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Stimulation of human cytotoxic T cells with HIV-1-derived peptides presented by recombinant HLA-A2 peptide complexes

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Abstract

HLA-A2 heavy chain and β_2-microglobulin were expressed in *Escherichia coli*, and refolded in the presence of peptides derived from HIV-1 RT and *gag* proteins. When recombinant HLA-A2 molecules were attached to cells lacking HLA-A2, the cells became susceptible to lysis by HLA-A2 restricted cytotoxic T lymphocyte (CTL) clones specific for peptides derived from RT and *gag* proteins. Limiting dilution analyses of peripheral blood mononuclear cells from HIV-1-infected individuals showed that the recombinant HLA-A2 peptide complexes covalently immobilized on microspheres stimulated the development of HLA-A2 peptide-specific CTL. Preformed HLA–peptide complexes may provide an alternative to immunization procedures that depend upon intracellular processing of antigen to elicit T cell responses.

Introduction

MHC-encoded class I molecules serve as peptide binding, transport and display proteins on the cell surface, and can evoke T cell responses when recognized by antigen-specific receptors on cytotoxic T lymphocytes (CTL). MHC class I molecules are glycoproteins consisting of two non-covalently associated polypeptide chains, a larger or heavy chain and a smaller or light chain, termed β_2-microglobulin (1). Human class I molecules have been refolded from subunits produced in *Escherichia coli* in association with synthetic peptides (2). The three-dimensional structures of several recombinant complexes, determined by X-ray crystallography (3), are virtually identical to the structure of the class I molecule isolated from human cells (4). However the MHC proteins produced in *E. coli* do not contain the complex carbohydrate that is normally attached to asparagine 86. The aim of this work is to determine if an MHC class I protein, specifically HLA-A2 (A2), produced in non-glycosylated form in *E. coli* and assembled with synthetic nonapeptides can (i) elicit cytolytic responses by $CD8^+$ T cell clones arising in the course of natural infection with HIV-1 and (ii) stimulate the proliferation of peptide-specific $CD8^+$ T cells in peripheral blood lymphocytes of HIV-1 seropositive donors.

The HLA-A2 (HLA-A*0201) heavy chain and β_2-microglobulin light chain were assembled in the presence of two antigenic peptides derived from HIV-1: a peptide from reverse transcriptase (RT-IV9, amino acids 476–484, ILKEPVHGV) (5) and a peptide from the *gag* protein (Gag-SL9, amino acids 77–85, SLYNTVATL) (6). Both peptides represent disease response targets in HLA-$A2^+$ HIV-1-infected patients (7), conform to the HLA-A2 consensus motif (8), and have the optimal sequence recognized by RT-IV9- and Gag-SL9-specific CTL clones obtained from asymptomatic HIV-1-seropositive patients (9,10). Both peptides are recognized by cytotoxic T cell clones at picomolar concentrations, with SD_{50} values (i.e. peptide concentration giving half-maximal specific lysis) of ~3 pM for Gag-SL9 and ~0.1 pM for RT-IV9 (5,9).

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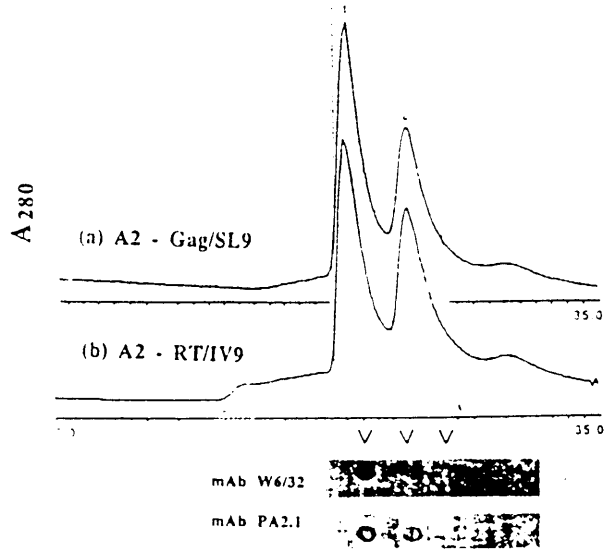


Fig. 1. Gel filtration HPLC profiles of HLA-A2 peptide complexes. HPLC profiles of HLA-A2 reconstitution from recombinant heavy chain and β_2-microglobulin in association with (a) HIV-1 Gag-SL9 peptide 77–85, SLYNTVATL and (b) HIV-1 RT-IV9 peptide 476–484, ILKEPVHGV. Specific binding of mAb W6/32 and mAb PA2.1 (both specifically reactive with native HLA-A2 complexes) to HPLC fractions was used to monitor refolded A2-peptide complexes.

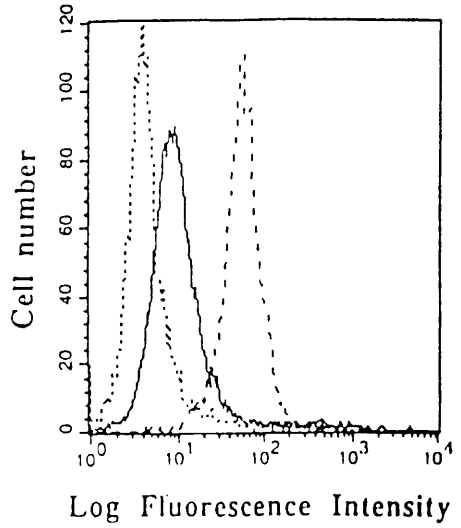


Fig. 2. Adsorption of purified soluble HLA-A2 peptide complexes to the surface of HLA-A2-negative cells monitored by FACS analysis. MHC class I expression on unmodified HLA-A2-negative C1R cells (HLA-B,C) was determined with mAb BB7.7–FITC conjugate (dotted line, BB7.7 anti-HLA-A,B,C). To adsorb the HLA–peptide complexes we covalently attached mAb BBM1 (anti-β_2-microglobulin) and mAb MA2.1 (anti-HLA-A2,B17) to the cell surface using a heterobifunctional cross-linker (SPDP). Surface bound mAb–SPDP conjugates were detected using FITC–goat anti-mouse $F(ab)_2$ fragments (dashed line). Adsorption of HLA-A2 peptide complexes to mAb-coated cells was shown by an increase in MHC class I expression using mAb BB7.7–FITC conjugate (solid line).

Methods

CTL clones and cell lines

CTL clones from asymptomatic HIV-1 seropositive patients and the corresponding (autologous) Epstein–Barr virus-transformed B lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (6,7). Subjects 115i, 15760 and 161J were previously shown to have significant *gag*-specific CTL activity (6 and unpublished). HLA-typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory using standard serological techniques (subject 161J: A2, A3; B7, B60; C3; DR2, DR4; subject 010–115i: A2, A28; B14, B52; Cw8; DR1,2; DQ1 and subject 15760: A2; B44, B51; C3; DR11). RPMI 1640 (Sigma, St Louis, MO) containing 20 % (v/v) heat-inactivated FCS (Sigma) was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) and used for all cell lines.

Gel filtration HPLC of HLA-A2 peptide complexes

Peptides were synthesized at the MIT Biopolymers laboratory. A2 refolding and complex formation was initiated by dilution of the two denatured subunits in the presence of peptides as previously described (2).

mAb binding to HPLC fractions

mAb binding to HPLC fractions was used to monitor refolded A2-peptide complexes. Samples of HPLC peak fractions (20 µl) were pipetted onto a nitrocellulose filter (Amersham, Santa Clearbrook, IL) and allowed to adsorb for 2 h at room temperature. The membrane was then incubated with 3% BSA in PBS followed by the respective mAb in 50 mM Tris–HCl, pH 8, 100 mM NaCl, 0.05% Tween. Specific binding of mAb W6/32 (11) (IgG2a specific for HLA-A,B,C) and PA2.1 (12) (IgG1 specific for HLA-A2 and A28), both reactive with native HLA-A2 complexes, was detected with goat anti-mouse Ig conjugated to horseradish peroxidase (1:5000; Amersham). After washing the nitrocellulose was incubated for 1 min with reagents for ECL immunodetection (1:1; Amersham) and exposed to X-ray film. HLA-A2 peptide concentrations were measured by micro-BCA (bicinchoninic acid) assay (Pierce, Rockford, IL), using BSA as a standard.

Covalent attachment of mAb to the cell surface

mAb BBM1 (13) (anti-β_2-microglobulin) and mAb MA2.1 (14) (anti-HLA-A2, B17) were covalently attached to cells using a heterobifunctional cross-linker, SPDP [*N*-succinimidyl 3-(2-pyridyldithio)propionate, Pierce], as described (15). SPDP (50 µl, 20 mM in ethanol) was added to mAb in PBS, pH 7.2, (1 mg/ml) for 30 min at room temperature and then dialyzed against PBS. Cells (10^6) were reduced with 50 µM dithiothreitol for 30 min, washed twice with PBS and incubated with 200 µl of mAb–SPDP at 0.5 mg/ml (1 h at room temperature), washed and subjected to FACS analysis.

Adsorption of soluble A2-peptide complexes to mAb pre-coated cells

Cells (10^6) with covalently attached mAb (using SPDP and dithiothreitol, see above) were incubated with 10 µg soluble HLA-A2 peptide complexes in 500 µl PBS for 30 min at 4°C, washed in 10 ml of cold RPMI, counted and adjusted to the appropriate cell number.

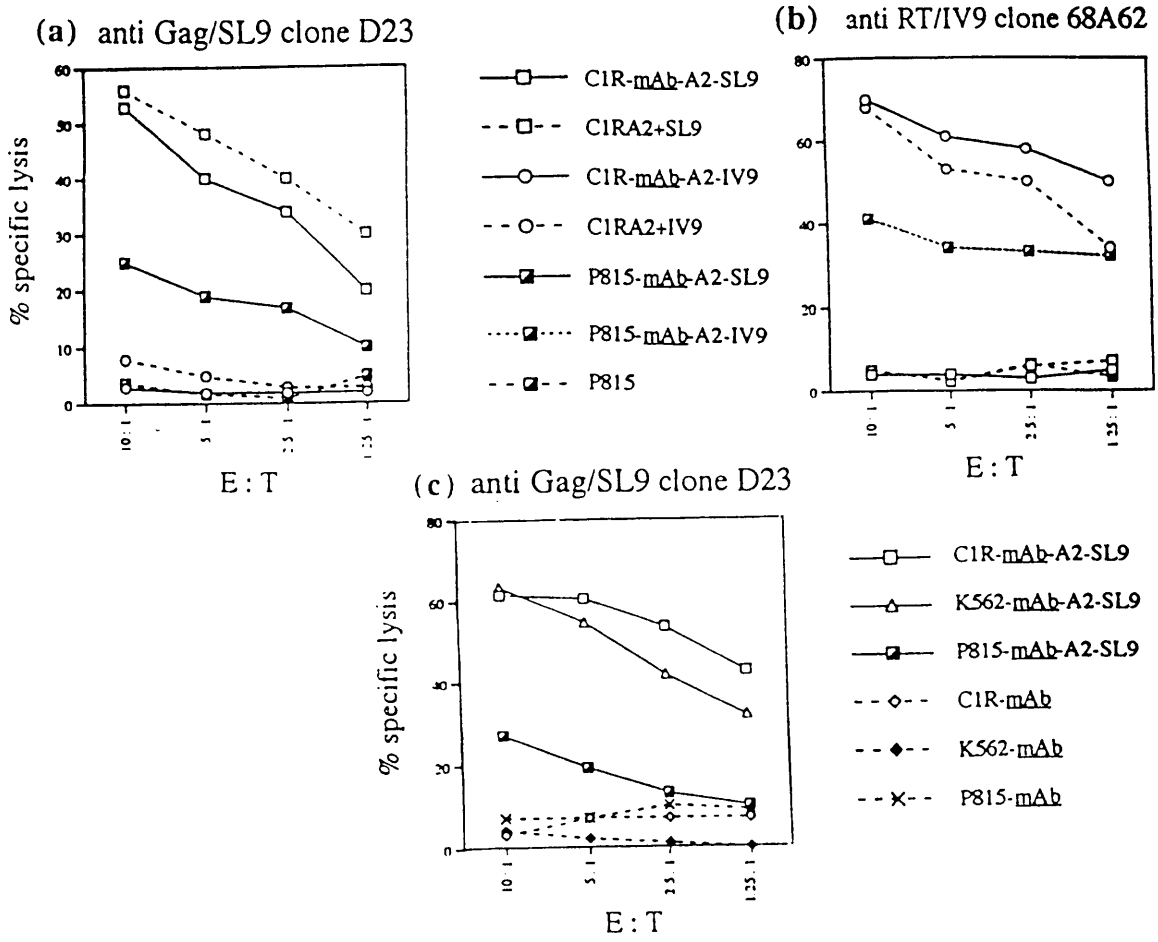


Fig. 3. Susceptibility of target cells coated with purified A2-peptide complexes to lysis by RT- and Gag-specific CTL clones. Cytotoxicity assays were carried out with the Gag-SL9-specific CTL clone D 23 (a and c) and the RT-IV9-specific CTL clone 18030 (b) on C1R cells, precoated with mAb and loaded with soluble A2-peptide complexes (C1R-mAb–A2-peptide), as well as C1R-A2-transfectants pulsed with peptide (C1R-A2 + peptide, 1 μg/ml). CTL clones were also tested against P815 cells and P815-mAb–A2-peptide as well as K562 and K562-mAb–A2-peptide. HLA-A2 peptide complexes were bound to ^{51}Cr-labeled target cells after covalent attachment of anti-HLA-A2-specific mAb MA2.1 (a and 3) or anti-β_2-microglobulin-specific mAb BBM1 to the cell surface (c). Background lysis for both T cell clones was determined by testing them against untransfected C1R and C1R cells pulsed with the respective peptide (data not shown).

FACS staining

MHC class I expression on unmodified C1R, K562 and P815 cells was determined with mAb BB7.7–FITC conjugate [BB7.7 (16), anti-HLA-A,B,C]. Surface bound mAb–SPDP conjugates were detected using FITC–goat anti-mouse F(ab)$_2$ fragments (Jackson Immunoresearch, Avondale, PA). Adsorption of HLA-A2 peptide complexes to mAb-coated cells was shown by an increase in MHC I expression using the mAb BB7.7–FITC conjugate. Immunofluorescent staining was performed according to standard protocols, using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Cytotoxicity assays

The ^{51}Cr-labeled target cells (10^7 cells) were washed twice with cold PBS, incubated with 50 μM dithiothreitol for 30 min, again washed with cold PBS and resuspended in 200 μl of SPDP–mAb at 0.5 mg/ml. After 30 min, the cells were washed twice with cold PBS, resuspended with 10 μg soluble HLA-A2 peptide in 500 μl PBS at 4°C, and after 30 min cells were washed with cold RPMI and adjusted to 2×10^5 cells/ml. Cytotoxicity assays were performed in duplicate with CTL clones added at effector-to-target ratios (E:T) of 10, 5, 2.5 and 1.25:1 to mAb–A2 peptide-coated ^{51}Cr-labeled cells and to peptide-pulsed cells (preincubated with a peptide concentration of 1 μg/ml for 30 min and washed). After 4 h at 37°C, cell supernatants were assayed for ^{51}Cr release. Total ^{51}Cr release was determined by detergent lysis and percent specific lysis was calculated as [(^{51}Cr sample release – spontaneous release)/(total release – spontaneous release)]×100. Spontaneous release was always <20% of maximal release.

Preparation of silica microspheres (beads) covalently modified with HLA–peptide complexes

We prepared silica beads (microspheres, 5 μm diameter) whose surfaces are covalently modified with the HLA-A2 peptide complexes using either the RT or *gag* nonapeptides. Beginning with commercially available (Bangs, Location??,

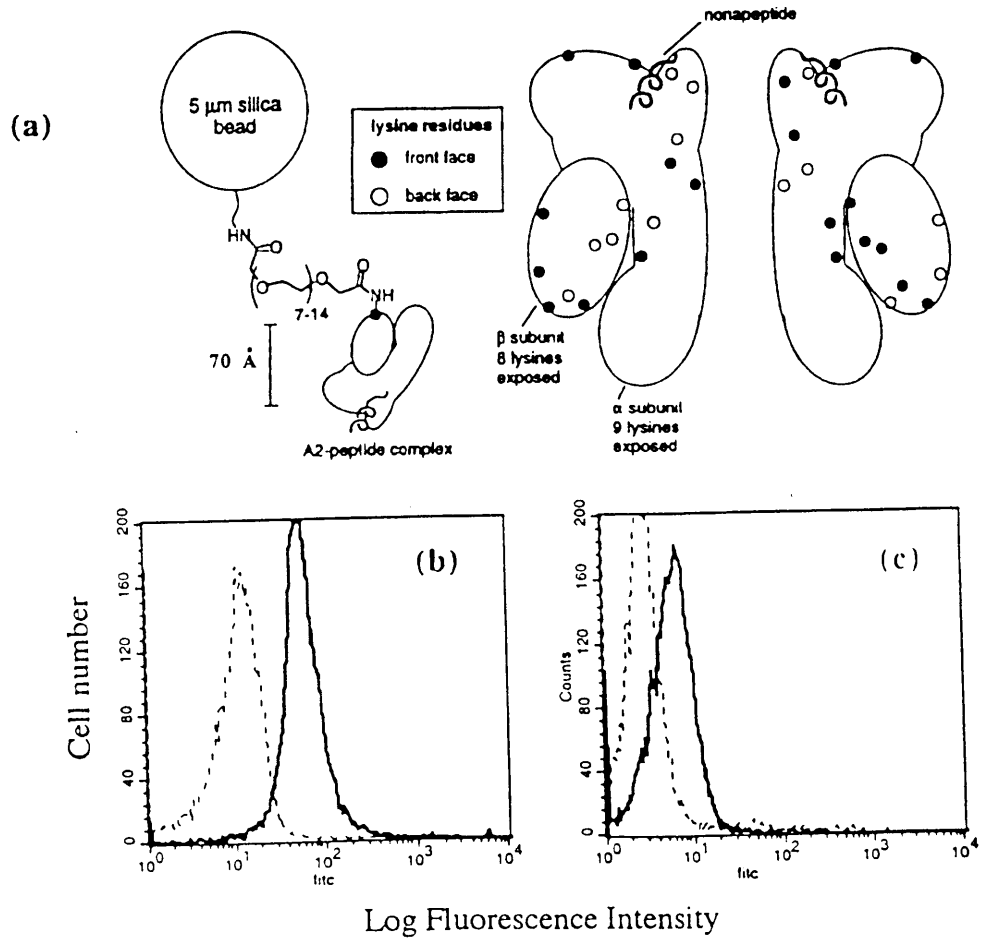
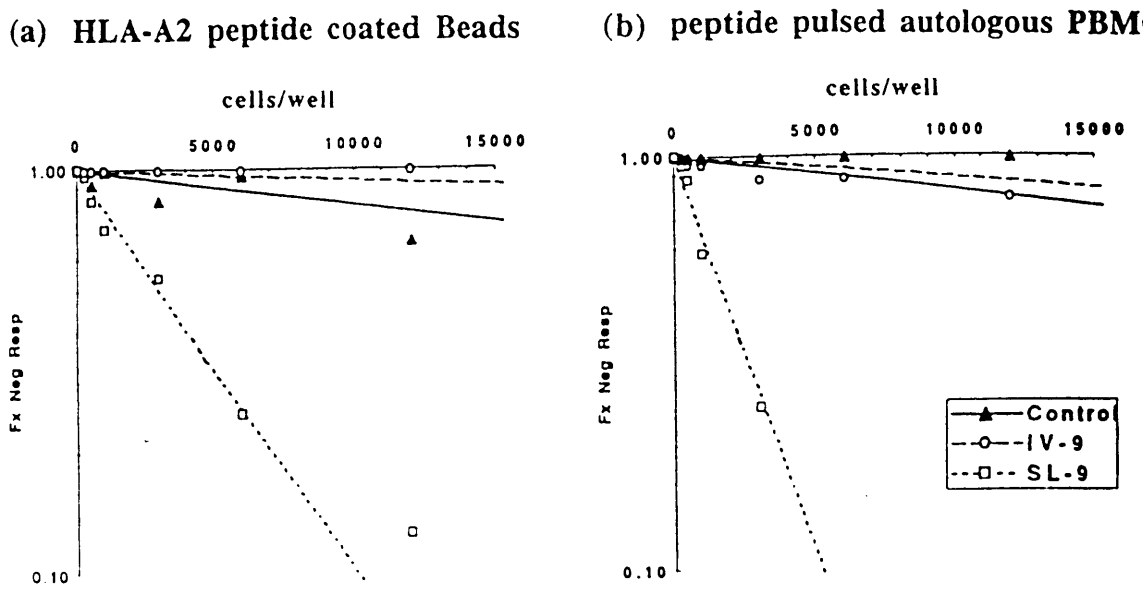


Fig. 4. Schematic representation of beads covalently modified with HLA-A2 peptide complexes and FACS analysis. HLA–peptide complexes were covalently coupled to 5 μm silica beads derivatized with active (NHS) ester groups at the terminal positions of oligoethyleneglycol (seven to 14 EG groups per chain). The distribution of potentially reactive ε-amino groups of lysine residues of the HLA-A2 peptide complex is shown on the right. The positions of the lysines were identified using the structure of the HLA-A2 peptide complex deposited in the protein data bank (23) and analysed using Quanta. HLA-A2 peptide-coated beads were prepared for FACS analysis exactly as were the cells. These beads (5×10^5) were incubated with a polyclonal rabbit serum (1:200) against heavy chain (b) and 1 μg of HLA-A2-specific mAb MA2.1 (c) and stained with FITC–goat anti-rabbit $F(ab)_2$ fragments (solid line, b) and goat anti-mouse $F(ab)_2$ fragments (solid line, c). Beads harboring only a OH-terminated oligoethyleneglycol were used as a negative control and stained as described above (dashed line in b and c). The fluorescence intensity of C1R cells with reduced MHC class I expression (HLA-B,C) stained with polyclonal rabbit serum (1:200) against heavy chain is similar to (b) (data not shown).

IN) aminopropyl silica beads (4 NH_2 groups/100 $Å^2$), we first prepared an activated surface using the bis *N*-hydroxysuccinimide derivative of a diacid of oligoethyleneglycol (Fig. 4a). The beads were first washed twice with deionized water, twice with ethanol, then resuspended in a small amount of anhydrous ethanol and added to a tube containing a solution of 10 mM bis(NHS)oligoethyleneglycoldicarboxylic acid (a mixture of diesters containing seven to 14 ethyleneglycol units) and 10 mM triethyl amine in anhydrous dimethyl formamide. The reaction tube was inverted gently (for mixing) for 24 h at room temperature. The beads were then washed three times with deionized water and modified in one of two ways:

(i) The activated beads were converted into non-interacting control beads by reacting them as above with 10 mM aminoethanol, yielding a OH-terminated oligoethyleneglycol-derivatized surface. It has been shown previously that surfaces modified covalently with short oligoethyleneglycols are often highly resistant to protein adsorption (17).

(ii) The activated beads were reacted with either the A2–RT peptide complex or the A2–*gag* peptide complex using ~50 μg of either of the A2-peptide complexes (1 nmol) dissolved in PBS (1.5 ml) with 1 mM triethylamine. Approximately 3 mg of the activated beads (10 nmol) were used per reaction mixture. Assuming a maximum coverage of 1 complex/30 nm^2, at most 0.1 nmol of protein would form a monolayer on the surface of 3 mg beads; therefore, the protein was added at 10-fold molar excess over reactive groups in the reaction. The solution was allowed to invert gently for 6 h at room temperature and the beads were washed as described above. They were finally stored at 4°C in PBS, 0.1% sodium azide and 1 μg/ml of the appropriate peptide. The residual concentration of soluble peptide after washing and resuspension of the beads was calculated to



(c) precursor CTL per 10^6 PBMC

	Control	IV-9	SL-9	subject
peptide pulsed autologous PBMC	1 (0-8)	19 (12-31)	326 (248-428)	
HLA-A2 peptide coated Beads	1 (0-8)	2 (0-8)	198 (153-256)	161 J
peptide pulsed C1R-A2 cells	4 (1-11)	2 (0-8)	110 (84-144)	
HLA-A2 peptide coated Beads	11 (6-20)	6 (3-14)	52 (37-72)	115i
peptide pulsed autologous PBMC	19 (11-31)	12 (6-22)	449 (336-591)	
HLA-A2 peptide coated Beads	20 (12-33)	6 (3-14)	229 (176-297)	15760

Fig. 5. Quantitation of Gag-SL9- and RT-IV9-specific CTL stimulated by multivalent recombinant HLA-A2 peptide complexes. Limiting dilution analysis was performed on PBMC from three HIV-1-infected subjects. PBMC (250–16000 per well, 24 wells each) were stimulated *in vitro* with HLA-A2 peptide complexes covalently attached to beads (15,000 HLA-A2 SL9 beads or HLA-A2 IV9 beads) and alternatively with peptide-pulsed (SL9 or IV9) γ-irradiated autologous PBMC or peptide-pulsed γ-irradiated C1R-A2 transfectants (10 μg/ml for 1 h). IL-2 was added at day 3 at a final concentration of 50 U/ml. The stimulated CTL were tested 12 days later against ^{51}Cr-labeled autologous B-LCL alone (▲) or autologous B-LCL incubated with 1 μg/ml SL9 (□) or IV9 (○) peptide. Regression lines were calculated by the method of maximum likelihood (18). A direct comparison of limiting dilution analyses performed with HLA-A2 peptide-coated beads (a) and peptide-pulsed autologous PBMC (b) is shown for subject 15760. Precursor frequencies of CTL determined in three subjects are summarized below. Ranges are given in parenthesis.

be 100-fold below the minimum concentration required to stimulate the corresponding T cells when added to these cells in the presence of feeder cells.

FACS staining of A2-peptide-coated beads

A2-peptide-coated beads were prepared for FACS analysis exactly as were cells. Beads were washed in cold RPMI and aliquots of 5×10^5 beads were incubated in a total volume of 100 μl for 30 min with a polyclonal rabbit serum (1:200) against HLA heavy chains or with A2-specific mAb MA2.1 (1 μg), washed, and finally stained with FITC–goat anti-rabbit $F(ab)_2$ fragments or goat anti-mouse $F(ab)_2$ fragments (Jackson) for 30 min at 4°C. Beads harboring only the OH-terminated oligoethyleneglycol chain were used as a negative control and stained as described above. FACS analysis was performed as with cells.

Limiting dilution analysis of precursor CTL (pCTL) stimulation by recombinant A2-peptide complexes on microspheres

Numbers of pCTL specific for peptides from the HIV-1 *gag* (SL9) and RT (IV9) proteins were determined by limiting dilution analysis (18). Peripheral blood mononuclear cells (PBMC) from HIV-1-positive subjects were stimulated *in vitro* using silica beads to which A2-peptide complexes were covalently attached. PBMC were cultured at 250 –16,000 cells/well in 24 replicate wells in 96-well microtiter plates (Costar, Cambridge, MA) with either 15,000 beads/well coated with A2-peptide; alternatively the PBMC were stimulated with 25,000 peptide-pulsed autologous antigen-presenting cells [γ-irradiated (3000 rad) PBMC pulsed with 10 μg/ml peptide for 1 h followed by removal of unbound peptide]. To each well was added 2.5×10^4 γ-irradiated (3000 rad) PBMC 'feeder' cells from an HIV-1-seronegative HLA-mismatched donor and

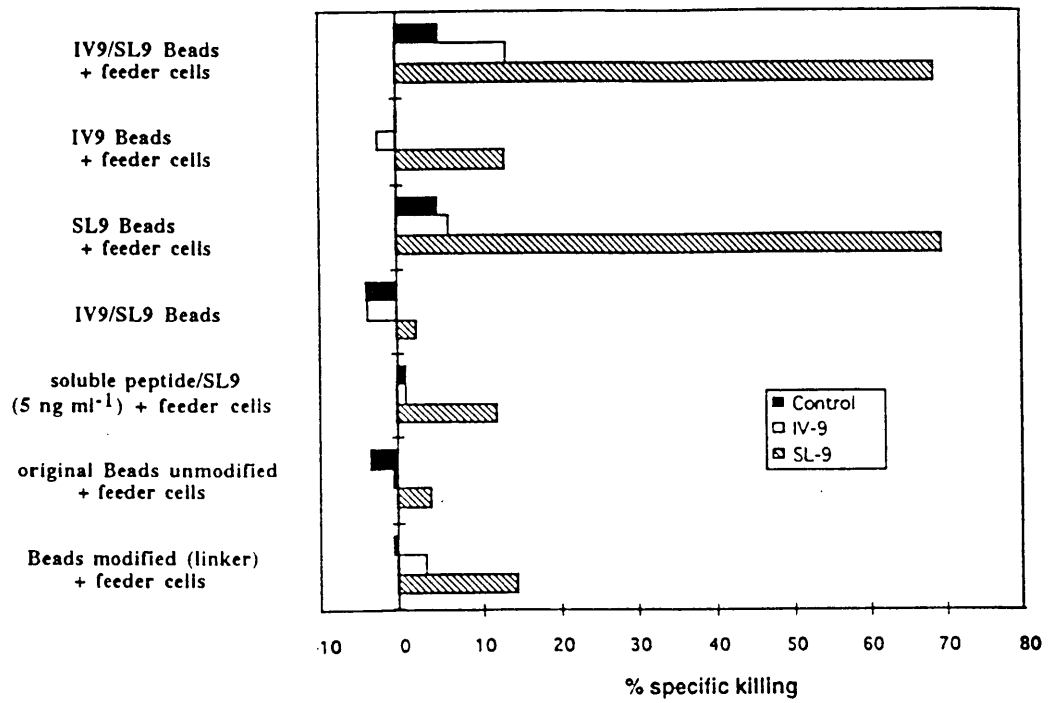


Fig. 6. T cell stimulation by multivalent recombinant HLA-A2 peptide complexes is specific. PBMC (20,000/well) from HIV-1-positive subject 15760 were placed in culture along with the 5 μm silica beads (20,000/well) modified covalently with the HLA-A2 peptide complexes in the presence or absence of HLA-mismatched γ-irradiated feeder cells (7×10^4/well). IL-2 was added at day 4 at a final concentration of 50 U/ml. After 12 days culture, lytic activity was assayed against ^{51}Cr-labeled autologous B-LCL alone (solid bars) or autologous B-LCL incubated with 1 μg/ml IV9 (open bars) or SL9 (shaded bars) peptide. Data are expressed as a percentage of specific ^{51}Cr release and values represent the average of four wells.

recombinant IL-2 (50 U/ml added on day 3). After 12 days each well was split and assayed for cytotoxicity on ^{51}Cr-labeled autologous B-LCL that were either incubated with synthetic SL9 or IV9 peptide (1 μg/ml peptide for 1 h and washed) or B-LCL without added peptide. The fraction of non-responding wells was the number of wells in which ^{51}Cr release did not exceed the mean + 3 SD of the release in the 24 control wells (no peptide) divided by the total number of assayed wells (19,20). pCTL frequency was estimated by the maximum likelihood method (18).

T cell stimulation by recombinant HLA–peptide complexes

PBMC (20,000/well) from HIV-1-positive subject 15760 were placed in 96-well microtiter plates along with the 5 μm silica beads (20,000/well) modified covalently with the A2-peptide complexes in the presence or absence of γ-irradiated HLA-mismatched feeder cells (3000 rad, 7×10^4/well). IL-2 (Hoffman La Roche) was added at day 3 at a final concentration of 50 U/ml. After 12 days lytic activity was assayed on peptide-pulsed ^{51}Cr-labeled target cells (autologous B-LCL pulsed with 1 μg/ml peptide as described above).

Results

Refolding of HLA-A2 in the presence of HIV-1-derived peptides

HLA-A2 complexes with RT-IV9 (A2-IV9) and Gag-SL9 (A2-SL9) were formed by dilution of recombinant heavy chain (1 μM) and β_2-microglobulin (2 μM) in the presence of each peptide (100 μM) (2). The complexes appeared as sharp peaks on HPLC gel filtration at elution times consistent with the mol. wt of the ternary complex (Fig. 1). HPLC fractions were pipetted onto nitrocellulose and stained with mAb specific for class I MHC proteins (MHC I). The first peak fraction, corresponding to the folded complex, was recognized by mAb W6/32 (11), which recognizes human MHC I molecules in general, and by mAb PA2.1 (12), which recognizes the native HLA-A2 molecule specifically. The recognition by mAb sensitive to conformation implies that the recombinant complexes contain native epitopes, consistent with the presence of correctly folded molecular complexes.

Adsorption of A2-peptide complexes to target cells

To determine if the A2-peptide complexes assembled *in vitro* can activate A2-restricted T cell clones, we coated a panel of A2-negative cells with the complexes and used them as targets for RT-IV9 and Gag-SL9 specific T cell clones derived from HIV-1-infected individuals (clone 68A62: anti-A2-IV9 and clone D23: anti-A2-SL9) in cytotoxic T cell assays. The modified target cells were the HLA-A negative human B cell line C1R (21), the MHC I negative human cell line K562, and P815, a mouse mastocytoma cell line. To bind the A2-peptide complexes on these cells, we first covalently attached the A2-specific mAb MA2.1 (14) to the surface of dithiothreitol-treated C1R and P815 cells using the heterobifunctional cross-linker SPDP. The antibody coated cells were subsequently incubated with a saturating concentration of soluble A2-

peptide complexes. The binding of A2 to the cell surface was demonstrated by flow cytometry, using mAb BB7.7 (16), which reacts with a combinatorial determinant of HLA-A,B,C heavy chain and β_2-microglobulin (Fig. 2).

Specific lysis of A2-peptide-coated target cells by CTL clones.

As shown in Fig. 3, the CTL clones lysed C1R cells coated with the relevant A2-peptide complexes, indicating that the HIV-1 peptides were effectively presented and recognized by T cells bearing receptors of the appropriate specificity. C1R cells coated with the reconstituted A2-peptide complexes were lysed essentially as well as were C1R cells transfected with A2 and pulsed with peptides (1 μg/ml). Both T cell clones also lysed the murine P815 cells to which the appropriate A2-peptide complex had been adsorbed, although to a lesser extent than human target cells modified with the same complexes. As expected, the T cells failed to lyse unmodified P815 cells and C1R-A2 transfectants pulsed with an irrelevant peptide.

Recognition of A2-peptide complexes by TCR was evidently not impaired by the binding of these complexes to mAb MA2.1, even though this antibody binds to the polymorphic region of HLA-A2 close to the peptide binding groove (22). HLA-A2 peptide molecules held by mAb MA2.1 at the cell surface might not be accessible for optimal engagement with the TCR. Accordingly mAb BBM1, an anti-β_2-microglobulin antibody (13), was covalently linked to C1R, K562 and P815 cells, and used to bind refolded complexes. A2-peptide complexes on each of these target cells were just as susceptible to lysis (Fig. 3c) as target cells that made use of the MA2.1 mAb.

Covalent coupling of A2-peptide complexes to microspheres.

A crucial question is whether the recombinant molecules can stimulate pCTL present in blood samples of HIV-1-seropositive subjects, i.e. in naturally primed individuals. To examine this possibility, we covalently coupled the A2-peptide complexes to silica beads derivatized with active ester groups at the free ends of oligoethyleneglycol chains that extended ~30–50 Å from the bead surface.

Figure 4 shows the coupling procedure and the distribution of potentially reactive ε-amino groups of lysine residues of the A2-peptide complex. The particular amino groups that react with activated ester groups on the surface of the bead determines the orientation of the MHC complex. Positive staining with a rabbit serum against human heavy chain (Fig. 4b) showed attachment of HLA complexes on the surface. Staining with the anti-HLA-A2 mAb MA2.1 (Fig. 4c) indicated that a significant fraction of the HLA–peptide complexes maintained a native configuration and were exposed to the solvent, and are thus presumably accessible to TCR on T cells.

Recombinant HLA–peptide complexes are immunogenic

To assess the immunogenic effectiveness of the A2-peptide complexes on beads, we used them to stimulate PBMC of three seropositive subjects (161J, 115I and 15760) measuring the frequency of peptide specific pCTL after 12 days by limiting dilution analyses (Fig. 5). The results show that the recombinant A2-peptide complexes were effective and as specific as peptide-pulsed autologous antigen-presenting cells in inducing functional responses that normally depend upon cell–cell interactions, i.e. they triggered antigen-dependent responses of primed pCTL. A direct comparison of limiting dilution analyses performed with A2-peptide complexes displayed on beads (Fig. 5a) and autologous peptide-pulsed PBMC (Fig. 5b) revealed that the responses were of comparable magnitude.

Expansion of pCTL is peptide specific

To rule out non-specific mitogenic effects of the bead surface, PBMC were stimulated with a mixture of A2-IV9- and A2-SL9-coated beads and tested for A2-IV9- and A2-SL9-specific responses (Fig. 6). Only SL9 specific responses were detectable. Culture of the same PBMC with beads bearing only A2-IV9 induced no response; only A2-SL9 beads stimulated a specific SL9 response. The apparent absence of IV9-specific pCTL in the responder population was confirmed using the same batch of beads as in the limiting dilution analysis (Fig. 5). The recovery of SL9-specific CTL suggests a true expansion of peptide-specific pCTL, confirming other data (6 and unpublished), that the dominant Gag-specific HLA-A2 restricted response is directed against the SL9 peptide in these subjects.

Titration of peptide revealed that a peptide concentration over 5 ng/ml SL9 was required to stimulate pCTL expansion from PBMC in the presence of feeder cells (Fig. 6 and data not shown). By contrast much less peptide is needed when it is presented by HLA-A2 on the microspheres. Thus with maximal packing a 5 μm microsphere could accommodate about 2×10^6 HLA-A2 peptide complexes in a monolayer. This number corresponds to 0.35 ng/ml peptide if all the A2 on the beads were loaded with the peptide originally and all the peptide adduct then dissociated (given 2×10^4 beads, 0.2 ml/well and a maximum of 2×10^6 A2-peptide complexes per bead). By comparing the fluorescence intensity of HLA-A2 peptide-coated microspheres with C1R cells stained with polyclonal antibodies to MHC I heavy chain it appears, however, that the actual amount of surface-bound A2-peptide complexes on the microspheres is ~50 times lower than the calculated maximum, i.e. the fluorescence intensity is similar to C1R cells with ~3×10^4 HLA B35 and Cw4 per cell (21). Thus it appears that the T cell response was elicited by far less peptide associated with HLA-A2 on the microspheres than by peptide added in solution. Non-specific mitogenic effects of the beads modified with only OH-terminated oligoethylenglycol groups were not observed (Fig. 6).

Discussion

This is the first demonstration of biological activity of *E. coli*-derived HLA-A2 peptide complexes. In contrast to native HLA-A2, the recombinant protein used here is not glycosylated at Asn-86 and its β_2-microglobulin subunit has an additional N-terminal methionine residue not cleaved following synthesis (3). These differences have previously been shown not to significantly affect the crystal structure of A2 in the form of A2-peptide complexes (3,4).

We show here that these differences also do not prevent recombinant A2-peptide complexes from eliciting specific cytolytic responses of $CD8^+$ CTL clones from HIV-1-infected

individuals. Thus, the antigen-specific receptor (TCR) of these clones can recognize recombinant *E. coli*-produced A2-proteins refolded in association with appropriate peptides. This study shows furthermore that microspheres coated with recombinant A2-peptide complexes can specifically stimulate T cells in PBMC from HIV-1-infected individuals. The stimulated cells, presumably primed by natural infection, develop into an expanded population of CTL that specifically responds to the A2-peptide complex that triggered this development. Since the A2-peptide-coated microspheres lack B7 and other co-stimulatory molecules, their immunogenicity has to be considered in the light of the large body of evidence that, in addition to TCR ligation, the activation of quiescent T cells depends upon secondary signals that trigger the expression of IL-2 receptors and the production of lymphokines (35). In the present system which makes use of PBMC from HIV-1-infected individuals, it is possible that the responding T cells already express IL-2 receptors and that the exogenous recombinant IL-2 substitutes for endogenous production of lymphokines. In addition, the HLA-mismatched feeder cells might provide a co-stimulatory function and, by eliciting an alloreaction, induce cells in the responding PBMC population to produce endogenous lymphokines. In any event, whatever the detailed mechanism may be, it seems clear that the beads coated covalently with A2-peptide complexes are active immunogenically with PBMC of HIV-1-infected individuals.

Several different systems have been developed for stimulating T cells with purified class I MHC molecules (24–26) including allogeneic MHC proteins (27, 28) and various methods have been used to assay T cell activation, e.g. release of BLT-esterase (29), production of IL-2 in T cell hybridomas and release of serotonin in basophils transfected with a TCR-ζ fusion (30). The MHC–peptide complexes used in these studies included purified native MHC I molecules, cleaved from the cellular surface (28,29), and 'empty' MHC I molecules produced in insect cells and loaded with peptides of interest (31,32). Soluble H-2K^d–peptide fusions were also produced in which heavy chain, β_2-microglobulin and peptide have been fused into a single chain protein and cells expressing these constructs were shown to be highly immunogenic (33). Genetically engineered soluble H-2D^d molecules coated on plastic were also found to present HIV-1 envelope peptide to an antigen-specific CTL clone, inducing it to produce IFN-γ (34).

Since the overexpression of the class I subunits in the *E. coli* system and the subsequent refolding provides an abundant source of homogenous HLA–peptide complexes, the recombinant soluble complexes described here offer new tools for exploring diverse problems and issues, such as signal transduction events elicited by agonist and antagonist peptide ligands (36,37), the identification and isolation of antigen-specific T lymphocytes in complex cell mixtures, and the development of vaccines aimed at stimulating particular antigen-specific T cells.

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Abbreviations

B-LCL	Epstein–Barr virus-transformed B lymphoblastoid cell line
CTL	cytotoxic T lymphocyte
PBMC	peripheral blood mononuclear cell
SPDP	*N*-succinimidyl 3-(2-pyridyldithio)propionate

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