Detection of Major Histocompatibility Complex Class I-Restricted, HIV-Specific Cytotoxic T Lymphocytes in the Blood of Infected Hemophiliacs

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VIRUS-SPECIFIC major histocompatibility (MHC)-restricted cytotoxic T lymphocyte (CTL) activity is an effective mechanism through which the immune system can lyse virally infected cells during acute infection. In addition, these cells are believed to be important in controlling viral replication in chronic or persistent virus infections such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV). Infection with human retroviruses also appear to stimulate a virus-specific CTL response. Cytotoxic T lymphocyte lines specific for HTLV-I have been established from peripheral blood mononuclear cells (PBMCs) of individuals infected with this retrovirus. It has been hypothesized that CTLs specific for human immunodeficiency virus type one (HIV-1), the causative agent of AIDS, may play a role in controlling replication and spread of this human retrovirus in asymptomatic seropositive individuals. Recently, Walker et al succeeded in demonstrating the presence of T cells in PBMCs of HIV-1-seropositive homosexual men that specifically lyse autologous EBV-transformed B-lymphoblastoid cell lines (B-LCLs) that have been infected with recombinant vaccinia virus (VV) vectors expressing HIV-1 envelope, gag, and polymerase gene products. These responses appear to be MHC restricted.

Vectors are excellent tools for measuring virus antigen-specific CTL responses and have been used extensively in other viral systems. In addition, VV expressing HIV-1 envelope gene products are capable of stimulating HIV-1 envelope-specific CTL responses in vaccinated humans, chimpanzees, and macaques.

In the present study, we used a system of recombinant VV vectors to detect MHC class I-restricted, HIV-1-specific CTL responses in the PBMC of HIV-1-seropositive hemophiliac patients. In addition, we attempted to correlate the level of these responses with other known determinants of HIV-1 disease progression.

MATERIALS AND METHODS

Patient population. Approximately 150 patients with hemophilia are currently being followed at the New England Area Comprehensive Hemophilia Center, Worcester Memorial Hospital, MA, as part of an ongoing prospective study of immunoregulatory defects in hemophilia. All patients give informed consent before entering the study and are followed yearly. Approximately 90% of these individuals are seropositive by Western blot for antibodies to HIV-1; most seroconverted before 1983.

Lymphocyte separation. PBMCs were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients. PBMC preparations contained 70% to 80% lymphocytes, 10% to 20% monocytes, and <20% polymorphonuclear leukocytes as determined by cell morphology.

Lymphocyte surface marker studies. The relative percentages of the CD4 and CD8 lymphocyte populations were enumerated using direct immunofluorescence with phycoerythrin-conjugated mouse monoclonal antibodies Leu3A and Leu2A (Becton Dickinson, Mountain View, CA). Samples were analyzed with a FACS 440 (Becton Dickinson). Absolute numbers of lymphocytes per microliter of blood were determined by multiplying the relative percentage by the absolute number of PBMCs as determined from complete blood counts.

Virus culture. HIV-1 was cultured from PBMC by a modification of previously published techniques. PBMCs were cocultivated with previously phytohemagglutinin (PHA)-stimulated PBMCs from normal healthy seronegative controls. Cells were maintained in RPMI 1640, supplemented with 20% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY) and 10% interleukin-2 (IL-2, Electronucleonics, Silver Spring, MD). Cultures were sampled twice weekly for HIV-1 p-24 antigen by electroimmunoassay (EIA) (Dupont deNemours, Wilmington, DE) and HIV-1 genome by a nonisotopic in situ hybridization technique. Fresh PHA-stimulated blasts were added weekly, and cultures were maintained for 3 weeks before being considered negative.

Target cell lines. Autologous B-LCL were created by incubation of PBMCs with supernatant from the EBV-producing marmoset cell line B95.8 (ATCC No. CRL-1612). Autologous T-lymphoblastoid cell lines (T-LCLs) were created by cocultivation of PBMCs with supernatant from the EBV-producing marmoset cell line B95.8 (ATCC No. CRL-1612).
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PHA-stimulated PBMCs with an equal number of γ-irradiated (10,000 rad) MT-2 cells (provided by Dr Miyoshi) according to the methods of Merl et al. All transformed cell lines were maintained in RPMI 1640 with 10% to 15% FCS. T-LCL cultures were also supplemented with 10% IL-2 to maintain optimum growth characteristics.

VV vectors. Molecular clones containing sequences encoding envelope and gag genes from HIV-1 strain BH10 were inserted into target cells at an MOI of 10, for four hours (BSC-40) or 16 hours (KPL) and the precipitating substrate BCIP/4Dl 2. 1 (monoclonal anti-p55; Epitope, Beaverton, OR) or with DNA hybridization of viral genomic DNA. VV strain NYCBH contains the entire HIV-1 envelope coding sequence as well as 96 base pairs (bp) of 5' proximal and 107 bp of 3' proximal untranslated sequences. Recombinant vAbTl41 (141/gag/55) contains the entire HIV-1 envelope coding sequence as well as 96 base pairs (bp) of 5' proximal and 107 bp of 3' proximal untranslated sequences. Recombinant vAbT144 (144/gag/55) contains the entire HIV-1 gag coding sequence beginning at the translation initiation codon and extending ~200 bp beyond the gag translation termination codon. The predicted molecular structure of the recombinant viruses was confirmed by restriction endonuclease analysis and DNA hybridization of viral genomic DNA. VV strain NYCBH was used as the control virus in all CTL studies.

Expression analysis. Expression of the HIV-1 antigens by the recombinant VVs was confirmed in BSC-40 cells by an in situ ELISA assay performed directly on viral plaques. Incubation with 4D12.1 (monoclonal anti-p55; Epitope, Beaverton, OR) or with NEA-9303 (monoclonal anti-gp41; DuPont) was followed by alkaline phosphatase-labelled goat anti-mouse IgG (AP-GAM; Kirkegaard & Perry Labs, KPL) and the precipitating substrate BCIP/ NBT (KPL).

Radioimmunoprecipitations were performed by infecting 1 x 10⁶ target cells at an MOI of 10, for four hours (BSC-40) or 16 hours (B-LCL) with 100 μCi ³H-glucosamine (140/env/160) or ¹H-leucine (141/gag/55). Harvesting and precipitation was performed as previously described using mouse monoclonal antibodies (MoAbs) against HIV-1 antigens or pooled HIV-1-positive vaccine-negative human sera. Cell culture supernatants were assayed for HIV-1 gag antigen by an antigen-capture ELISA assay with a MoAb system (Dupont). Surface expression of HIV-1 envelope glycoprotein was assessed by the ability of VV-infected cells to form syncytia with c8166 cells, in a four-hour cocultivation.

CTL assay. Autologous B-LCLs or T-LCLs were infected or mock infected with VVs (NYCBH, 140/env/160, 141/gag/55) at a multiplicity of infection of 10 according to the methods of McMichael et al. Sixteen hours later, the cells were washed and incubated with 100 μCi Na2 (⁵¹CrO₄) (New England Nuclear) at 37°C for 60 minutes, washed twice with phosphate-buffered saline (PBS), and resuspended, after viability determination with trypan blue exclusion, to 1 x 10⁷ cells/mL in RPMI 1640 with 10% FCS. One hundred-microliter aliquots of target cell suspension were then dispersed into wells of 96-well round-bottom microtiter plates. To these wells 100 μL effector cell suspension containing 5.0, 2.5, or 1.25 x 10⁶ freshly isolated PBMC from hemophilia patients was then added [effector to target ratio (E:T) = 50, 25, 12.5:1]. Spontaneous release wells using RPMI 1640 with 10% FCS in place of effector cells were included in all assays. Test plates were centrifuged at 200 g for five minutes and incubated for six hours at 37°C in a humidified, 5% CO2 environment. Plates were then recenterfuged at 200 g for five minutes, and 100 μL assay supernatant was collected from each well and counted in a Packard γ-counter. Maximal incorporation was determined by counting the cpm in a 50-μL sample of target cell suspension. All tests were performed in triplicate.

Percentage of specific cytolysis was calculated by the formula 100 x [(test cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]. Percentage of HIV-1-specific cytolysis was calculated by subtracting percentage of specific cytolysis against NYCBH-infected targets from percentage of specific cytolysis against targets expressing HIV-1 antigens.

Effector cell depletions. Effector PBMCs were depleted of cells expressing a NK phenotype with a cocktail of the MoAbs Leu11b and Leu7 (Becton Dickinson) and rabbit complement (Cedarlane Laboratories, Ontario, Canada) by previously published techniques. Adequacy of the depletions was assessed by inhibition of killing of the NK-sensitive erythroleukemia cell line K-562 (ATCC-CCL243) in parallel assays. Percentage of decrease in cytolysis after depletions was calculated by the formula 100 x % of specific cytolysis of complement-treated effector cells - percentage of specific cytolysis of depleted effector cells)/percentage of specific cytolysis of complement-treated effector cells.

Antibody inhibition studies. MoAbs OKT1 (CD5), OKT3 (CD3), OKT8 (CD8), and W6/32 were prepared from ascites following intraperitoneal (IP) injection of 1 to 1.5 x 10⁶ hybridoma cells into previously pristane-treated CAF1 (OKT3, OKT8) or Balb/C (OKT1, W6/32) mice. MoAb OK1a 1 was purchased as a 1:3.3 dilution of ascites (Ortho Diagnostics) and dialyzed twice against PBS to remove azide, resulting in a final dilution of ascites of 1:5. Fifty microliters of a 1:20 dilution of each of these ascites fluids was added to wells of a 96-well round-bottom microtiter plate containing 1 x 10⁶ chromium-label target cells in 50-μL aliquots. One hundred microliters of previously NK cell-depleted effector cells were added to each well for a final E:T ratio of 50:1. After incubation and harvesting as described previously, percentage of specific cytolysis was calculated as previously described.

Statistical analysis. Data expressed in Figs 1 through 4 represent means and n refers to the number of samples tested. Where present, error bars represent SEM. Significance was determined when appropriate by analysis of variance (ANOVA), and statistical significance was defined as P < .05.

RESULTS

141/gag/55 was clearly demonstrable in the PBMCs of 17 HIV-1-seropositive hemophilia patients (Fig 1). These responses were significantly greater than the responses against NYCBH-infected targets (P < .01) and were clearly demonstrable in 15 of the 17 (88%) seropositive patients tested to date. PBMCs from seven of the 17 seropositive patients mediated greater cytotoxicity against gag-expressing B-LCL targets than against envelope-expressing targets. Only one patient showed greater cytotoxicity against envelope than against gag-expressing targets; the rest of the patients showed similar cytotoxicity against both HIV-1 protein-expressing targets. Assays were discarded from analysis if spontaneous release was >30%.

**HIV-1-specific cytolysis against T-LCLs.** Since B-LCLs from HIV-1-seropositive individuals secrete HIV-1-specific IgG27 which could mediate cytolysis against targets expressing HIV-1 proteins through antibody-dependent cell-mediated cytotoxicity (ADCC), PBMCs from six seropositive hemophilia patients were assayed for their ability to mediate cytolysis against autologous T-LCLs infected with recombinant VV vectors. As shown in Fig 2, T-LCL targets expressing HIV-1 gag or envelope antigens also serve as appropriate targets for HIV-1-specific cytolysis. Although the degree of specific cytolysis noted against HIV-1 protein-expressing T-LCL targets was not statistically significantly greater than the cytolysis noted against vaccinia-expressing T-LCL targets in this small number of assays and was less than that noted against B-LCL targets, other work in our laboratory has shown T-LCLs to be fairly resistant to T cell lysis and extremely resistant to NK cell lysis (B. Tomkinson, R. Koup, unpublished observations, November 1987). Since B-LCL targets appear to be more sensitive to cytolysis than T-LCL targets, all further reported study results involve autologous B-LCL targets only.

**NK cell resistance of measured cytolysis.** To ensure that the measured cytolysis in these assays was not mediated solely by NK cells, either through ADCC or lymphokine-activated killer (LAK) cell lysis, PBMCs from ten seropositive patients were depleted of NK cells by antibody-dependent, complement-mediated lysis. Of these ten patients, seven had adequate (>20%) HIV-1 gag-specific cytolysis and three had adequate HIV-1 envelope-specific cytolysis to assess the effect of NK cell depletions. As shown in Fig 3, although NK cell depletion had a significant effect on lysis of K562 cells, HIV-1-specific cytolysis against gag- and envelope-expressing B-LCL targets was only inhibited 23% and 15%, respectively, indicating that most of these cytolytic responses were not mediated by

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**Fig 1.** Cytotoxicity v B-LCL targets. Cumulative data from CTL assays on six HIV-1-seronegative and 17 HIV-1-seropositive hemophilia patients whose PBMCs were reacted against autologous B-LCLs infected with NYCBH (VAC), 141/gag/55 (GAG), or 140/env/160 (ENV), in a six-hour chromium release assay.

**Fig 2.** Cytotoxicity v B-LCL and T-LCL targets. Cumulative data from CTL assays on HIV-1-seropositive hemophilia patients, six of whose PBMCs were reacted against autologous T-LCLs infected with NYCBH (VAC), 141/gag/55 (GAG), or 140/env/160 (ENV), and 17 of whose PBMCs were reacted against autologous B-LCLs similarly infected. Left panel is a duplicate of right panel in Fig 1 and is reproduced for comparison.

**Fig 3.** Effect of NK cell depletion of effector cells on specific cytolysis. Effector cells were depleted of NK cells through MoAb-dependent, complement-mediated lysis. All effector cells were reacted against autologous B-LCLs infected with VV vectors, and K-562 cells were included as controls. Mean virus-specific cytolysis before depletion was as follows: gag, 38% (n = 7); envelope, 24% (n = 3). Mean cytotoxicity before depletion was 43% (n = 7) against K562 targets. All assays were performed at an E:T ratio of 50:1.
NK cells. In addition, the low level of cytolysis noted against NYCBH-infected B-LCL targets was inhibited almost completely by NK cell depletion (75%) and probably represents a slight NK cell sensitivity of these virally infected cell (data not shown).

**MoAb inhibition studies.** To determine the cellular origin and MHC restriction of the cytolytic responses remaining after NK cell depletions, MoAbs were added to the CTL assays to inhibit specific cytolysis. PBMCs from four patients had >15% gag-specific cytolysis, and PBMCs from one patient had ≥15% envelope-specific cytolysis after NK cell depletion; the results of MoAb inhibitions on these five individuals are shown in Fig 4. MoAbs used were OKT3 which is directed against the CD3 protein of the T-cell receptor and inhibits antigen-specific T-cell–dependent killing,28 OKT8 which is directed against the CD8 surface antigen, W6/32 which is directed against common determinants on MHC class I molecules and inhibits cytolysis by class I-restricted CTL clones,28,29 and OKIa1 which is directed against common determinants on MHC class II molecules and inhibits cytolysis by class II-restricted CTL clones.29 OKT1 was used as a control, being directed against a pan T-cell marker, CD5, and does not affect T-cell–mediated cytolysis.28,30

As is expected of cytolysis mediated by virus-specific, MHC class I-restricted CTLs, the HIV-1–specific cytolysis against autologous targets expressing gag proteins was inhibited by MoAbs OKT3, OKT8, and W6/32, but not OKT1 or OKIa1, and the specific cytolysis against envelope-expressing targets appear to follow a similar pattern (Fig 4). This inhibition of cytolysis does not represent a nonspecific phenomenon as cytolysis by CD4-positive, class II-restricted, dengue virus-specific CTL clones is inhibited by these same OKT3 and OKIa1 preparations, but not by the W6/32 or OKT8 preparations (I. Kurane, personal communication, April 1988).

**Correlations to clinical predictors of disease progression.** All 17 of the seropositive hemophilic patients described in this study were asymptomatic, falling into CDC group II or III classifications.31 When the level of gag– or envelope-specific CTL responses in these individuals was compared with absolute CD4 or CD8 lymphocyte counts or ratios of helper to suppressor cells, no positive correlations could be determined. However, when we compared the level of gag– or envelope-specific CTL with the ability to detect HIV-1 in PBMC cultures, PBMCs that did not yield HIV-1 in culture appeared to have more vigorous CTL responses than PBMCs that did yield virus on cocultivation (Table I). This difference did not reach statistical significance (P < .1, gag: P < .3, env) in this small patient population, however.

**DISCUSSION**

The present study was designed to confirm and extend the observations of Walker et al1 that HIV-1–specific CTL responses directed against envelope, and to a lesser degree, gag gene products were detectable in PBMCs of HIV-1–seropositive subjects in the absence of in vitro stimulation. Our results support the observation of Walker et al with some significant differences. Our studies have detected much more vigorous CTL responses against gag-expressing targets in this hemophilic population than in the homosexual population studied by Walker et al.1 This could relate to differences in the immune response to HIV-1 in these two distinct populations, differences in the stage of disease at which the responses were measured, or subtle differences in the level of gag antigen expression by the different VV vectors used in these two studies. We therefore believe this to be the first description and characterization of high-level gag-specific CTL activity in PBMCs of seropositive individuals.
The level of virus-specific CTL measured in these studies is without precedent in other persistent viral infections, including the related human retrovirus HTLV-1.7,8 These high and sustained responses may be a result of constant in vivo stimulation of CTL by ongoing viral replication, or the measured responses may not represent true MHC-restricted CTL responses but instead represent the activity of either NK cells or killer cell-mediated ADCC.26,32 As would be expected in bulk PBMC assays, more than one mechanism may be contributing to the measured cytolyis. The responses measured by Walker et al appear to be T cell mediated,5,8 whereas similar responses measured against targets absorbed with gp-120 by Weinhold et al appear to be NK cell mediated.32 Our studies indicate that a small but distinct proportion (15% to 25%) of the measured HIV-1-specific cytolyis can be eliminated by depleting effectors of cells bearing the CD16 phenotype. Cells of this phenotype mediate cell killing through direct mechanisms or through ADCC.26,32 We believe that the former mechanism is most likely responsible for the low level of NK-cell sensitivity observed in this system since we previously showed that B-LCLs infected with 141/gag/55 do not present viral antigens on their surface capable of mediating ADCC.26

The remainder of the cytolyis response against targets expressing HIV-1 proteins does appear to follow the characteristics of a classical virus-specific MHC class I-restricted CTL response, being inhibited by MoAbs to CD3, CD8, and MHC class-I, but not CD5 or MHC class II. In our MoAb inhibiting system, we found it important first to deplete effectors of NK cells to avoid augmenting cytolyis through the action of cells bearing FC-receptors.33 Although a restriction was noted by Walker et al5 against targets expressing HIV-1 envelope proteins, the class I or class II restriction pattern was not readily apparent from the data and suggests that both CD4+ and CD8+ clones may have been responsible for the T-cell–mediated killing measured in his assays. HIV-1 gp-120–specific CTL clones derived from lymphocytes in the CSF of HIV-1–infected individuals have both MHC class I and MHC class II restriction patterns.24 Most of the HIV-1 gag-specific CTL responses measured in our population, however, appear to be mediated by CD8+, MHC class I-restricted CTL, as evidenced by strong inhibition by antibodies to CD8 and class I antigens, whereas the envelope-specific responses may be mediated by both class I- and class II-restricted CTL since incomplete inhibition of killing was noted with class I-specific antibodies.

Several prognostic indicators of disease progression from the asymptomatic state to AIDS-related complex (ARC) or AIDS have been described, including loss of antibody to p-24,25 appearance of p-24 antigen in serum,20 loss of CD4 lymphocytes,26 and decrease in helper to suppressor ratio.26 We previously described the ability to isolate HIV-1 from the PBMCs of asymptomatic HIV-1–seropositive hemophilia patients as being strongly predictive of disease progression.14 The inability to isolate HIV-1 from the PBMCs of asymptomatic seropositive hemophilia patients may correlate with more vigorous HIV-1–specific CTL responses. Although this association does not yet reach statistical significance, we hope to confirm or deny this initial observation as we continue to follow this patient population. The presence of circulating HIV-1–specific CTL in these virus culture-negative but seropositive hemophilia patients indicates that they have been infected with live virus and not just immunized with inactive virus. In addition, the high level of CTL response in these patients from whom HIV-1 cannot be isolated may be indicative of low-level virus replication that is controlled by an effective immune response.

In summary, we confirmed the findings of previous investigators and detected HIV-1–specific CTL responses in PBMCs of infected hemophilia patients in the absence of in vitro stimulation. Most of these cytolytic responses appear to be NK cell resistant, T cell mediated, and MHC class I restricted. These responses are directed against both HIV-1 gag and envelope antigens, and the generation of these responses in vivo may be one way in which the human immune system can adequately deal with this devastating infection.

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REFERENCES


7. Walker BD, Flexner C, Paradis TJ, Fuller TC, Hirsch MS, Schooley RT, Moss B: HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. Science 240:64, 1988


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