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$CD8^+$ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity

(cytotoxic T lymphocyte/T-cell abnormalities)

GIUSEPPE PANTALEO*†, ANDREA DE MARIA*, SCOTT KOENIG*, LUCA BUTINI*, BERNARD MOSS‡, MICHAEL BASELER§, H. CLIFFORD LANE*, AND ANTHONY S. FAUCI*

*Laboratory of Immunoregulation and ‡Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and §Program Resources, Incorporated, Frederick, MD 21701

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ABSTRACT **In this study, we have investigated the potential mechanisms responsible for the loss of human immunodeficiency virus type 1 (HIV-1)-specific cytolytic activity in the advanced stages of HIV-1 infection. We have demonstrated that HIV-1-specific cytotoxic T lymphocytes are predominantly contained within the $CD8^+DR^+$ subset. Furthermore, we have shown by a redirected killing assay that there is a dichotomy between HIV-1-specific cytolytic activity and broad cytolytic potential since the cytolytic machinery of $CD8^+DR^+$ cells is still functioning even in patients with AIDS who have lost their HIV-1-specific cytolytic activity. In addition, by comparative analysis of these two types of cytolytic activity over time we have demonstrated a progressive loss of HIV-1-specific cytolytic activity in the advanced stages of the disease, whereas the cytolytic potential remained unchanged regardless of the clinical stage. As previously shown in patients with AIDS, even in asymptomatic HIV-1-seropositive patients, $CD8^+DR^+$ cells from the same patient, compared to $CD8^+DR^-$ lymphocytes, showed a substantial reduction in their ability to proliferate *in vitro* in response to different stimuli, such as mitogens (phytohemagglutinin and phorbol 12-myristate 13-acetate) and monoclonal antibodies directed against CD3, CD2, and CD28 molecules, and displayed a defective clonogenic potential. Thus, on the basis of these results we propose that the loss of HIV-1-specific cytolytic activity in HIV-1-infected individuals may result at least in part from a progressive decrease in the pool of HIV-1-specific cytotoxic T lymphocytes belonging to the $CD8^+DR^+$ subset whose ability to expand has been impaired.**

Major histocompatibility complex class 1-restricted human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) have been documented in humans (1–7), primates (8), and mice (9). These HIV-1-specific CTLs belong predominantly to the $CD8^+$ subset of T lymphocytes (1, 6, 7). It has been suggested that a progressive decrease of HIV-1-specific cytolytic activity parallels the progression of HIV-1 infection (2, 4). It is still unclear, however, whether the defective HIV-1-specific cytolytic function is caused by a selective loss of HIV-1-specific CTL precursors or is due to a more general impairment of the cytolytic machinery of $CD8^+$ cells. In addition, a defective generation *in vitro* of CTLs against influenza virus and cytomegalovirus has been described in HIV-1-infected individuals (10, 11).

We have recently demonstrated that in AIDS patients there exists a population of cells with a reduced ability to undergo clonal proliferation (nonclonogenic), which predominantly belong to the $CD8^+$ subset that is phenotypically defined by the surface expression of HLA-DR antigens (12). In the present study, we demonstrate that the HIV-1-specific cytolytic activity is predominantly confined to this subset of circulating $CD8^+DR^+$ cells with decreased clonogenic potential. In addition, by a redirected killing assay we have also analyzed the cytolytic potential $CD8^+$ T-cell subsets in HIV-1-seropositive individuals.

By comparative analysis over time of the HIV-1-specific cytolytic activity and of the cytolytic potential of $CD8^+$ T cells, we demonstrate that in patients with AIDS there is a loss of HIV-1-specific cytotoxicity. However, in these same patients, $CD8^+$ cells totally retained their cytolytic potential as measured in the redirected killing assay.

These results suggest that the loss of HIV-1-specific cytolytic activity in patients with AIDS results at least in part from a defect in the HIV-1-specific CTL population to expand *in vivo*.

MATERIALS AND METHODS

Study Subjects. Three asymptomatic seropositive homosexual men, five patients with AIDS, and one symptomatic patient (stage 5B according to the Walter Reed classification) were studied.

Monoclonal Antibodies (mAbs). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAbs used in this study for phenotypic analysis were Leu4 (anti-CD3), Leu2a (anti-CD8), anti-HLA-DR, and Leu7 (anti-CD57), which were purchased from Becton Dickinson.

Isolation of Lymphoid Cells. Lymphocytes were isolated from peripheral blood over a Ficoll/Hypaque density gradient.

Flow Cytofluorometric Analysis. Analysis of total peripheral blood mononuclear cells (PBMCs) for the distribution of CD8, HLA-DR, and Leu7 antigens was performed by two-color cytofluorometric analysis on an EPICS profile (Coulter). Cells were stained with FITC-conjugated and PE-conjugated mAbs.

Cell Sorting. Cell sorting was performed as described (13). To isolate $CD8^+DR^+$ and $CD8^+DR^-$ cells, PBMCs were stained with a FITC-conjugated anti-HLA-DR mAb and a PE-conjugated anti-CD8 mAb. The purity of sorted populations ranged between 96% and 98%.

Target Cells and Cytotoxicity Microassay. Lymphoblastoid cell lines (LCLs) were generated from the PBMCs of the

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Abbreviations: HIV, human immunodeficiency virus; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; LCL, lymphoblastoid cell line; EBV, Epstein–Barr virus; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

†To whom reprint requests should be addressed at: National Institutes of Health, Building 10, Room 11B-13, Bethesda, MD 20892.

patients (autologous) by transformation with culture supernatant derived from marmoset cells infected with the B95-8 strain of Epstein–Barr virus (EBV) and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. To assess HIV-1-specific cytolytic activity LCL cells (2×10^6) were simultaneously infected for 8 hr with three recombinant vaccinia vectors expressing the env, gag, pol, and nef proteins of HIV-1_{IIIB}, labeled with 100 μCi (1 Ci = 37 GBq) of sodium [^{51}Cr]chromate (Amersham), washed three times, and then resuspended to the appropriate concentration for use as target cells. LCL cells infected with recombinant vaccinia virus containing the bacterial *lacZ* gene were used as controls. To assess the cytolytic potential, we performed a redirected killing assay using as target cells the P815 murine mastocytoma cell line. P815 cells (1×10^6) were labeled for 1 hr at 37°C with 100 μCi of sodium [^{51}Cr]chromate, washed three times, and then resuspended to the appropriate concentrations. Anti-CD3 mAb (1:50,000 dilution of ascitic fluid) was added at the time of the assay. Control wells contained effector and target cells in the absence of anti-CD3 mAb. In both types of cytolytic assays, multiple effector/target cell ratios were examined in duplicate. Percentage specific cell lysis was calculated as described (13).

Recombinant Vaccinia Viruses. Four recombinant vaccinia vectors were used in these experiments. Vac/env (vPE16) expresses the HIV envelope glycoprotein in the absence of other HIV structural or regulatory proteins (2). The Vac/vVK2 was derived from the HXB2 clone of HIV-1. The *gag–pol* genes were placed in the same reading frame to produce a gag–pol fusion protein (V. Karacostas and B.M., unpublished data). The Vac/nef (vVTFnef) contains the complete nef open reading frame of HIV-1 constructed as described (14). Vac/lac (vSC8), a recombinant vaccinia vector containing the bacterial *lacZ* gene, was used as a control (2).

Monocyte Preparation. Leukapheresis was performed on HIV-seronegative healthy donors and the mononuclear cells were then separated by low-speed centrifugation over LSM (Organon Teknika–Cappel) as described (12).

Stimulation of Lymphocytic Populations and Assay of DNA Synthesis. Stimulation of lymphocytic populations was performed as described (12). Briefly, sorted $CD8^+DR^+$ and $CD8^+DR^-$ cells were stimulated with different stimuli, including anti-CD3 mAb (1:50,000 dilution of ascitic fluid), a stimulatory combination of anti-CD2 mAbs (anti-$CD2_2$ + anti-$CD2_3$) (1:1000 dilution of ascitic fluid), anti-CD28 mAb (Kolt-2) (1:5000 dilution of ascitic fluid), and phytohemagglutinin (PHA) (0.2 μg/ml) alone or in combination with phorbol 12-myristate 13-acetate (PMA) (0.5 ng/ml). Cells were incubated in sterile U-bottomed microtiter plates for 72 hr in the presence of the appropriate stimulus and 10^4 allogeneic irradiated (4000 rad; 1 rad = 0.01 Gy) monocytes. Purified interleukin 2 (10%) (64 units/ml) was added 24 hr after initiating the cultures. Cell proliferation was evaluated by [^{3}H]thymidine incorporation after a 16-hr pulse with 0.5 μCi of [^{3}H]thymidine.

Microcultures and Statistical Analysis. For cloning of T lymphocytes, we used the limiting-dilution microculture technique of Moretta *et al.* (15) and statistical analysis was performed as described (15, 16).

RESULTS AND DISCUSSION

We have recently identified in patients with AIDS an expansion *in vivo* of a $CD8^+$ T-cell subset (30–50% of total $CD8^+$ T lymphocytes) characterized phenotypically by the surface expression of HLA-DR antigens (12). As compared to $CD8^+DR^-$ T cells, $CD8^+DR^+$ T cells showed a dramatic defect in their capacity to proliferate *in vitro* in response to various stimuli such as mitogens (i.e., PHA and PMA) or mAbs directed against the CD3/T-cell receptor complex, CD2 and CD28 molecules, and in their clonogenic potential (12).

To delineate the potentially protective role of $CD8^+$ lymphocytes during the progression of HIV-1 infection and to understand the mechanisms responsible for the progressive loss of HIV-1-specific cytolytic activity occurring in the advanced stages of HIV-1 infection, we characterized the cytolytic properties of this $CD8^+DR^+$ T-cell subset.

To this end, we initially studied three asymptomatic HIV-1-seropositive individuals who manifested an *in vivo* expansion of the $CD8^+DR^+$ subset (30–60%). Freshly isolated PBMCs from the three patients were incubated with a FITC-conjugated anti-HLA-DR mAb and PE-conjugated anti-CD8 mAb and were then separated into $CD8^+DR^+$ and $CD8^+DR^-$ cells by cell-sorter analysis. These sorted populations were examined either for their capacity to proliferate in response to different stimuli or for HIV-1-specific cytotoxicity against env, gag, pol, and nef proteins of HIV-1_{IIIB}. Similar to our previous observation in AIDS patients (12), the $CD8^+DR^+$ T cells in these healthy HIV-1-seropositive individuals showed, although to a lesser extent, a consistent defect compared to $CD8^+DR^-$ T cells in their ability to proliferate in response to anti-CD3 mAb (Fig. 1). A similar decreased potential for proliferation was also observed after stimulation with mitogens (i.e., PHA and PMA) or anti-CD2 and anti-CD28 mAbs (data not shown). In addition, $CD8^+DR^+$ cells also showed a severe defect in their clonogenic potential (57–63% reduction compared to $CD8^+DR^-$ cells in the three patients studied) (data not shown). To assess the HIV-1-specific cytolytic activity of these cells, autologous EBV-transformed LCLs

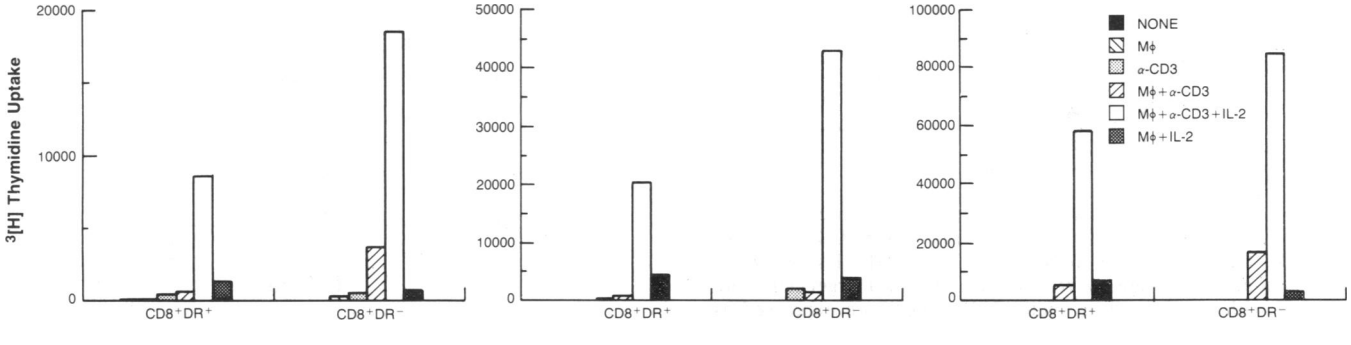

FIG. 1. Comparative analysis of the potential for proliferation of $CD8^+DR^+$ and $CD8^+DR^-$ cell subsets. Sorted $CD8^+DR^+$ and $CD8^+DR^-$ cell populations from three healthy HIV-1-seropositive individuals were stimulated with anti-CD3 mAb (1:50,000 dilution of ascitic fluid) in the presence of irradiated (4000 rad) allogeneic exogenous monocytes (10^4 cells per well are shown). Purified interleukin 2 (final concentration, 10%) was added to the microcultures stimulated with anti-CD3 mAb 24 hr after initiating the cultures. At the end of the period of stimulation (72 hr), cell proliferation was evaluated by [^{3}H]thymidine uptake (16-hr pulse). Each bar represents mean [^{3}H]thymidine uptake from duplicate cultures (<15% variability per culture).

were infected with HIV vaccinia recombinant viruses and used as target cells in a chromium-release assay. The results for each subject are presented in Fig. 1. In the three asymptomatic seropositive patients studied, the HIV-1-specific cytolytic activity was predominantly restricted to the $CD8^+DR^+$ subset (Fig. 2 *Ia*, *IIa*, and *IIIa*). No consistent HIV-1-specific cytolytic activity was mediated by the $CD8^+DR^-$ subset. To exclude the possibility that the lack of HIV-1-specific cytolytic activity in the $CD8^+DR^-$ subset was due to a functional defect of the cytolytic machinery in these cells, we performed a comparative analysis of the cytolytic potential in the two $CD8^+$ subsets by using a redirected killing assay (17). This assay involves the use of appropriate $Fc\gamma R^+$ target cells and mAbs of the appropriate IgG isotype. By the use of anti-CD3 mAb, crosslinking between target and effector cells occurs and this leads to triggering of the lytic machinery of CTLs and target cell lysis if the effector cell is functional. As shown in Fig. 2 (*Ib*, *IIb*, *IIIb*), $CD8^+DR^+$ cells displayed significant levels of cytotoxic activity in the redirected killing assay. Furthermore, the levels of redirected cytotoxic activity displayed by $CD8^+DR^-$ cells were similar to those of $CD8^+DR^+$ cells despite the fact that there was substantial disparity between the two subsets in HIV-1-specific cytolytic activity (Fig. 2 *Ia*, *IIa*, and *IIIa*). These results clearly indicate that the HIV-1-specific CTLs are contained within the $CD8^+DR^+$ subset and that very little HIV-1-specific cytolytic activity is present within the $CD8^+DR^-$ cells despite intact cytolytic potential in the latter subset (Fig. 2 *Ib*, *IIb*, and *IIIb*). In three normal donors, the levels of redirected cytotoxic activity displayed by sorted $CD8^+$ cells ranged between 40% and 60% at the same effector target cell ratio (data not shown).

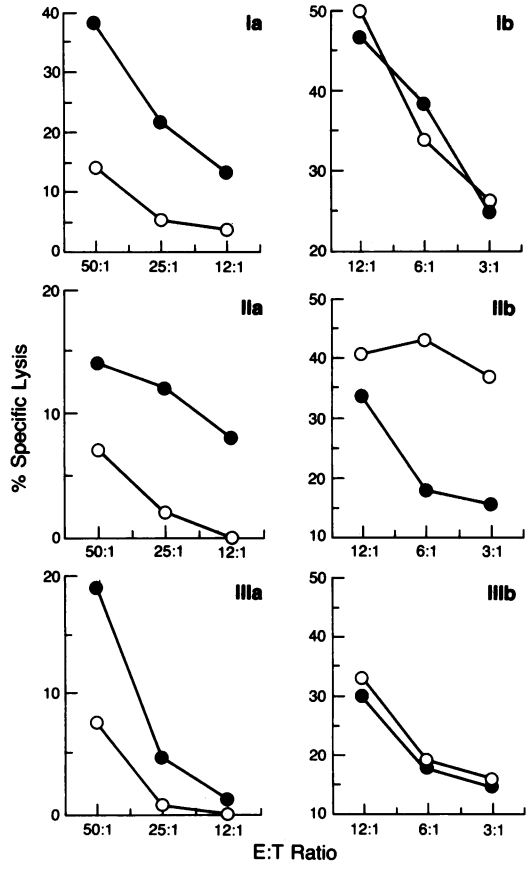

FIG. 2. Cytolytic activity against target cells expressing HIV-1_{IIIB} env, gag, pol, and nef proteins by fresh sorted $CD8^+DR^+$ (●) and $CD8^+DR^-$ (○) subsets from three different HIV-1-seropositive healthy homosexual men (*Ia*, *IIa*, and *IIIa*). Control target cells were infected with vaccinia virus vector VSC8. Data are expressed as percentage specific lysis after subtraction of the background levels. (*Ib*, *IIb*, and *IIIb*) Cytolytic activity against P815 target cells in the presence of anti-CD3 mAb (1:50,000 dilution of ascitic fluid) by the same $CD8^+$ subsets. In the control microcultures, cytolytic activity was measured in the absence of anti-CD3 mAb. Data are expressed as percentage specific lysis after subtraction of background levels. E:T, effector/target cell.

Several mechanisms have been proposed to explain the decrease and ultimately the loss of HIV-1-specific cytolytic activity in AIDS (2, 4): (*i*) quantitative deficiency in $CD4^+$ T cells by a defective production *in vivo* of cytokines required for the optimal growth and function of CTLs (18); (*ii*) progressive decrease in the frequency of HIV-1-specific CTLs (4); (*iii*) cell-mediated suppression of HIV-1-specific CTLs (19); (*iv*) *in vivo* selection of HIV mutants (20).

To better characterize the nature of the defect in HIV-1-specific cytolytic activity, we have investigated the status of broad cytolytic functional capability in AIDS patients. To this end, freshly sorted $CD8^+DR^+$ and $CD8^+DR^-$ cells from a representative patient with AIDS were tested in a redirected killing assay. As shown in Fig. 3*A*, and in contrast to the findings in the asymptomatic seropositive individual, neither $CD8^+DR^+$ nor $CD8^+DR^-$ subsets from this patient with AIDS displayed HIV-1-specific cytolytic activity. On the contrary, both $CD8^+$ subsets were strongly cytolytic in the redirected killing assay (Fig. 3*B*), thus indicating that, even in patients with AIDS, the broad cytolytic potential of CTLs is not impaired. Similar results were observed in three additional patients (data not shown).

These results were further confirmed in a series of experiments in which we had the opportunity to perform a comparative analysis over time of the HIV-1-specific cytolytic activity and of the cytolytic potential assessed in the redi-

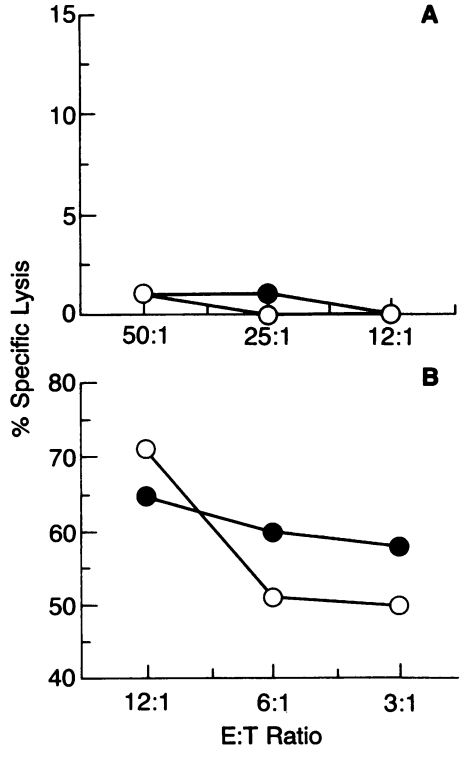

FIG. 3. HIV-1-specific cytolytic activity and broad cytolytic potential in $CD8^+DR^+$ and $CD8^+DR^-$ T-cell subsets in an AIDS patient. (*A*) Cytolytic activity against target cells expressing env, gag, pol, and nef proteins of HIV-1_{IIIB} by fresh sorted $CD8^+DR^+$ (●) and $CD8^+DR^-$ (○) cells. (*B*) The same $CD8^+$ subsets were tested against P815 target cells in the presence of anti-CD3 mAb. E:T, effector/target cell.

rected killing assay. In Fig. 4*A*, the levels of HIV-1-specific cytolytic activity displayed by $CD8^+DR^+$ cells at different time points separated by 3 years during the progression of HIV-1 infection are shown. Consistent HIV-1-specific cytolytic activity was detected in the early stages of HIV-1 infection (asymptomatic), whereas $CD8^+DR^+$ cells were not cytolytic in the advanced stages of disease (AIDS). On the contrary, no major changes in the levels of cytolytic activity measured in the redirected killing assay between the early and advanced stages in HIV-1 infection were detected within this $CD8^+DR^+$ subset (Fig. 4*C*). As mentioned above, the $CD8^+DR^-$ cells manifested minimal, if any, HIV-1-specific cytolytic activity at either time point (Fig. 4*B*), whereas broad cytolytic potential was intact at both times (Fig. 4*D*).

As shown in Fig. 5, similar results were obtained in another patient who became symptomatic (Walter Reed stage 5B) after 26 months of observation. The decrease in HIV-1-specific cytolytic activity paralleled the progression of HIV-1 infection (Fig. 5*A*), whereas the levels of cytolytic activity of both $CD8^+DR^+$ and $CD8^+DR^-$ subsets measured in the redirected killing assay were unchanged despite progression in the clinical stage of HIV-1 infection (Fig. 5 *B* and *C*).

We have also investigated whether in the group of patients included in this study there was an expansion of the $CD8^+CD57^+$ subset. $CD8^+CD57^+$ lymphocytes have been shown previously to suppress B-cell differentiation and T-cell proliferative responses to soluble antigens and mitogens (19, 20). In addition, it has been recently shown that $CD8^+CD57^+$ mediate suppression *in vitro* of HIV-1-specific cytolytic activity (21). In the two patients with AIDS presented in this study and in another group ($n = 7$) not included here, a high percentage of $CD8^+$ cells did indeed express CD57 antigen (up to 50% of $CD8^+$ cells) (data not shown). However, the percentage of $CD8^+CD57^+$ cells ranged between 30% and

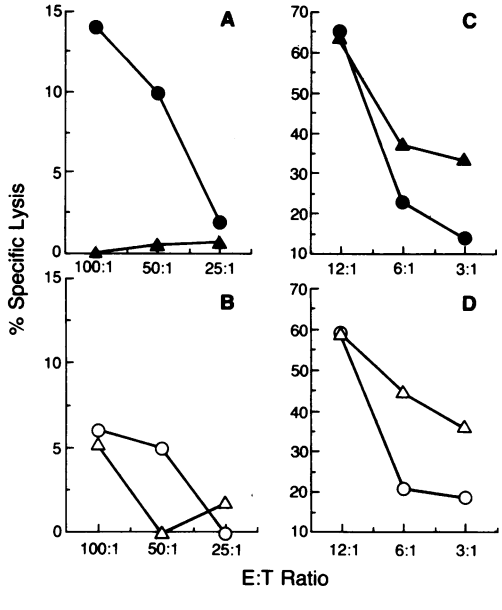

FIG. 4. Comparative analysis over time of the HIV-1-specific cytolytic activity and of the cytolytic potential of $CD8^+DR^+$ and $CD8^+DR^-$ cells from the same patient during the progression of HIV-1 infection (healthy seropositive in 1986 and AIDS in 1989). (*A*) Cytolytic activity against target cells expressing $HIV\text{-}1_{IIIB}$ env, gag, pol, and nef proteins by sorted $CD8^+DR^+$ cells (●, 1986; ▲, 1989). (*B*) HIV-1-specific cytolytic activity by $CD8^+DR^-$ cells (○, 1986; △, 1989). (*C*) Cytolytic activity against P815 target cells in the presence of anti-CD3 mAb by $CD8^+DR^+$ (●, 1986; ▲, 1989). (*D*) Cytolytic activity against P815 target cells in the presence of anti-CD3 mAb by $CD8^+DR^-$ (○, 1986; △, 1989) subsets. In this experiment, frozen cells, thawed 24 hr before performing the cytolytic assay, were used as the source of effector cells. E:T, effector/target cell.

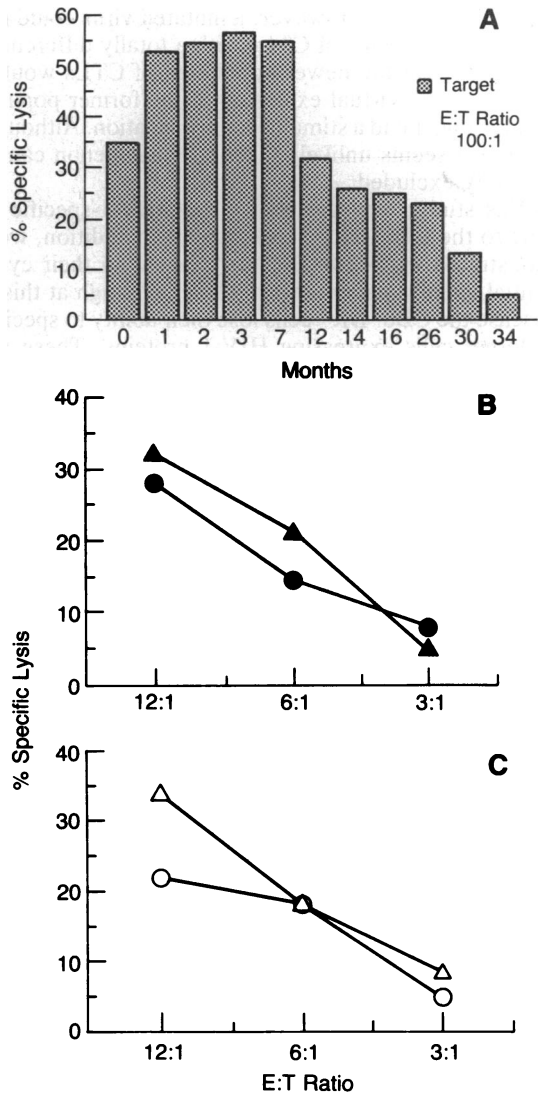

FIG. 5. HIV-1-specific cytotoxicity by fresh unfractionated PBMCs from a HIV-1-seropositive homosexual man over a 3-year period (*A*) (healthy seropositive in 1986 and AIDS in 1989). Cytolytic potential of $CD8^+DR^+$ (●, 1986; ▲, 1989) and $CD8^+DR^-$ (○, 1986; △, 1989) subsets during the progression of HIV-1 infection are shown in *B* and *C*, respectively. In the experiments shown in *B* and *C*, frozen cells were used as the source of effector cells. In this experiment, LCL cells were infected with only the vaccinia vector expressing the env protein since no cytolytic activity was detected against LCL cells bearing the other HIV-1 proteins. E:T, effector/target cell.

40% (data not shown) in the three healthy HIV-1-infected individuals in the present study with significant levels of HIV-1-specific cytolytic activity. In addition, the percentage of $CD8^+CD57^+$ cells was >70% during the first 12 months of observation of the HIV-1-specific cytolytic activity of the patient presented in Fig. 5*A*. Therefore, on the basis of our results there is little to support the hypothesis of cell-mediated suppression of HIV-1-specific CTLs as a mechanism responsible for the loss of HIV-1-specific cytolytic activity.

In vivo selection of HIV mutants has been proposed as a potential mechanism by which HIV-infected target cells could escape specific killing (22). Since the identical HIV-1 proteins expressed by vaccinia constructs were used in the target cells employed at the time points when HIV-specific cytolytic activity was present early in the course of infection and when the activity was markedly diminished at a later time, mutated virus *in vivo* would unlikely explain the loss of

HIV-specific CTLs. However, a mutated virus could indeed trigger the emergence of CTLs with a totally different specificity, such that the newer population of CTLs would predominate to the virtual exclusion of the former population, which no longer had a stimulus for propagation. Although this explanation seems unlikely, such a phenomenon cannot be completely excluded.

In this study, we have shown that HIV-1-specific CTLs belong to the $CD8^+DR^+$ T-cell subset. In addition, we have demonstrated that $CD8^+DR^+$ cells maintain their cytolytic potential even in patients with AIDS, although at this stage of disease the $CD8^+DR^+$ cells lose their ability to specifically lyse target cells expressing HIV-1 proteins. These results indicate that impaired functioning of the cytolytic machinery is not the mechanism responsible for the loss of the HIV-1-specific cytolytic activity, and that $CD8^+DR^+$ T cells are primarily involved in the antiviral immune response. In this context, it is important to point out that the expansion *in vivo* of $CD8^+DR^+$ cells is a finding common to other viral infections such as EBV and cytomegalovirus (23, 24) as well as in bone marrow transplantation (25) and certain chronic inflammatory diseases such as systemic lupus erythematosus (26) and rheumatoid arthritis (27). Thus, the appearance in the circulation of this $CD8^+$ subset may not be specific for HIV infection, but it may be a feature of certain conditions in which a chronic stimulation of the immune system occurs. The particular relevance of this phenomenon to HIV infection may lie in the fact that HIV-specific $CD8^+$ cytolytic cells are critical to the containment of the virus and any functional impairment or decrease in the ability of this population to expand may have serious consequences for the host.

We have previously shown (12) that the $CD8^+DR^+$ cells in patients with AIDS showed a severely reduced capability to proliferate in response to different stimuli and to undergo clonal expansion *in vitro*. In the present study, we have demonstrated that (*i*) these $CD8^+DR^+$ cells obtained from healthy HIV-1-infected individuals showed similar characteristics and (*ii*) the HIV-1-specific CTLs predominantly belong to this same poorly clonogenic $CD8^+DR^+$ cell population. In this regard, it is of interest that it has been recently shown that the frequency of HIV-1-specific CTL precursors decreases as HIV-1 infection progresses (4).

Thus, our findings suggest that the defect of HIV-1-specific cytolytic activity in infected individuals may be due to a progressive loss over the course of HIV infection of HIV-1-specific CTLs, which belong predominantly to the $CD8^+DR^+$ subset of T cells. Since this subset manifests markedly decreased clonogenic potential, progressive loss in the ability to expand this subset may ultimately contribute at least in part to the depletion of HIV-1-specific CTLs from the T-cell repertoire at the same time that the broad cytolytic capability of $CD8^+$ T cells remains intact.

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