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Staphylococcal Exotoxin Superantigens Induce Human Immunodeficiency Virus Type 1 Expression in Naturally Infected CD4$^+$ T Cells

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A high proportion of *Staphylococcus aureus* strains of human origin produce one or more exotoxins. In vivo, these toxins may give rise to a variety of clinical syndromes. In vitro, staphylococcal exotoxins have been shown to bind both to human leukocyte antigen (HLA) class II molecules on antigen-presenting cells and to the T-cell receptors on large fractions of T cells. The result of this interaction may be proliferation of the T cells, T-cell anergy, or apoptosis, depending on several factors, including the state of the responding cells and the presence of accessory molecules. Using naturally infected peripheral blood mononuclear cells depleted of CD8$^+$ T cells, we have shown that staphylococcal exotoxins are powerful inducers of human immunodeficiency virus type 1 expression and that they induce expression at low concentrations and with greater efficiency than other T-cell mitogens. Human immunodeficiency virus type 1 was produced entirely by CD4$^+$ T cells in this model; monocytes were expendable both as a source of virus and as a source of HLA class II molecules as long as other cells expressing HLA class II molecules were present. The results suggest that infection by *S. aureus* may be a cofactor in the immunopathogenesis of AIDS.

During the asymptomatic phase of human immunodeficiency virus type 1 (HIV-1) infection, fewer than 1 in 5,000 CD4$^+$ T cells are normally found to be harboring HIV-1 provirus (3, 20). These are mostly resting cells and must be activated in order to produce infectious HIV-1. In vivo activation of CD4$^+$ T cells is thought to occur predominantly in response to peptide fragments of antigen presented by human leukocyte antigen (HLA) class II molecules (15). However, the frequency of T cells which specifically recognize a given peptide-HLA class II complex is only on the order of 1 in 10,000 or less (1). It follows that the likelihood that a particular antigenic peptide will activate an HIV-1 provirus-containing CD4$^+$ T cell is small.

Staphylococci produce exotoxins (staphylococcal exotoxins [SE]) which are among the most potent T-cell mitogens known (16). They have been shown to bind to HLA class II molecules outside the peptide-binding groove, and each exotoxin stimulates T cells bearing any of a number of sequences encoded by the T-cell receptor Vβ (16). Thus, in contrast to antigenic peptides, each SE may stimulate as many as 20% of all T cells, a property which has earned them the term "superantigens" (12, 16). Staphylococci are frequently isolated from HIV-1-infected individuals (14). More than 40% of *Staphylococcus aureus* strains produce one or more exotoxins (8). Thus, SE superantigens may frequently be available for activation of large subsets of T cells in HIV-1-infected individuals. If able to induce HIV-1 replication, such superantigenic T-cell activation might be an important mechanism behind the ongoing production of new HIV-1 virions in vivo (11). In this study, we showed that SE superantigens are powerful inducers of HIV-1 expression in naturally infected CD4$^+$ T cells in vitro. CD4$^+$ T-cell proliferation and the expression of HIV-1 were critically dependent on the presence of cells expressing HLA class II molecules.

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MATERIALS AND METHODS

Study participants. Patients 1 and 2 had previously participated in a study to determine the frequency of HIV-1-infected circulating CD4$^+$ T cells and were selected as the two participants with the highest proportions of infected cells (3). Patients 3 and 4 were randomly selected among patients attending an outpatient clinic for HIV-1-infected individuals. They were all free of clinical signs and symptoms at the time of the study (Centers for Disease Control group II; CD4$^+$ T cell counts, 0.38×10^9 to 0.64×10^9 per liter). Blood from HIV-seronegative donors was obtained from the Red Cross Blood Bank, Oslo, Norway.

MAb and immunomagnetic beads. Monoclonal antibodies (MAb) specific for CD4 (clone 66.1) and the T-cell receptor αβ heterodimer (clone T10/B9) were generous gifts from J. Hansen, Fred Hutchinson Cancer Center, Seattle, Wash., and J. S. Thompson, University of Kentucky Medical Center, Lexington, Ky., respectively. Anti-HLA class II MAb HKB-1 and anti-HLA DR MAb B8.11 were generously provided by S. Funderud, The Norwegian Radium Hospital, Oslo, Norway, and B. Malissen, Centre d'Immunologie, Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseille, France, respectively. Anti-CD8 MAb ITI-5C2 and anti-CD14 MAb 1D5 were produced in our laboratory. Anti-CD4-CD8 MAb (Leu 3/2 Simultest) and their irrelevant isotypic control MAb were purchased from Becton Dickinson, San Jose, Calif. Anti-CD3 MAb OKT3 was obtained from Ortho Pharmaceuticals, Raritan, N.J. Fluorochrome-conjugated goat anti-mouse immunoglobulin G and M γ-, μ-, and light-chain antibodies were purchased from TAGO Inc., Burlingame, Calif. MAb were coated onto immunomagnetic beads (Dynabeads M-450; Dynal, Oslo, Norway) as described previously (3, 4).

Flow cytometry. Cells were stained for CD4 and CD8 in two-color fluorescence studies using fluorescein isothiocyanate-conjugated Leu-3a and phycoerythrin-conjugated Leu-2a, respectively. Fluorescein isothiocyanate-conjugated

TABLE 1. Proliferation and HIV-1 production by PBMC depleted of $CD8^+$ T cells in response to activation by SE or T-cell mitogens

SE or T-cell mitogen[a]	Result for cells from patient[b]:											
	1 (1:8,000; 1.4 × 10^6)			2 (>1:500; 1.6 × 10^6)			3 (1:20,000; 0.9 × 10^6)			4 (1:70,000; 0.84 × 10^6)		
	Proliferation (cpm)[c]	Day of onset[d]	Peak concn[e]	Proliferation (cpm)	Day of onset	Peak concn	Proliferation (cpm)	Day of onset	Peak concn	Proliferation (cpm)	Day of onset	Peak concn
SEA	14,200	19	188	5,376[f]	3	1,045	22,667	–	–	10,030	–	–
SEB	8,428	19	169	5,357	3	1,225	13,711	–	–	6,721	–	–
SEC1	10,043	15	439	7,548	3	1,548	11,175	26	1.1	7,588	–	–
SEC2	5,878	19	393	7,910	3	1,441	14,061	–	–	6,272	–	–
SEC3	6,540	23	1.1	4,897	3	1,628	14,412	26	7.6	7,633	–	–
SED	40,510	15	329	47,864	3	1,228	27,440	19	1,161	40,185	19	742
SEE	30,922	8	353	41,727	3	1,608	34,914	26	3.5	37,875	23	191
TSST-1	17,845	15	163	6,993	3	734	24,081	–	–	7,818	–	–
ET	11,200	15	1,383	6,267	3	814	12,310	19	1,519	7,972	–	–
MAb OKT3	ND	ND	ND	16,363	3	677	32,387	12	461	12,944	–	–
PHA	7,789	–	–	5,214	7	18	15,784	30	4.3	4,283	–	–
None	2,213	19	0.1	460	7	0.5	2,120	–	–	2,978	–	–

[a] TSST-1, toxic shock syndrome toxin 1; ET, exfoliative toxin; PHA, phytohemagglutinin; none, RPMI medium with fetal calf serum and no toxin.
[b] Numbers in parentheses are ratios of infected to uninfected $CD4^+$ T cells and numbers of cells per well. –, negative; ND, not done.
[c] Measured as [^{3}H]thymidine incorporation.
[d] First day of supernatant HIV p24 positivity.
[e] Peak supernatant HIV p24 concentration, in nanograms per milliliter.
[f] Cells were cultured in the absence of rIL-2.

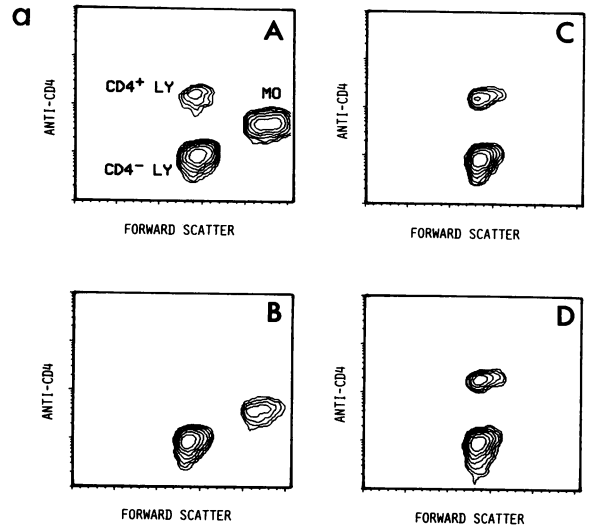
anti-HLA DR MAb B8.11 was used in single-color fluorescence studies. Cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, Calif.). The signals were collected and stored as list mode files and were analyzed with the Lysis II program.

Cell cultures. Cell subsets were depleted from peripheral blood mononuclear cells (PBMC) by using immunomagnetic beads coated with the relevant MAb, at a ratio of 10 beads per cell as described previously (9). Following depletion, the remaining cells always contained less than 2% of the depleted subset. Cells were established as single cultures in 24-well plates (Costar, Cambridge, Mass.) or as triplicate cultures in 96-well plates in RPMI 1640 (GIBCO, Paisley, Scotland) containing 10% (vol/vol) fetal calf serum supplemented with 10 U of recombinant interleukin-2 (rIL-2) (Amersham International, Amersham, England) per ml. The cells were activated by an SE (Toxin Technology, Sarasota, Fla.) at 1 μg/ml, by the anti-CD3 MAb OKT3 at 1 μg/ml, or by phytohemagglutinin at 5 μg/ml. The culture medium was changed twice a week, and the supernatants were tested for the concentration of HIV-1 p24 antigen by means of an antigen capture enzyme-linked immunosorbent assay (Abbott). The cells were restimulated by the original activating agent at the same concentration once a week. The cultures, except those from patient 2, were maintained for 30 days. Supernatant HIV-1 p24 in the cultures of cells from patient 2 peaked on days 7 through 11, and therefore the cultures were terminated on day 14. Cell proliferation was determined by transferring volumes corresponding to 10^5 cells according to the initial cell count to 96-well plates on day 3, incubating with [^{3}H]thymidine (2 μCi) for 24 h, and then determining the amount of incorporated radioactivity. In some experiments, separate triplicate cultures containing 5 × 10^4 cells each were established for the isolation of HIV and for [^{3}H]thymidine incorporation.

Limiting dilution assays. For the determination of the frequency of HIV-1-infected cells, $CD4^+$ T cells were positively selected by using Dynabeads coated with the anti-CD4 MAb 66.1 at a ratio of four beads per cell (3). The cells were separated from the beads by incubation with anti-Fab antiserum (DETACHaBEAD; Dynal), washed twice, and left overnight at 37°C. Serially diluted patient $CD4^+$ T cells were cocultured with $CD4^+$ T cells from HIV-seronegative donors, and the cells were activated with beads coated with the anti-T-cell receptor MAb T10/B9 at a ratio of four beads per cell in the presence of 10 U of rIL-2 per ml as described previously (3). On the basis of the Poisson distribution formula, the frequency of infected cells is given as the number of cells per well required to give 63% HIV-1-producing wells (18).

RESULTS AND DISCUSSION

PBMC were depleted of $CD8^+$ T cells in order to remove the cells responsible for the suppression of HIV-1 replication while retaining HLA class II-expressing cells (4, 22). These cells were activated in culture by SEA, SEB, SEC1 through -3, SED, SEE, toxic shock syndrome toxin 1, exfoliative toxin, MAb OKT3, or phytohemagglutinin. With the exception of exfoliative toxin, a final concentration of 1 μg of toxin per ml was well above the saturating concentration required for the proliferation of PBMC depleted of $CD8^+$ T cells (data not shown). A summary of the results is given in Table 1. The most important determinant for the detection of HIV-1 p24 in the culture supernatants was the frequency of HIV-1 provirus-containing $CD4^+$ T cells. Thus, HIV-1 replication was induced by all the SE in cultures of cells from the two individuals with the highest frequencies of HIV-1-infected $CD4^+$ T cells (patients 1 and 2). When the proportion of $CD4^+$ T cells infected by HIV-1 was low, only those exotoxins which gave high proliferative responses were able to induce HIV-1 expression (patient 4). The time required for culture supernatants to become HIV-1 p24 positive varied considerably and seemed to depend mainly on the frequency of HIV-1 provirus-containing $CD4^+$ T cells and on the proliferative response to each of the staphylococcal superantigens. However, the time required for culture positivity could also depend on the phenotype of the HIV-1 quasispecies infecting each individual (17). Patient 2, whose exotoxin-stimulated cell culture supernatants were all HIV-1



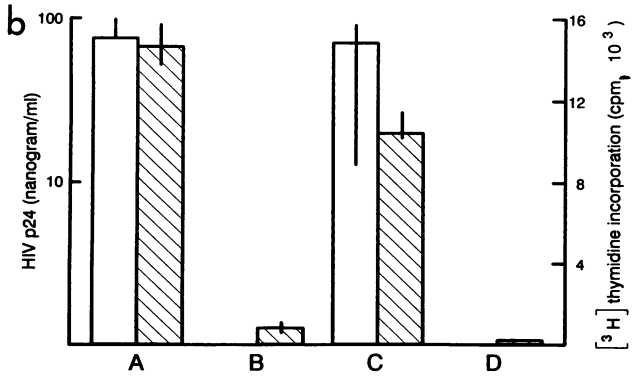


FIG. 1. Results of flow cytometric studies (a) and HIV-1 p24 concentrations in cell supernatants on day 8 (open bars) and [^{3}H]thymidine incorporation (hatched bars) (all values are medians and ranges of triplicate experiments) (b). PBMC from patient 2 were depleted of CD8$^+$ T cells (A), CD8$^+$ and CD4$^+$ T cells (B), CD8$^+$ T cells and monocytes (C), and CD8$^+$ T cells and HLA class II-expressing cells (D). The cells were examined by using anti-CD4 and anti-CD8 MAb in two-color fluorescence studies, but the populations are presented here as functions of anti-CD4 MAb staining (y axis) and size (forward scatter) (x axis).

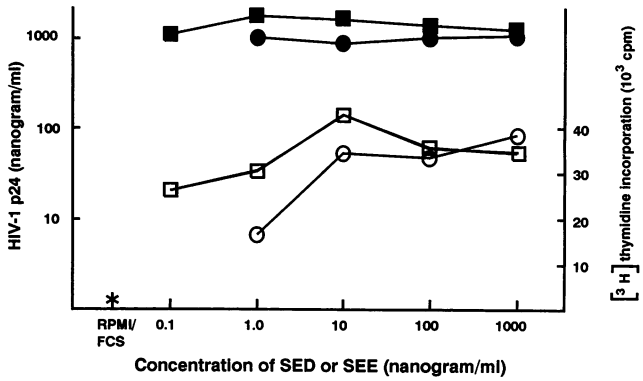


FIG. 2. Cell proliferation and HIV-1 production in response to different concentrations of SED and SEE. PBMC depleted of CD8$^+$ T cells from patient 2 were cultured in the absence of rIL-2. ○ and □, proliferation in response to SED and SEE, respectively. ● and ■, supernatant HIV-1 p24 concentrations on day 7 in response to SED and SEE, respectively. In unstimulated cells, the p24 concentration was 0.2 ng/ml and the proliferative response was 235 cpm (*). FCS, fetal calf serum.

p24 positive on day 3, has been extensively studied. All bulk virus isolates as well as each of several viral clones have replicated in cell lines and induced cytopathology consistent with a rapid-high phenotype (1a).

In order to identify the cell subsets involved in the SE-mediated induction of HIV-1 expression from PBMC depleted of CD8$^+$ T cells, cell subset depletion experiments with cells from patient 2 were performed. The results are presented in Fig. 1. When CD4$^+$ T cells were depleted, cell proliferation was considerably reduced, and HIV-1 expression was completely abrogated (compare the results from experiments B and A). In contrast, depletion of monocytes left the expression of HIV-1 unchanged and caused only a slight reduction of [^{3}H]thymidine incorporation (compare the results from experiments C and A). The results show that the source of expressed virus in these experiments was the CD4$^+$ T cells. Theoretically, monocytes could have contributed to the concentration of HIV p24 in the cell supernatants. Monocytes have been shown to contain HIV-1 in some individuals, and the binding of SE to their HLA class II molecules has been shown to cause transcriptional activation of certain genes (21). Experiment C demonstrated, however, that monocytes were expendable both as a source of HIV-1 and as a source of HLA class II. The importance of the presence of cells expressing HLA class II molecules was demonstrated in experiment D. In this experiment, HLA class II-expressing cells were depleted, removing all the monocytes and leaving less than 0.2% HLA DR-expressing lymphocytes. Both cell proliferation and the expression of HIV-1 were completely abrogated, although CD4$^+$ T cells were present at a proportion comparable to those in experiments C and A (19 versus 17% in experiment C and 9% in experiment A). Thus, free soluble SE were unable to induce [^{3}H]thymidine incorporation and the expression of HIV-1 in the CD4$^+$ T cells.

Long-term T-cell cultures require exogenous IL-2. We took advantage of the rapid kinetics of HIV-1 production in cells from patient 2 to examine whether SE might induce HIV-1 expression in the absence of exogenously added cell growth factors. No differences in HIV-1 p24 concentration between cell cultures established with and without exogenous IL-2 were found (the results of the experiment performed in the absence of rIL-2 are presented in Table 1). Subsequent experiments involving cells from several more HIV-infected individuals have shown that the proliferative response of PBMC depleted of CD8$^+$ T cells is not changed by the addition of rIL-2 and that SE-activated PBMC depleted of CD8$^+$ T cells from uninfected donors produce supersaturating levels of IL-2 (5). This suggests that endogenous IL-2 produced by the CD4$^+$ T cells in response to SE is sufficient to sustain these cells through 2 weeks of continuous HIV-1 production in vitro.

Dilutions of SED and SEE as low as 100 pg/ml still resulted in cell proliferation and high levels of HIV-1 replication (Fig. 2). In cell titration experiments, the frequencies of HIV-1 provirus-containing PBMC depleted of CD8$^+$ T cells were found to be 1:2,000 for SED-responsive and 1:3,000 for SEE-responsive cells (Table 2). Assuming that these exotoxins activate 20% of the CD4$^+$ T cells at most

TABLE 2. Limiting dilution analysis of the occurrence of HIV-1-producing cells in cells from patient 2

No. of cells/well	No. of positive cultures/total no.[a]		
	SED (1:2,000)	SEE (1:3,000)	$CD4^{+b}$ (>1:500)
8×10^2	0/4	0/4	10/10
4×10^3	4/4	3/4	10/10
2×10^4	4/4	4/4	8/8
1×10^5	4/4	4/4	ND[c]

[a] PBMC depleted of $CD8^+$ T cells were activated by SED or SEE in the absence of rIL-2 and allogeneic feeder cells. Numbers in parentheses are results espressed as ratios of infected to uninfected cells.

[b] For details of limiting dilution analysis of HIV-infected $CD4^+$ T cells, see Materials and Methods.

[c] ND, not done.

(12), this indicates that each exotoxin-binding, HIV-1 provirus-containing cell was induced to support active HIV-1 replication. This conclusion is supported by the results of the experiments using cells from patient 4. PBMC depleted of $CD8^+$ T cells usually contain less than 50% $CD4^+$ T cells (2). If this was the case also for patient 4, then SED and SEE induced HIV-1 expression in wells containing fewer than 6 HIV-1 provirus-containing $CD4^+$ T cells (Table 1).

Our results contrast with those in a recent article, which reported that the PBMC depleted of $CD8^+$ T cells from all asymptomatic HIV-1-infected individuals studied failed to proliferate and instead showed evidence of death by apoptosis in response to activation by SEB (10). That study involved a large number of HIV-infected individuals, and the methods employed were quite similar to the ones used in the present study. Although this study involved only a small group of HIV-1-infected individuals, we show by [^{3}H]thymidine incorporation that PBMC depleted of $CD8^+$ T cells are capable of proliferation, and by the measurement of supernatant p24 concentration we show that these cells are able to continuously express HIV-1 for at least 3 weeks in culture. Cell numbers were greatly increased in these long-term cultures (data not shown). In subsequent studies, involving other HIV-1-infected individuals, we have found that the SE-induced proliferation of PBMC depleted of $CD8^+$ T cells, measured as [^{3}H]thymidine incorporation (counts per minute) per $CD4^+$ T cell present in the well, was as strong for cells from the HIV-1-infected study participants as for cells from HLA DR- and age-matched uninfected controls (2). The discrepancy in results between the two studies must be left unexplained at the present time.

One unexpected observation made during these experiments was that SED and SEE gave considerably higher proliferative responses than the other exotoxins or mitogens (Table 1), suggesting binding by SED and SEE to a high proportion of T cells in our patients. This contrasted with results obtained by testing PBMC depleted of $CD8^+$ T cells obtained from nine HIV-1 seronegative donors, where SED and SEE gave proliferative responses similar to the other exotoxins and mitogens (data not shown). In other studies of HIV-1 seronegative individuals, neither SED nor SEE has been shown to bind to a disproportionately large fraction of T-cell receptor Vβ-expressing cells (6, 16). The significance of these observations remains to be established.

Certain microbial gene products, notably from cytomegalovirus, Epstein-Barr virus, and human T-cell leukemia virus type I (HTLV-I), have been shown to transactivate HIV-1 long terminal repeat (7, 13, 19). Theoretically, such transactivating agents might bypass the requirement for cell activation by specific antigen in order for HIV-1 production by infected cells to occur. However, these agents either are rare (HTLV-I) or require coinfection of the same cell for transactivation of HIV-1 to occur (Epstein-Barr virus and cytomegalovirus). They are thus likely to be of limited importance as cofactors for the development of AIDS. In contrast, staphylococci are common pathogens, and more than 40% of all strains produce one or more exotoxins (8, 14). Our experiments demonstrate that these exotoxins, at very low concentrations and without exogenously added cell growth factors, can induce virus expression from each HIV-1-infected, superantigen-responsive $CD4^+$ T cell in vitro. Such superantigenic T-cell activation may also induce HIV-1 replication in vivo and thus may represent an important cofactor for the development of AIDS.

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