Peripheral Blood Cytotoxic γδ T Lymphocytes from Patients with Human Immunodeficiency Virus Type 1 Infection and AIDS Lyse Uninfected CD4+ T Cells, and Their Cytocidal Potential Correlates with Viral Load

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Progression of human immunodeficiency virus type 1 (HIV-1) infection in humans is marked by declining CD4+-T-cell counts and increasing virus load (VL). Cytotoxic T lymphocytes (CTL) play an important role in the lysis of HIV-infected cells, especially during the early phase of asymptomatic infection. CTL responses in the later phase of disease progression may not be as effective since progressors with lower CD4+-T-cell counts have consistently higher VL despite having elevated CTL counts. We hypothesized that, apart from antiviral effects, some CTL might also contribute to AIDS pathogenesis by depleting CD4+ T cells and that this CTL activity may correlate with the VL in AIDS patients. Therefore, a cross-sectional study of 31 HIV-1-infected patients at various clinical stages was carried out. Purified CTL from these donors as well as HIV-seronegative controls were used as effectors against different human cell targets by using standard 51Cr release cytolytic assays. A direct correlation between VL and CTL-mediated, major histocompatibility complex (MHC)-unrestricted lysis of primary CD4+-T-cell, CEM.NK6, and K562 targets was observed. CD4+-T-cell counts and duration of infection also correlated with MHC-unrestricted cytolytic activity. Our data clearly show that γδ CTL are abnormally expanded in the peripheral blood of HIV-infected patients and that the Vδ1 subset of γδ T cells is the main effector population responsible for this type of cytolysis. The present data suggest that γδ CTL can contribute to the depletion of bystander CD4+ T cells in HIV-infected patients as a parallel mechanism to HIV-associated immunopathogenesis and hence expedite AIDS progression.
(23–26) have focused mainly on the phenomenon of CD4$^+$ T-cell depletion, we investigated the pathological role of various CTL in AIDS infection by analyzing cytolytic responses in relation to CD4$^+$-T-lymphocyte counts, VL, and the duration of clinical infection. Here, we present evidence showing the following: (i) γδ CTL can lyse cells other than CD4$^+$ T lymphocytes, which implies that the pathogenic ramifications attributable to CTL-linked cytosis may be even more diverse than previously thought; (ii) CTL-mediated lysis of target cells is directly correlated with the VL of patients; and (iii) γδ CTL are abnormally expanded in the blood of patients (interestingly, the effector cells responsible for the lysis we observed were found to be mainly of the V61 phenotype).

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MATERIALS AND METHODS

Subjects. Thirty-one HIV-1-infected patients classified as being asymptomatic or symptomatic or as having AIDS according to the guidelines of the Centers for Disease Control and Prevention (15) were recruited through the University of Montreal Medical Center’s AIDS clinic (at Hotel-Dieu Hospital, Montreal, Canada) for this cross-sectional study. The subjects were found to be seropositive for HIV-1 infection by enzyme-linked immunosorbent assay, with confirmation by Western blotting. Their infections were at different stages of clinical progression (asymptomatic or symptomatic or as having AIDS according to the guidelines of the Centers for Disease Control and Prevention, Vancouver, Canada). All antibodies were used according to the manufacturer’s instructions. Briefly, in vitro-activated PBMC were washed thrice and the pellet was resuspended in 1 ml of RPMI 1640 medium containing 2% FBS. Respective antibodies for purification of CTL (i.e., CTL enrichment cocktail containing MAbs to CD4, CD16, CD19, CD36, and CD56 cell surface antigens) and of CD4$^+$ T lymphocytes (CD4$^+$-T-cell enrichment cocktail containing MAbs to CD8, CD16, CD19, CD36, and CD56 cell surface antigens) were added separately (100 μl/ml concentration each) to the cell suspension, followed by mixing and then incubation on ice for 30 min. Magnetic cobalt (microbeads precoated with anti-mouse IgG) was then added (60 μl/ml), and the cells were reincubated on ice for 30 min with intermittent shaking every 10 min. The cell suspension was then passed through a magnetic column, and the purified cells (unlabeled fraction) were eluted. The purities of separated cell populations for the selection markers (89% ± 5% [mean ± standard deviation] for CD8$^+$ cells and 91% ± 4% for CD4$^+$ T cells) were determined by flow cytometry (data not shown).

To isolate γδ and γδ CTL (81 and 82 populations, two-step and three-step immunomagnetic column purifications, respectively, were carried out using PBMC according to the manufacturer’s instructions. Briefly, purified CTL (i.e., following removal of CD4$^+$-, CD16$^+$-, CD19$^+$-, CD36$^+$-, and CD56$^+$-positive cells) were treated with anti-human γδ pan-TCR MAbs to elute γδ CTL through the column. The other aliquot was labeled with γδ pan-TCR MAbs to obtain the γδ CTL fraction, which, at the third step of purification, yielded V61 and V82 subsets when anti-V62 and anti-V61 TCR-specific MAbs, respectively, were used as two separate treatments. The purities of the CTL preparations thus obtained were as follows: 99.08% ± 1.09%, 97.10% ± 1.42%, and 96.46% ± 0.81% for γδ, 81, and 62 CTL, respectively.

CD4$^+$-T-cell killing in plasma VL. The peripheral blood CD4$^+$-T-lymphocyte counts of the patients were determined by flow cytometry by using the whole-blood lysis method and MAbs from Becton Dickinson (San Jose, Calif.). The plasma VL (log 10 HIV RNA copies per milliliter) was quantified by an Amplicor HIV monitor test (Roche Diagnostic System Inc., Somerville, N.J.) following the manufacturer’s instructions. Briefly, a 1:42-μl region in the gag gene of HIV-1 was amplified by reverse transcription-PCR, and enzyme-linked immunosorbent assay was used to detect biotinylated HIV-1 and standard ampli ons. Finally, the RNA copy number was determined by following a standard protocol.

Cytotoxicity (51Cr release) assay. The standard procedure for the 51Cr release assay (1, 7) was followed with minor modifications. Briefly, effector cells were prepared as described above (with RPMI 1640 medium containing 2% FBS) and resuspending them in RPMI 1640 medium with 10% FBS at a concentration of 106 cells/ml. CD4$^+$-T-lymphocyte targets were also washed in the same way and chromium labeled by incubation with Na2CrO4 (New England Nuclear, Boston, Mass.) at a dose rate of 100 μCi/106 cells for 90 min. The cells were later washed four times and resuspected at a concentration of 2 × 106 cells/ml. Other cell targets, i.e., CEM.NK 6 cells (a CD4$^+$-T-cell line resistant to NK cell-mediated lysis and lacking expression of HLA class II antigens) and K562 cells (an erythroleukemic cell line lacking CD4 molecules and MHC class I antigens), were similarly labeled and prepared. First, each target cell suspension was added (50 μl/well) to triplicate wells of 96-well, V-bottom microtiter plates (Nunclon, Roskilde, Denmark). Effector cell suspension was then added to triplicate wells in amounts of 50 and 100 μl/well to achieve E/T ratios of 20:1 and 40:1, respectively. No effectors were added to the negative (i.e., spontaneous) control wells. The volume was increased to 200 μl/well by the addition of medium to the experimental and control wells. HCl (0.1 N) was added to the maximum control wells. The plates were subsequently incubated at 37°C in a humidified, 5% CO2 incubator for 8 h. Supernatants from triplicate wells were drawn (100 μl each) for gamma counting, and the percentage of specific lysis was calculated using the following standard formula: percentage of specific lysis = [(experimental 51Cr release − spontaneous release) × 100]/maximum 51Cr release − spontaneous release).

Statistical analysis. Determination of the correlation (r) between the level of specific cytosis and each of the different clinical parameters was carried out with Pearson’s rank correlation test using the Microsof Excel program. The Shapiro-Wilk’s test was used to check the normality of distributions. The differences between mean values (Mann-Whitney nonparametric r test) were consid-
RESULTS

Lysis of autologous and allogeneic CD4⁺ T, CEM.NK®, and K562 cells by purified CTL from AIDS patients. Experiments were first carried out by using CTL effectors from four AIDS patients against autologous primary CD4⁺ T cells and two target cell lines at E:T ratios of 20:1 and 40:1 (Fig. 1). Given that autologous CD4⁺ T cells were lysed by CTL, the study was then extended by including allogeneic CD4⁺ T cells from HIV-1-seronegative healthy donors. Target cytolysis was analyzed in relation to three clinical parameters of CTL donors, i.e., peripheral CD4⁺-T-cell counts, VL, and duration of infection. For each of the three clinical parameters, CTL donors were arbitrarily subdivided as follows. One subgroup included individuals with CD4⁺-T-cell counts of ≥300/μl, while the counterpart group consisted of subjects with counts of <300/μl. With respect to the VL of the patients at the time of blood sampling, those regarded as having low copy numbers of plasma viral RNA (≤log_{10} 2.70 copies/ml) formed one group and those regarded as having high copy numbers of plasma viral RNA (>log_{10} 2.70 copies/ml) formed another group. Similarly, subjects having a duration of infection of ≥3 years were considered as one subgroup and the rest were considered as the counterpart group. Experimental controls were run simultaneously by using purified in vitro expanded CTL from age-matched HIV-1-seronegative individuals. The obtained data on CTL-mediated lysis of allogeneic CD4⁺-T-cell, CEM.NK®, and K562 targets with respect to the three clinical parameters are shown in Fig. 2A. The mean differences between the data for each target and that for the respective controls were statistically significant (P < 0.05). The data on the correlation between the specific lysis of each target and CD4⁺-T-cell counts, VL, and duration of infection are summarized in Fig. 2B, C, and D, respectively. For CD4⁺-T-cell targets, the percentage of cytolytic activity correlated negatively with CD4⁺-T-cell counts (rₑ = −0.79) and positively with both VL and the duration of infection (rₑ = 0.92 and rₑ = 0.76, respectively). As for CEM.NK® targets, the correlations (rₑ values) with CD4⁺-T-cell counts, VL, and duration of infection were −0.97, 0.50, and 0.59, respectively. The specific lysis of K562 cells also correlated with the CD4⁺-T-cell counts (rₑ = −0.96), VL (rₑ = 0.47), and duration of infection (rₑ = 0.59) of the CTL donors (P value of <0.05 in each case).

Hence, both the CEM.NK® and K562 cell lines were as susceptible to CTL from AIDS patients as were the autologous and allogeneic CD4⁺ T lymphocytes. Moreover, unlike primary CD4⁺-T-cell targets, both the CEM.NK® and K562 cell lines did not require activation for CTL-mediated cytolysis in vitro (data not shown). We thus found that CTL-mediated lysis of these targets was related neither to the presence or absence of MHC class I and II surface antigens nor to the CD4⁺ receptors on them.

CTL immunophenotypes and subtypes in HIV-infected patients. Since CTL from AIDS patients showed MHC-unrestricted killing of various cell types, we further determined the TCR phenotypes in the peripheral blood of these donors. The data were statistically analyzed by grouping donors as low virus load (LVL; i.e., VL of <log_{10} 2.70 copies/ml), medium virus load (MVL; i.e., VL between log_{10} 2.70 and log_{10} 3.70 copies/ml), and high virus load (HVL; i.e., VL of >log_{10} 3.70 copies/ml) patients. As shown in Fig. 3A, the mean percentage of expression of αβ TCR⁺ CTL decreased and that of γδ TCR⁺ CTL increased with increasing VL in HIV-infected patients. Compared with the results for the control individuals, however, only the HVL group showed overall significant alterations (for αβ CTL, P < 0.0092; for γδ CTL, P < 0.0005). The CTL effector populations from 10 HVL patients were also used to determine the δ1 and δ2 γδ CTL subsets by fluorescence-activated cell sorter analysis. Figure 3B shows that the Vδ1 γδ CTL subset was predominant in HIV-infected patients while the Vδ2 subset was predominant in the HIV-seronegative controls (P < 0.05).

Lysis is precluded following TCR blockade by specific mAbs and elimination of the Vδ1 subset from γδ T cell effectors. Because the above-mentioned data showed that peripheral blood γδ CTL are expanded in HIV progressors with HVL and that the CTL from these individuals displayed relatively higher killing activity, we sought to determine which subset of γδ CTL was the principal effector of this cytolytic activity. Therefore, anti-human αδ and γδ pan-TCR mAbs were used to pretreat CTL before they were mixed with the targets. The data obtained indicate that anti-γδ pan-TCR MAb suppressed target cytolysis (P < 0.0001) (Fig. 4A). A similar suppression of lysis was observed after either CTL pretreatment with anti-human
V61 γδ TCR MAb or removal of this particular subset from the effector population (Fig. 4B, autologous CD4⁺-T-cell targets). Interestingly, the decrease in cytolysis observed when anti-αβ TCR MAb was used was insignificant in most cases and CTL labeling with anti-human V62 γδ TCR MAb also produced less cytolysis (data not shown). As expected, mock-treated and untreated CTL effectors showed comparable cytolytic activities for the targets used.

DISCUSSION

The depletion of CD4⁺ T lymphocytes is a hallmark of HIV-related pathogenesis in humans, and different scenarios have been proposed to explain this phenomenon. Since the massive replication of HIV during progression has been ascribed as being only one of the contributive mechanisms of CD4⁺-T-cell depletion, more information is required to validate any of the other suggested hypotheses. It is well known that chronic immune activation associated with HIV-1 infection is the major reason for the induction of various CTL subsets and that HIV-1-specific CTL are primarily involved in suppressing virus replication (9, 35, 48). Nonetheless, a positive correlation between CTL expansion and rapid progression to AIDS (24) has also been reported, and previous studies have shown that CTL effectors from AIDS patients lyse CD4⁺ T lymphocytes (22, 24, 55, 56) and are of the αβ TCR⁺ phenotype (23). However, no study has evaluated simultaneously the roles of αβ and γδ CTL effectors in the depletion of CD4⁺ T cells in HIV-positive patients. Therefore, the present study adds a new element to these scenarios by showing that γδ CTL from HIV-infected patients can lyse not only autologous CD4⁺ T cells but also allogeneic primary CD4⁺ T cells, as well as human lymphoid cell lines. We compared the cytoidal...
effector function of different CTL subsets either by specific MAb-mediated TCR blocking or by elimination of the counterpart subset(s) from the effector population. Our data clearly indicate that peripheral blood γδ T cells in HIV-infected patients are abnormally expanded and are predominantly respon-
sible for MHC-unrestricted lysis of various human cell types, whereas the αβ TCR effectors display only a minor level of cytolytic activity. Our data also show that the V61 CTL subset represents the main effectors of this cytolysis and that targets with and without CD4+ or MHC class I or II molecules are susceptible to their cytolytic activity.

Normally, γδ T cells constitute only 5 to 10% of human peripheral blood CTL (39), but abnormal expansions, especially of the V61 γδ T-cell subset, in several disease conditions

FIG. 3. CTL immunophenotypes and their relationship to HIV infection and VL. (A) αβ and γδ CTL populations in the activated CTL from HIV-infected patients and HIV-seronegative controls. As described in the legend to Fig. 1, CTL effectors were analyzed for expression of TCR phenotypes. Enriched T cells (depleted of CD4-, CD16-, CD19-, CD36-, and CD56-positive populations) obtained from HIV-positive and HIV-seronegative (age-matched, healthy) donors were washed thrice in cold RPMI 1640 medium with 2% FBS and labeled with αβ and γδ pan-TCR specific MAbs according to standard protocols. FACScan data, presented as means plus SEM, were obtained from five independent determinations that included three groups as follows: LVL (patients with VL of <log10 2.70 copies of HIV-1 RNA/ml), MVL (patients with VL between log10 2.70 and log10 3.70 copies of HIV-1 RNA/ml), and HVL (patients with VL of >log10 3.70 copies of HIV-1 RNA/ml). Asterisks represent statistically significant mean difference from the respective control value for each TCR phenotype. (B) V61 and V62 γδ CTL subsets in HIV-positive patients and HIV-seronegative controls. For these determinations, PBMC from 10 HVL HIV-positive individuals and six HIV-seronegative controls were purified for γδ CTL and stained with anti-γδ and anti-γδ-pan TCR specific MAbs as well as isotype control antibodies. FACScan data are presented as the percentages of positive cells of each subset for HIV-infected patients and control individuals. For each phenotype, the differences between the values for HIV-infected patients and those for the controls were statistically significant (P < 0.05; Mann-Whitney nonparametric t test).

FIG. 4. Analyses of anti-CD4+ effector CTL phenotypes. (A) Suppression of target cytolysis following CTL labeling by TCR-specific MAbs. In vitro-activated and purified CTL from six HIV-infected patients were mixed with 51Cr-labeled targets in the cytotoxicity assays. The effectors (CTL) were pretreated with anti-human αβ and γδ pan-TCR-specific isotype control antibodies by using recommended optimum concentrations before the CTL were mixed with the targets. The percentage of specific lysis (cytotoxicity) was determined from the amount of radioactivity released into the supernatants after 8 h of incubation at 37°C. Each bar in the figure represents mean cytotoxicity plus SEM from triplicate experiments. Asterisks indicate statistically significant differences (P < 0.05) from the values for the respective controls for each target used. The percentage of specific lysis (cytotoxicity) was determined from the amount of radioactivity released into the supernatants after 8 h of incubation at 37°C. Each bar in the figure represents mean cytotoxicity plus SEM from triplicate experiments. Asterisks indicate statistically significant differences (P < 0.05) from the values for the respective controls for each target used. (B) Comparative lysis by purified TCR αβ, V61, and V62 γδ CTL effectors. Shown is the percentage of specific lysis of allogeneic CD4+ T lymphocytes by purified CTL effectors from four HIV patients at an E:T ratio of 20:1, as determined by 8-h microculture cytolytic assays. The difference (nonparametric t test) between the mean percentage of lysis by the 61 and 62 effectors and αβ CTL-mediated lysis was found to be statistically significant (P < 0.05).
including HIV-1 infection (53) and neoplasia (29) have been reported. Although selective expansion of peripheral blood γδ T cells has been reported during HIV-1 infection (8, 53), the immunological consequences of this phenomenon have not yet been explained. We speculate that γδ T cells induced in the early stages of acute HIV-1 infections are maintained as precursors of their effector subsets during progression. Consequently, ex vivo expansion of these precursors would yield reactivated effectors that may cause depletion of various cell targets, as demonstrated in our microculture cytotoxicity assays. Since these CTL lyse both infected and uninfected targets, it seems obvious that their effector function is directed not towards a virus-specific antigen(s) but rather against the cellular ligand(s) commonly expressed on targets as a sequel to immune activation and/or dysregulation. It may be noted here that, as part of the indirect consequences of HIV infection, sustained overstimulation of the immune system during infection has been reported as a factor leading to cumulative, detrimental effect (i.e., CD4+ T-cell depletion) (Feinberg et al., letter). Given the potential of immortalized human lymphoid cell lines for continuous division, the target ligand(s) may already be highly expressed on CEM.NKR and K562 cells compared with the primary CD4+ T-cell targets. This is perhaps the reason why, as our results indicate, these cell lines (unlike CD4+ T cells) do not require further reactivation for their CTL-mediated cytolysis.

To our knowledge, there are no reports presently available that define either the cause or the mechanism of selective expansion of γδ T cells in the peripheral blood of HIV progressors or identify the target molecule(s) mediating this cytolytic activity. Most human γδ T cells (Vγ2Vδ2 or Vγ9Vδ2 subset) recognize non-peptide phosphorylated antigens, isoprenyl phosphate, and related prenyl pyrophosphate molecules (46, 47) as well as alkylamine antigens (13). Both the phosphate and amine antigens are important products of microbes and self-antigens, and their presentation does not involve MHC class I or class II peptide antigen-presenting molecules (31, 33, 38). It might be speculated that these antigens may be recognized much as haptons are recognized by immunoglobulins and TCRs of cytotoxic T cells (12). We show that both activated autologous and allogeneic primary CD4+ T cells, as well as the CEM.NKR and K562 cell lines, are lysed by γδ CTL. Which target molecules are involved in this cytolyis by γδ CTL from HIV-positive individuals is not known. However, it has previously been reported (37) that CD4+ T cells activated with either PHA or anti-CD3 antibodies express MHC class I-related surface MICA and MICB (28, 29, 44), whereas the CEM.NKR and K562 cell lines express ULBPs, with the expression of ULBPs being independent of the expression of surface MHC class I and class II antigens on these cells (17, 45). Therefore, it is probable that MICA and MICB and/or ULBP ligands are expressed on these targets and mediate cytolysis by delivering both TCR-dependent signal 1 and NKG2D-dependent signal 2 (5) to the V61 γδ T effectors (54) and signal 2 to the Vγ9Vδ2 CTL effectors (18). Widely expressed nonpolymorphic CD1c molecules may also be involved in TCR-mediated recognition and target cytolysis by the V61 γδ CTL (6, 42). In any event, it remains to be determined which type of ligand molecules are specifically induced or modified on the target cells during HIV infection and to establish whether these molecules play a role in γδ CTL-mediated cytolyis.

In view of their broad-scale ex vivo killing potential as shown by this study, the V61 γδ CTL effectors may be associated, at least in part, with the immunopathology observed in HIV progressors. Furthermore, it is interesting that functional anergy of the V62 T subset in as many as 60% of asymptomatic HIV-infected patients has also been reported (53). Our results showing the correlation of CTL-mediated killing with the various clinical parameters are also corroborated by previous studies (10, 34). Persistent decline in CD4+ T-cell counts is both a hallmark and a prognostic indicator of HIV progression. Subsequent to the onset of symptomatic HIV infection, sharp declines in CD4+ T-cell counts and consistent ascension of VL are the major features indicating progression. Based on our results, it is tempting to suggest that continued selection of diverse progeny of viral variants (25) may not be sufficient to account for the ineffectiveness of existing CTL repertoires in containing viral replication and that the progressive immune dysregulation observed in HIV-infected patients may lead to (or result from) the induction and expansion of V61 γδ CTL. These CTL seem to be capable of contributing to immunopathogenesis through depletion of vital cell types involved in the adaptive immune response to infection. Interestingly, lack of full-blown AIDS in chimpanzees has been associated in part with the failure to develop MHC-unrestricted CTL in blood (55) as well as the absence of a Th1-to-Th2 cytokine shift (30). This clearly contrasts with the characteristic features of HIV infection in humans.

Finally, it is noteworthy that although this study was carried out with patients on HAART, MHC-unrestricted, CTL-mediated cytolyis of HIV-infected and bystander cells in individuals both on and not on HAART (23, 24) has been reported; however, γδ T-cell-mediated cytolytic activity was not investigated in those previous studies. It is not known at present whether different therapeutic regimens prescribed to HIV-1-infected patients and AIDS patients have any effect on CTL-mediated cytopathicity. Previous observations, together with the present data, clearly emphasize the need for further studies on this matter. Such studies could provide important insights on the pathogenetic mechanisms involved in the progression of HIV infection and AIDS. Taken together, our data suggest that abnormal expansion of γδ CTL, especially of the V61 subset, in HIV-1-infected patients may contribute to lysis of bystander CD4+ T lymphocytes as well as other cells, leading eventually to exacerbation of immunopathology and AIDS in these individuals.

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REFERENCES