

Human Immunodeficiency Virus-Specific Cytotoxic Responses of Seropositive Individuals: Distinct Types of Effector Cells Mediate Killing of Targets Expressing *gag* and *env* Proteins

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By using target cells that expressed isolated *env*, *gag*, p27^{nef}, or p23^{vif} molecules introduced by recombinant vaccinia viruses containing genes encoding these polypeptides, it was possible to identify *env*, *gag*, p27^{nef}, and p23^{vif} as cytolytic target antigens for freshly isolated blood cells from human immunodeficiency virus 1 (HIV-1) seropositive patients. Most of the patients tested (95%) manifested a specific cytotoxic activity against vaccinia virus-*env*-infected target cells. The *env*-specific cytotoxic activity was not restricted by the major histocompatibility complex and was not mediated by T lymphocytes, as shown by the absence of blocking effect with an anti-CD3 monoclonal antibody and by the inefficiency of CD3⁺, CD8⁺, or CD4⁺ and CD8⁺ depletion to reduce the cytotoxic activity against the *env*-expressing target cells. In the same conditions, the cytotoxic activity specific for *gag* was abrogated and *gag* major histocompatibility complex-restricted cytotoxic T lymphocytes were detected in 85% of the subjects tested. Therefore, in a HIV-1 seropositive subject, distinct types of effector cells mediate the lysis of target cells expressing *gag* and *env* proteins.

The acquired immunodeficiency syndrome (AIDS), first described in 1981, is the consequence of infection with the human immunodeficiency virus (HIV). HIV has a selective tropism for CD4⁺ helper/inducer thymus-derived (T) lymphocytes, but the depletion of CD4⁺ cells cannot be explained totally by the direct cytopathic effect of the virus. It has been proposed that an (auto)immune phenomenon plays a role in the depletion of the CD4⁺ lymphocytes that is associated with HIV infection (8, 14, 27).

Cell-mediated immune responses, particularly the generation of specific cytotoxic lymphocytes, are of importance in the pathogenesis of various viral infections. The development of effector cells with cytotoxic activity can either be useful in the recovery from a viral infection (26) or, in contrast, can contribute to pathological changes as in the case of adult mice infected with lymphocytic choriomeningitis virus (6, 11, 22). Therefore, characterization of the cellular immune response to HIV antigens in infected people may be important in explaining disease progression and designing immune therapies.

Here, we report the existence of HIV-specific cytotoxic lymphocytes in 20 of 20 seropositive HIV-1 subjects and show that the p27^{nef} (F) and p23^{vif} (Q) antigens can serve as targets for cytotoxic effector cells in addition to the *env* (gp160) and the *gag* precursor (p55) proteins. We show that while the predominant HIV *gag*-specific cytotoxicity is mediated by T cells restricted by major histocompatibility complex (MHC) antigens, the predominant HIV envelope-specific cytotoxicity is not restricted by MHC antigens and is not mediated by T cells.

MATERIALS AND METHODS

Media and reagents. RPMI 1640 (GIBCO BRL, Cergy, France) supplemented with L-glutamine (2 mM), penicillin

(100 U/ml), streptomycin (100 µg/ml), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (20 mM), and 10% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Haemek, Israel) (RPMIc) was used for most assays. The mouse EL4 cell line was a gift from G. Milon (Institut Pasteur, Paris, France), the mouse P815 cell line was a gift from D. Juy (Institut Pasteur, Paris, France), and the human K562 cell line was a gift from E. Gomard (Hôpital Cochin, Paris, France).

Monoclonal antibodies and complement. Monoclonal antibody anti-Leu11b, (which recognizes the CD16 antigen) was purchased from Becton Dickinson, Grenoble, France. Monoclonal antibodies directed against the CD3, CD4, and CD8 antigens were from Ortho Diagnostics Systems, Roissy, France (OKT3, OKT4, and OKT8, respectively) or Immunotech, Marseille, France (IOT3, IOT4, and IOT8a, respectively). The low Tox H rabbit complement was purchased from Cedarlane, Tebu, France.

Viruses. The different recombinant viruses used to infect the target cells have been previously described, except for the vaccinia virus (VV) Q recombinant (12, 19-21; G. Rautmann et al., in press). Briefly, the viruses used were the wild-type (WT) VV, strain Copenhagen, or various recombinants encoding either the middle T antigen of the polyomavirus (as a control) or the *env* (gp160), *gag* (p55), p27^{nef} (F or 3' open reading frame) or p23^{vif} (Q or *sor*) antigens of the HIV-1/BRU isolate (recombinant VV TG 1139, VV TG 1144, VV TG 1147, or VV TG 1160, respectively). The VV Q recombinant was constructed as follows: the *EcoRI* fragment from the plasmid pJ 19-13 (37) containing the coding sequence from the *vif* (Q) gene of HIV-1 was cloned in a M13 vector, and a *BglII* site was created upstream of the initiation codon by site-directed mutagenesis by using the oligonucleotide 5'TCCCTAAAGATCTTT3'. The resulting *BglII-EcoRI* fragment was subcloned in pTG186 (20) and trans-

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ferred into the VV genome. The resulting recombinant is VV TG 1160.

Patients. Among the 20 HIV-1 seropositive patients (Western blot [immunoblot] confirmed), 13 were healthy seropositive subjects (PIP2, PIP9, PIP11, PIP12, LOS9, LOS21, LOS25, LOS29, LOS45, LOS47, LOS49, LOS61, and LOMB), 5 had persistent generalized lymphadenopathy (PIP1, LOS1, LOS5, LOS19, and LOS59), and 2 had AIDS-related complex (PIP3 and PIP5).

The nine seronegative controls were healthy laboratory workers. For each subject, a B-cell line was derived after infection (of peripheral blood cells) with Epstein-Barr virus (EBV) and tissue typing was performed by established procedures.

Preparation of effector cells. Effector cells were autologous fresh peripheral blood mononuclear cells (PBMCs) isolated from blood by Ficoll-metrizoate density gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway).

Antibody plus complement-mediated lysis. Antibodies were added to 10×10^6 PBMCs in a total volume of 1 ml of RPMI-1% fetal calf serum. The final dilutions (in micrograms per milliliter) were: anti-Leu11b, 1.7; OKT3, 1; OKT4, 1.7; OKT8, 1.7; IOT4, 5; and IOT8a, 5. After 45 min at room temperature, 0.4 ml of rabbit complement was added and the mixture was incubated at 37°C for 45 min; the cells were then washed twice and suspended in RPMIc. The efficiency of depletion was at least 95%, as determined by surface immunofluorescence.

Inhibition of specific cytotoxicity with monoclonal antibodies. PBMCs (5×10^6 cells per ml) were incubated for 30 min at 37°C with monoclonal antibody to CD3 (IOT3, clone X-35 [4 µg/ml]) or with monoclonal antibody to CD16 (Leu11b; 1 µg/ml) as a control, washed, and suspended in RPMIc before addition to the chromium-labeled target cells.

Preparation of target cells. EBV-transformed autologous or heterologous B-cell lines (8×10^6 cells in 300 µl of RPMI-20 mM HEPES) were mock infected or infected with the appropriate VV recombinant at a multiplicity of infection of 5, incubated at 37°C for 1 h, washed, and suspended at 10^6 cells per ml in fresh RPMI medium plus 10% fetal calf serum for 16 h at 37°C.

Cytotoxic assays. At 16 h after infection or mock infection, 2×10^6 target cells were pelleted and suspended in 300 µl of RPMIc containing 3.7 MBq of $\text{Na}_2^{51}\text{CrO}_4$ (CJS1, Amersham, France) for 60 min at 37°C with gentle agitation every 15 min. The cells were then washed four times, and suspensions of 5×10^5 target cells in 100 µl of RPMIc were added to round-bottom 96-well microtiter plate wells (Costar 3799, OSI, France). Various concentrations of effector cells in 0.1-ml aliquots (in quadruplicate) were then added to yield the indicated effector cell/target cell ratios. Plates were centrifuged at $100 \times g$ for 1 min and then incubated for 4 h at 37°C in 5% CO_2 . After incubation, the plates were centrifuged at $150 \times g$ for 5 min, and an aliquot of supernatant was collected from each well with a Skatron harvesting frame (7072 SCS SKATRON; OSI). The percent ^{51}Cr release was calculated by using the following formula: percent ^{51}Cr release = $100 \times [(\text{experimental radioactivity} - \text{spontaneous radioactivity}) / (\text{total radioactivity} - \text{spontaneous radioactivity})]$ where spontaneous release was that obtained from target cells incubated with RPMIc alone, total release was that obtained from target cells incubated with 10% Triton X-100 detergent, and radioactivity is measured in counts per minute.

In our assays, spontaneous release from uninfected cells (including K562 target cells) or VV-infected target cells was

between 15 to 25% and never exceeded 35% of total release. For each assay, two sets of fixed target cells were established for an indirect immunofluorescence assay; in such assays, more than 90% of the VV-infected cells were stained with a polyclonal anti-VV serum and more than 90% of the VV recombinant-infected cells were stained with the specific corresponding monoclonal antibodies (The *env*- and the *gag*-specific monoclonal antibodies were monoclonal antibodies 110-4 and 18-3 from Genetic Systems, Seattle, Wash., and the $p27^{nef}$ - and $p23^{vif}$ -specific monoclonal antibodies were from Transgène, S.A., Strasbourg, France.)

RESULTS

PBMCs of seropositive patients exhibited specific cytotoxic activity against autologous target cells presenting HIV-1 antigens. Fresh PBMCs from 20 seropositive patients and 9 seronegative controls were tested for specific cytotoxic activity, using as targets autologous B-EBV-transformed cells that either were not infected or were infected by VV containing either no insertion (WT) or expressing *env* (gp160), *gag* (p55), $p27^{nef}$, or $p23^{vif}$ genes of HIV-1. No specific cytotoxic activity was detected at different effector cell/target cell ratios for the seronegative control N17 (Fig. 1a); a similar result was obtained with eight other seronegative controls tested (data not shown). In contrast, a significant cytotoxic activity was detected in the peripheral blood of HIV-1 seropositive donor LOS61 (Fig. 1b); both an HIV-1 *env* (gp160)- and HIV-1 *gag* (p55)-specific cytotoxic response was observed in this patient, whereas no cytotoxic activity was detected against the WT-infected autologous target cells. Moreover, no cytotoxic activity was detected against the HIV-2 *gag* VV-infected autologous target cells; this suggests an HIV-1-specific response for the *gag* antigen, despite the relatively high (60%) conservation in the *gag* gene between HIV-1 and HIV-2 (13). In some experiments, a VV recombinant containing a polyomavirus gene was used to infect the target cells and no specific lysis was observed (results not shown).

In the same experimental conditions, a specific cytotoxic activity was observed in 19 of 20 HIV-1 seropositive patients when *env* (gp160) was expressed (Fig. 2a and b) and in 17 of 20 HIV-1 seropositive patients when *gag* (p55) was expressed in the target cells (Fig. 2c and d). In most cases, the cytotoxic activity was higher when the *env* gene was expressed. For five subjects, however, the activity against *gag*-expressing target cells was higher than that for *env*. There was no apparent correlation between the stage of the disease and the cytotoxic activity measured; the two patients with AIDS-related complex had a positive cytotoxic activity against *env*-expressing targets (PIP3 and PIP5), and one patient (PIP3) had no activity against *gag*, whereas 12 of 13 healthy seropositive subjects reacted against *env* and 11 of 13 reacted against *gag*. Therefore, at least at this stage of analysis, the most severely affected individuals in this group did not significantly differ from the less affected. However, the clinical status of the seropositive donors is being followed in parallel with their cell-mediated immunity to more fully analyze any correlations.

In addition, three of eight patients manifested a low but positive activity when the $p27^{nef}$ protein was expressed and three of seven patients did so when the $p23^{vif}$ protein was expressed in the target cells (Fig. 3). Only five patients were tested simultaneously for cytotoxic activity against *env*, *gag*, $p27^{nef}$, and $p23^{vif}$: one showed an activity against *env* only (LOS25), two showed activity against *env* and *gag* (LOS47

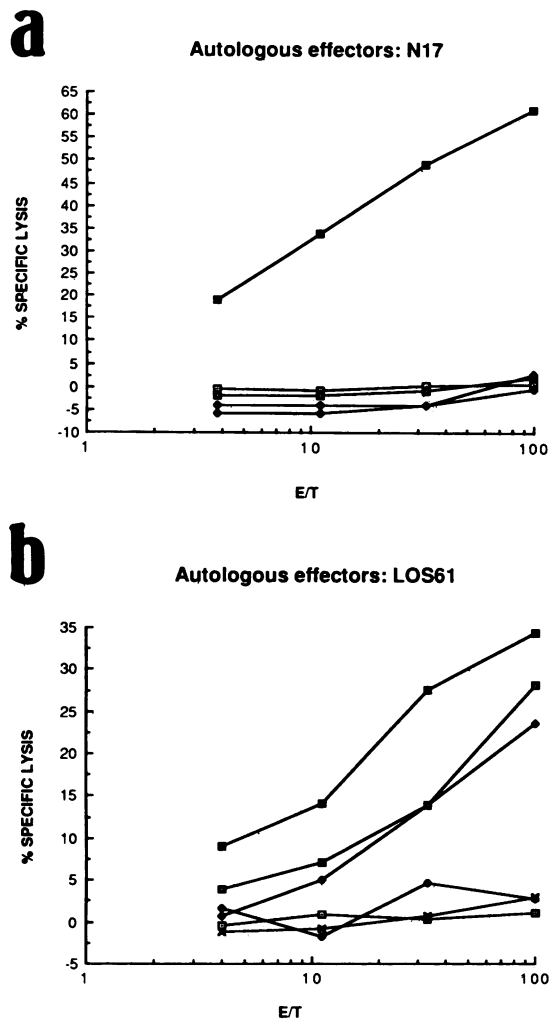


FIG. 1. Cytotoxic activity of fresh PBMCs from a seronegative (N17) (a) and a seropositive (LOS61) subject (b). Cytotoxicity of fresh PBMCs was determined as described in Materials and Methods in a 4-h ^{51}Cr release assay with four different effector cell/target cell ratios (E/T). Autologous ^{51}Cr -labeled B-EBV target cells were either not infected (\square) or infected with the WT VV (\blacklozenge) or with the VV-*env*-HIV-1 (\square), VV-*gag*-HIV-1 (\blacklozenge), or VV-*gag*-HIV-2 (\times) recombinant. The K562 cell line was used as a control to evaluate the NK activity (\blacksquare).

and LOS49), one showed activity against *env*, *gag*, and $p23^{\text{vir}}$ (LOS59), and one showed activity against *env*, *gag*, and $p27^{\text{nef}}$ (LOMB). Thus, in addition to the *env* and *gag* antigens, the $p27^{\text{nef}}$ and $p23^{\text{vir}}$ antigens can serve as targets for cytotoxic effector cells in seropositive individuals.

The natural killer (NK) activity against the K562 cell line was measured in most assays. In the experiments in Fig. 1, the NK activity was higher in the control seronegative subject than in the seropositive subject. However, the mean value of the two groups at a 50/1 effector cell/target cell ratio was 48.2% (range, 34 to 61%) and 43.8% (range, 17 to 72%) for the control subjects and the seropositive subjects, respectively. Therefore, in our experiments there was no difference in the NK activity between seronegative and seropositive subjects.

HIV *gag*-specific cytotoxic activity was restricted by MHC antigens while HIV *env*-specific activity was not. In a separate

set of experiments, the ability of effector cells from seropositive subjects to kill human leukocyte antigen (HLA) non-identical target cells expressing *env* or *gag* HIV-1 antigen was investigated (Fig. 4). An *env*-specific killing of human heterologous targets was observed in each case tested (Fig. 4a and c). In these two experiments, there were no HLA class I antigens shared between the effector cells and the target cells. In five different experiments in which in addition to the autologous target cells, a mouse cell line (EL4 or P815 lymphoma) was used as heterologous targets, specific killing was noted when these heterologous targets were infected by the HIV-1 *env* VV recombinant (Fig. 4b). Thus, because the *env*-specific killing occurred in the absence of shared HLA antigens, the *env* activity was unrestricted. This does not exclude, however, the presence of a minor population with HLA-restricted *env*-specific cytotoxic activity.

In contrast, in the same experiment, there was no heterologous *gag*-specific killing when there was no HLA class I shared antigens (Fig. 4c). The PBMCs of donor 65 were able to lyse the VV-*gag*-infected target cells of donor 65, but they did not lyse the VV-*gag*-infected target cells of donor 67, an unmatched class I B-EBV cell line. In another experiment (with donor PIP5), a cytotoxic activity was observed against the VV-*gag*-infected autologous targets but not against the VV-*gag*-infected EL4 mouse cell line (results not shown). Therefore, the major *gag* response differed from the major *env* response in its MHC restriction.

The HIV *gag*-specific cytotoxic activity was inhibited by monoclonal antibodies to the CD3 antigen, whereas HIV *env*-specific cytotoxicity was not. To identify the cell type mediating the *env*- and the *gag*-specific cytotoxic activity, we attempted to inhibit the cytotoxicity with monoclonal antibodies to the CD3 and the CD16 surface proteins. Preincubation of effector cells with a CD3-specific monoclonal antibody inhibited the *gag*-specific, but not the *env*-specific, cytotoxic activity in the two seropositive subjects tested (Fig. 5). This indicates that the lysis of autologous target cells expressing *gag* antigen was T cell mediated. In comparison, the effect of anti-CD3 on cytolytic activity against *env*-infected targets was variable at different effector cell/target cell ratios; however, there was a consistent failure to block *env*-specific lysis with this antibody. In the experiment in Fig. 5b, there was a decreased level of cytotoxic activity when effector cells were treated with the anti-Leu11b monoclonal antibodies as a control, but this was not observed in three other experiments.

HIV *gag*-specific cytotoxic activity was mediated by CD8⁺ T lymphocytes. The nature of effector cells recognizing the *gag* and the *env* antigen was further investigated in depletion experiments. The complete depletion of the cytotoxic activity against the VV-*gag*-infected autologous target cells was obtained when PBMCs were treated simultaneously with monoclonal antibodies against CD4 and CD8 plus complement (Fig. 6a). As a control, the depletion with monoclonal antibodies anti-Leu11b plus complement had no effect on the cytotoxic activity against the VV-*gag*-infected autologous target cells. Anti-Leu11b could, however, remove most of the NK activity against the K562 cells (results not shown). In all experiments, the depletion of CD4⁺ and CD8⁺ cells had no effect on the cytotoxic activity against the VV-*env*-infected autologous target cells. However, in some experiments, when the CD16⁺ cells were removed, we were able to observe a partial decrease of the cytotoxic activity against the *env*-expressing target cells. Therefore, the *gag*-specific cytotoxic activity is mediated by T lymphocytes, but the *env*-specific cytotoxicity is not. To further investigate this

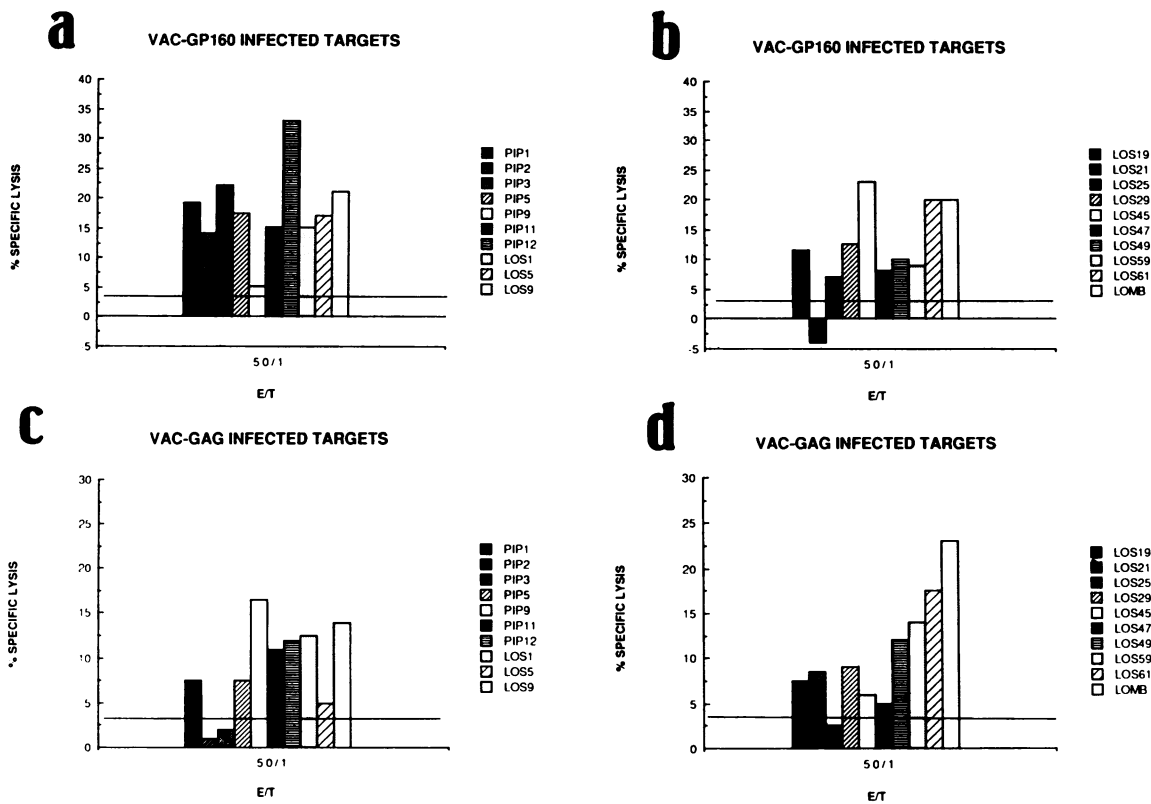


FIG. 2. Specific cytotoxicity at a fixed effector cell/target cell (E/T) ratio (50/1). Most of the seropositive donors present an activity against *env* (95%) and *gag* (85%). Autologous B-EBV cell lines infected with the VV-*env* (VAC-GP160) recombinant (a and b) or with the VV-*gag* (VAC-GAG) recombinant (c and d) are shown. The horizontal line represents the mean plus 2 standard deviations of the level of the lysis obtained when the autologous B-EBV cell lines were infected with the WT virus or with the polyomavirus middle T VV. For each assay, two sets of fixed target cells were established for an indirect immunofluorescence assay; in such assays, more than 90% of the VV-infected cells were stained with a polyclonal anti-VV serum and more than 90% of the VV recombinant-infected cells were stained with the specific corresponding monoclonal antibodies. The cytotoxic activity was determined as described in Materials and Methods.

point, an additional experiment was therefore performed. The cytotoxic activity against the VV-*gag*-infected autologous target cells was depleted when either CD3⁺ or CD8⁺ cells were removed and no change was observed with the removal of CD4⁺ or CD16⁺ cells from PBMCs (Fig. 6b). In this same experiment, the cytotoxic activity against the VV-*env*-infected autologous target cells was not affected by such treatments (not shown). The results from these experiments indicate that treatment of PBMCs with anti-CD4- and-CD8, anti-CD3, or anti-CD8 plus complement reduces the cytotoxic activity against VV-*gag*-infected target cells, but not against VV-*env*-infected targets, allowing us to conclude that the *gag*-specific cytotoxicity is mediated by CD3⁺ and CD8⁺ T lymphocytes. The nature of the *env*-specific effectors has not yet been determined, although they are not CD3⁺, CD4⁺, or CD8⁺ T lymphocytes. The role of the CD16⁺ cell subset in the *env*-specific cytotoxicity remains to be clarified.

DISCUSSION

To characterize the host immune response to HIV, recombinant HIV-VV were used to express HIV-1 antigens in EBV-transformed B-cell lines from 20 HIV-1 seropositive subjects and 9 seronegative controls. We have shown that in the same HIV-seropositive individual, distinct types of effector cells mediate killing of targets expressing *env* and *gag*

proteins. The *env* cytotoxic activity that was present in 19 of 20 (95%) seropositive subjects (Fig. 2) was not restricted by MHC antigens (Fig. 4) and not mediated by T lymphocytes, as shown by the absence of blocking effect of an anti-CD3 monoclonal antibody (Fig. 5) and by the inefficiency of CD3⁺, CD8⁺, or CD4⁺ and CD8⁺ depletion to reduce the cytotoxic activity against *env*-expressing target cells. On the other hand, in the same conditions, the *gag*-specific cytotoxic activity was abrogated upon depletion of CD8⁺ T cells (Fig. 5 and 6), and *gag* MHC-restricted cytotoxic T lymphocytes (CTLs) were detected in 85% of the subjects tested (Fig. 2 and 4).

Our results demonstrate that HIV-1 *gag* is a target for MHC-restricted CD3⁺, CD8⁺ cytotoxic lymphocytes, as shown in 17 of 20 (85%) seropositive subjects (Fig. 2, 4, 5, and 6). A *gag*-specific cytotoxic response has been reported by others (38), but to our knowledge, this is the first report showing the nature of the *gag*-specific effector cells. The high percentage (85%) of seropositive donors with a *gag*-specific cytotoxic activity suggests that *gag* is a major protein for CTL recognition. In other viral infections, such as influenza virus (4, 18), respiratory syncytial virus (3), and vesicular stomatitis virus (30), the CTL responses are directed mainly against internal viral proteins. In one seropositive subject (Fig. 1) with an HIV-1 *gag*-specific lysis, no lysis was detected against HIV-2 p56 *gag* VV-infected autologous target cells, suggesting a type specificity for the

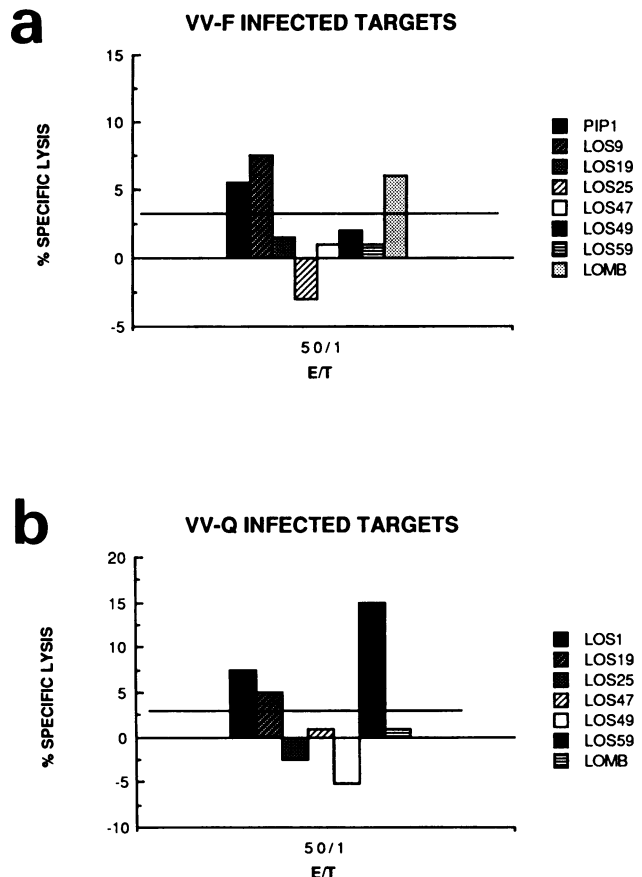


FIG. 3. Specific cytotoxicity at a fixed effector cell/target cell (E/T) ratio (50:1). The p27^{nef} (F) and p23^{vir} (Q) antigens can serve as targets for cytotoxic effector cells. (a) Autologous B-EBV cell lines infected with the F recombinant. The mean percent specific lysis of targets infected with WT virus was 0.1%, with a range from -4 to 3%. (b) Autologous B-EBV cell lines infected with the Q recombinant. The mean percent specific lysis of targets infected with WT virus was 0.6%, with a range from -1.7 to 3%. The horizontal line represents the level of the lysis obtained when the autologous B-EBV cell lines were infected with the WT virus or with the polyomavirus middle T VV (mean plus 2 standard deviations). The cytotoxic activity was determined as described in Materials and Methods.

gag antigen recognition despite the cross-reactivity in Western blot analysis between HIV-1 and HIV-2 *gag* (5). The fact that a *gag* MHC-restricted CTL activity is present in 85% of seropositive patients may imply continuous antigenic stimulation in those subjects. However, in our experience, HIV was isolated by coculture from fresh lymphocytes in only 1 of 30 cases (results not shown). In addition, we were not able to correlate the *gag*-specific activity to a positive core antigenemia in 10 subjects tested using a commercial solid-phase type enzyme immunoassay (Diagnostics Pasteur, France). However, small amounts of antigens, which may be sufficient for continuous cell stimulation, would not be detected with such techniques.

In contrast with the predominant *gag*-specific cytotoxic activity that is mediated by MHC-restricted CTLs, the predominant HIV *env*-specific cytotoxicity is not restricted by MHC antigens and is not mediated by T cells (Fig. 2, 4, and 5). Our results appear in contradiction with the recent data of Walker et al. (38) who reported that B-EBV-trans-

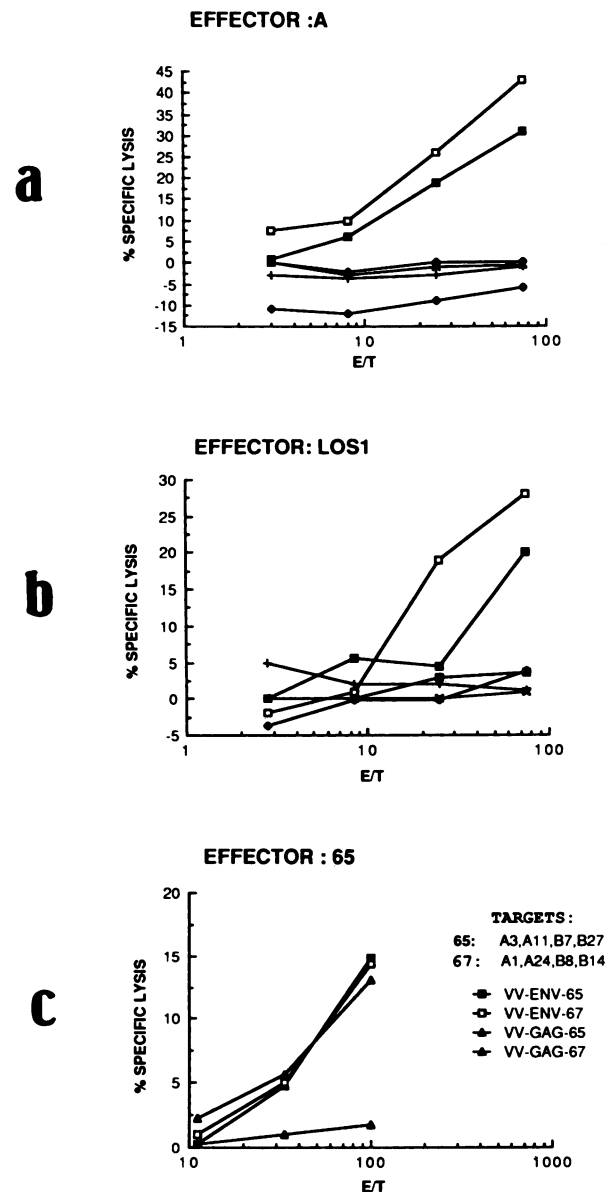


FIG. 4. HIV-specific cytotoxicity on autologous and heterologous targets. Cytotoxicity assays were performed as described in Materials and Methods, using effector cells from three different seropositive donors. (a) Effector cells: donor A. Autologous target cells of the HLA serotype were A1, A9(23), B8, B12(45). Heterologous target cells of the HLA serotypes were A10, A19, and B16(38 & 39). Symbols: \square , not infected, autologous target; \blacklozenge , infected with VV WT, autologous target; \blacksquare , infected with VV *env*, autologous target; \times , not infected, heterologous target; \diamond , infected with VV WT, heterologous target; \square , infected with VV *env*, heterologous target. (b) Effector cells: LOS1 donor. The heterologous target was the mouse EL4 cell line, and the autologous target was B-EBV. Symbols: \square , not infected, autologous target; \times , not infected, heterologous target; \blacksquare , infected with VV WT, autologous target; \diamond , infected with VV WT, heterologous target; \blacksquare , infected with VV *env*, autologous target; \square , infected with VV *env*, heterologous target. (c) Effector cells: donor 65. The targets for donor 65 were A3, A11, B7, and B27; the targets for donor 67 were A1, A24, B8, and B14. Symbols: \blacksquare , infected with VV *env*, donor 65; \square , infected with VV *env*, donor 67; \blacktriangle , infected with VV *gag*, donor 65; \triangle , infected with VV *gag*, donor 67. There was no lysis when the targets were uninfected or infected with the WT VV.

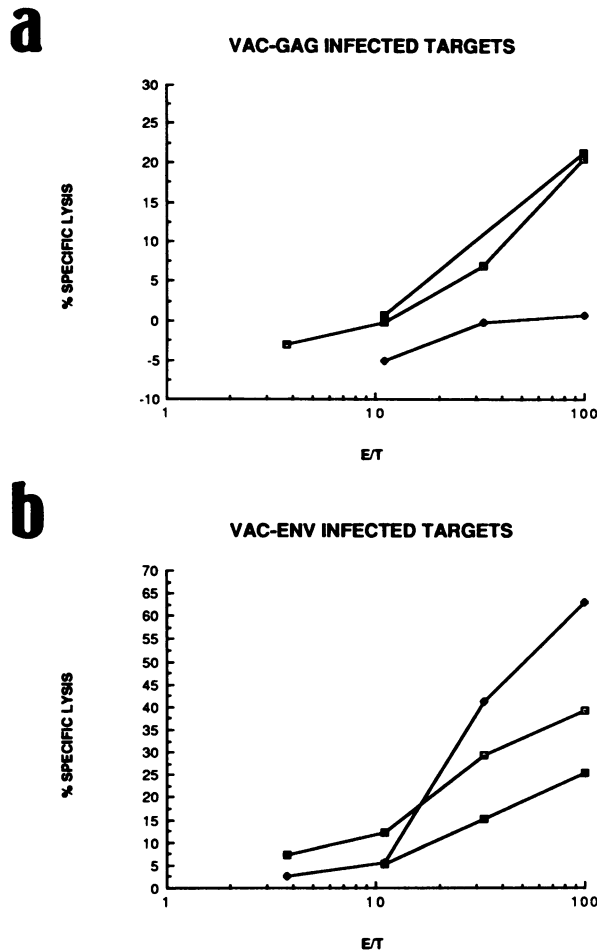


FIG. 5. HIV-*gag* specific cytotoxicity activity was inhibited by monoclonal antibody to the CD3 antigen (a), while HIV-*env* specific cytotoxicity was not (b). The cytotoxic activity of fresh PBMCs from a seropositive donor (PIP12) was measured as described in Materials and Methods. Before the addition to the chromium-labeled VV-*gag* (a) or VV-*env* (b) infected target cells, PBMCs were incubated for 30 min at 37°C with the IOT3 monoclonal antibody (monoclonal antibody against CD3, clone X-35) (◆) or with the Leu11b monoclonal antibody (monoclonal antibody against CD16) (■) or were not treated (□).

formed autologous target cells infected with a recombinant VV encoding the gp160 *env* gene were killed by MHC-restricted T lymphocytes. Their results, however, reflected a preferential killing of the *env*-expressing autologous target cells, rather than a strong MHC restriction. On the other hand, the unrestricted activity seen in our experiments against the *env*-expressing target cells and observed in seven of seven subjects tested does not exclude the existence of a minor population of HLA-restricted *env*-specific T cells. The fact that some investigators were able to select an HLA-restricted *env*-specific activity after in vitro secondary stimulation reflects the existence of such a population (34). As a matter of fact, this situation may occur in vivo and it is likely that HLA-restricted *env*-specific cytotoxic activity of alveolar lymphocytes from HIV-positive patients with lymphocytic alveolitis may result from a local in vivo antigenic stimulation of the CD8⁺ lymphocytes by autologous HIV-infected macrophages (1, 28).

In our experiments, the nature of the *env*-specific effector cells remains to be clarified; the results indicate that the *env*

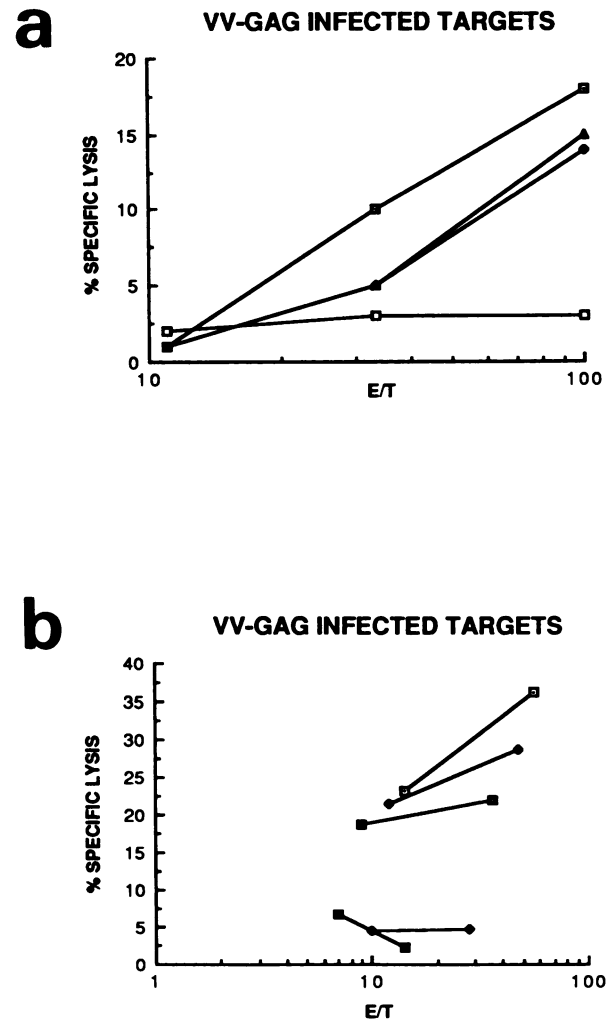


FIG. 6. The effector cells with activity against *gag* were T cells. (a) Depletion of effector cells from a seropositive donor (LOS59) with monoclonal antibody specific for CD4 (IOT4), CD8 (IOT8a), or CD16 (Leu11b) plus rabbit complement. Effector cells were not treated (□), treated with C' alone (△), treated with IOT4 plus IOT8a plus C' (□), or treated with Leu11b plus C' (◆). (b) Depletion of effector cells from a seropositive donor (PIP9) with monoclonal antibodies specific for CD3 (OKT3), CD4 (OKT4), CD8 (OKT8), or CD16 (Leu11b) plus rabbit complement. Effector cells were not treated (□), treated with Leu11b plus C' (◆), treated with OKT3 plus C' (■), treated with OKT8 plus C' (◇), or treated with OKT4 plus C' (▣). In both panels a and b, target cells were EBV-transformed autologous B cells infected with VV-*gag*.

cytotoxic activity detected in our assay was probably not due to T lymphocytes bearing CD3, CD4, or CD8 antigens. In HIV seropositive patients, the presence of an antibody-dependent cellular cytotoxicity against HIV-infected or -coated cells has been reported by several investigators (24, 32, 40; J. D. Katz, P. Nishanian, and B. Bonavida, FASEB J. A471, 1988), and anti-gp120 antibodies from a HIV seropositive individual were shown to mediate anti-HIV antibody-dependent cellular cytotoxicity (25). K cells responsible for an antibody-dependent cellular cytotoxicity could therefore be involved in the *env*-specific cytotoxic activity. One must consider also the possibility that the cellular anti-*env* cytotoxicity could be mediated by NK cells (33), as is the case for herpes simplex virus or cytomegalovirus

32, 40; J. D. Katz, P. Nishanian, and B. Bonavida, *FASEB J.* A471, 1988), and anti-gp120 antibodies from a HIV seropositive individual were shown to mediate anti-HIV antibody-dependent cellular cytotoxicity (25). K cells responsible for an antibody-dependent cellular cytotoxicity could therefore be involved in the *env*-specific cytotoxic activity. One must consider also the possibility that the cellular anti-*env* cytotoxicity could be mediated by NK cells (33), as is the case for herpes simplex virus or cytomegalovirus infection (2, 23). Although a decrease in nonspecific NK activity has been reported in AIDS patients (17), we found no difference in these activity between 14 seropositive and seronegative patients studied. Our group of seropositive patients, however, did not yet include patients who have advanced to AIDS. In two experiments, a partial decrease of the *env* cytotoxic activity was found after depletion by anti-Leu11b monoclonal antibodies plus complement, which is known to deplete the NK population (results not shown). Whatever the nature of the *env*-specific killer cells, several reports indicate that a majority of the seropositive patients show a cell-mediated cytotoxicity against target cells expressing the *env* antigen (28, 34, 38, 40), and it is likely that the discrepancy of the results concerning the nature of these effector cells may reflect the diversity of the possible mechanisms involved in *env*-specific cytolysis.

During several viral infections, it has been shown that CTLs recognize viral glycoproteins (15, 29, 41–43) or internal viral proteins, including structural (3, 4, 15, 18, 29, 30, 36, 39, 42) or nonstructural proteins (4, 31). Specific CTLs that recognize both *gag* gene- and *env* gene-encoded specificities have been described in other retrovirus infections (15, 29), and recent reports have identified HIV cytotoxic activity of PBMCs or alveolar lymphocytes against *env*, *gag*, or polymerase products of HIV (28, 38, 39). We show here that p27^{nef} (F or 3' open reading frame) and p23^{vif} (Q or *sor*), neither of which have been shown to be structural virion proteins, can serve as target for cytotoxic effector cells, but the nature of these effectors remains to be identified. To our knowledge, this is the first report describing a specific cytotoxic activity against these two regulatory proteins of HIV. The *vif* gene has been shown to influence virus infectivity (10, 35), and the significance of p23^{vif} cytotoxic activity is not well understood. Because p27^{nef} protein synthesis can continue in the absence of the *rev* protein, in contrast with that of *env* and *gag* proteins (7, 9), latently infected lymphocytes that do not express structural proteins (16) may still be the target for p27^{nef} specific cytotoxic effector cells.

For certain virus infections, CTLs may contribute to pathological change (6, 11, 22). Among the 20 seropositive subjects tested in this study, the most severely affected (PIP3 and PIP5) did not significantly differ from those less affected. The number of patients with overt disease was not sufficient to correlate the stage of the disease following HIV infection with a quantitative or qualitative difference in cytotoxic activity. Longitudinal analysis of these patients will be necessary to address this question.

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