Naturally Processed Viral Peptides Recognized by Cytotoxic T Lymphocytes on Cells Chronically Infected by Human Immunodeficiency Virus Type 1

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Summary

We have established long-term cultures of several cell lines stably and uniformly expressing human immunodeficiency virus type 1 (HIV-1) in order to (*a*) identify naturally processed HIV-1 peptides recognized by cytotoxic T lymphocytes (CTL) from HIV-1–seropositive individuals and (*b*) consider the hypothesis that naturally occurring epitope densities on HIV-infected cells may limit their lysis by CTL. Each of two A2-restricted $CD8^+$ CTL specific for HIV-1 gag or reverse transcriptase (RT) recognized a single naturally processed HIV-1 peptide in trifluoroacetic acid (TFA) extracts of infected cells: gag 77-85 (SLYNTVATL) or RT 476-484 (ILKEPVHGV). Both processed peptides match the synthetic peptides that are optimally active in cytotoxicity assays and have the consensus motif described for A2-associated peptides. Their abundances were ≈400 and ≈12 molecules per infected Jurkat-A2 cell, respectively. Other synthetic HIV-1 peptides active at subnanomolar concentrations were not present in infected cells. Except for the antigen processing mutant line T2, HIV-infected HLA-$A2^+$ cell lines were specifically lysed by both A2-restricted CTL, although infected Jurkat-A2 cells were lysed more poorly by RT-specific CTL than by gag-specific CTL, suggesting that low cell surface density of a natural peptide may limit the effectiveness of some HIV-specific CTL despite their vigorous activity against synthetic peptide-treated target cells.

MHC-restricted CTL play a central role in immune responses against many viruses by destroying virus-infected cells (1). Unlike antibodies, $CD8^+$ CTL recognize conserved sequences from intracellular viral proteins in addition to sequences from virus-encoded cell surface glycoproteins (2). $CD8^+$ CTL specific for diverse HIV products (e.g., gag, pol, nef, env) appear within 1–2 wk of infection, and the importance of these CTL is suggested by their unusually high frequencies: they can often be detected in freshly isolated PBMC without the in vitro antigenic stimulation usually required to demonstrate CTL activity in other viral infections (3–14).

It has been proposed that the $CD8^+$ CTL response to HIV is one of the main factors contributing to a long asymptomatic period in infected individuals (15, 16). Recent studies documenting HIV-specific T cells in HIV-exposed but uninfected individuals further support a role for these cells in controlling infection (17–19). Yet CTL usually do not succeed in eradicating HIV (20, 21). Two possible mechanisms for this failure are viral mutation leading to escape from CTL recognition (22–25) and altered lymphokine production secondary to depletion of $CD4^+$ T cells. Another possibility is that the density of many HIV epitopes (i.e., particular peptide-MHC complexes) on infected cells is too low to trigger effective lysis of these cells by mature $CD8^+$ CTL in vivo.

In considering this third possibility, it is important to distinguish between acute infection by viruses such as influenza or vaccinia, in which viral genes are overexpressed at the expense of host cell protein synthesis, and chronic infection by HIV, in which cells continue to express their normal complement of self proteins (as evidenced by their ability to grow and proliferate for long periods; see below). In the latter case, HIV-derived peptides must compete with a much larger excess of host cell peptides for presentation by class I MHC (MHC-I)[1] proteins to $CD8^+$ T cells. Can a sufficient

[1] *Abbreviations used in this paper:* MHC-I, class I MHC; RT, reverse transcriptase; SD_{50}, peptide concentration that sensitizes target cells for half-maximal lysis; TFA, trifluoroacetic acid; VSV, vesicular stomatitis virus.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/10/1283/11 $2.00
Volume 180 October 1994 1283–1293

number of HIV peptides succeed in occupying MHC-I binding sites in order to render infected cells good targets for destruction by CTL? The presence of a vigorous and specific CTL response in HIV infection implies that the density of HIV epitopes is sufficient to stimulate the differentiation of $CD8^+$ precursor T cells into mature CTL (at least on those cells involved in CTL induction). However, it remains unclear whether the number of specific peptide–MHC-I complexes on most HIV-infected cells (i.e., $CD4^+$ T cells, macrophages) is adequate for efficient killing of these cells by mature $CD8^+$ CTL.

The density of an HIV epitope on infected cells cannot be measured without first knowing the precise identity of the HIV peptide involved. Prior work characterizing HIV epitopes has relied on vaccinia recombinants (26) and synthetic peptides up to ≈25 amino acids in length (27, 28). However, the peptides produced by cellular antigen processing pathways may or may not be identical to the synthetic peptides used in target cell sensitization assays (29), and so the exact identities of HIV epitopes in infected cells are not known. To reveal both the identities and abundances of HIV peptides presented endogenously by MHC-I molecules, as well as other properties such as their MHC binding affinities and immunogenicities, we have established an assay system based on several newly developed cell lines that stably and uniformly express HIV-1 and CTL obtained from HIV-seropositive individuals.

Materials and Methods

Cells. The following human cell lines were used for transfections and infections (MHC-I molecules expressed by each cell are in parentheses): Jurkat (HLA-A9, A25, B7, B41); Jurkat stably transfected with the gene for HLA-A2 and here designated JA2 (kindly supplied by Dr. Linda Sherman, The Scripps Research Institute, La Jolla, CA) (30); H9 (HLA-A1, Bw62, Cw6); MOLT4; the TxB hybridoma T1 (HLA-A2, B5); the antigen processing mutant T2 derived from T1 (both kindly provided by Dr. Peter Cresswell, Yale University, New Haven, CT) (31); the EBV-transformed B cell line JY (HLA-A2, B7, Cw7); and the promonocytic cell line U937. All cell lines were grown in "K" medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME). CTL were derived from PBMC obtained from asymptomatic HIV-1–seropositive subjects and maintained as described (32, 33).

Transfection with Cloned HIV-1 DNA. The establishment of cell lines stably expressing HIV-1 proviral DNA was achieved by transfection with plasmid vectors R7neo or R7hyg, modifications of pHXB2gpt (34) in which selectable marker genes conferring resistance to G418 or hygromycin B, respectively, were inserted in place of a 255 bp fragment located at the 5′ end of the *nef* gene (35). The resulting plasmid DNA, which includes the entire HIV-1 genome except for *nef*, was purified and linearized by digestion with XbaI, and transfections were carried out by electroporation as described (35). 10^7 cells (Jurkat, JA2, H9, MOLT4, T1, T2, JY, or U937) were washed and resuspended in 0.4 ml cold PBS in 0.4-cm electroporation cuvettes, mixed with 20 μg linearized plasmid DNA, placed on ice for 5 min, subjected to a single pulse of 250 V, 960 μF (Bio-Rad apparatus; Bio-Rad Laboratories, Hercules, CA), and returned to ice for 10 min. Measured time constants were 30–40 ms. Cells were then washed and resuspended in K medium for incubation at 37°C in 5% CO_2.

After 48 h, an aliquot of 200 μl from each culture supernatant was assayed for HIV-1 production by p24 ELISA (DuPont NEN, Boston, MA). Depending on the plasmid used, R7neo or R7hyg, cells were selected with 0.5 mg/ml G418 (GIBCO BRL, Gaithersburg, MD) or hygromycin B (Calbiochem-Novabiochem Corp., San Diego, CA), a concentration shown to result in the death of over 50% of untransfected cells within 6 d; JA2 cells already under selection with 250 μg/ml G418 for A2 expression, were transfected only with R7hyg and subjected to dual selection. After several weeks of stable growth and evidence of HIV expression in all cells by immunofluorescence (see below), cells were maintained and expanded in 162 cm^2 cell culture flasks (Costar Corp., Cambridge, MA) in the presence of 0.25 mg/ml G418 and/or hygromycin B. HIV-expressing cell lines were named according to the plasmid vector used: JA2/R7hyg, H9/R7neo, H9/R7hyg, etc.

Immunofluorescence Microscopy and Flow Cytometry. To test for HIV expression, acetone-fixed cells were stained with a mouse anti-p24 monoclonal antibody for 30 min at room temperature (36). After the slides were washed with PBS, cells were incubated with the $F(ab')_2$ fragment of FITC-conjugated goat anti–mouse IgG (Cappel Laboratories, Durham, NC or Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature in the dark. Cells were then washed, counterstained with 0.5% Evans blue dye in PBS (Sigma Chemical Co.) for 10 min, washed again, and examined under a fluorescent microscope. For purposes of visualizing all cells and calculating the proportion of cells expressing HIV, cells that had been stained with anti-p24 and FITC-conjugated antibodies were also examined by phase contrast.

All cells were checked to confirm A2 status before transfection and, for $A2^+$ cells, several weeks after being grown under selection for HIV expression. 5×10^5 cells were stained with 1 μg of the anti-A2 monoclonal antibody PA2.1 and with 5 μg of the $F(ab')_2$ fragment of FITC-conjugated goat anti–mouse IgG (Pierce Chemical Co., Rockford, IL) and were then analyzed on an EPICS C cytofluorimeter (Coulter Corp., Hialeah, FL) or by fluorescence microscopy (for HIV^+ cells). CD4 expression before transfection was also determined using an anti–human CD4 monoclonal antibody (Becton Dickinson & Co., Mountain View, CA).

Peptide Synthesis and Chromatography. All peptides were synthesized by standard t-Boc chemistry on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA), analyzed by reverse phase HPLC and amino acid analysis, and found to be highly pure. Peptides were named according to the protein source (gag or reverse transcriptase [RT]), the first and last amino acid residues, and the peptide length, e.g., gag-SL9 = SLYNTVATL and RT-IV9 = ILKEPVHGV. Peptide concentrations measured by Micro BCA Assay (Pierce Chemical Co.) ranged from 10 to 100% of the concentrations calculated from dry weights; for peptides gag-SL9 and RT-IV9, precise concentrations were determined by quantitative amino acid analysis. HPLC separations of synthetic peptides were performed using a 4.6 × 250 mm C18 reverse phase column (model 218TP104; VYDAC, Hesperia, CA). The gag peptides were separated on a gradient of 0.25% B/min at a flow rate of 1 ml/min, with A = 0.1% trifluoroacetic acid (TFA) (Pierce Chemical Co.) in H_2O and B = 0.1% TFA in isopropanol, and the RT peptides were separated on a gradient of 1% B/min at a flow rate of 1 ml/min, with A = 0.1% TFA in H_2O and B = 0.085% TFA in acetonitrile.

Natural Peptide Extraction and Fractionation. Cultured cells were harvested by centrifugation, washed with cold PBS, and stored in batches of 10^9 at −70°C. Cells were lysed by adding 15 ml 1%

aqueous TFA on ice (and additional TFA if needed to bring the pH below 2), and douncing with 50 strokes. The material was transferred to a Centriprep 10 ultrafiltration device (Amicon Corp., Beverly, MA) and subjected to centrifugation at 2,600 *g* for several hours at 4°C (37). The ultrafiltrate (molecular mass <10 kD) was removed (>12 ml), an equal volume of 1% TFA was added to the retentate, and centrifugation was repeated twice in order to maximize the recovery of extracted peptides. Pooled filtrates were dried by lyophilization and redissolved in 0.1% TFA for fractionation by HPLC using the conditions described above for either the gag or the RT peptides. 40 fractions were collected at 1 min intervals in presiliconized microfuge tubes, dried by SpeedVac (Savant Instruments, Inc., Farmingdale, NY), and redissolved in H_2O with vortexing.

Cytotoxicity Assay. CTL assays were performed as described (38), using ^{51}Cr-labeled 115EBV cells (an autologous EBV-transformed B cell line) for CTL line 115Ip, or ^{51}Cr-labeled JY cells for CTL clone 68A62 when testing synthetic peptides or HPLC fractions.

Results

Establishment of Long-term Cell Lines Stably and Uniformly Expressing HIV-1. Several human cell lines were chosen for transfection with HIV-1 proviral DNA linked to a drug resistance gene, including T cells (Jurkat, JA2, H9, and MOLT-4), a TxB hybrid (T1) and its derivative antigen processing mutant cell line (T2), an EBV-transformed B cell line (JY), and a promonocytic cell line (U937). 2 d after transfection of each line by electroporation with plasmid R7neo or R7hyg (35), cell culture supernatants were shown to contain the HIV-1 p24 core antigen by ELISA. Selection was initiated either at this time or 3 wk later in order to permit infection of untransfected cells with virus produced by successfully transfected cells in the same culture; such infected (as opposed to transfected) cells also acquired the drug resistance gene. For convenience, HIV-expressing cell lines are referred to as HIV-infected even though they may include both transfected and infected cells. After several weeks during which fresh medium was supplied two to three times per week, all eight cell cultures were found to be 100% HIV$^+$ by p24 immunofluorescence (Fig. 1).

JA2/R7hyg (JA2 cells transfected with plasmid R7hyg) and H9/R7neo were readily expanded to >10 liters over a period of several weeks by maintaining the cells in log phase. They have remained HIV$^+$ for over 1.5 yr, showing that HIV expression is compatible with long-term cell viability and proliferative activity, at least under special circumstances. H9/R7hyg, Jurkat/R7hyg, and T1/R7hyg were also maintained and expanded for several months, whereas MOLT-4/R7hyg, JY/R7hyg, and U937/R7hyg grew more slowly, possibly reflecting their greater susceptibility to cytopathic effects of the virus and the gradual emergence of variants resistant to these effects. Only T2/R7hyg suffered steadily declining viability, and these cells did not survive beyond a few months despite evidence of successful transfection and initial selection of an HIV$^+$ population. In light of CD4-dependent syncytia formation as a mechanism for HIV-mediated cytopathicity (39), the high CD4 levels in T2 cells (70–80% higher than T1 and U937, the next highest) were considered a possible explanation for the singular failure of these cells to survive infection. However, when T2 cells were first sorted by FACS® (Becton Dickinson & Co.) for a CD4^{low} subset (stable for at least 1 mo at a level 30–40% higher than T1) and then transfected, the resulting T2/R7hyg was still only a short-term cell line. Nevertheless, these cells were uniformly HIV$^+$ and could be tested as targets for lysis by specific CTL along with the other stably transfected lines.

Lysis of HIV-infected Cell Lines by HIV-specific CTL. HIV-infected cells were tested as targets for direct lysis by several HIV-specific CD8$^+$ CTL, including the A2-restricted gag-specific line 115Ip and the A2-restricted RT-specific clone 68A62. CTL line 115Ip readily lysed the A2$^+$ HIV-infected target cells JA2/R7hyg, JY/R7hyg, and T1/R7hyg but not their untransfected counterparts (Fig. 2, *A–C*). CTL clone 68A62 specifically lysed target cells T1/R7hyg and JY/R7hyg (to a lesser extent), but JA2/R7hyg was hardly lysed more than uninfected JA2 cells (Fig. 2, *E-G*). The fourth available A2$^+$ HIV-infected target cell, T2/R7hyg, is a mutant cell line lacking genes for the peptide transporter subunits TAP-1 and TAP-2; it was not lysed by CTL 115Ip despite high levels of p24 expression (Fig. 2 *D*). Consistent with this result, previous studies showed that T2 cells infected with influenza virus, vaccinia virus, or vesicular stomatitis virus (VSV) failed to present known viral epitopes to appropriate CD8$^+$ CTL (40–43); however, these same cells were shown to present epitopes from Sendai virus (43), HIV-1 env (44), and minigene expression vectors encoding certain viral peptides (45), apparently via a TAP-independent pathway for loading MHC-I molecules with cytosolic peptides. Failure to detect lysis of T2/R7hyg cells under conditions where T1/R7hyg and other HIV$^+$ cells were efficiently lysed suggests that the TAP pathway is required for normal presentation of the gag peptide recognized by CTL 115Ip.

Identification of the Optimal Synthetic Peptides Recognized by Two HIV-specific CTL. Two sets of peptides based on previously described gag (33, Johnson, R. P., unpublished data) and RT (32, 38) sequences were synthesized and tested for their ability to sensitize uninfected A2$^+$ target cells for lysis by the A2-restricted CTL 115Ip and 68A62, respectively. The peptide with the lowest peptide concentration that sensitizes target cells for half-maximal lysis (SD_{50}) value (38), i.e., peptide concentration giving 50% of maximal specific lysis, was defined as the optimal synthetic peptide for each CTL. Results for CTL 115Ip are shown in Fig. 3 *A*. Peptides gag-SL9 and gag-RL10 were both active at picomolar concentrations, and gag-SL9 consistently had the lowest SD_{50} ($\approx$30 pg/ml). The closely related 9-residue peptides gag-LY9 and gag-RT9 were 10,000-fold less active than gag-SL9, although at micromolar concentrations even these peptides resulted in >50% specific lysis of A2$^+$ target cells. For CTL clone 68A62, SD_{50} data from the screening of 18 synthetic peptides derived from RT residues 469-485 were previously tabulated (38), and some of the titrations are shown in Fig. 3 *B*. RT-IV9, the optimal synthetic peptide for this CTL clone (SD_{50} $\approx$1 pg/ml), was previously shown to associate tightly and specifically with target cell A2, dissociating with a half-life of 200–600 h (as its iodinated derivative).

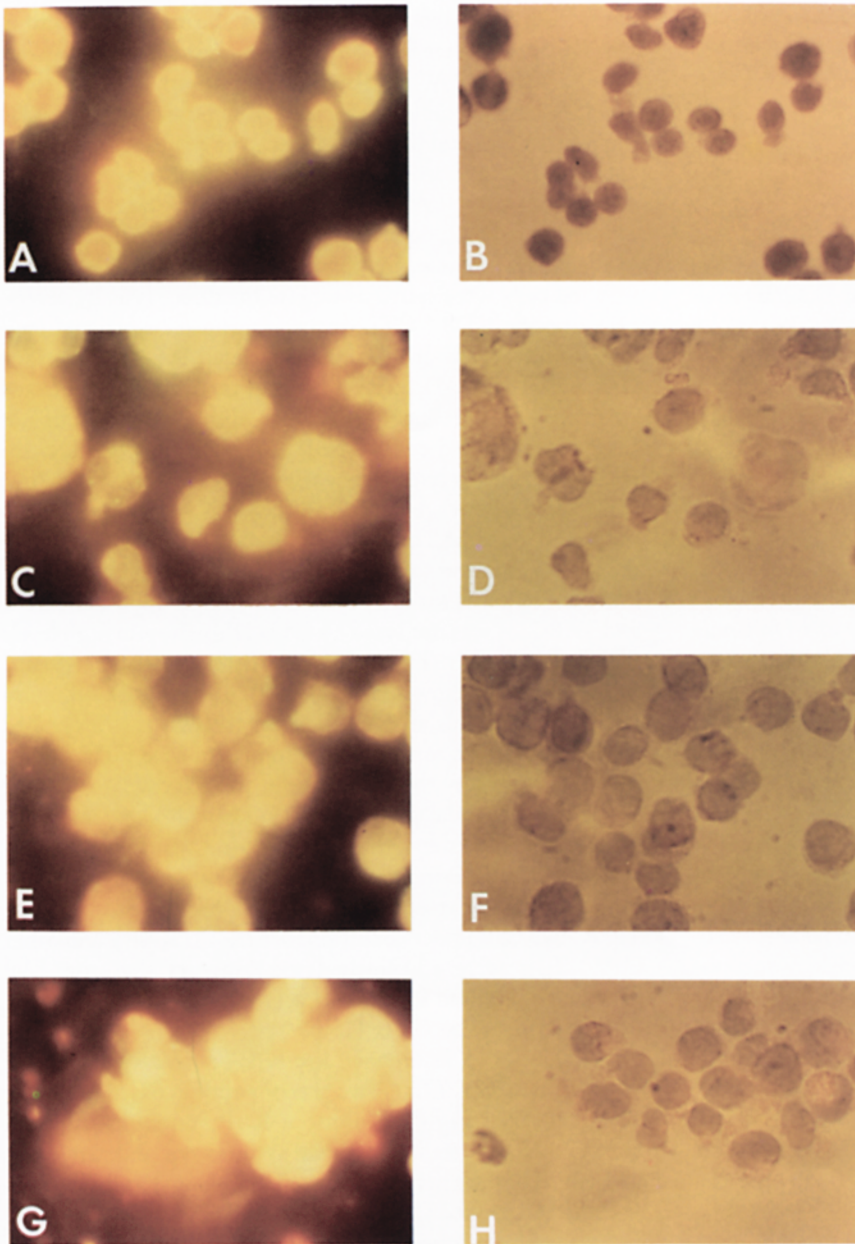

Figure 1. Lymphocyte cell lines transfected with HIV-1 DNA stably and uniformly express viral antigen while continuing to proliferate. Cells were transfected with plasmid R7hyg, grown in the presence of hygromycin B for at least 1 mo, and stained with anti-p24 monoclonal antibody and FITC-labeled secondary antibody as described in Materials and Methods. For each cell line, a single field is visualized by fluorescence (*left*) and phase contrast (*right*) microscopy. Cell lines shown are JA2/R7hyg (*A* and *B*), H9/R7hyg (*C* and *D*), T1/R7hyg (*E* and *F*), and JY/R7hyg (*G* and *H*). For H9/R7hyg cells, HIV-induced syncytia can be seen. Untransfected control cells exhibited no fluorescence under the staining conditions used.

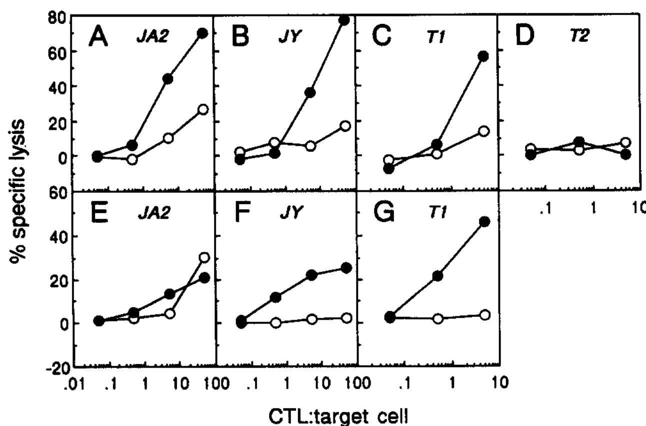

Figure 2. HIV-expressing cell lines are susceptible to lysis by HIV-specific CTL. Cytotoxicity assays were carried out using the gag-specific CTL line 115Ip (*A–D*) or the RT-specific CTL clone 68A62 (*E–G*) and the ^{51}Cr-labeled target cells indicated at the top of each panel. Lysis of T2 cells by CTL clone 68A62 was not tested. Target cells were either untransfected (○) or had been transfected with plasmid R7hyg at least 1 mo before and were uniformly positive for HIV expression by p24 immunofluorescence (●). In addition, the Bw62-restricted gag-specific CTL lines 35C18 and 35C44, obtained by stimulating PBMC from an HIV-1–seropositive individual with the 25-residue peptide p17/2 (33), efficiently lysed H9/R7hyg cells but not untransfected H9 cells (not shown).

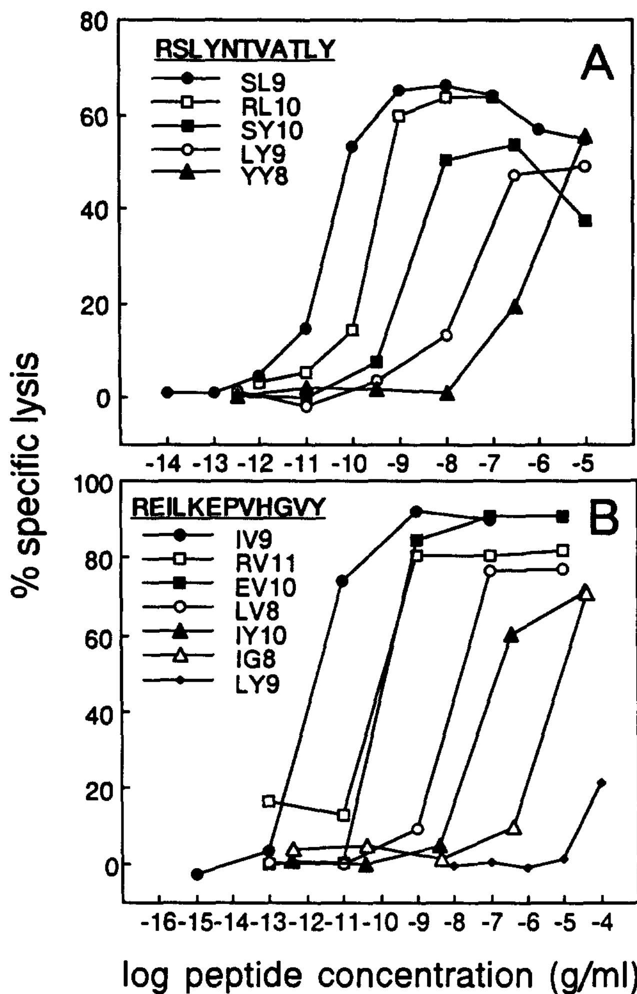

Figure 3. Synthetic peptides define the optimal epitopes recognized by HIV-specific CTL. Cytotoxicity assays were carried out with (*A*) the gag-specific CTL line 115Ip and ^{51}Cr-labeled autologous 115EBV target cells or (*B*) the RT-specific CTL clone 68A62 and ^{51}Cr-labeled JY target cells. Various concentrations of the synthetic peptides shown were added to target cells for 1 h at 37°C and remained in the assay during the 4-h incubation of CTL and target cells. Cells were then pelleted and supernatants were assayed for ^{51}Cr release. All peptides were negative for toxicity (<5% specific lysis) and were titrated at least twice, with results always within one order of magnitude. Also tested with CTL 115Ip, but not shown, were gag peptides LL8, RT9, and ST8, each of which was less active than gag-LY9. SD_{50} values (peptide concentrations giving 50% of maximal specific lysis [38]) for the optimally active synthetic peptides gag-SL9 (*A*) and RT-IV9 (*B*) were 30 pg/ml and 1 pg/ml, respectively.

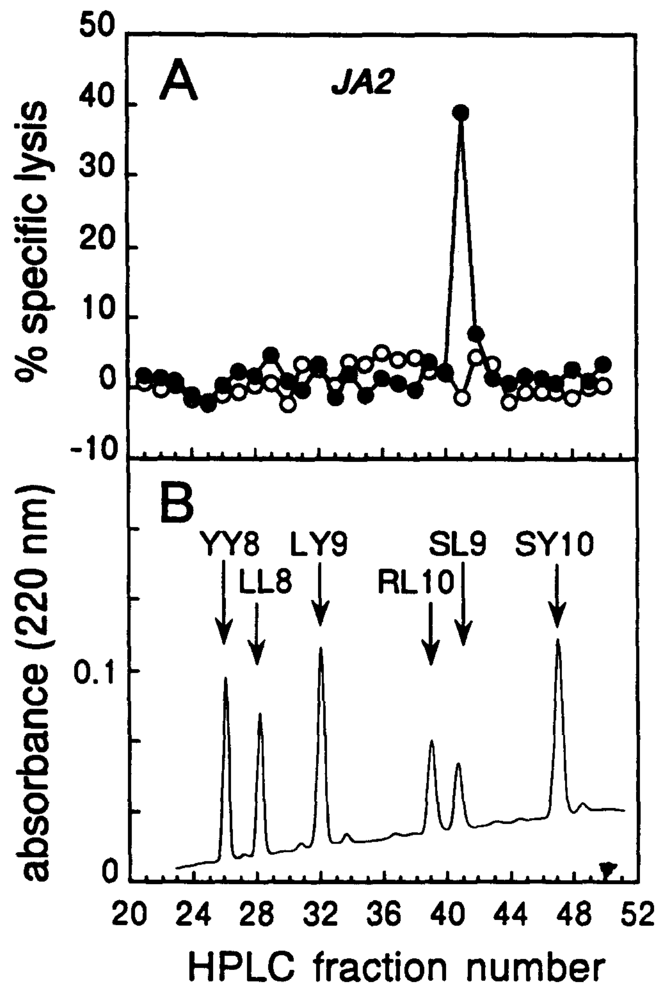

Figure 4. HIV-expressing cell line JA2/R7hyg produces a single peptide, gag-SL9, for recognition by CTL line 115Ip. (*A*) 10^9 JA2/R7hyg (●) or untransfected JA2 (○) cells were lysed in 1% TFA, homogenized by douncing, subjected to ultrafiltration in order to isolate a peptide-containing fraction (<10 kD), and fractionated by C18 HPLC using a gradient of 0.25% isopropanol per min at a flow rate of 1 ml/min. Fractions were collected at 1 min intervals, dried, and tested for their ability to sensitize ^{51}Cr-labeled 115EBV target cells for lysis by CTL 115Ip in a 4-h cytotoxicity assay. Toxicity controls consisting of target cells incubated with each fraction in the absence of CTL were negative (not shown). (*B*) HPLC retention times of the synthetic peptides that can be recognized by CTL 115Ip are shown under the same chromatographic conditions used in *A*. In addition, gag peptides ST8 and RT9 both eluted in fraction 19, which does not correspond to an active fraction from TFA-extracted JA2/R7hyg cells.

Identification of Two HIV-1 Peptides Produced by Endogenous Antigen Processing in Infected Cells. To identify the naturally processed HIV-1 peptides present in HIV-infected cells, JA2/R7hyg cells were expanded to several liters, and MHC-bound peptides were isolated by TFA extraction, ultrafiltration, and reverse phase HPLC fractionation. When the HPLC fractions were added to uninfected A2$^+$ target cells in cytotoxicity assays, only a single HPLC fraction had sensitizing activity for each of the two A2-restricted HIV-specific CTL examined: fraction 41 for CTL 115Ip (Fig. 4 *A*) and fraction 32 for CTL 68A62 (Fig. 5 *A*).

To identify the active peptides in these fractions, their retention times were compared with those of all the synthetic peptides that could be recognized by the same CTL. Under an appropriate set of HPLC conditions (column, solvents, gradient, etc.), peptides differing even slightly in length or sequence can be resolved and therefore differentiated based on characteristic retention times. Thus, of all the active synthetic gag peptides tested using CTL 115Ip (including all possible 8-, 9-, and 10-mers contained within a longer active synthetic peptide), only gag-SL9 eluted in fraction 41 under the same HPLC conditions used for the JA2/R7hyg cell extract

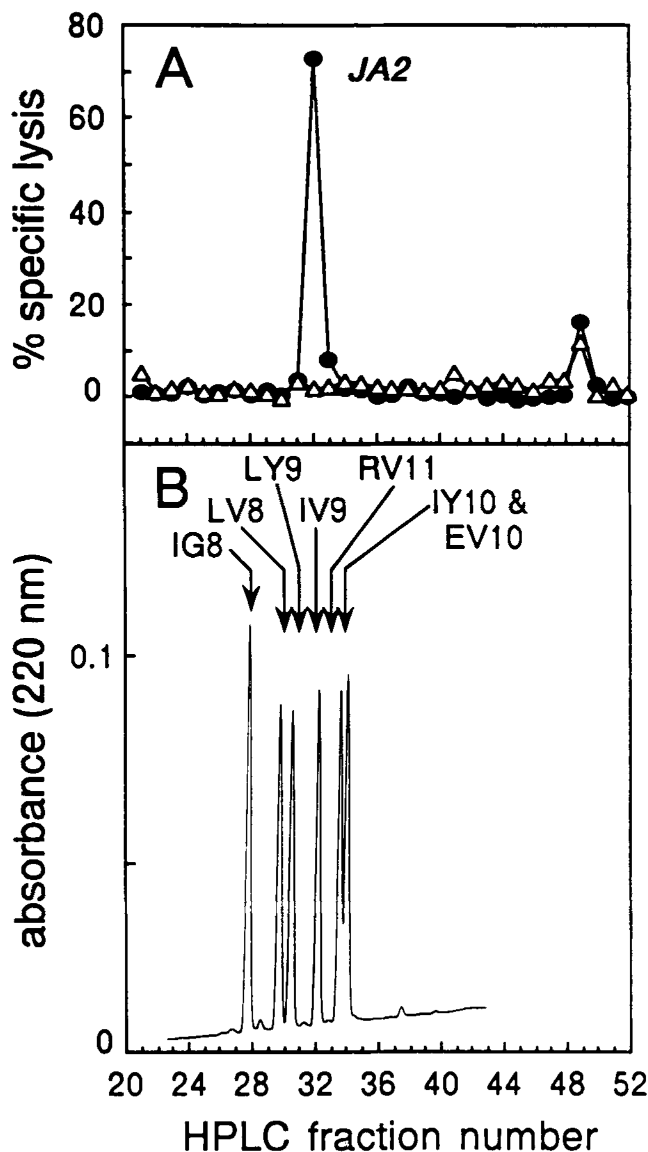

Figure 5. HIV-expressing cell line JA2/R7hyg produces a single peptide, RT-IV9, for recognition by CTL 68A62. (*A*) 10^9 JA2/R7hyg cells were lysed in 1% TFA and prepared as described in Fig. 4 *A*, except that HPLC fractionation was performed using a gradient of 1% acetonitrile per min. Fractions were added to ^{51}Cr-labeled JY target cells and tested for toxicity (△) and for lysis by CTL 68A62 (●). (*B*) HPLC retention times of the synthetic peptides that can be recognized by CTL 68A62 are shown under the same chromatographic conditions used in *A*.

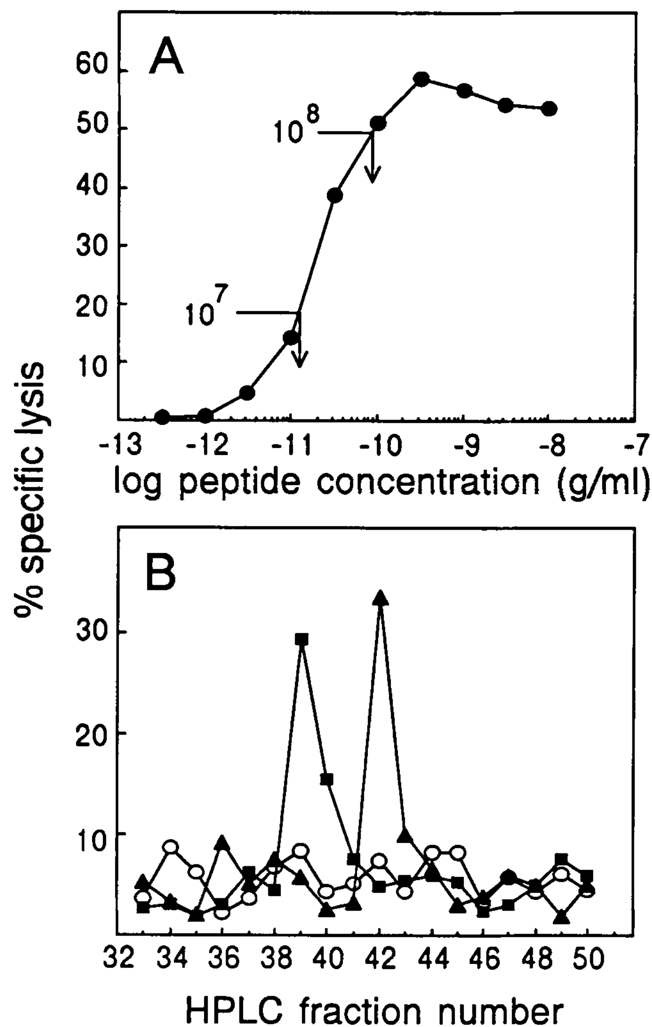

Figure 6. Each JA2/R7hyg cell contains approximately 400 molecules of peptide gag-SL9. (*A*) 10^9 JA2/R7hyg cells were lysed in 1% TFA and prepared as described in Fig. 4 *A*. The active HPLC fraction recognized by CTL 115Ip was titrated in a standard cytotoxicity assay using ^{51}Cr-labeled JY target cells. Percent specific lysis values obtained using 10^7 or 10^8 JA2/R7hyg cell equivalents per well (i.e., 1 or 10% of the active fraction) are indicated by arrows on a standard curve performed with synthetic peptide gag-SL9 and JY target cells in the same assay. The calculated recovery was ~100 gag-SL9 molecules per JA2/R7hyg cell. (*B*) 10^9 untransfected JA2 cells were lysed as described (*open circles*) or were first "spiked" with 100 pg synthetic gag-SL9 (*solid triangles*) or 1 ng gag-RL10 (*solid squares*). From a comparison of percent specific lysis values due to TFA-extracted cells and standard curves for each of these synthetic peptides, their overall yields for all steps from cell lysis through cytotoxicity assay were found to be 25 and 30%, respectively.

(Fig. 4 *B*), indicating that the peptide recognized by CTL 115Ip on the surface of HIV-infected cells is gag-SL9. Similarly for CTL 68A62, only synthetic peptide RT-IV9 had the same retention time as the active HPLC fraction isolated from JA2/R7hyg cells (Fig. 5 *B*), indicating that CTL 68A62 recognizes RT-IV9 on HIV-infected cells.

The Abundance of Two Naturally Processed HIV-1 Peptides. To measure the abundance of the naturally processed peptide gag-SL9, 10^9 JA2/R7hyg cells were lysed and treated as described above, and the active HPLC fraction was titrated in a cytotoxicity assay using CTL 115Ip. As shown in Fig. 6 *A*, the amount of peptide recovered from 10^7 or 10^8 JA2/R7hyg cells gave 19 or 50% specific lysis, respectively. These values, compared with the standard curve from a cytotoxicity assay carried out under identical conditions with synthetic gag-SL9, correspond to a recovery of ≈100 peptide molecules per JA2/R7hyg cell. To estimate the overall efficiency of peptide recovery, a "spiking" experiment was performed in which 100 pg synthetic gag-SL9 was added to 10^9 untransfected JA2 cells. Subsequent extraction and fractionation

steps were identical to the treatment of JA2/R7hyg cells, culminating in cytotoxicity assay of HPLC fractions using CTL 115Ip (Fig. 6 *B*). From the percent specific lysis due to either 5 or 20% of the sample in fraction 42 (5 pg or 20 pg if the overall yield was 100%), comparison to a standard curve for synthetic gag-SL9 led to an estimated 25% overall yield (e.g., 20% of the sample gave 33% specific lysis, corresponding to 5 pg synthetic gag-SL9 on a standard curve generated in the same assay; standard curve not shown). Similarly, for synthetic gag-RL10 added to untransfected JA2 cells, the overall yield was 30%. Since even a very tightly A2-binding peptide (radiolabeled RT-IV9) completely dissociated from A2 after 60 min of exposure to 1% TFA (38), the calculated yield likely reflects all steps in the isolation of naturally processed gag-SL9, including the release of peptide from preexisting peptide–MHC complexes.

The abundance of naturally processed peptide RT-IV9 was established similarly by comparison with a standard curve of synthetic RT-IV9. The amount of peptide recovered from 2×10^8 JA2/R7hyg cells gave the same degree of lysis as 6×10^8 molecules of synthetic RT-IV9 when added to uninfected A2$^+$ target cells (i.e., a synthetic peptide concentration of 5×10^{-12} M in a total volume of 200 μl; not shown). Therefore only ≈3 molecules of RT-IV9 per HIV-infected cell were recovered, for a calculated abundance of ≈12 molecules per infected cell (assuming a similar overall yield to gag-SL9 and gag-RL10).

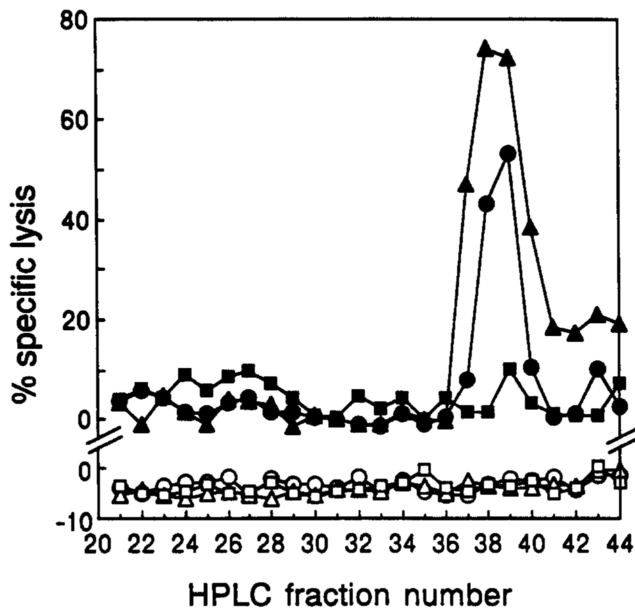

Figure 7. Generation of peptide gag-SL9 in HIV-expressing cells is independent of HLA-A2. JA2/R7hyg cells (*solid circles*), H9/R7neo cells (*solid triangles*), or untransfected JA2 cells (*solid squares*) were lysed in 1% TFA and prepared as described in Fig. 4 *A*. Fractions were tested for their ability to sensitize ^{51}Cr-labeled 115EBV target cells for lysis by CTL 115Ip in a 4-h cytotoxicity assay. Toxicity controls consisting of target cells incubated with each fraction in the absence of CTL are included (*corresponding empty symbols*). H9/R7neo cells are HLA-A2$^-$. Retention time of the active fraction does not agree precisely with Fig. 4 *A* because a different C18 column was used for this separation. Untransfected JA2 cells fractionated before H9/R7neo gave no activity, ruling out contamination of the HPLC column.

Expression of an HLA-A2–binding HIV-1 Peptide Occurs Independently of HLA-A2. Given numerous findings by Rammensee et al. (46) that peptides recognized by CTL accumulate to detectable levels only in cells expressing the restricting MHC molecule, we were surprised to find a strong peak of activity corresponding to gag-SL9 in TFA extracts of the A2$^-$ infected cell line H9/R7neo (Fig. 7). However, it remains unknown whether gag-SL9 binds to a different HLA molecule expressed by H9 cells, e.g., HLA-A1, Bw62, or Cw6.

Discussion

The reasons for incomplete elimination of HIV-infected cells by CTL in vivo are not yet understood (20, 21). One possibility is that high mutation rates in viral proteins could impair recognition and lysis of infected cells by virus-specific CTL (22), much as mutations in viral surface glycoproteins can result in escape from antibody recognition (47). To date, evidence both for and against the emergence of CTL escape mutants in HIV infection has been published (23, 24, 48, 49). Another mechanism for diminished CD8$^+$ CTL activity in HIV infection may be altered lymphokine production by "helper" T cells as a result of steadily declining CD4$^+$ T cell counts and aberrant CD4$^+$ T cell function in infected individuals. However, some individuals with low CD4$^+$ T cell levels retain vigorous CTL activity (50); furthermore, intact CD8$^+$ CTL function in CD4-deficient "knockout" mice argues against an absolute dependence of CTL on CD4$^+$ T cells (51). Here we raise a third possibility: CTL of HIV-infected individuals may be incompletely effective in eradicating the virus because of a paucity of viral epitopes expressed on infected cells.

When cells are infected by lytic viruses (including influenza, vaccinia, Sendai, and VSV), host cell protein synthesis shifts toward virus-encoded products at the expense of endogenous ("self") protein synthesis. This overproduction of viral proteins gives rise to relatively high levels of viral peptides, which in turn form high cell surface densities of peptide–MHC-I complexes and facilitate lysis of the infected cells by virus-specific CTL. Accordingly, previous studies of naturally processed viral peptides seen by T cells have made use of lytically infected cells (52–54). However, infection by HIV (or by certain other viruses, such as EBV and CMV) can result in chronic expression of viral proteins for months or years, along with the full complement of normal self proteins necessary for cell viability and function. In these cases, a relatively small number of viral proteins (i.e., ≈10 for HIV) must compete with all the proteins normally expressed in a cell (perhaps 5,000–10,000) for presentation by a limited number of MHC-I proteins to CD8$^+$ T cells. Can HIV-derived peptides compete with such a vast excess of self peptides and occupy sufficient numbers of MHC-I binding sites to result in effective killing of HIV-infected cells by CTL?

Previous studies of peptides recognized by HIV-specific T cells have relied on vaccinia virus recombinants containing HIV genes or gene segments and synthetic peptides corresponding to HIV sequences (27, 28). While these studies have led to the delineation of numerous T cell epitopes, oper-

ationally defined as synthetic peptides that trigger T cell activities in vitro, and to the identification of candidate peptides for vaccine trials (55), little is known about which of these peptides are actually generated within HIV-infected cells, their respective abundances, or their likely importance in the anti-HIV CTL response.

To reveal the identities and abundances of HIV-1 peptides expressed by infected cells and recognized by HIV-specific CTL, we developed a panel of stably HIV-infected cell lines from which naturally processed peptides could be isolated directly. Adding infectious HIV-1 directly to $CD4^+$ cells as a method of establishing infection tends to result in cultures which are only partially infected and which are difficult to sustain, presumably due to cytopathic effects of the virus. Therefore to obtain cultures in which all of the cells are productively infected and can be expanded to large numbers, we introduced the viral genome by transfection of cloned proviral DNA containing the entire HIV-1 genome except for *nef*, which is not required for viral replication in cell culture and which was replaced by an antibiotic resistance gene (neomycin or hygromycin) (35). Cell cultures under continuous antibiotic selection were uniformly HIV^+ (Fig. 1) and were expanded to many liters while continuing to express HIV for long periods (over 1.5 yr for JA2/R7hyg and H9/R7neo). Establishment of the infected B cell line JY/R7hyg, $CD4^-$ by flow cytometry, was also made possible by this technique of transfection and direct selection. Other HIV-infected A2-negative cell lines have previously been established for various studies, including derivatives of A3.01, H9, HeLa-$T4^+$, and U937 cells (56).

The RT-specific CTL clone 68A62 lysed cell lines T1/R7hyg and JY/R7hyg reasonably well, but hardly lysed JA2/R7hyg even at a CTL/target cell ratio of 50:1. Since JA2/R7hyg was lysed well by CTL 115Ip, these results suggested that levels of the naturally processed peptide(s) seen by 68A62 may be low on JA2/R7hyg cells. To address this issue directly, we applied techniques described by Rammensee et al. (46) to determine the precise identity and abundance of this naturally processed peptide(s). Briefly, HIV-infected target cells were treated with TFA, the extracted peptides were fractionated by reverse phase HPLC, and individual fractions were added to uninfected $A2^+$ cells for cytotoxicity assay. Because the naturally processed peptides from, say, 10^8 cells can be loaded onto 10^4 $A2^+$ target cells in a cytotoxicity assay, the sensitivity of peptide detection is amplified by as much as 10,000-fold (or even more if more cells are extracted, subject to solubility and toxicity limitations). Another advantage of this assay system is that both $A2^+$ and $A2^-$ infected cells can be examined for the presence of a particular peptide, since $A2^+$ target cells are used in the final assay (e.g., Fig. 7). Because the retention time of a given peptide under well-defined HPLC conditions is highly reproducible, comparison with synthetic peptide standards allows the naturally processed peptide(s) to be identified and quantitated. (In contrast, the amount of peptide recovered from 10^8–10^{10} cells is typically in the low or subfemtomole range, well below what is usually required for direct chemical sequencing by Edman degradation or mass spectrometry.) By these methods it was established that the single naturally processed peptides recognized by CTL 115Ip and 68A62 were gag-SL9 and RT-IV9, respectively (Figs. 3–5). Both peptides matched the optimally active synthetic peptides recognized by these CTL, and both conform to the A2 consensus motif (57).

The abundance of naturally processed gag-SL9 was found to be ≈400 molecules per infected cell by measuring the recovery of this peptide from JA2/R7hyg cells (≈100 molecules/cell) and the efficiency of the isolation procedure (25%) (Fig. 6). Assuming that half the losses are associated with the HPLC step (50% yield), repeated rounds of HPLC fractionation will result in much more severe losses. For example, the purification of two overlapping peptides from the enzyme α-ketoglutarate dehydrogenase that are recognized by an alloreactive CTL clone was accomplished by five (58) or six (59) successive HPLC rounds before sequencing; if each round was associated with a 50% yield, the cumulative yield would have been only 1–3%. In this scenario, the natural abundance of these two peptides is far greater than that of the HIV-1 gag peptide reported here: since ≈400 (58) or 7,000 (59) peptide molecules per allogeneic cell were actually recovered, their abundances are likely to be as high as 12,000 and 400,000 molecules per cell, allowing for the expected yields. Such high abundances of naturally processed peptides in the alloreactive system may result from high levels of their precursor, a normal cell protein, and probably account for the appearance of a family of peptides rather than the single peptide seen by two HIV-specific (as well as other virus-specific [52–54]) CTL.

The extent of lysis by CTL 115Ip (as much as 80% specific lysis of JY/R7hyg target cells) indicates that HIV-1 peptides can compete with the myriad self peptides generated in the same cells for binding to MHC-I proteins and transport to the cell surface. In fact, the levels of gag peptide (≈400 molecules per infected cell) were not much lower than the recoveries of two influenza peptides from influenza virus–infected cells (220–540 molecules/cell, uncorrected for extraction efficiencies [60]), despite the significantly different nature of the two infections (lytic vs. chronic). For VSV-infected cells, however, van Bleek and Nathenson reported that 5–10% of MHC-bound peptides were of viral origin, and that these were dominated by a single octamer, implying a significantly higher abundance in this system (52). A naturally processed *Listeria monocytogenes* peptide epitope similarly was present at >3,000 copies per infected cell (61). But in cells transfected with the ovalbumin gene and recognized by ovalbumin-specific CTL, only ≈100 molecules of a sensitizing ovalbumin peptide were recovered (no correction for extraction efficiency) (62).

In contrast to CTL 115Ip, CTL 68A62 exhibited lower levels of activity against HIV-infected target cells, and lysed JA2/R7hyg only poorly even though the optimally active peptide RT-IV9 was shown to be present in these same cells. This result can be explained by the relatively low abundance of RT-IV9 on JA2/R7hyg cells (≈12 molecules per cell), consistent with the 10–20-fold lower expression of RT than gag protein in HIV-infected cells due to a ribosomal frameshift required for RT expression (63). Since growth of JA2/R7hyg cells under selective pressure for HIV expression might have

resulted in higher than normal levels of HIV-1 peptides, RT-IV9 could be even more sparse on naturally infected cells. T1/R7hyg cells, which are lysed better than JA2/R7hyg cells by CTL 68A62, probably have a higher epitope density, as suggested by higher levels of p24 antigen (not shown) and by the fact that uninfected T1 and JA2 cells are similarly sensitive to exogenous RT-IV9 (SD_{50} = 10–20 pg/ml). Thus, in spite of the extreme sensitivity of CTL 68A62 to synthetic RT-IV9 (Fig. 3 *B*) and the demonstration of RT-IV9 in JA2/R7hyg cell extracts (Fig. 5), CTL 68A62 lyses JA2/R7hyg cells inefficiently (relative to their lysis by CTL 115Ip, Fig. 2), emphasizing the critical role of epitope density in lysis of these infected target cells and raising a serious question as to the likely effectiveness of this CTL clone in vivo.

In this context it is worth noting that when synthetic peptides are added to target cells in cytotoxicity assays, very high peptide concentrations are often used, e.g., 10–100 μM. At such concentrations, peptides with even modest affinities for the restricting MHC molecule (K_A = 10^6–10^8 M^{-1}) will occupy a large fraction of available MHC binding sites at equilibrium (>99%), resulting in far greater numbers of specific peptide-MHC complexes than the few hundred per cell often seen for naturally processed peptides (53, 60, 62, 64). For example, JY cells have ≈10,000 A2 molecules available for binding to exogenous peptides (38), so peptide RT-IV9 (equilibrium constant for binding to A2 ≈10^7 M^{-1} [Kageyama et al., manuscript submitted for publication]) will form up to 10,000 peptide–MHC complexes per target cell when added at micromolar concentrations. Similarly, other synthetic peptides can efficiently sensitize target cells by loading cell surface MHC-I molecules even though these peptides are not detectable in infected cells and are therefore not actually relevant to the immune response (e.g., gag-RL10, RT-EV10). Thus epitope identification based on synthetic peptides, while useful as a preliminary guide, may not accurately represent CTL-target cell interactions as they occur in vivo.

Overall, our results suggest that HIV-infected cells can be good targets for lysis by HIV-specific CTL, but that CTL effectiveness depends critically upon epitope density: the RT-specific clone 68A62 exhibited poor killing of a target cell (JA2/R7hyg) that was shown to express only a few copies per cell of the optimally active peptide. Because the affinities of antigen-specific T cell receptors for their peptide–MHC ligands are likely to influence the density of epitopes required for efficient lysis of target cells, it is still possible that CTL bearing high-affinity receptors may lyse infected target cells having very low epitope densities (Kageyama et al., manuscript submitted for publication; and Sykulev et al. [64a]); however, no information is currently available regarding T cell receptor affinities for HIV-specific CTL. The outcomes of adoptive therapy trials using CTL in HIV-infected humans (65, 66) may therefore depend on T cell receptor affinities as well as on the numbers and specificities of the CTL used. The most effective CTL against HIV-infected cells ultimately may be those that can recognize the most abundant naturally processed HIV epitopes; using the stably infected cell lines described here, it should be possible to identify and quantitate these epitopes.

We thank Dr. Linda Sherman for Jurkat cells transfected with HLA-A2 (here termed JA2); Dr. Peter Cresswell for cell lines T1 and T2; Richard F. Cook and Michael Kelley of the M.I.T. Biopolymers Laboratory for peptide synthesis; and Drs. Glenn Dranoff, Jun Liu, and Hans-Gustaf Ljunggren for carefully reading the manuscript.

This work was supported by National Institutes of Health (NIH) grants R35 CA-42504, AI-26463, AI-34247, CA-60686, CA-14051 (Cancer Center Core Grant), and T32 CA-09255 (Training grant). R.P. Johnson acknowledges NIH grant AI-33327 and a Burroughs Wellcome Foundation/Infectious Disease Society of America Young Investigator Award and is a Scholar of The American Foundation for AIDS Research.

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Received for publication 1 March 1994 and in revised form 27 May 1994.

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