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Effective Lysis of HIV-1–Infected Primary CD4$^+$ T Cells by a Cytotoxic T-Lymphocyte Clone Directed Against a Novel A2-Restricted Reverse-Transcriptase Epitope

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Summary: Most HIV-specific cytotoxic T-lymphocyte (CTL) epitopes have been identified using peptide-pulsed and recombinant vaccinia virus–infected targets. These systems may not accurately reflect the ability of epitopes to be presented by HIV-infected T cells. Recent studies suggest, in fact, that some CTL epitopes are poorly presented on HIV-infected cells. In this study, we have identified a novel A2.1-restricted HIV reverse-transcriptase (RT) epitope and investigated the presentation of this epitope by HIV-infected primary CD4$^+$ T cells and T-cell lines. A CD8$^+$ CTL clone, isolated from a seropositive subject that recognized a novel A2-restricted epitope KYTAFTIPSI (aa 293-302) in RT, was used for these studies. Primary CD4$^+$ T cells and the CD4$^+$ T-cell line T1 were infected with virus from T1-nPLAP, a cell line stably transfected with HXB-nPLAP, a molecular construct of HIV linked to a placental alkaline phosphatase (PLAP) marker gene. A uniformly infected cell population, obtained by immunomagnetic selection for PLAP expression, was used as targets in CTL assays. HIV-infected T cells were lysed by CTL recognizing this RT epitope as effectively as peptide-pulsed targets. This suggests that some RT epitopes are good targets for CTL recognition. **Key Words:** Cytotoxic T lymphocyte—CD4 T-cell lysis—A2-restricted reverse-transcriptase epitope.

HIV infection stimulates a vigorous major histocompatibility complex (MHC) class I restricted viral-specific cytotoxic T-lymphocyte (CTL) response, which may play an important role in host defense (1–3). A rational approach to effective vaccine design for HIV might include antigenic epitopes in conserved regions of the virus that are restricted by prevalent class I alleles. Sequence motifs and anchor residues have been identified for peptides binding to prevalent class I alleles, which makes it possible to predict CTL epitopes restricted by these alleles (4–7). However, the molecular basis for the immunodominance of certain epitopes in the CTL response is not clear. After experimental manipulation or in vivo peptide immunization, CTL responses can be induced against potential CTL epitopes (cryptic or subdominant) identified on the basis of motif and human leukocyte antigen (HLA) binding affinity, which may not be recognized during natural infection (8,9).

HLA A2 is the most prevalent class I allele worldwide (10). Recognition of HIV epitopes restricted by A2 has been elicited in seropositive subjects immunized with recombinant HIV gp160 protein (8). However, only a few A2-restricted epitopes have been identified in natural infection with HIV despite the presence of many HLA-A2 motifs in the viral sequence (3,11–14). This may reflect suboptimal presentation of certain CTL epitopes on infected CD4$^+$ T cells despite their ability to induce a CTL response if presented in the context of vaccination.

Even for HIV-specific CTL epitopes recognized in natural infection, it has been suggested that the low density of certain epitopes on infected CD4$^+$ T cells makes

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them ineffective as target epitopes despite the induction of a CTL response against them (15). The systems that have been used to monitor anti-HIV CTL activity employ vaccinia virus–infected or peptide-loaded target cells. These systems are both highly artificial because they provide massive quantities of peptide antigens for MHC binding that are unlikely to be achieved in HIV-infected cells. Cell lines transfected with molecular clones of HIV have been used as targets in a few studies (15,16), but these also may not accurately mimic antigen presentation by HIV-infected primary T cells.

In this study, we looked at the lysis of HIV-infected T1 cells (a TxB hybrid cell line [17]) and primary $CD4^+$ T cells by a CTL clone directed against a novel A2-restricted HIV reverse-transcriptase (RT) epitope in a conserved region of the virus. We used a molecular clone of HIV, HXB-nPLAP, which expresses a placental alkaline phosphatase gene in place of the nef gene (18). Stable transfection of an A2-expressing T1 cell line could be obtained by immunomagnetic selection of nPLAP-expressing cells with a monoclonal antibody (mAb) directed against PLAP. Use of virus derived from this stably transformed cell line T1-nPLAP, allowed us to infect and select immunomagnetically a population of uniformly infected primary $CD4^+$ T cells that were used as target cells in cytotoxicity assays. HIV-infected primary T-cell targets are effectively lysed by the clone, suggesting an adequate level of epitope display on infected $CD4^+$ T cells.

MATERIAL AND METHODS

Isolation of HIV Reverse-Transcriptase–Specific Cytotoxic T-Lymphocyte Clones

Reverse-transcriptase–specific CTL clones were isolated from Ficoll Hypaque–separated peripheral blood mononuclear cells (PBMC) from HIV-seropositive subject 307. The subject gave informed consent and the study was approved by the Center for Blood Research Human Investigation Review Board. At the time of study, the subject had been HIV-seropositive for 6 years, was not receiving any antiretroviral therapy, and had stage B2 disease (according to the U.S. Centers for Disease Control and Prevention [CDC] classification) with a CD4 count of 360/mm^3 and viral titer below the level of detection. In bulk lines and clones generated from this subject, a CTL response was directed against HIV gag, env, pol, and nef (19). PBMCs were cultured in 96-well plates at five cells per well in the presence of 1×10^5 allogeneic irradiated feeder cells and 0.1 μg/ml of the CD3 mAb 12F6 in RPMI-1640 culture supplemented with 15% heat-inactivated fetal calf serum (FCS) and 600 IU/ml of recombinant interleukin-2 (rIL-2; Chiron, Emeryville, CA, U.S.A.). Only 30% of wells demonstrated growth, of which five exhibited HIV-specific CTL activity and were further expanded. After 2 to 3 weeks, clones were transferred to 24-well plates and restimulated with anti-CD3 mAb and allogeneic feeder cells. CTL activity of the clones was tested against autologous EBV-transformed B-lymphoblastoid cell line (B-LCL) targets infected with vaccinia virus expressing HIV env, gag, RT, and lacZ control as described (20). The HIV-specific clone BR21 was maintained in culture by periodic restimulation for >1 year without change in specificity.

Synthetic HIV-1 Reverse-Transcriptase Peptides

Synthetic peptides corresponding to aa 168-315 of the HIV_{IIIB} RT protein (amino acid numbering as described by Ratner et al. [21]) were synthesized on an Advanced Chemtech peptide synthesizer (Louisville, KY, U.S.A.) by FMOC chemistry and evaluated for purity by reverse-phase analytical high-performance liquid chromatography (HPLC). A series of 20 mer peptides with 10 aa overlaps were synthesized for initial screening and for finer mapping, 10 mer peptides with 2 aa overlaps were synthesized corresponding to the region defined with the 20 mer peptides. Lyophilized peptides were reconstituted at 1 mg/ml in phosphate-buffered saline (PBS) with 10% dimethyl sulfoxide (DMSO) and used in assays at a final concentration of 25 μg/ml.

Epitope Mapping

After determining the specificity of the clone with vaccinia virus expressing HIV-RT (vCF21), vaccinia vectors expressing nested truncations of the same RT isolate (vCF32, vCF33, vCF34, vCF35, vCF36, and vCF37) were used to infect autologous B-LCL to localize the CTL epitope (14). For finer epitope mapping, overlapping 20 mer peptides and shorter 10 mer peptides were incubated with targets as described (13,20). Cytotoxicity assays were performed against target cells that were either infected with vaccinia constructs encoding complete or truncated HIV-1 RT protein or preincubated with relevant peptides. B-LCL target cells were labeled with ^{51}Cr for 1 hour at 37°C, washed three times and resuspended at 10^5/ml. Labeled targets (10^4) were added to triplicate wells of U bottom microtiter plates. Effector cells were suspended at various E:T ratios in 100 μl and added to target cells and the plates incubated at 37°C over CO_2 for 4 hours. Supernatants (35 μl) were counted on a Packard Microplate reader (Meriden, CT, U.S.A.), and percentage-specific cytotoxicity was calculated from the average cpm as ([average cpm – spontaneous release]/[total release – spontaneous release]) × 100.

HLA Tissue Typing and MHC Restriction Analysis

Peripheral blood mononuclear cells were typed for HLA class I antigens by serologic methods by the Immunology Laboratory of New England Medical Center. In some cases, the expression of HLA-A2.1 was verified by flow cytometry staining with the A2.1-specific mAb PA2.1. MHC restriction of lysis was determined using a panel of B-LCL targets matched for one or more class I alleles.

Flow Cytometric Analysis of A2.1 Expression on Peptide-Treated T2 Cells

T2 is a peptide transport–defective mutant cell line derived from the T1 (HLA-A2.1, B5) cell line (22). Cell surface expression of A2.1 on T2 cells is reduced unless cells are incubated in the presence of a peptide capable of binding to A2.1. T2 cells were maintained in culture in serum free AIM V medium (Gibco, Paisley, U.K.). Cells were incubated overnight at 37°C with the 10 mer A2-restricted RT epitope KYTAFTIPSI identified in the CTL assay or the 9 mer predicted epitope YTAFTIPSI within it, at a concentration of 50 μg/ml in the presence of 3 μg/ml human β_2-microglobulin (Sigma, St. Louis, MO,

U.S.A.). Cells were also incubated with irrelevant peptides that do not bind to A2.1 (LFCASDAKA and FCASDAKAY) or with a well-characterized A2-binding peptide from the matrix protein of influenza A virus (GILGFVFTL) (23). Increase in cell surface expression of A2.1 was determined by staining with A2.1-specific mAb PA2.1 (1:50 dilution) for 30 minutes at 4°C. Cells were washed and incubated for another 30 minutes at 4°C with 1:50 dilution of phycoerythrin (PE)-conjugated secondary goat anti-mouse Ig antibody (Dako, Carpenteria, CA, U.S.A.). The samples were fixed with 1% formaldehyde and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

HIV-Infected Target Cells

A long-term HIV-1–expressing cell line T1-nPLAP was established by calcium phosphate transfection with the HIV-1 molecular clone, HXB-nPLAP in which the nef gene had been replaced with the placental alkaline phosphatase reporter gene (18). Uniformly stably infected cells were obtained by immunomagnetic selection using a 1:25 dilution of mAb MO858 against placental alkaline phosphatase (PLAP) (Dako) and Miltenyi beads coated with anti-mouse Ig. The cell line was monitored regularly for HIV-1 expression by flow cytometry using FACScalibur flow cytometer with KC57 p24 mAb conjugated to fluorescein isothionate (FITC, Coulter Corp., Miami, FL, U.S.A.). T1-nPLAP was used as a source of virus and as target cells in cytotoxicity assays. To obtain uniformly HIV-infected primary $CD4^+$ T cell targets, autologous or allogeneic A2-matched PBMC were depleted of CD8 T cells and stimulated with phytohemagglutinin (PHA; 2 μg/ml) for 2 days before infection with viral supernatant from the T1-nPLAP cell line. After overnight incubation, cells were washed and cultured in RPMI-1640 culture medium with 10% FCS and 60 IU/ml IL-2. On day 4 after infection, nPLAP-expressing cells were selected as already described. HIV expression in the selected cells was confirmed by flow cytometry before their use as targets in cytotoxicity assays. Cytotoxicity assays with HIV-infected cells were performed as stated except that 60 IU/ml IL-2 was present in the culture medium.

Flow Cytometry

For p24 staining, cells were resuspended in 50 μl Hank's balanced salt solution (HBSS) and permeabilized using Caltag Laboratories (Burlingame, CA, U.S.A.) Fix and Perm kit according to the manufacturer's protocol. Fixed cells were incubated for 15 minutes at room temperature with 2 μl of p24 mAb KC57 conjugated to FITC. After washing with 5 ml of HBSS, cells were resuspended in PBS with 1% formaldehyde for analysis. Flow cytometric analysis was performed on events in the live cell gate as determined by forward and side scatter profile. To assess placental alkaline phosphatase expression on HIV-infected cells, cells were suspended in 50 μl FACS buffer (2% FCS, 0.02 % NaN_2 in PBS) to which 2 μl of mAb MO858 was added. After incubation for 30 minutes at 4°C, cells were washed twice with 1 ml of FACS buffer and stained with 2 μl of FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (Dako) for 20 minutes. Cells were washed with 1 ml of FACS buffer and resuspended in FACS buffer with 1% formaldehyde for analysis.

RESULTS

Identification of a Novel A2-Restricted Reverse-Transcriptase Epitope From an HIV-Infected Donor

The RT-specific $CD4^-$ $CD8^+$ clone BR21 was identified by preliminary screening with B-LCL targets infected with vaccinia constructs expressing envelope, gag, and RT proteins of HIV (data not shown). For epitope characterization, autologous B-LCL infected with vaccinia vectors expressing truncations of the HIV-RT gene were used as targets in cytotoxicity assays. The epitope was found to be contained within aa 168-315 and identified as KYTAFTIPSI (aa 293-302) (Fig. 1). The class I restriction of the RT-specific CTL clone was defined with a panel of recombinant vaccinia–RT–infected B-LCL target cells matched at one or more class I alleles. The clone lysed three different allogeneic targets matched for HLA-A2.1 with no significant lysis of non–A2.1-expressing targets. Specific lysis of A2.1-transfected Jurkat cells infected with vaccinia-RT also occurred (Fig. 2).

Upregulation of A2.1 Expression on T2 Cells Incubated With the A2-Restricted Reverse-Transcriptase Epitope

T2 is a mutant cell line, derived from the T × B hybrid T1 cell line, which has a peptide transport defect resulting in cell surface HLA-A2.1 expression that is only 30% to 50% of the level of that of T1 cells. A2.1 cell surface expression in this cell line can be upregulated by culture in the presence of exogenous A2-binding peptides. To verify that the identified peptide epitope binds to HLA-A2.1, T2 cells were incubated with the 10 mer peptide KYTAFTIPSI or with the shorter 9 mer peptide YTAFTIPSI, which is the likely minimal peptide epitope predicted from the consensus anchor motifs of A2.1-binding peptides (5–7). Both were able to increase cell surface expression of A2.1 to the level seen with the known A2-restricted influenza peptide GILGFVFTL (23) (Fig. 3). Incubation with non–HLA-A2–restricted peptides did not increase levels of A2.1 on the surface of T2 cells.

Enriched Population of HIV-Infected T-Cell Targets for Cytotoxic T-Lymphocyte Assays

Technical problems associated with obtaining a uniform and viable population of HIV-infected primary target cells have hampered using HIV-infected T-cell lines and primary T cells to examine CTL recognition of infected cells. For this reason, autologous Epstein-Barr virus (EBV)–transformed B-cell lines pulsed with peptide or infected with recombinant vaccinia virus have been widely used to study HIV-specific CTL. However, antigen processing and presentation are not likely to be the same as in HIV-infected T cells, which are primary targets of HIV infection. We therefore developed a simple method to select for uniformly HIV-infected cells in T-

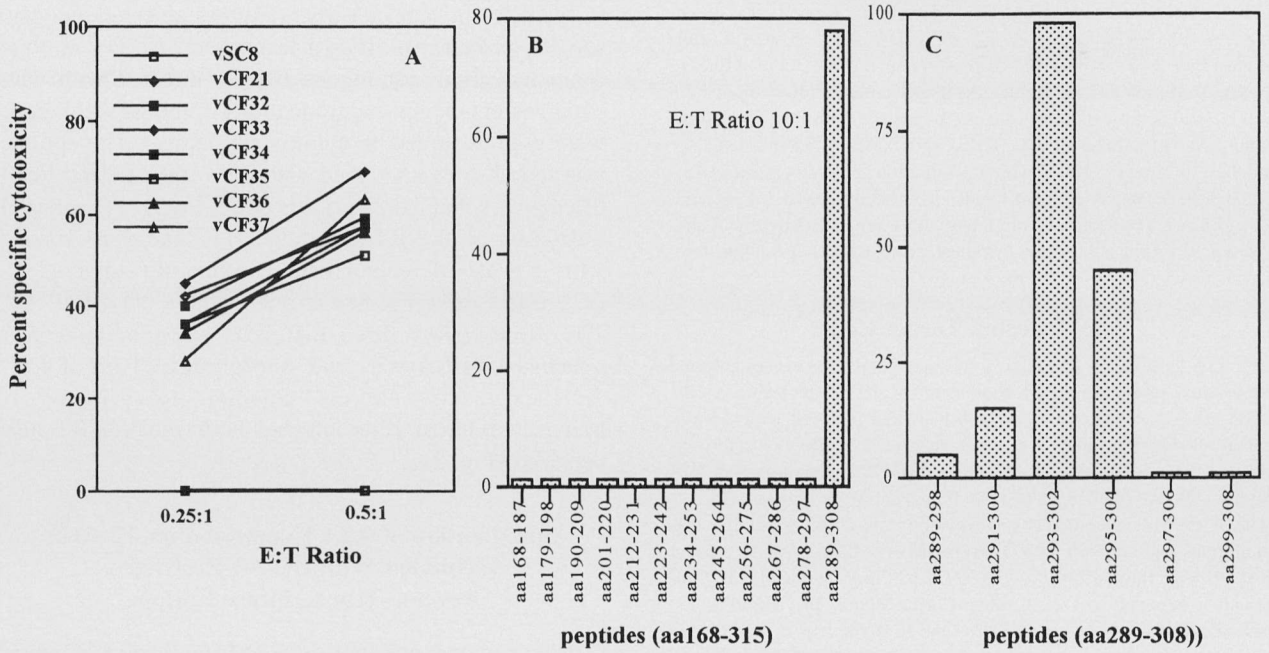

FIG. 1. Epitope mapping of HIV reverse-transcriptase (RT)–specific cytotoxic T-lymphocyte (CTL) clone BR21, using recombinant vaccinia virus and synthetic peptides. (**A**) CTL assay against autologous B-LCL targets infected with vaccinia virus vectors expressing serial truncations of HIV-1 RT (vCF21 and vCF32 encode for aa 168-706 of RT; vCF33: aa 168-598; vCF34: aa 168-531; vCF35: aa 168-480; vCF36: aa 168-422 and vCF37: aa 168-315; amino acid numbering as in reference 21) or the lacZ control (vSC 8) identified an epitope within RT aa 168-315. (**B**) B-LCL targets pulsed with overlapping 20 mer peptides spanning aa 168-315 of RT were used in a cytotoxicity assay to identify an epitope in aa 289-308. (**C**) For fine mapping of the epitope, B-LCL targets pulsed with 10 aa peptides with 2 aa overlaps were used as targets. The 10 mer epitope corresponding to aa 293-302 is KYTAFTIPSI.

cell lines and primary T cells. T1 cells were transfected with a molecular clone of HIV that was linked to the placental alkaline phosphatase gene. A stably transfected cell line was derived by repeated immunomagnetic selection with mAb antibody against nPLAP (Dako) and anti-mouse IgG coated Miltenyi beads (Fig. 4). Virus containing culture supernatants from this line were used to infect T-cell targets and infected cells were then obtained by immunomagnetic selection. Using this method, primary HIV-infected $CD4^+$ T cells could be obtained in sufficient numbers for use in cytotoxicity assays. Infected primary T cells did not expand well in culture after selection and showed extensive cell death and syncytia formation. However, cell viability was not a problem when infected cells were used for CTL assays on the day of selection when IL-2 was added to the CTL assay medium. Spontaneous release was within the acceptable limits of 15% to 20%.

Recognition of HIV-Infected and Peptide-Pulsed T1 Cells by the Reverse-Transcriptase–Specific Cytotoxic T-Lymphocyte Clone

To assess the ability of the HIV-RT–specific clone BR21 to lyse HIV-infected targets, T1 cells were infected with HIV_{IIIB} virus or with the HXB-nPLAP virus at a multiplicity of infection (MOI) of 5 (Fig. 5*A*). Uninfected T1 cells were used as control targets. For all targets, cytotoxicity was also tested in the presence of the cognate peptide. The lysis of infected cells was somewhat higher in the presence of the cognate peptide. This increase with peptide could be attributed to the presence of uninfected T1 target cells. The level of infection was about 60% for either HIV_{IIIB}-infected or HXB-nPLAP–infected cell lines as determined by p24 expression (Fig. 6). No increase in cytotoxicity was observed when the cognate peptide was added to the uniformly infected targets, obtained by immunomagnetic selection with PLAP mAb (Fig. 5*B*).

Efficient Lysis of Uniformly Infected Primary $CD4^+$ T-Cell Targets

To obtain a uniform population of HIV-infected primary $CD4^+$ T-cell target cells, allogeneic A2-matched CD8-depleted PBMC were stimulated with PHA for 2 days and infected with viral supernatants from the T1-nPLAP cell line. Three days after infection, HIV-infected primary T cells were positively selected as de-

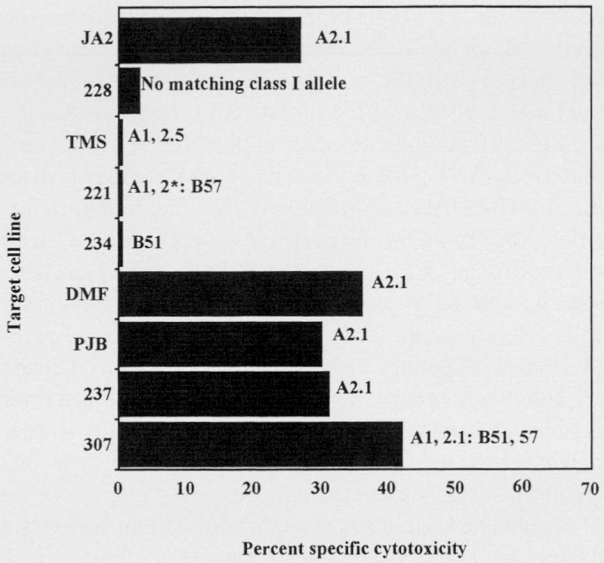

FIG. 2. Cytotoxic T-lymphocyte (CTL) clone BR21 is restricted by human leukocyte antigen (HLA)-A2.1. Autologous and allogeneic Burkitt's lymphoma cell line (B-LCL) targets matched at one or more class I alleles or the HLA-A2.1–transfected Jurkat cell line JA2 were infected with recombinant vaccinia expressing HIV reverse-transcriptase (RT) or the lacZ control. RT-specific cytotoxicity (defined as the difference in percentage of specific lysis of the RT-expressing and control targets) was tested at an E:T ratio of 2.5:1. Note that target cell line 221 expresses an undefined A2 subtype distinct from A2.1 because it does not stain with mAb PA2.1.

scribed earlier. Virus expression was confirmed by internal staining with p24 mAb and about 60% to 70% of cells were positive for p24 Ag (Fig. 7). The mean fluorescence of p24 staining was less uniform and less intense in the primary infected T cells than in the T1 cell line. To ensure acceptable levels of spontaneous release, infected cells were used in CTL assays immediately after selection and the assay was done in medium containing IL-2. As was the case with the T1 line, an increase in CTL lysis was seen with addition of the cognate peptide when primary infected cells were directly used as targets, but not with PLAP-selected, uniformly infected target cells (Fig. 8). This suggests that most of these immunomagnetically selected cells were infected and expressed adequate levels of the epitope to be recognized by CTL.

DISCUSSION

HIV infection stimulates a vigorous and broad-based CTL response, which may be important in host defense at all stages of the disease (1–3,24). Although it is widely recognized that an effective vaccine against HIV should stimulate viral-specific CTL, the complexity of the response resulting from sequence variability and class I polymorphism has presented a formidable challenge. To overcome these barriers, CTL epitopes in relatively conserved regions of the virus, which are restricted by common HLA haplotypes, have been sought. An additional requirement, which is only now being appreciated, is the representation of the epitope on virus-infected cells. Although HLA-A2–restricted gp160 CTL epitopes identified on the basis of binding affinity have elicited a CTL response in gp160 immunized individuals, it is not clear whether these epitopes are targeted in natural infection with HIV (8). Thus far, only four A2-restricted HIV epitopes have been definitively identified as recognized in natural infection with HIV despite the presence of many more A2 consensus sequences in the virus (3,11, 12,15,25). In this paper, we document a novel

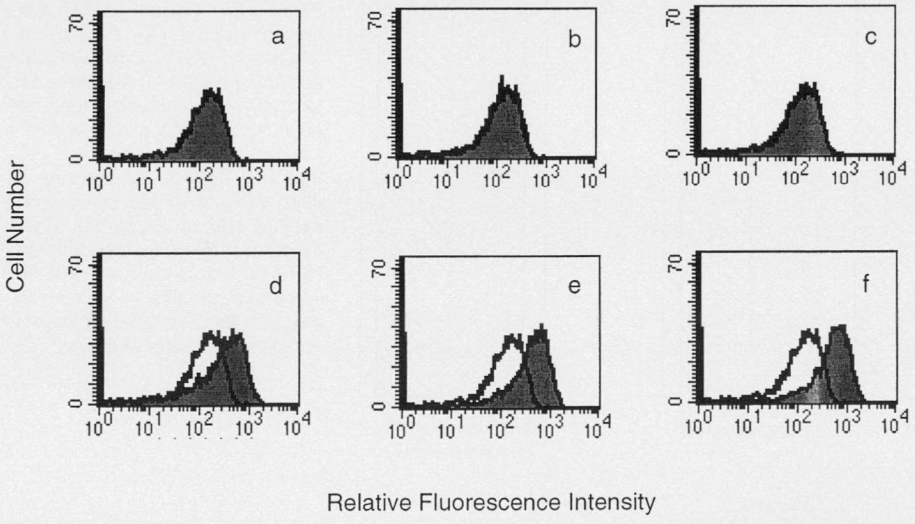

FIG. 3. Flow cytometric analysis shows increased human leukocyte antigen (HLA) A2.1 expression on T2 cells treated with the novel reverse-transcriptase (RT) epitope peptide. The transporter-deficient T2 cell line was cultured overnight alone (**a**) or in the presence of the 10 mer A2-restricted RT peptide KYTAFTIPSI (**e**) or the putative 9 mer epitope YTAFTIPSI (**f**). Irrelevant peptides LFCASDAKA and FCASDAKAY (**b,c**) and the influenza virus type A matrix peptide GILGFVFTL (**d**) served as negative and positive controls, respectively. In parts **b** through **f,** the histogram for T2 cells not exposed to peptide is white, whereas the histogram for staining of peptide-incubated cells is filled.

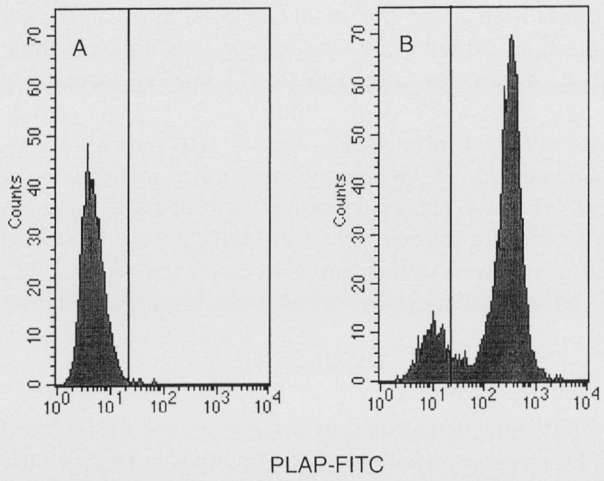

FIG. 4. Stable transfection of T1 cells with a molecular clone of HIV expressing the placental alkaline phosphatase in place of the nef gene. T1 cells transfected with plasmid HXB-nPLAP and immunomagnetically selected for nPLAP expression were stained with mAb against PLAP and fluorescein isothiocyanate–conjugated anti-mouse goat Ig. Flow cytometric analyses of (**A**) untransfected control and (**B**) nPLAP-transfected T1 cells are shown.

A2-restricted HIV-RT epitope in a conserved region of the virus. The epitope was fine mapped at the 10 mer level to a region spanning aa 293-302 of RT. Based on anchor motifs for A2-restricted recognition, the 9 aa epitope within the 10 mer peptide KYTAFTIPSI can be predicted to be YTAFTIPSI. The predicted sequence only loosely fits the motif at the second position (the tolerated T instead of the preferred L or M) but has the putative A2 binding residue at position 9 (L, V, or I preferred) (5,7). Although we have not tested for restriction by Cw alleles, it is unlikely that the epitope is restricted by Cw because specific lysis was observed with three different A2.1-expressing B-LCL targets presenting this epitope as well as with A2.1 transfected Jurkat cells. Moreover, the 10 mer peptide as well as the putative 9 mer A2 epitope deduced from the 10 mer sequence are able to upregulate A2.1 expression on the transporter deficient T2 cells. This confirms that the 9 and 10 mer peptides bind to HLA-A2.1 and helps validate their designation as A2.1-restricted epitopes.

A published study suggests that for certain epitopes a dichotomy may exist between induction of effector T cells and effective lysis of HIV-infected cells even in natural infection. A well-characterized A2-restricted RT epitope was found to be ineffective in the lysis of an HIV-infected T-cell line despite vigorous activity against peptide-pulsed and recombinant vaccinia virus–infected target cell lines (15). Using CTL clones directed against the same RT epitope, Yang et al. found that RT-specific CTL that recognized this epitope were less efficient than

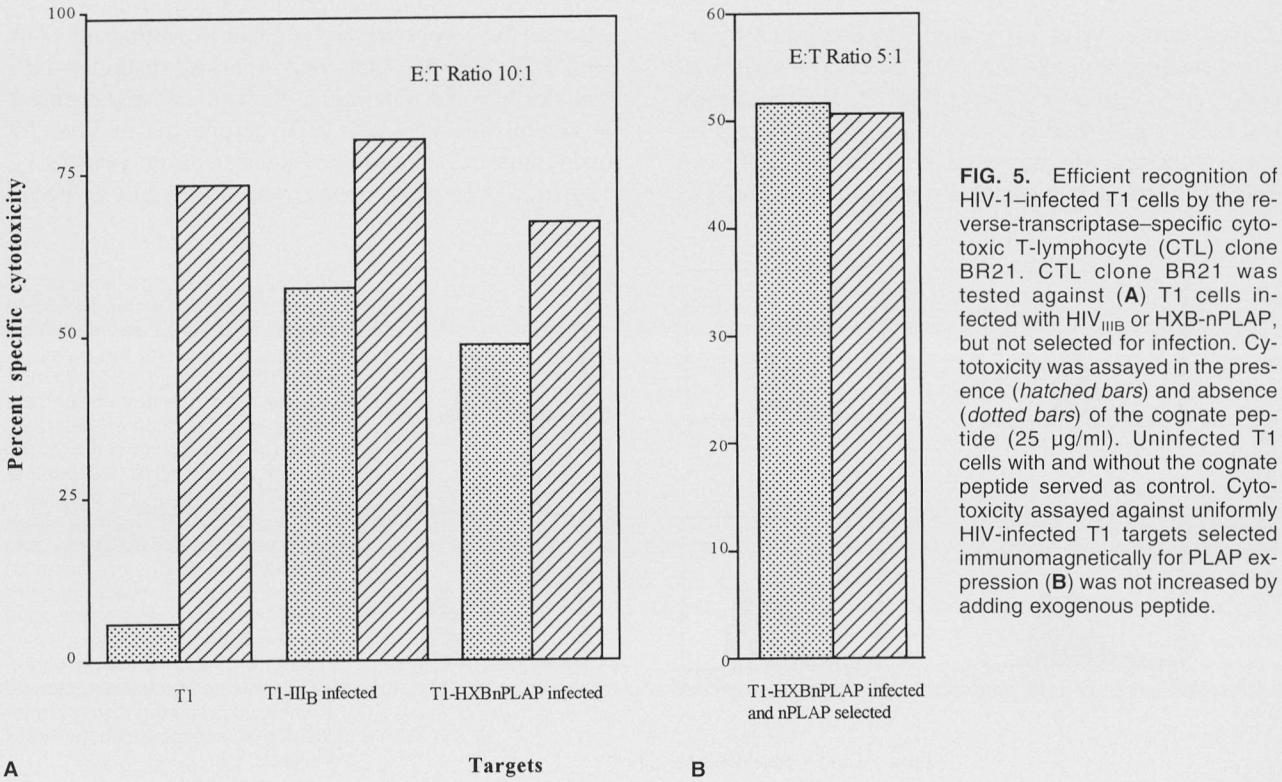

FIG. 5. Efficient recognition of HIV-1–infected T1 cells by the reverse-transcriptase–specific cytotoxic T-lymphocyte (CTL) clone BR21. CTL clone BR21 was tested against (**A**) T1 cells infected with HIV_{IIIB} or HXB-nPLAP, but not selected for infection. Cytotoxicity was assayed in the presence (*hatched bars*) and absence (*dotted bars*) of the cognate peptide (25 μg/ml). Uninfected T1 cells with and without the cognate peptide served as control. Cytotoxicity assayed against uniformly HIV-infected T1 targets selected immunomagnetically for PLAP expression (**B**) was not increased by adding exogenous peptide.

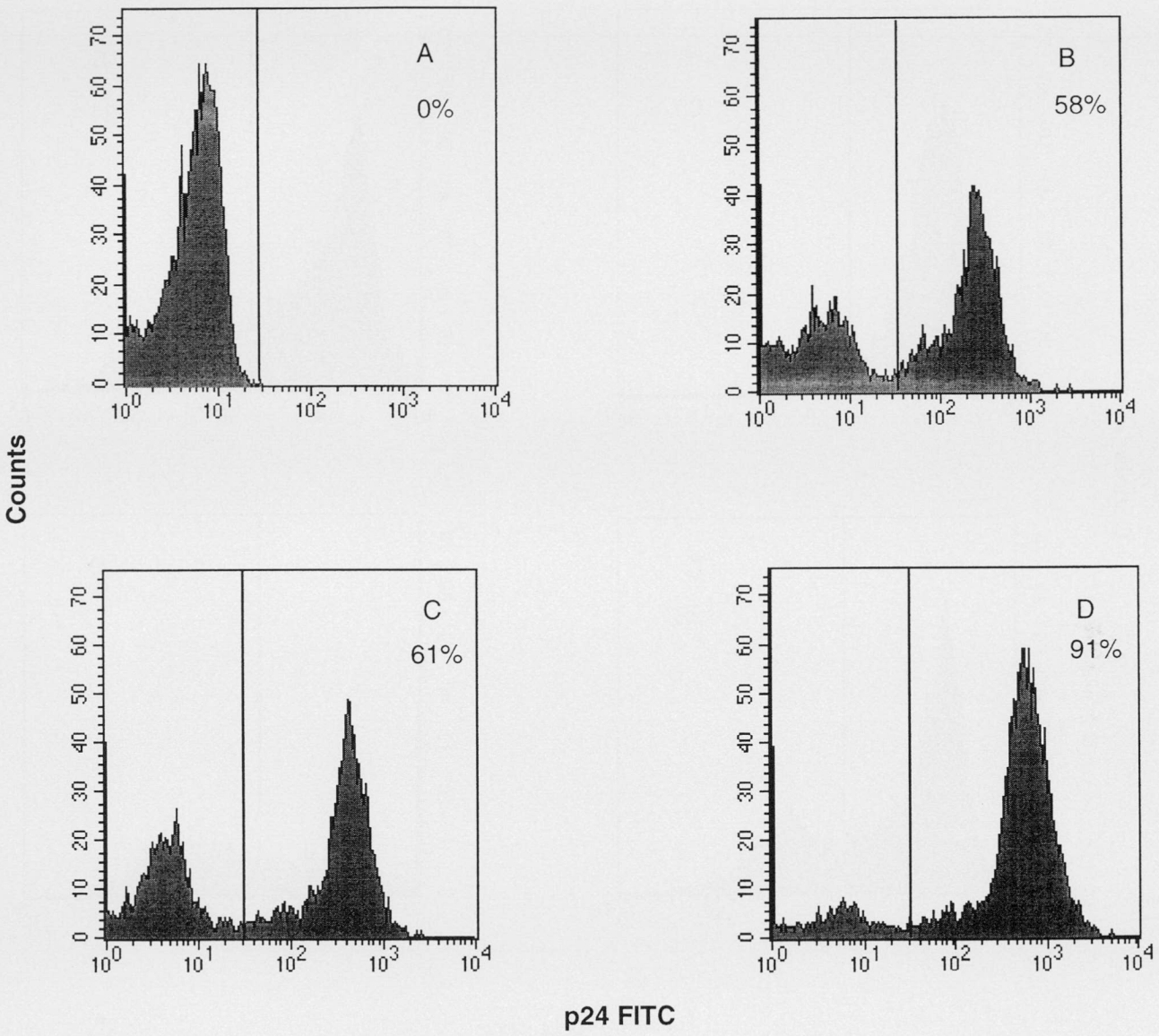

FIG. 6. Flow cytometric analysis of intracellular p24 in T1 cells after infection with HIV_{IIIB} or HXB-nPLAP. Three days after infection, T1 cells were permeabilized and stained with p24 mAb. P24 expression is shown for (**A**) uninfected control T1 cells, (**B**) HIV_{IIIB}-infected T1 cells, (**C**) HXB-nPLAP–infected T1 cells, and (**D**) HXB-nPLAP–infected T1 cells selected for nPLAP expression.

envelope and gag-specific CTL in lysing the HIV-infected T-cell line T1 (16). From our data, it appears that this may not be a general property of RT-directed CTL, because we found efficient lysis of both T1 cells and HIV-infected primary T cells by the CTL clone directed against a novel A2-restricted RT epitope. However, this RT peptide may have higher affinity for A2.1 or the T-cell receptor (TCR) of the CTL clone used in the present study may have higher affinity for its MHC-peptide ligand, enabling efficient lysis of the naturally processed peptide despite a low density on infected cells. Studies by Sykulev et al. suggest that CTLs bearing high affinity receptors can efficiently lyse targets even when the epitope is present at only a few copies per target cell (26). In fact, a proportion of the infected target cells expresses such low levels of p24 that it is not detectable above background by flow cytometry. Nonetheless, the lysis of these cells was not enhanced by adding peptide in excess, suggesting that CTL are exquisitely sensitive to low levels of peptide antigen.

The system that we have used to demonstrate lysis of HIV-infected primary T cells provides a simple feasible method to obtain adequate numbers of HIV-infected cells for CTL assays to assess the relevance of identified

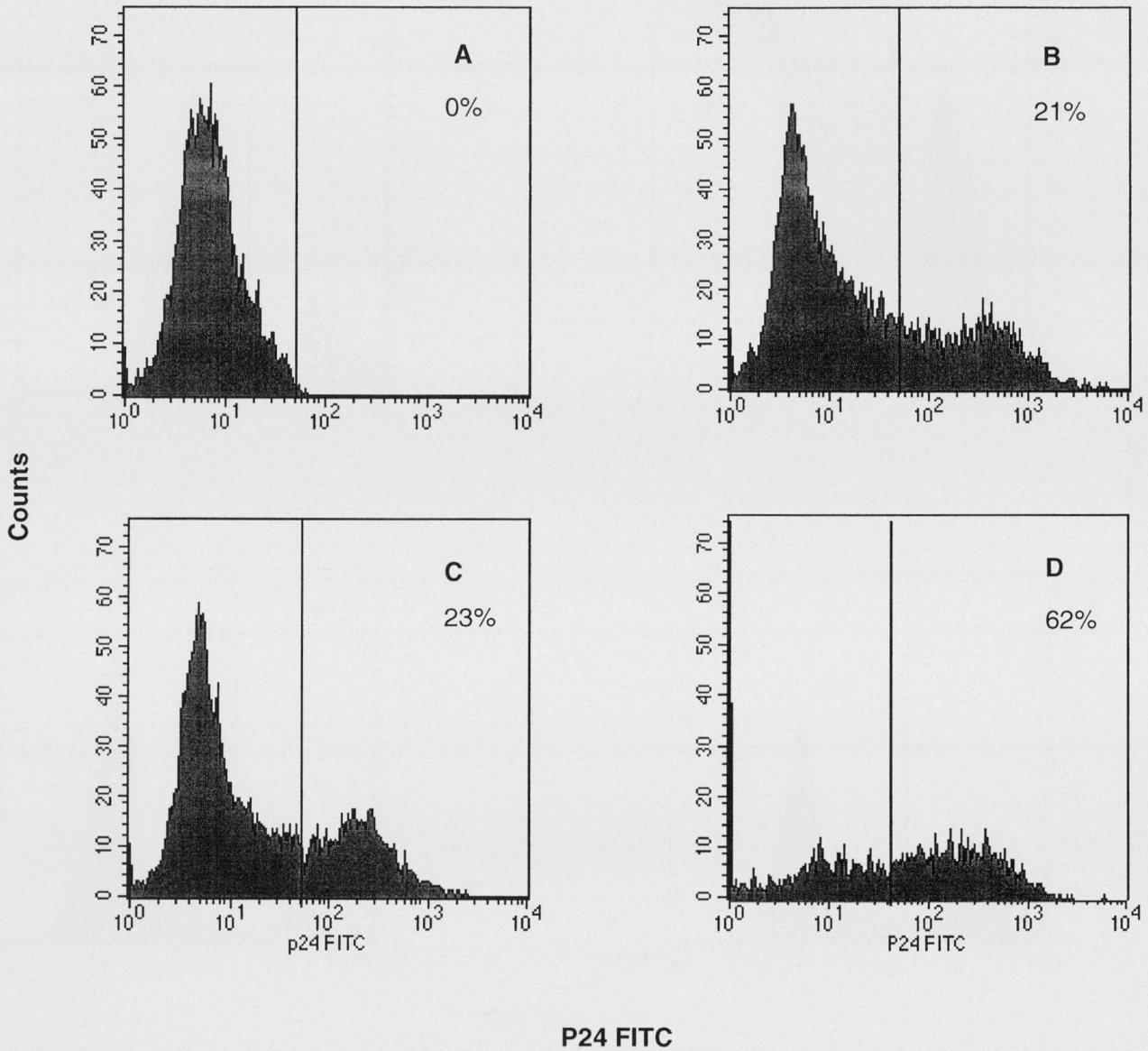

FIG. 7. Flow cytometric analysis of intracellular p24 in HIV-infected primary CD4+ T cells. CD8-depleted phytohemagglutinin (PHA) blasts were infected with HIV_{IIIB} or HXB-nPLAP virus. Three days later, the cells were permeabilized and stained for p24. P24 expression is shown for (**A**) uninfected control CD4 PHA blasts, (**B**) HIV_{IIIB}-infected CD4 PHA blasts, (**C**) HXB-nPLAP–infected CD4+ PHA blasts, and (**D**) HXB-nPLAP–infected CD4+ PHA blasts immunomagnetically selected for nPLAP expression.

epitopes in the context of physiologically relevant HIV-infected targets. We found that p24 expression in HIV-infected primary CD4+ T-cell blasts is lower than in a stably transfected T×B cell line. For some CTL of lower affinity TcR than clone BR21, it may be that lysis of primary T cells may not occur even when infected transformed cell lines are readily lysed. Although our method comes close to approximating the condition of antigen presentation in natural infection of primary T cells, it is only suitable for target cell infection by genetically modified molecular clones of HIV-1. It is possible that target cells infected by primary HIV isolates might present antigen differently than target cells infected with modified cloned virus. CTL recognition of type-specific epitopes not present in HIV_{IIIB} is also missed by this assay. Another method to use HIV-infected primary T cells as targets in CTL assays was recently described (27). Because HIV-1 nef induces downmodulation of CD4, infected target cells were purified by immunomagnetic depletion of CD4-expressing cells.

It has recently been suggested that nef expression

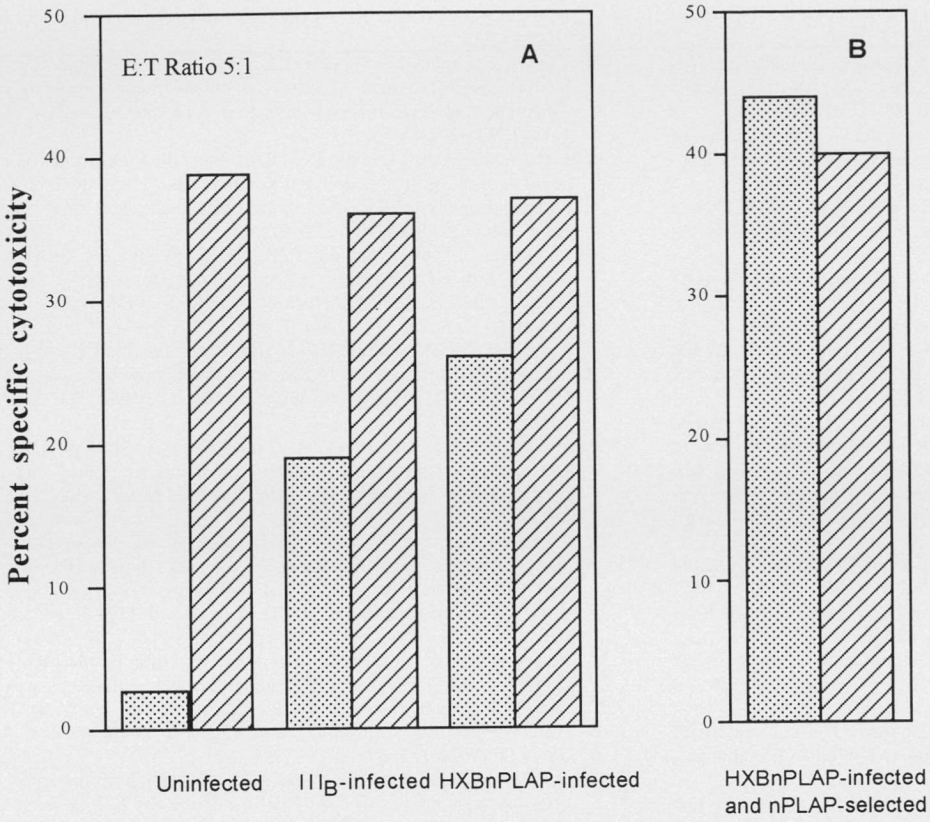

FIG. 8. Primary HIV-infected $CD4^+$ T cells are recognized for cytotoxic T-lymphocyte (CTL) lysis. Allogeneic A2-matched CB8-depleted peripheral blood mononuclear cells (PBMC) were activated with phytohemagglutinin (PHA) and infected with HIV_{IIIB} or HXB-nPLAP. Four days later, the infected primary CD4 T cells were used in cytotoxicity assays directly (**A**) or after enriching for HIV-infected cells by immunomagnetic selection (**B**). Cytotoxicity was assayed against targets in the presence (*hatched bars*) or absence (*dotted bars*) of the cognate peptide. Controls were uninfected CD8-depleted PHA blasts with and without peptide.

leads to downmodulation of cell surface MHC expression, which makes HIV-infected cells poor targets for CTL lysis (28,29). For most of our assays, we used virus derived from a molecular clone of HIV in which the nef gene has been replaced with the placental alkaline phosphatase gene. We therefore could not evaluate the contribution of nef-induced MHC downmodulation to HIV-specific lysis. Class I expression was not downregulated on primary T cells infected with the HIV-nPLAP virus (data not shown). We did observe some downmodulation of class I expression on HIV_{IIIB}-infected T1 cells (mean fluorescence intensity for A2.1, 595 versus 372), but this was also seen on vaccinia virus–infected T1 cells and did not seem to render them resistant to CTL lysis. In the experiments in which HIV_{IIIB}-infected targets were compared with HXB-nPLAP–infected targets (Figs. 4 and 7), the lysis of cells infected with nef-containing HIV_{IIIB} was comparable with that of nef-deleted HXB-nPLAP.

In summary, we identified a novel A2-restricted RT epitope recognized by a CTL clone from a seropositive subject that adds to the repertoire of useful epitopes for potential vaccine and immune based therapies. This epitope was presented and recognized as efficiently in primary infected T cells as it was in peptide-loaded or recombinant vaccinia virus–infected target cell lines. We have also developed a simple method to examine CTL lysis of infected primary $CD4^+$ T cells, which are natural targets of HIV infection.

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