Response to Superantigen Stimulation in Peripheral Blood Mononuclear Cells from Children Perinatally Infected with Human Immunodeficiency Virus and Receiving Highly Active Antiretroviral Therapy

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Our understanding of the pathogenesis of perinatal human immunodeficiency virus (HIV) infection is still evolving. We sought to characterize the response to the bacterial superantigen Staphylococcus enterotoxin B (SEB) of lymphocytes from HIV-infected children receiving treatment with highly active antiretroviral therapy (HAART). Using the flow cytometric methodology, we quantified apoptosis, proliferation, cytokine production, and activation antigen upregulation in CD4 and CD8 T lymphocytes following in vitro stimulation of peripheral blood mononuclear cells (PBMCs) with SEB. The levels of proliferation, CD4 interleukin-2 (IL-2) production, CD8 gamma interferon (IFN-γ) production, and upregulation of CD69 expression by cells from HIV-infected children were indistinguishable from those by cells from controls. However, stimulation with SEB dramatically decreased the ratio of resting apoptotic cells to cycling apoptotic cells in the controls but not in the patients. In addition, unstimulated spontaneous apoptosis of CD4 T cells remained greater in the patients than in the controls. The percentages of IL-2-positive CD8 T cells and IFN-γ-positive CD4 T cells following SEB stimulation were significantly lower in the patients than in the controls. Our multiparameter approach was able to demonstrate differences in lymphocyte superantigen responsiveness in HIV-infected children receiving HAART in comparison to that in uninfected controls, notably, an apoptotic versus a proliferative response to stimulation.

Infection with human immunodeficiency virus (HIV) results in a progressive depletion of immune function which, in the absence of treatment, leads to AIDS. The majority of children infected with HIV acquire the disease from their mothers (21) through perinatal transmission. Acquisition of HIV during birth at a time when the immune system has not yet matured results in a virus-host encounter unique from that in adults. Interventions aimed at interruption of mother-to-child transmission have dramatically reduced the incidence of HIV in children, and most HIV-infected pediatric patients receiving clinical care in the United States have chronic established infections. With potent combination antiretroviral therapies, many children are able to maintain viremia at low or undetectable levels concomitant with a rebound of the CD4 T-lymphocyte population. An important question is whether functional reconstitution of the lymphocyte compartment can be achieved in children by use of highly active antiretroviral therapy (HAART). In this regard, in a study of patients with cancer, the capacity to produce new CD4 T lymphocytes after chemotherapy was inversely related to the patient’s age (11). Thus, while untreated HIV infection rapidly causes disease in children, therapeutic intervention in this population holds great reward. In this study, we sought to quantify the level of lymphocyte responsiveness to a bacterial superantigen achieved by a group of children infected with HIV perinatally and receiving antiretroviral therapy according to the present standard of care. To this end, we used flow cytometric technology and measured the levels of apoptosis, proliferation, cytokine production, and upregulation of the activation marker CD69 in both CD4 and CD8 T-lymphocyte populations in response to stimulation with Staphylococcus enterotoxin B (SEB). Superantigens bind with a high affinity to the major histocompatibility complex class II molecule at a region distinct from the conventional antigen binding groove and cause proliferative activation of T cells (23). While effective HAART treatments may lead to the numerical reestablishment of the lymphocyte compartment, the critical determinant of immune reconstitution is lymphocyte function. Our multiparametric approach to determination of the functional competence of T lymphocytes from children infected with HIV revealed a normalization of some responses, while other responses remained different from those of the control population.

MATERIALS AND METHODS

Study population. We initially analyzed lymphoproliferation data for 125 HIV-infected children collected prior to the advent of HAART. These children were all infected perinatally but had various viral loads and CD4 counts. Our secondary, detailed analysis was performed with seven HIV-infected children receiving HAART (Table 1) who were being monitored at the Pediatric Immunology Clinic at North Shore University Hospital and was based on normalization of their lymphoproliferative responses. At the time of the study, two patients had suppressed virus to levels below detection limits and four children had CD4 T-cell counts which classified them as immune category 1 (3). In addition, healthy, unexposed, uninfected adult volunteers (n = 6; age range, 32
to 40 years) were used as controls. All samples were obtained following attainment of informed consent according to institutional guidelines and were conducted under a protocol approved by an institutional review board.

**Sample preparation and cell isolation.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood from controls or HIV-positive patients by density gradient sedimentation with Ficoll (Accurate Chemical and Scientific, Westbury, N.Y.). The cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 2 mM glutamine (Gibco BRL, Life Technologies, Gaithersburg, Md.), and 1% penicillin-streptomycin. The cells were cultured in the presence or the absence of 200 ng of SEB (Toxin Technology, Sarasota, Fla.) per ml.

**Lymphoproliferative responses.** PBMCs were cultured as described above for 3 days in the presence or the absence of SEB. Cultures were pulsed with 1 µCi of [3H]thymidine (Amersham Biosciences, Piscataway, N.J.) for 16 h and were then harvested on a Skatron cell harvester (Skatron Instruments, Lier, Norway). Proliferation was measured by determination of the amount of [3H]thymidine incorporated on a liquid scintillation counter (Pharmacia, Alameda, Calif.).

**Determination of cytokine production.** Samples with and without SEB in tubes slanted at 5° were incubated at 37°C in a humidified 5% CO2 atmosphere. After 2 h of incubation, 5 µg of brefeldin A (Sigma, St. Louis, Mo.) per ml was added, and the samples were incubated overnight. The samples were washed with Hanks balanced salt solution (HBSS) and labeled with fluorescently conjugated monodonal antibodies for the surface expression markers CD4 phycoerythrin (PE)-cyanine 5 (Cy5) (Coulter-Immunotech) and CD8 fluorescein isothiocyanate (FITC; Becton Dickinson) for 10 min at room temperature. The cells were washed with HBSS and fixed and permeabilized with Cytofix/Cytoperm (Pharmingen), according to the instructions of the manufacturer. Samples were labeled with fluorescently conjugated mononodal antibodies for intracytoplasmic cytokine with either anti-interleukin-2 (anti-IL-2) PE or anti-interferon gamma (anti-IFN-γ) PE (Pharmingen). Samples were stored at 4°C until flow cytometric analysis.

**Simultaneous assessment of proliferation and apoptosis.** The binding of annexin V, which reveals phosphatidylserine exposure on the outer cell membrane, was used to indicate those cells undergoing apoptosis. The cell cycle distribution revealed by DNA staining with the dye 4′,6-diamidino-2-phenylindole (DAPI) was used to assess cycling cells, which were considered those cells in S, G2, and M phases of the cell cycle. Importantly, labeling with annexin and DAPI was performed in the same tube, yielding information about the cell cycle distribution of apoptotic cells. PBMCs cultured with or without SEB were harvested after 4 days and washed with annexin binding buffer (Pharmingen). Annexin FITC (Pharmingen) and surface marker antibody CD4 PE-Cy5 or CD8 PE-Cy5 (Coulter-Immunotech) were added. After 10 min, the cells were washed with annexin binding buffer and Permeafix (Ortho Diagnostics) was added according to the directions of the manufacturer. The cells were washed with HBSS, and DAPI (Molecular Probes, Eugene, Oreg.) was added at a final concentration of 1 µg/ml. The samples were stored at 4°C until analysis on the flow cytometer.

**Plasma membrane antigen expression.** Samples were labeled with the fluorescently conjugated anti-CD4 PE-FITC monoclonal antibody in combination with anti-CD8 PE and anti-CD8 perCP (Becton Dickinson) in order to determine changes in the levels of expression of the activation molecule CD69 on the cell surface.

**Statistical analysis.** Analysis of the data was performed with SigmaStat software (Jandel Scientific, San Rafael, Calif.). Following assessment of the normality of the distribution of the data, parametric or nonparametric statistical tests were chosen, as appropriate. The Wilcoxon signed rank test or the Mann-Whitney rank-sum test was used to compare the results for the two groups, with P values <0.05 considered significant. The Spearman rank order correlation test was used to determine relationships between variables, with P values <0.008 considered significant, following Bonferroni adjustment for the number of comparisons.

**RESULTS**

Our bulk lymphoproliferation study analyzed data from 125 HIV-infected children prior to the introduction of combined-therapy regimens. These measurements revealed a significant decrease in the replicative responses of patient cells to SEB (controls, 15,941 ± 7,318; patients, 8,586 ± 8,034 [P < 0.0001]).

By using the lymphocyte response to SEB as a model system, our findings for the subset of HIV-infected children receiving HAART indicated that for many of the parameters measured, the response of the patient group to SEB was indistinguishable from that of the control group. Both CD4 and CD8 T lymphocytes proliferated in response to SEB stimulation in both groups (Tables 2 and 3). In a similar manner, the ability of CD4 cells to produce IL-2 and the ability of CD8 cells to produce IFN-γ were intact in the patient group. The cells of both groups retained their abilities to become activated, as indicated by upregulation of the surface protein CD69.

However, our multiparametric approach was able to identify certain immune defects in the HIV-infected children. The ability of CD4 T lymphocytes to produce IFN-γ (3% for the HIV-infected children versus 8% for the controls) and the ability of CD8 T cells to produce IL-2 (2% for the HIV-infected children versus 8% for the controls) were compromised in the patient group. The levels of spontaneous CD4 T-cell apoptosis in the patients (50%), measured following culture without any additional stimulus, remained above those in the controls (16%).

An important aspect of this study was that the combined DAPI-annexin assay allowed resting apoptotic cells to be distinguished from those apoptotic cells which were actively cycling prior to their death, as shown in Fig. 1. The ratio of the percentage of resting apoptotic cells/percentage of cycling apoptotic cells in response to SEB dramatically decreased in both the CD4 and the CD8 populations from the controls (Fig. 2) while it was unchanged in the cell populations from the patients. This observation shows that the normal control response to SEB is cell division followed by cell death, while in patients, many resting, nondoning lymphocytes died in response to an activating stimulus.

The strength of the multiparametric approach to this inves-
CD4 T-lymphocyte responses

<table>
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<tr>
<th>Patient no.</th>
<th>% Prolif.</th>
<th>% Apop. spon.</th>
<th>% Apop. stim.</th>
<th>% Cells positive for:</th>
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Patient group mean: 31±32
Control group: 19±18

Control group range: 9–53

Patient group range: 5–72

- Abbreviations: % Prolif., the percentage of proliferating cells in response to SEB; % Apop. spon., the percentage of cells undergoing apoptosis following culture in the absence of any additional stimulation; % Apop. stim., the percentage of cells undergoing apoptosis following culture in the presence of SEB.
- * Statistically significant difference (P < 0.05) between patient and control groups.

We designed an assay system using flow cytometry to assess the level of response to superantigen attained in peripheral blood lymphocytes from children infected with HIV. A previous study measuring proliferation demonstrated that the response of lymphocytes to SEB was highly reproducible and provided the best means of discrimination of the lymphocyte responses between HIV-infected and control subjects (1). In our hands, SEB also provided consistent and reproducible results and demonstrated proliferative defects in untreated HIV-infected children in bulk lymphoproliferation assays. This led us to choose SEB as a model system, in combination with sensitive and specific flow cytometry-based single-cell assays, to investigate lymphocyte responsiveness in children receiving antiretroviral therapy. Our goal was to determine to what extent the responses of treated children mirrored those of controls and where, if any, differences were located.

HIV infection is a worldwide epidemic among children and is one of the leading causes of pediatric morbidity and mortality. Infection with HIV in children most often occurs perinatally and in the absence of therapy manifests a wide range of symptoms, including opportunistic infections, malignancy, and neurologic deterioration (13). The advent of potent antiretroviral compounds has transformed HIV infection into a chronic disease in many individuals. We observed several functional lymphocyte responses in treated patients that were not different from the responses of the controls, namely, the levels of lymphocyte proliferation, IL-2 production in CD4 cells, and IFN-γ production in CD8 cells and the escalation of CD69 expression upon activation. Defects in the upregulation of CD69 have been reported in HIV-infected adults (14) and have been correlated with defects in the lymphocyte proliferative response (16), as the capacity to upregulate CD69 following mitogenic challenge was shown to be restricted to previously unactivated lymphocytes. Our finding of normal CD69 upregulation in response to SEB may reflect abatement of the chronic inflammatory conditions which typify HIV infection and partial reestablishment of a resting lymphocyte pool.

However, even in the absence of problems with toxicity or viral resistance and even with the most effective treatment

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### TABLE 3. CD8 T-lymphocyte responses

<table>
<thead>
<tr>
<th>Patient no.</th>
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<th>% Apop. stim.</th>
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</table>

Patient group mean: 12±12
Control group: 18±20

Control group range: 7–57

- Abbreviations: % Prolif., the percentage of proliferating cells in response to SEB; % Apop. spon., the percentage of cells undergoing apoptosis following culture in the absence of any additional stimulation; % Apop. stim., the percentage of cells undergoing apoptosis following culture in the presence of SEB.
- * Statistically significant difference (P < 0.05) between patient and control groups.
currently available, a reservoir of HIV exists during HAART (6), and this reservoir provides a mechanism for the lifelong persistence of virus (7). In fact, evidence exists for continuing HIV transcription in patients who are receiving HAART and who have undetectable levels of virus (8). Our laboratory (4) and others (18) have demonstrated decreases in lymphocyte apoptosis upon the introduction of treatment; in fact, Mohri and colleagues (12) showed that elevated rates of both proliferation and death of CD4 T cells in HIV-infected individuals normalized following initiation of antiretroviral therapy. While our findings indicated normal lymphocyte proliferation in the HIV-infected children, we were able to identify immune defects; for example, the levels of spontaneous apoptosis of CD4 T cells remained elevated in the patients, in agreement with findings from other laboratories (2, 15). Grivel et al. (9) showed that productive infection of lymphoid tissue ex vivo with HIV isolates induced apoptosis of CD4 T cells but not that of CD8 T cells, indicating that the virus alone can trigger death of the CD4 subset, while additional factors are required for CD8 death. Renno and coworkers (17) demonstrated that apoptosis of superantigen-activated T cells is restricted to cells which have undergone a discrete number of divisions, consistent with our results for the controls. The pattern was different in the patients, however. Changes in the ratio of resting apoptotic cells to cycling apoptotic cells were absent in the HIV-infected children, whose cells may be in a preapoptotic state which precludes a normal response pattern of activation, division, and then apoptosis. Instead, when T lymphocytes from an HIV-infected child are exposed to stimulation, they undergo cell death. This observation may have uncovered the driving force underlying the total breakdown of the immune system due to HIV infection. Even in patients receiving optimal therapy, lymphocytes lack that window of response time when they are protected from death and in which they are able to perform...
their function of pathogen elimination. This death-prone state of peripheral lymphocytes may manifest itself as unresponsiveness to a novel antigen, i.e., a different pathogen. Instead of responding in a manner positive for the host, the stimulus of infection may trigger an apoptotic cascade within the very cells responsible for host protection.

SEB has previously been used as a stimulus for cytokine induction; in fact, it was used for in vivo activation in a simian immunodeficiency virus macaque system and resulted in a 3-log decrease in viral load (5) in association with increased levels of IL-2 and IFN-γ production. Sieg and colleagues (19) showed a selective defect in IL-2 production in CD4 T cells in response to SEB in a cohort of HIV-infected adults with relatively high viral burdens, while these patients exhibited normal levels of IFN-γ production. This observation contrasts with our findings for HIV-infected children receiving optimal antiretroviral therapy, as the CD4 T cells in these children exhibited normal IL-2 production and defective IFN-γ production. Trimble and colleagues (20) showed that HIV-specific CD8 T cells produce IFN-γ but not IL-2, a result similar to what we observed for the entire CD8 population in response to SEB stimulation.

It is conceivable that the accelerated lymphocyte division rate during the course of HIV infection (22) deprives these cells of certain functional characteristics. These deficiencies or holes in the immune system may persist indefinitely and may not be restored to normal levels, despite prolonged effective therapy. Using the upregulation of CD69 as an indicator of immune function, Hsieh et al. (10) assessed antituberculosis cellular immunity after tuberculin stimulation in a cohort of HIV-infected individuals following 12 months of successful HAART and showed that either the CD4 or the CD8 T cells of certain patients failed to regain responsiveness to purified protein derivative, despite adequate control of HIV. Given the fact that eradication of HIV is thus far unattainable, the achievement of complete normality of lymphocyte function may be an unrealistic goal.

FIG. 2. The ratio of resting apoptotic cells to cycling apoptotic cells was determined with samples simultaneously stained with DAPI and labeled with annexin for controls (upper panel) and patients (lower panel). The ratio represents the percentage of apoptotic cells in G_{0} or G_{1} phase of the cell cycle versus the percentage of apoptotic cells in S, G_{2}, and M phases. Box plots represent the median and 10th and 90th percentiles for the group. Statistically significant differences for SEB-stimulated samples compared to the results for samples in medium alone (MED) are indicated with asterisks and were determined by the Wilcoxon signed-rank test.

FIG. 3. Statistically significant relationships among parameters measured for the patient group in response to SEB stimulation. Correlation coefficients, as determined by the Spearman rank order correlation test, are indicated. The Bonferroni adjustment was used to correct for the number of variables analyzed, with $P$ values <0.008 considered significant.
REFERENCES


