep erythrocytes. Human-alloreactive or antigen-specific $CD4^+$ or $CD8^+$ T cell clones were obtained as described previously (8, 9). Several $CD4^+8^-$ T cell clones lacking the TCR α/β chain dimer (WT31 $^-$) but expressing CD3 (10, 11) and $CD4^+8^+WT31^+$ T cell clones were obtained from liver biopsies of patients with acute viral hepatitis. Northern blot analysis showed that the $CD4^+8^-WT31^-$ T cells express the TCR chain. $CD4^+$ or $CD8^+$ populations of resting T cells were obtained by positive selection in an Epics V cell sorter.

A T cell line (FH-T) was established from PHA blasts of a patient (FH) with a selective deficiency in HLA class II antigen expression (12). This cell line consisted of 99% $CD8^+$ T lymphocytes and had the capacity of lectin-dependent proliferation and cytotoxicity. The cell line was completely devoid of HLA class II antigen expression.

Two sublines of the Jurkat T cell leukemia line were used, one expressing CD3 and the TCR at the surface, and the other negative for the TCR complex but expressing CD2.

A panel of established cell lines was used as AC or target cells. HLA class II antigen positive cell lines were several EBV-transformed B cell lines (B-LCL), the Sezary T cell line HUT78, the Burkitt's lymphoma Daudi, and the Hodgkin's cell line L428 (these latter two do not express HLA class I antigens). HLA class II antigen negative cell lines included the monocytic cell lines U937 and HL60, the erythroid cell line K562, and several acute T cell leukemia cell lines, such as Molt4 or Jurkat. An EBV-transformed B cell line, FH-B, was established from the patient with HLA class II antigen deficiency. This cell line had all functional and phenotypical characteristics of normal EBV-transformed B cells with the exception of HLA-class II antigen expression. Expression was not inducible by $IFN-\gamma$.

Assay for Proliferative Responses. Proliferative responses were measured in triplicates of 200 μ l containing 10^4 cloned T cells or 3×10^4 purified resting T cells as responder and 2×10^4 monocytes or mitomycin C-treated tumor cells as AC in U-shaped 96-well microtiter plates (8). Assays using TLC as responder were pulsed with [3H]TdR (NEN Chemicals, Dreieich, Federal Republic of Germany) after 30 h, assays using purified T cells were pulsed after 48 h for 16 h. The results are expressed as mean cpm of triplicates. The SE of mean was always $<10\%$, except for low values, and is not given in the tables.

Fixation of AC to prevent antigen processing and presentation was performed using paraformaldehyde as described (13). Briefly, mitomycin C-treated AC were fixed for 5 min at 37°C with 1% paraformaldehyde. The reaction was stopped by addition of 0.15 M glycine/HCl, PH 7.2, and the cells were washed several times in culture medium. This treatment completely abrogated proliferation of the fixed cells, even without mitomycin C treatment.

Assay for Cytotoxic Activity. A standard 4-h ^{51}Cr release assay was performed in 96-conical-well microtiter plates as described (9). Pretreatment of target cells with chloroquine to inhibit antigen processing (14) was performed as described (15). B-LCL cells were incubated with SE in the presence of chloroquine (0.25 mM) for 2 h, were washed, and then used as target cells.

Determination of Intracellular CA^{2+} Concentration. Monocyte-depleted, resting T cells or cloned T cells were loaded with 1 μ M of the acetoxymethylester of Fura-2 (Calbiochem-Behring Corp., Frankfurt, FRG) at 5×10^7 cells/ml in RPMI 1640. After a 30-

min incubation at 37°C in the dark, the cells were diluted 10-fold and incubated for a further 30 min. The cells were washed in Hepes-buffered HBSS, and the fluorescence of the cell suspension (2.5×10^6 /ml) was monitored with a spectrofluorimeter in a quartz cuvette. The cell suspension was excited at 335 nm and fluorescence was measured at 510 nm at 37°C (16).

Results

Stimulation of T Cells with Enterotoxins Requires HLA Class II Positive AC. PBMC showed a proliferative response with peak proliferation to all enterotoxins after 2 d. Remarkably, SE were active even in picomolar concentrations. This response was entirely AC dependent but monocytes as AC could be replaced by autologous or allogeneic B-LCL cells (not shown). Purified T cells of both CD4⁺ or CD8⁺ subsets proliferated in the presence of monocytes or B-LCL cells as AC (not shown). To investigate the requirements for stimulation of CD4⁺ or CD8⁺ T cells by SE, we used cloned T cell lines as responder and defined tumor cells as AC or target cells. As shown in Table I for a CD4⁺ CTL clone, SEA could induce proliferation or trigger the lethal hit similar to the lectins, PHA or Con A. However, in contrast to triggering by lectins, stimulation of proliferation or cytotoxicity correlated with the expression of HLA class II antigens on AC or target cells. An experiment with only several representative cell lines as AC or target cells is shown, and a much larger number of cell lines was tested with identical results. Most stringent evidence that the differential capacity to stimulate or serve as targets was due to the expression of HLA class II molecules came from the use of the mutant cell line FH-B derived from the HLA class II antigen-deficient patient. These cells did not function as stimulator for SE-induced activation, although, besides lack of HLA class II expression, they were indistinguishable from other EBV-transformed B cells.

TABLE I
Proliferative and Cytotoxic Response of Cloned CD4⁺ T Cells to SEA Correlates with Expression of HLA Class II Antigens on AC or Target Cells

| AC/target cell | Class II expression | [³ H]TdR incorporation* | | | % lysis* | | |
|---------------------------|---------------------|-------------------------------------|------|------|----------|-----|-----|
| | | Med | SEA | PHA | Med | SEA | PHA |
| — | — | 0.2 | 0.6 | 0.9 | — | — | — |
| Autologous E ⁻ | + | 0.4 | 17.6 | 24.2 | ND | ND | ND |
| Allogeneic B-LCL | + | 0.2 | 11.9 | 14.2 | 0 | 63 | 39 |
| FH-B | — | 0.2 | 0.3 | 16.6 | 0 | 0 | 36 |
| Daudi | + | 0.3 | 8.5 | 9.1 | 0 | 46 | 74 |
| U937 | — | 0.3 | 0.8 | 6.3 | 0 | 0 | 45 |
| K562 | — | 0.3 | 0.8 | 6.3 | 0 | 0 | 51 |
| HUT78 | + | ND | ND | ND | 0 | 51 | 68 |
| MOLT4 | — | ND | ND | ND | 6 | 6 | 48 |

A CD4⁺ helper-independent CTL clone (specific for influenza virus plus HLA-DR1) was tested for proliferative and cytotoxic response to various cells in the presence of SEA (1 ng/ml) or PHA (5 μg/ml).

* Data are presented as cpm [³H]TdR incorporation ($\times 10^{-3}$) or percent lysis of targets at an effector/target cell ratio of 4:1.

The requirement for class II antigens in activation by SE was not confined to CD4⁺ T cells. An identical pattern of stimulation was observed with several CD8⁺ helper-independent CTL clones and with CD4⁻8⁻ T cell clones, lacking the TCR α and β chain dimer but expressing the chain (Table II).

Inhibition of SE-induced T Cell Activation by Antibodies to HLA Class II Antigens. mAbs to HLA class II molecules inhibited the proliferative response of T cells and T cell clones to SE in the presence of AC (not shown) and blocked the SE-induced lysis of class II positive target cells (Table III). To exclude that the anti-class II antibodies had an inhibitory effect on the CTL, we used the mutant FH-T CTL line that does not express HLA class II molecules. PHA-induced cytotoxicity or proliferation was not affected by the mAb. The same mAb blocked the antigen-specific response of the class II-specific clones used, but had no effect on antigen-specific activation of class I-specific CTL clones (not shown). Inhibition was dose dependent and best detectable at low concentrations of SE that still induced nearly optimal cytotoxicity or proliferation. This inhibition was found with all clones tested, regardless of their CD4⁺ or CD8⁺ phenotype or specificity for HLA class I or II antigens. Antibodies against multiple class II antigens inhibited stronger than DR-specific antibodies.

Role of Class II Antigens in SE-induced Triggering. We considered two possible functions of class II antigens in T cell triggering by SE; either the stimulant is processed and presented by the AC in the context of class II antigens, or a physical association takes place by binding of SE to class II molecules on the cell surface. To discriminate between these models, we assayed the effect of inhibition of antigen processing in the AC on T cell activation. If the AC were fixed with paraformaldehyde before the assay, stimulation by SE was still as effective as by PHA (Table IV). The concentration of paraformaldehyde used was 10-fold

TABLE II
HLA Class II-dependent Response to SEA of CD4⁻8⁺ and CD4⁻8⁻ T Cell Clones

| Responder | Response | Mitogen | AC/target cells | | |
|---------------------------------|---------------------------|---------|-----------------|--------|--------|
| | | | B-LCL | FH-B | L428 |
| CD4 ⁻ 8 ⁺ | Proliferation* | — | 110 | 150 | 120 |
| | | SEA | 14,440 | 220 | 8,070 |
| | | PHA | 9,420 | 11,670 | 16,180 |
| | Cytotoxicity [†] | — | 1 | -1 | 2 |
| | | SEA | 31 | 1 | 48 |
| | | PHA | 33 | 42 | 63 |
| CD4 ⁻ 8 ⁻ | Proliferation* | — | 200 | 190 | 300 |
| | | SEA | 8,110 | 160 | 10,550 |
| | | PHA | 7,560 | 3,260 | 17,340 |

An HLA-A2-specific alloreactive CD4⁻8⁺ CTL clone and a CD4⁻8⁻WT31⁻ TCR chain⁺ clone of unknown specificity were assayed for response to the mitogens SEA (1 ng/ml) and PHA (5 μ g/ml). An HLA-A2⁻ B-LCL, the class II negative mutant B cell line FH-B, and the class I negative, class II positive cell line L428 were used as AC or target cells.

* cpm [³H]TdR incorporation

[†] Percent lysis at an effector/target cell ratio of 1.

TABLE III
Inhibition of Triggering Via SEA by Anti-class II Antibodies

| mAb | Specificity | % Inhibition of lysis by the CD8 ⁺ CTL line FH-T | |
|---------|--------------------------|---|-----|
| | | SEA | PHA |
| DA6.231 | HLA-DR, DQ, DP | 64 | -18 |
| L227 | HLA-DR, DQ | 47 | 0 |
| L243 | HLA-DR | 30 | -19 |
| BBM.1 | β_2 -microglobulin | -17 | -54 |

The CD8⁺ T cell line FH-T derived from patient FH and deficient in HLA class II antigen expression was used as effector cells at a CTL/target ratio of 4. Target cells were allogeneic B-LCL cells in the presence of SEA (0.01 ng/ml) or PHA (5 μ g/ml). mAbs against monomorphic determinants of HLA class II antigens were added to target cells and mitogen at a concentration of 1:1,000 of ascitic fluid. Lysis in the absence of mAb was 47% induced by SEA and 27% by PHA.

higher than required to prevent presentation of soluble or viral antigens by these cells to the clones (not shown). Furthermore, pretreatment of target cells with SE in the presence of chloroquine to inhibit class II-dependent antigen presentation (14) did not lead to a reduction of SE-induced target cell lysis (data not shown). This indicates that the class II dependence of T cell stimulation by SE is due to a selective physical association with class II molecules on the surface of AC or target cells.

The T Cell Response to SE is Clonally Expressed. When a large number of T cell clones was tested for the response to different enterotoxins, different patterns in the response to SE were noticed.

Approximately 50% of all CTL clones could be triggered by SEA and SEB to proliferate, but not to kill, class II positive targets, although they could kill the same targets in the presence of the lectin PHA. Furthermore, many clones showed a preferential reactivity to a distinct type of enterotoxin. An example of this clonal variation is demonstrated with five CD4⁺ clones in Table V. Whereas most clones showed proliferation in response to all enterotoxins, cytotoxicity was triggered not in all clones and not with all enterotoxins. Thus, some clones were induced to kill in the presence of SEA but not SEB, some clones vice versa,

TABLE IV
Effect of Fixation of Accessory Cells on T Cell Responses to SEA and PHA

| AC | Proliferative response of a CD4 ⁺ clone to | | |
|--------------|---|--------|--------|
| | Medium | SEA | PHA |
| — | 68 | 2,677 | 967 |
| B-LCL | 89 | 10,636 | 16,030 |
| B-LCL, fixed | 73 | 10,156 | 13,490 |

The proliferative response of a CD4⁺ T cell clone to SEA (1 ng/ml) or PHA (2.5 μ g/ml) was measured in the presence of untreated B-LCL cells or B-LCL fixed with 1% paraformaldehyde for 5 min.

TABLE V
Clonal Patterns in the Response to Staphylococcal Enterotoxins

| Response | Mitogen | Response by CD4 ⁺ clone | | | | |
|---|---------|------------------------------------|-------|--------|--------|-------|
| | | PB13 | PB24 | PB64 | PB86 | PB30 |
| Proliferation (cpm ^{[3} H]TdR incorporation) | — | 150 | 192 | 612 | 413 | 122 |
| | SEA | 3,150 | 8,558 | 19,650 | 15,234 | 146 |
| | SEB | 1,200 | 7,839 | 27,053 | 25,667 | 4,954 |
| | PHA | 5,800 | 9,102 | 17,635 | 14,904 | 2,589 |
| Cytotoxicity (% lysis) | — | 4 | 3 | 0 | 0 | ND |
| | SEA | 6 | 36 | 0 | 40 | ND |
| | SEB | 27 | 3 | 0 | 63 | ND |
| | PHA | 32 | 24 | 51 | 39 | ND |

A B-LCL line was used as AC or target cell for activation of proliferation or cytotoxicity by 5 CD4⁺ T cell clones derived from the same individual. Mitogens SEA and SEB were used at 1 ng/ml and PHA at 5 µg/ml.

and other clones did not kill with any enterotoxin. Rarely, clones were identified that did not respond to SEA even with proliferation. Of 110 clones with lectin-inducible cytotoxic activity that were studied, 41% were triggered by SEA to kill targets, 12% by SEB, 5% by SEA and SEB, and 42% showed only a proliferative response to SEA and SEB. An example with 59 clones obtained from one individual is shown in Fig. 1.

Evidence for a Direct Action of SE on T Cells. The clonal expression of reactivity to SE could indicate that enterotoxins may stimulate T cells via the clonally expressed T cell antigen receptor. Since binding of labeled SE to peripheral blood T cells was only weakly detectable above background, we tried to obtain circumstantial evidence for a direct effect of SE on T lymphocytes. Highly purified resting T cells could be induced to proliferate by SEA in the absence of AC if the protein kinase C activator 12-0-tetradecanoylphorbol-13-acetate (TPA) was added (Table VI). The response to anti-CD3 mAb was restored by TPA in

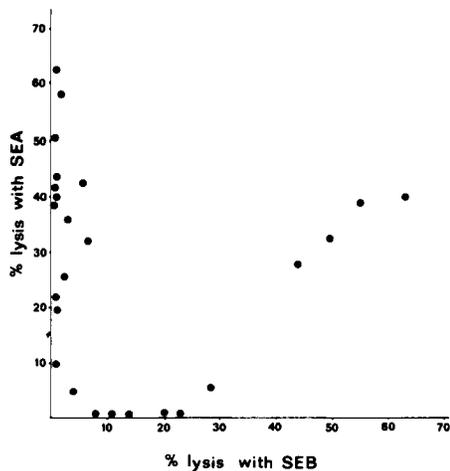


FIGURE 1. Clonal heterogeneity of the cytotoxic response of T lymphocytes to SE. 59 T cell clones randomly derived from one individual donor were tested for cytotoxicity against the autologous B-LCL cells in the presence of SEA (4 ng/ml) or SEB (4 ng/ml). 24 clones that showed cytotoxicity <5%-specific lysis are not shown in the figure. No clone lysed the B-LCL cells in the absence of SE.

TABLE VI
Replacement of AC by Phorbolsters in the Proliferative Response of Resting T Cells

| AC | cpm [³ H]TdR incorporation in response to | | | |
|--------|---|--------|--------|--------|
| | Medium | SEA | PHA | OKT3 |
| — | 210 | 299 | 471 | 281 |
| —, TPA | 1,062 | 7,494 | 12,968 | 19,212 |
| B-LCL | 578 | 17,706 | 20,380 | 680 |
| U937 | 132 | 145 | 7,889 | 3,785 |

Purified resting T cells were incubated with SEA (1 ng/ml), PHA (5 μ g/ml), or OKT3 (1 μ g/ml) with AC or TPA (10 ng/ml). Accessory cells were an HLA class II antigen positive, Fc receptor negative B-LCL, or the HLA class II antigen negative Fc receptor positive monocyte cell line U937.

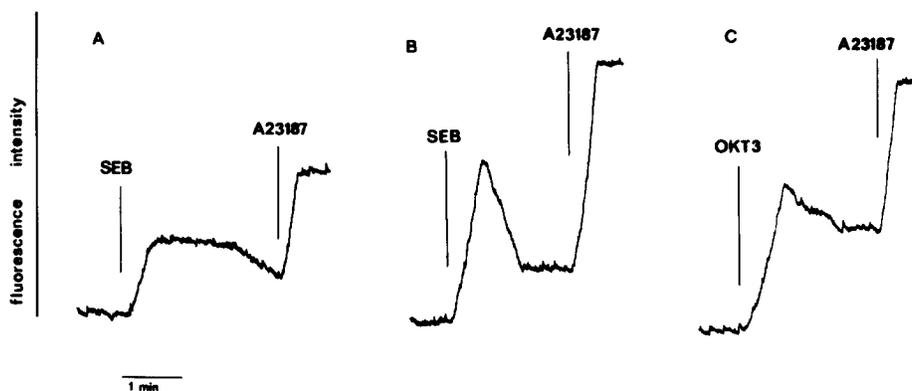


FIGURE 2. Rise in intracellular free Ca^{2+} concentration after triggering of T cells with SE or OKT3. Peripheral blood monocyte-depleted T cells (A) and cloned CD4^+ T cells (B and C) loaded with Fura-2 were triggered with 5 μ g/ml SEB (A and B) or a 1:400 dilution of OKT3 mAb (C), and the increase in fluorescence intensity at 510 nm was recorded. Maximal response was determined with the ionophore A23187 (4 μ g/ml).

same way, whereas TPA in the presence of anti-CD2 or anti-CD5 mAb had no activating effect (not shown). Furthermore, another functional effect of SE directly on the T cells could be detected by measuring intracellular calcium concentrations. As shown in Fig. 2, addition of SEB to peripheral T cells or cloned T4^+ T cells induced a rise in intracellular Ca^{2+} concentration similar to the anti-CD3 mAb OKT3. To provide further evidence that this rise in intracellular calcium was mediated via the TCR, we used the TCR-expressing Jurkat line and a variant that still expresses CD2 but not CD3. Although not shown here, only the CD3^+ Jurkat cells responded to SEB.

Discussion

AC-dependent polyclonal T cell activation by mitogens is widely used as a model for antigen-specific triggering. However, except for mitogenic mAbs, the molecular structures via which T cells are triggered by mitogens are unknown.

A proposed mechanism for T cell activation by mitogenic lectins is the cross-linking of the TCR complex (17, 18) or CD2 (19) with glycoproteins on the AC surface. The presence of MHC molecules on the AC is not required (8, 20). As an alternative hypothesis, it has been suggested that polyclonal T cell activation, in some cases, may be due to an immunological recognition of mitogen-modified MHC molecules (21). The I-E restricted activation of murine T cells by a soluble mitogen from *Mycoplasma arthritidis* has been suggested to fall into this category (22).

The AC-dependent T cell activation by Staphylococcal enterotoxins seems to be a novel principle. Although class II molecules on the AC or target cell are directly involved in SE-induced T cell stimulation, T cell activation by SE is not due to a restricted immunological recognition of SE bound to class II antigen, but SE molecules act directly, i.e., in the absence of class II antigens, with the target structure on the T cell. The nature of the target structure on the T cell is still unclear. The extensive clonal heterogeneity in the response to SE indicates a clonally variable structure. The finding that in many clones unresponsiveness to only one enterotoxin, e.g., SEA, was found, whereas the response to other enterotoxins, e.g., SEB, was present indicates that nonresponsiveness to an enterotoxin exhibits clonal specificity and is not due to a general failure of a clone to respond to enterotoxins.

Heterogeneity was most pronounced in the ability to respond with cytotoxicity to SE. A similar heterogeneity between activation to proliferation vs. activation to cytotoxicity can often be observed with antigen-specific triggering of CD4⁺ T cell clones; some CD4⁺ clones show a proliferative and cytotoxic response to a given stimulator cell, others show only a proliferative response but can nevertheless be induced to kill the same stimulator cell with lectin. A possible explanation for these findings is that triggering of cytotoxicity requires a higher activation threshold than triggering of proliferation. Evidence has been presented that T cells recognizing an antigen with low affinity can be induced to proliferate only whereas high affinity recognition can lead to triggering of cytotoxicity as well (23). Thus, the clonally variable reactivity to SE by T cells could indicate that different TCR clonotypes are bound with different affinity by different enterotoxins. That TCR chain-expressing T cells responded to SE could indicate that this alternative TCR is functionally similar to the classical $\alpha\beta$ TCR dimer.

The effects of SE are very similar to responses elicited by anti-TCR or anti-CD3 antibodies that require Fc receptor-dependent crosslinking to stimulate a full cytotoxic or proliferative response, but induce proliferation in the presence of phorbol esters (24) or a rise in intracellular Ca²⁺ by themselves (25). Since monovalent Fab fragments of anti-CD3 antibodies do not trigger cytoplasmic calcium increase (24), the activation by SE indicates that the enterotoxin molecule is multivalent and able to crosslink TCR molecules on the surface of the T cell similar to a divalent mAb.

A possible interpretation of these observations is that SE are functionally multivalent molecules that activate T cells by crosslinking variable parts of their antigen receptors with MHC class II molecules on AC or target cells. Thus, compared with activation by all other mitogens, activation via SE most closely

resembles the physiological MHC-restricted interaction of TCR and antigen bound to MHC molecules.

Summary

Staphylococcal enterotoxins (SE) are the most potent mitogens for T lymphocytes known; concentrations of $<10^{-9}$ M are sufficient for T cell activation. The mechanism of T cell activation by SE is unknown. We have used cloned human cytotoxic and proliferative T lymphocytes to dissect the molecular mechanism of T cell activation by SE. With rare exceptions, all TCR α/β chain-expressing T cell clones of CD4⁺ or CD8⁺ phenotype, as well as CD4⁻8⁻ TCR α/β chain negative chain-expressing T lymphocyte clones, respond with proliferation and/or cytotoxicity to SE. For triggering of all these clones, the presence of autologous or allogeneic MHC class II molecules on accessory or target cells is necessary. This requirement for class II antigens is not due to an immunological recognition of processed SE, since inhibition of antigen processing has no influence on the T cell response to SE. SE acts on the T cells directly since (a) they stimulate a rise in intracellular calcium concentration in T cell lines or purified T cells, and (b) accessory cells can be replaced by phorbol esters in the proliferative activation of resting T cells by SE. Furthermore, the T cell response to SE shows extensive clonal heterogeneity. These results suggest that SE are functionally bivalent mitogens binding highly selectively to HLA class II molecules and the TCR. Thus, compared with other polyclonal T cell activating agents, activation with SE most closely mimicks the physiological way of MHC-restricted antigen recognition by T lymphocytes.

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