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Presentation of peptides by cultured monocytes or activated T cells allows specific priming of human cytotoxic T lymphocytes *in vitro*

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Abstract

The conditions favouring effective specific cytotoxic T lymphocyte (CTL) priming have been exploited to set up a simple and reproducible method to induce a primary CTL response *in vitro*. We report that cultured monocytes, as well as activated T cells, pulsed with exogenous HLA-A2 binding immunogenic peptides, can induce primary peptide-specific CTL responses *in vitro* in a T_h-independent manner. Primary viral peptide-induced CTL were HLA-A2 restricted, and recognized both peptide-pulsed target cells and targets infected with recombinant vaccinia virus expressing viral endogenous antigens. In addition, both cultured monocytes and activated T cells primed peptide-specific $CD8^+$ T cells depleted from the $CD45RO^+$ memory cell fraction. The efficiency of CTL priming by monocytes was dependent upon the strong up-regulation of class I, adhesion and co-stimulatory molecules occurring spontaneously upon *in vitro* culture. The inability of unseparated peripheral blood mononuclear cells to mount a peptide-specific CTL response could be reverted by direct co-stimulation of responding $CD8^+$ T cells by soluble B7.1 or a stimulatory anti-CD28 antibody, that allowed a specific response to take place. Although co-stimulation via the B7–CD28 interaction appeared sufficient to trigger CTL responses, it was not essential for CTL priming, since neither anti-B7.1 mAb nor soluble CTLA-4 inhibited induction of primary CTL response. This new method for induction of specific $CD8^+$ T cell response *in vitro* may be exploited in adoptive immunotherapy in cancer or in HIV-infected patients.

Introduction

Recent advances in understanding the interaction between naturally processed peptides and MHC molecules allow the identification of the likely immunogenic peptides in a protein sequence on the basis of the presence of specific anchors (1). Peptides binding to MHC class I molecules, which are in general 9–10 residues long, usually contain within their sequence two anchor residues interacting with corresponding binding pockets in the MHC molecule (2–4). The binding can be measured *in vitro* by demonstrating the capacity of the synthetic peptide to assist the folding of a particular class I molecule (1,5–7). Definition of specific MHC motifs allows us to predict those peptides derived from viral or tumor antigens, potentially immunogenic for CTL.

Synthetic peptides have been used not only to identify the epitopes of a protein recognized by specific cytotoxic T lymphocytes (CTL), but also as vaccines to induce protective CTL in mouse (8–20). Moreover, peptides conjugated to either a helper epitope or lipid have been used to induce protective CTL responses (21,22). Alternatively, with regard to adoptive immunotherapy, i.e. in cancer patients or in severe immuno-

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compromised HIV carriers, one of the two following approaches may be considered: either the *in vivo* transfer of *in vitro*-primed, well characterized peptide-specific CTL, or peptide vaccination using well characterized peptides, that could be delivered on professional APC.

However, it would be desirable to set up a simple and reproducible method to induce primary antigen-specific CTL response *in vitro*. Indeed, such an approach may be essential to identify not only immunogenic peptides, but also the most appropriate professional APC capable of triggering CTL precursors. The prerequisite for such APC is to present a high density of peptide–MHC molecule complexes (signal 1) and to simultaneously deliver the co-stimulatory signals (signal 2) required for T cell activation, which has been defined as B7.1 or B7.2 interacting with CD28/CTLA-4 on T cells (23–26).

The strategies to induce primary CTL responses *in vitro* and the conditions that determine effective CTL priming as well as unfavourable conditions are reported herein.

Methods

Identification of HLA-A2.1 binding peptides

Peptides of HIVgp120 and hepatitis B envelope antigen (HBenvAg; subtype ADW2) carrying HLA-A2.1 motif (1) were synthesized by the solid-phase method on an automated multiple peptide synthesizer (AMS 422; Abimed, Langenfeld, Germany) using Fmoc chemistry. The purity of peptides was determined by reverse-phase HPLC. Peptides were diluted to a concentration of 2 mg/ml and stored at −20°C. The peptides were screened for their ability to stabilize HLA-A2.1 molecules on the surface of transporter defective mutant T2 cells (27,28). T2 cells were cultured in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% FCS (HyClone), 2 mM L-glutamine, 1% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml fungizone (Flow, Irvine, UK) (complete medium). T2 cells were washed twice and resuspended in either serum-free medium in the presence of 10 μg/ml human β_2-microglobulin (β_2m) (Sigma, St Louis, MO) or complete medium and incubated in 96-well flat-bottom plates in the presence or absence of different concentrations of peptide overnight at 37°C, 5% CO_2. Cells were washed and stained with an anti-HLA-A2.1 mAb (IgG2a, BB7.2; ATCC, Rockville, MD) for 30 min at 4°C followed by FITC–F(ab)'$_2$ goat anti-mouse Ig (GAM). Cells were washed twice and analysed using a FACScan (Becton Dickinson, Mountain View, CA).

Peptides showing high levels of stabilization of class I on T2 cells were studied for their ability to bind HLA-A2.1 by a direct binding assay, that is based on serologic detection of the conformational change of HLA class I α-chains induced by binding to specific peptides in the presence of β_2m, as described (29). Briefly, Epstein–Barr virus transformed-B (EBV-B) cells were lysed in Tris–HCl, pH 7.5, containing 0.5% NP-40 and protease inhibitors, and the lysates were denaturated by alkaline dissociation. The unfolded α-chain was separated from β_2m and peptides by gel filtration. The fractions containing the first major protein peak were pooled and incubated with test peptides and excess β_2m for 16 h at 25°C. The increase in folded α-chain activity induced by peptide binding was quantitated by a specific radioimmunoassay involving a rabbit anti-HLA class I serum and ^{125}I-labelled purified HLA-A2.1 molecules.

Purification of CD8$^+$ T cells

Human peripheral blood mononuclear cells (PBMC) were isolated from HLA-A2$^+$ healthy donors on Lymphoprep cushions (LSM; Organon Teknika, Durham, NC). Donors were negative for HBV and HIV serological markers. PBMC were then washed in serum-free medium and allowed to adhere to 24-well plates (Falcon) in RPMI–1% human AB serum. After 90 min at 37°C the non-adherent cells were removed and used for cell purification as described (30). Briefly, CD8$^+$ T cells were isolated by immunomagnetic separation with anti-CD8 mAb attached to Dynabeads (Dynal, Oslo, Norway). Positively selected cells were detached from magnetic beads by incubation with Detachabead (Dynal) according to the manufacturer's instructions. After the treatment, purified CD8$^+$ and CD8$^-$ T cells were >98% CD8$^+$ and <1% CD8$^-$ respectively. In some experiments, a CD45RA$^+$CD45RO$^-$ population was isolated from purified CD8$^+$ T cells by depletion of CD45RO$^+$ cells with anti-CD45RO$^+$ mAb attached to magnetic beads.

In vitro priming with peptide

PBMC (4–5×10^6) were incubated in RPMI–1% human AB serum in culture 24-well culture plates (Falcon) for 90 min at 37°C, 5% CO_2; the non-adherent cells were removed, and the adherent fraction was pulsed with different concentrations of peptide in serum-free medium for 4 h and used as APC. Adherent cells were then incubated with 1.5×10^6 responding purified CD8$^+$ T cells in the presence or absence of different concentrations of an anti-B7.1 mAb (31) or a fusion protein between human CTLA-4 and human IgG1 (huCTLA-4–huIgG1) (32). In some experiments we used as APC either an irradiated (3000 rad) autologous T cell clone, or irradiated (3000 rad) autologous PBMC or irradiated (13,000 rad) autologous EBV-B cells. In some experiments, either a fusion protein between human B7.1 and IgM (huB7.1–IgM) or an anti-CD28 mAb were added to cultures in which irradiated PBMC were used as APC.

In all cases after 2 days of culture, 50 U/ml rIL-2 (Proleukin; Eurocetus, Emeryville, CA) was added and after a further 5 days, CD8$^+$ T cells were re-stimulated with irradiated autologous phytohaemagluttinin (PHA)–T cell blasts pulsed with 10 μg/ml peptide. After a second administration of IL-2, viable CD8$^+$ T cells from each culture were tested for specific cytotoxicity on day 7 from the secondary stimulation.

Generation of CTL clones

T cell clones were isolated and maintained as previously described (33). Briefly, primary peptide-specific CTL were cloned by limiting dilution at 0.3 cells/well onto 96-well U-bottom plates in the presence of 0.5 μg/ml PHA-P (Wellcome Beckenham, UK), 50 U/ml rIL-2 and irradiated allogeneic feeder cells. After 2–3 weeks, cell growth was detected using an inverted microscope and growing cultures were tested for their capacity to mount a specific cytotoxic response to peptide-pulsed ^{51}Cr-labelled target cells. Peptide-specific CTL clones were then expanded in rIl-2-containing medium

and maintained in culture with 2 week cycles of re-stimulation with PHA plus allogeneic APC.

CTL assay

Cytotoxicity of $CD8^+$ T cells, primed with peptide-pulsed APC, was tested in a 6 h ^{51}Cr-release assay. T cells were used as effector cells at an E:T ratio ranging from 50:1 to 20:1. Effector cells were incubated in triplicate in U-bottom microtitre wells (Falcon), containing 5×10^3 ^{51}Cr-labelled homozygous HLA-$A2.1^+$ EBV-B cells. Target cells were labelled with 100 μCi of $Na^{51}Cr$ (Amersham, Buckinghamshire, UK) for 2 h and then pulsed with 10 μg/ml peptide for 1 h at 4°C or left unpulsed. In some experiments anti-HLA-A2 mAb or an anti-HLA-B27 mAb were added at the initiation of the assay.

In some experiments, EBV-B cells infected with recombinant vaccinia virus (rVV) expressing gp160 (VPE16) or with VSC8 as control were used as target cells. The rVV were kindly donated by Andrea De Maria (University of Genova, Italy). EBV-B cells (1.5×10^6) were incubated with 1 p.f.u./1×10^6 cells of the different preparations of rVV at 4°C for 10 min, washed and resuspended in 5 ml complete medium and incubated at 37°C, 5% CO_2, for 12 h before being used as target cells.

FACS analysis

The following purified specific mAb were used: anti-CD3 (IgG1, TR66), anti-CD4 (IgG1, 6D10), anti-HLA-A2.1 (IgG2a, BB7.2), anti-HLA-DR (IgG2a, L243), anti-HLA-DQ (IgG2a, SPVL3), anti-HLA-DP (IgG1, B7.21) and anti-B7.1 (IgG2a, B7.24); anti-CD28 (IgM, CK248) was kindly donated by Sandro Poggi (IST National Institute for Cancer Research, Genova, Italy) (34); anti-HLA-B27 (IgG1, MEI) was kindly donated by Rossella Sorrentino (Department of Experimental Medicine, University of L'Aquila, Italy); anti-CD19 (IgG1, J4.119), anti-CD13 (IgG1, SJ.1D1), anti-CD14 (IgG2a, RMO52), anti-CD45RA (IgG1, ALB11), anti-CD45RO (IgG2A, UCHL1), anti-CD1a (IgG1, BL6), anti-CD1b (IgG2a, 4.A7.6) and anti-CD1c (IgG1, L161) were purchased from Immunotech (Marseille, France); anti-CD11a (LFA-1, IgG, TEC-NK2) was purchased from TechnoGenetics (Milan, Italy); anti-HLA-ABC (IgG2a, W6.32) and anti-CD54 (ICAM-1, IgG1, 15.2) were purchased from Sera-Lab (Crawley Down, UK); and FITC–$F(ab)'_2$ GAM was purchased from TechnoGenetics.

Cells were labelled with mAb on ice for 30 min, washed four times, incubated for 30 min on ice with FITC–$F(ab)'_2$ GAM Ig, washed again and immediately analysed on FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW air-cooled 488 nm argon-ion laser. The cytometer was calibrated using three different types of CaliBRITE (Becton Dickinson) beads of ~6.6 μm in diameter. Gating was performed using a combination of forward and orthogonal light scatter (linear amplification). Propidium iodide was used to exclude dead cells. Data of 10,000 events was acquired and stored in list mode using the FACScan research software. The Lysys II 1.1 program was used for analysis of data.

Soluble human B7.1–IgM molecule

The generation and characterization of the soluble huB7.1–IgM will be described in detail elsewhere (P. Dellabona and G. Casorati, manuscript in preparation). Briefly, total cytoplasmic RNA extracted from an EBV-transformed cell line was reverse transcribed into cDNA, and the sequence encoding for the extracellular domains of the human B7.1 molecule was amplified by PCR using the following oligonucleotide: 5′-CCTGAGCTCCTGAAGCCATGGGCCACACACGG-3′ and 5′-TCTGACTTACCATCAGGAAAATGCTCTTGCTT-3′ (underlined is a consensus splicing donor site). The PCR was run with the following conditions: 20 s at 94°C, 30 s at 60°C and 30 s at 72°C for 20 cycles. The amplified product was cloned blunt ended into the pBluescript vector, sequenced to rule out PCR errors and subcloned into the *SstI–SalI* digested pCD4-hCu expression vector. The huB7.1–IgM was transfected by protoplast fusion into the mouse plasmocytoma cell line J558L as described (35) and the clones secreting the highest amount of proteins (3–5 μg/ml) were selected for expansion.

The approximate size of the B7.1–IgM was determined on an SDS–PAGE gel. Briefly, the B7.1–IgM protein was precipitated from culture supernatant using the anti-B7.1 mAb B7.24 (31) and Protein G (Pharmacia, Uppsala, Sweden), run on an SDS–PAGE both in reducing and non-reducing conditions, and blotted on a nitrocellulose filter. The filter was decorated with alkaline phosphatase-labelled GAM Ig (Southern Biotechnology, Birmingham, AL) followed by ECL (Amersham). Under reducing conditions, the B7.1–IgM migrated as a single molecular species of ~130 kDa, while under non-reducing condition it migrated with an apparent M_r of 700, consistent with the pentameric structure determined by the IgM Fc portion. To prepare the B7.1–IgM containing supernatant, the J558L clone secreting it was grown in standard RPMI medium (Hyclone) containing 7% FCS in a Miniperm fermenter (Haereus, Germany). We determined as 1:10 the optimal dilution of B7.1–IgM containing supernatant giving the highest proliferative response on purified human $CD4^+$ CD45RO T cells, stimulated with a sub-optimal dose of anti-CD3 mAb. The proliferative response was measured by [^{3}H]thymidine incorporation in a standard 72 h assay, and for each B7.1–IgM dilution was 1:10 = 47,000 c.p.m., 1:20 = 40,000 c.p.m., 1:40 = 38,000 c.p.m., 1:80 = 32,000 c.p.m. and 1:100 = 25,000 c.p.m. A spare culture supernatant used as a control gave the same background incorporation of 2000 c.p.m. at all dilutions tested.

Results

Identification of HLA-A2 binding peptides

T cell determinants to be used for the induction of CTL responses *in vitro* were identified using the known HLA-A2 binding motif (5,6). Forty-eight nonamers carrying the A2 motif were screened for their capacity to stabilize HLA-A2 molecules on the surface of the transporter defective mutant T2 cells essentially as described (36). A peptide of HIVgp120 (121–129; KLTPLCVSL) and a peptide of HBenvAg (335–343; WLSLLVPFV), that showed high levels of binding, were selected for functional experiments, according to our preliminary experiments demonstrating that CTL specific for those peptides efficiently cross-reacted on endogenous antigen presenting target cells. As shown in Fig. 1, these peptides can stabilize cell surface A2 molecules on T2 cells and can

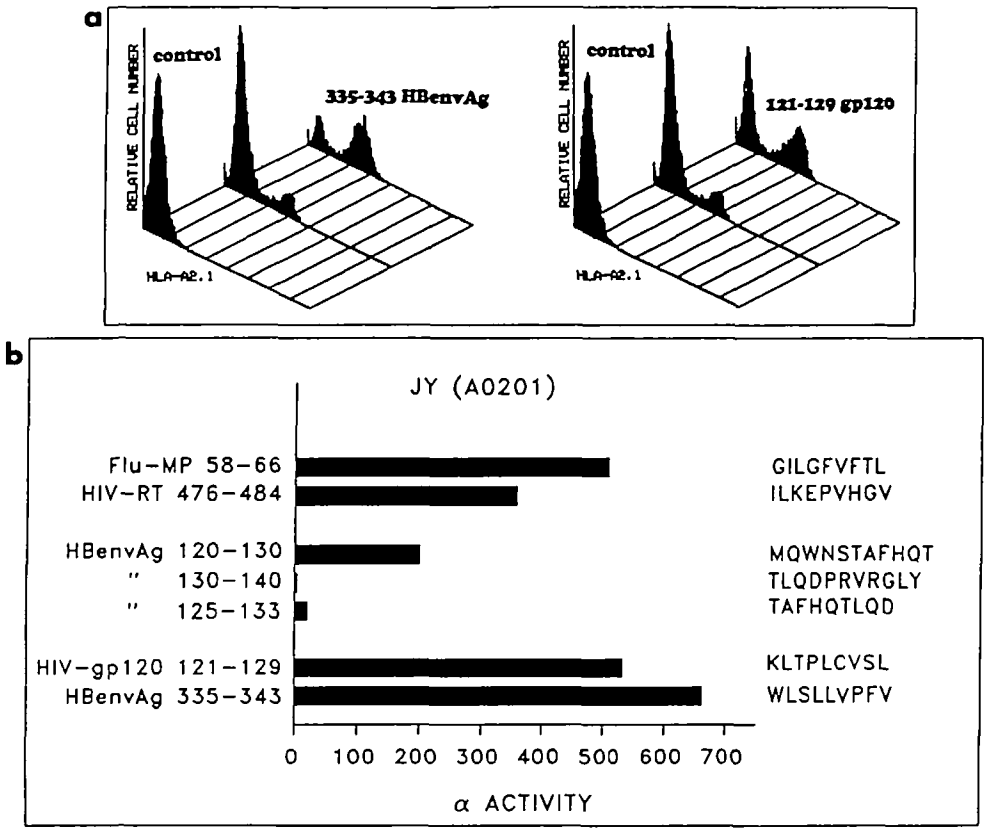


Fig. 1. Identification of HLA-A2 binding peptides. (a) Stabilization of surface HLA-A2 molecules in T2 cells by HIVgp120(121–129) and by HBenvAg(335–343) peptides as detected by surface staining with anti-HLA-A2 antibody. (b) Effect of peptide and β_2m on refolding of HLA-A2 α-chains isolated from the HLA homozygous cell line, JY (A0201, B7, X), as detected by a specific radioimmunoassay. One unit of activity detected by the radioimmunoassay is defined as the amount of test sample that induces a 50% inhibition of the specific binding involved in the assay system. The results are presented as α activity/ml of test sample. Bars indicate α activity above the control (α-chain – β_2m only). Flu-MP58–66 and HIV-RT476–484 represent positive controls, while HBV120–130, 130–140 and 125–133 represent negative controls.

assist the refolding of isolated A2 α chains in the presence of β_2m.

Characterization of APC for in vitro priming

Initial attempts to induce primary CTL responses by directly adding the HLA-A2 binding peptides to PBMC of HLA $A2^+$ donors were unsuccessful (data not shown). We reasoned that the failure to induce a specific response might be due either to insufficient number of sites available on APC or to inappropriate presentation, i.e. presentation on non-professional APC (26) or on the responding T cells themselves (37). We therefore asked whether selective display of the peptide on professional APC may favour stimulatory interactions and lead to CTL priming.

We tested different sources of APC: PBMC, EBV-B cells, the transporter mutant T2 that can be efficiently loaded with peptide, autologous T cell clones and adherent cells. Consistent stimulation of a peptide-specific response was obtained only when either adherent cells or T cell clones were used as APC. EBV-transformed autologous B cells and to a higher extent, T2 cells induced a very strong non-peptide-specific response that obscured a specific response.

Table 1. Phenotypic analysis of adherent cells at different times of *in vitro* culture

	Time of *in vitro* culture (h)[a]			
	0	2	6	24
CD13	20[b]	67	143	346
CD14	126	193	322	282
CD4	9	14	24	9
HLA-ABC	113	508	550	392
HLA-A2	52	181	194	154
HLA-DR	56	545	663	151
HLA-DP	20	50	156	142
HLA-DQ	9	20	45	33
CD1a	7	11	46	38
B7.1	0	7	27	38
CD54	19	42	96	236
CD11a	165	217	344	264
CD3	<1%[c]			
CD19	<1%			

[a]Adherent cells from HLA-$A2^+$ donor were analysed for surface markers at different times of *in vitro* culture.

[b]Mean fluorescence intensity. The background was subtracted.

[c]Values are expressed as percentage because they represent contaminating cells.

The population of adherent cells was characterized by surface staining (Table 1). This population consisted mainly of $CD13^+CD14^+$ monocytes. It should be noted that upon *in vitro* culture, monocytes underwent dramatic changes in surface expression of various markers within the first few hours of culture (Table 1). The surface expression of HLA-A2 molecules increased at least three times. A similar increase was evident for total class I as well as class II molecules. B7.1 was undetectable on freshly isolated monocytes, but was rapidly up-regulated after *in vitro* culture. The expression of ICAM-1 increased five times over 24 h while LFA-1 was unchanged. These results show that the simple *in vitro* culture of fresh monocytes is sufficient to up-regulate MHC, adhesion and co-stimulatory molecules, thus explaining their efficient

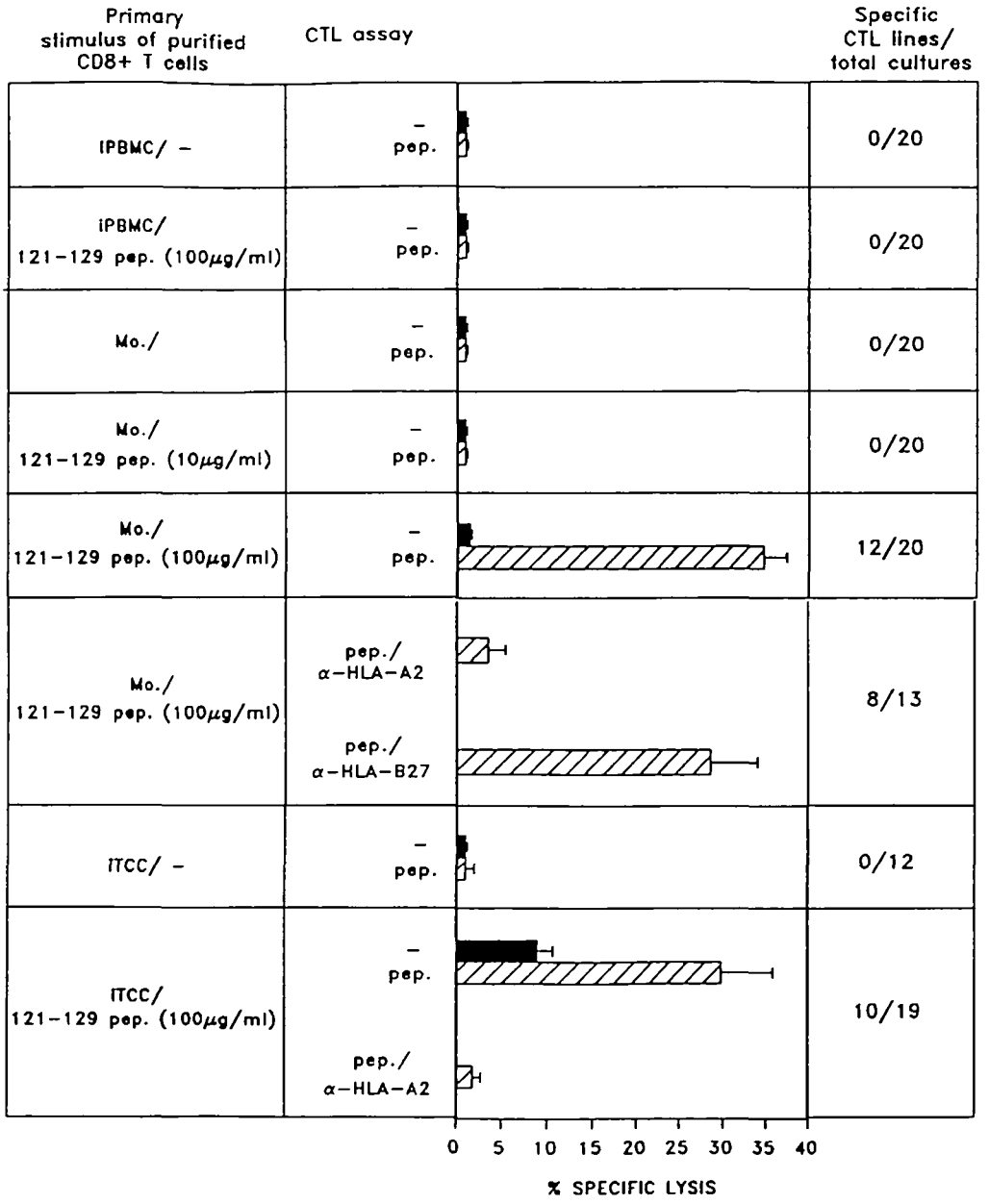


Fig. 2. Induction of primary *in vitro* peptide-specific CTL responses by peptide-pulsed activated monocytes. Representative experiments are shown, in which purified $CD8^+$ T cells from an HLA-$A2^+$ donor were stimulated in replicate cultures with either irradiated (i) PBMC or adherent monocytes or autologous activated (i) T cell clones pulsed or not with different concentrations of HIV gp120 (121–129 peptide). After 7 days, cultures were re-stimulated with peptide-pulsed (i) autologous T cell blasts and after a further 7 days the cytotoxic activity of the individual cultures was measured against ^{51}Cr-labelled target cells pulsed (hatched bars) or not (solid bars) with 10 μg/ml peptide, in the presence or absence of anti-HLA-A2 or anti-HLA-B27 mAb at an E:T ratio 25:1. On the far right of the figure the number of specific CTL lines above the total cultures is reported. Similar results were obtained using HBenvAg(335–343). Results represent the percentage mean of specific lysis expressed by the specific CTL lines.

presentation of peptide and T cell priming. As previously reported, human activated T cell clones express high levels of the same molecules and display 'professional' antigen presenting capacity (38,39).

Conditions for the generation of primary CTL responses using peptide-pulsed APC

A general protocol for the generation of specific CTL responses *in vitro* was developed. PBMC ($4–5\times10^6$) were incubated in 24-well culture plates for 90 min; the non-adherent cells were removed and the adherent fraction was extensively washed to deplete contaminating T and B cells and pulsed with different concentrations of peptide. Due to the low frequency of peptide-specific T cells in an unprimed donor, we set up several replicate 2 ml cultures containing adherent cells and 1.5×10^6 responding purified (>98%) $CD8^+$ T cells. Alternatively, responding $CD8^+$ T cells were cultured with the aforementioned APC (1×10^6), that had been previously pulsed with different concentrations of peptide. Anyway, after 2 days, IL-2 (50 U/ml) was added and the cultures were incubated for an additional 5 days before secondary stimulation with 10^6 irradiated autologous T cell blasts that had been pulsed with 10 μg/ml peptide. After a further 7 days, the cultures were individually tested for specific cytotoxicity against homozygous $HLA\text{-}A2^+$ EBV-B cells pulsed or not with peptide.

As evident from a typical experiment reported in Fig. 2, monocytes pulsed with 100 μg/ml peptide induce a peptide-specific cytotoxic response detectable in 12 out of 20 replicate cultures. When autologous activated T cell clones were used as a source of peptide-pulsed APC a similar cytotoxic response was obtained (Fig. 2). In contrast, no specific response was obtained when either peptide-pulsed PBMC (Fig. 2), T2 cells or EBV-B cells (not shown) were used as APC. In all cases the responding T cells were peptide specific and HLA-A2 restricted, as shown by the inhibition of killing by an anti-HLA-A2 antibody. Similar results were obtained in 10 out of 14 healthy donors tested. Figure 3 clearly shows that CTL activity was specific for the peptide used in the priming, since target cells incubated with an HLA-A2.1 binding control peptide were not lysed.

Further evidence for selective priming of CTL by activated monocytes was obtained from cytofluorimetric analysis. When unfractionated $HLA\text{-}A2^+$ PBMC were cultured with peptide-pulsed monocytes a selective expansion of $CD8^+$ T cells was detected on day 7 (not shown).

Interestingly, we noticed that the optimal timing for peptide loading on monocytes coincides with the first 4 h of *in vitro* culture when there is a maximum increase of surface class I molecules (Fig. 4 and Table 1).

We conclude that (i) when the only interactions allowed are those between specific CTL precursor and monocytes or T cell clones, a CTL response can be readily induced; (ii) in this case, induction of a primary CTL response triggered by professional APC does not require $CD4^+$ T cells; and (iii) failure of total PBMC to mount a CTL response to soluble peptide cannot be explained by insufficient peptide loading, but rather by the presence of inappropriate interactions.

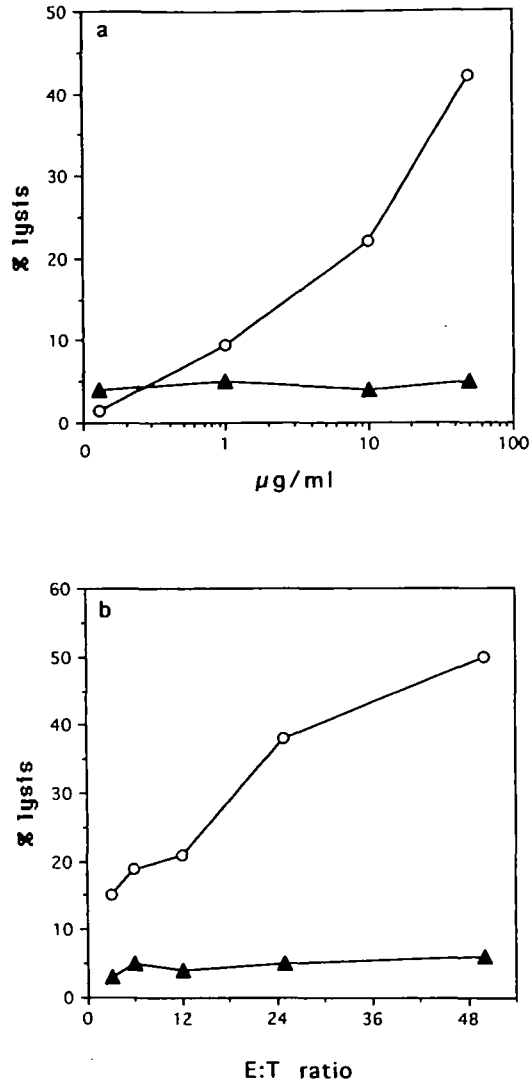


Fig. 3. Dose–response curves of *in vitro*-primed CTL. (a) HIVgp120(121–129)-primed CTL showed cytotoxic activity, at an E:T ratio 25:1, against ^{51}Cr-labelled target cells, previously pulsed with increasing concentrations of HIVgp120(121–129) peptide (O), but not when pulsed with a non-relevant HLA-A2-binding peptide, as the HBenvAg(335–343) (▲); (b) HIVgp120(121–129)-primed CTL were compared in their lytic responses against either HIVgp120(121–129)-sensitized (O) or HBenvAg(335–343)-sensitized (▲) target cells at different E:T ratios.

Co-stimulatory requirements for the induction of a primary CTL response

The fact that peptide presentation by monocytes or T cell clones is effective, while presentation by total PBMC (which include monocytes as well) is not, suggests that total PBMC contain cells capable of presenting the peptide in a 'suppressing' fashion. To test whether the inability of PBMC to mount a peptide-specific CTL response was determined by presentation of peptide on cells that lack co-stimulatory signals, we asked whether soluble molecules that directly

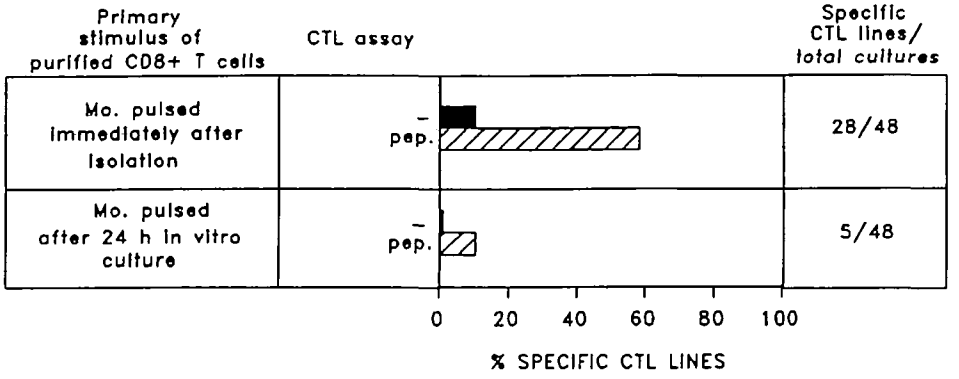


Fig. 4. Peptide pulsing of monocytes is most effective in the first hours of *in vitro* culture. Highly purified $CD8^+$ T cells were primed with monocytes that were pulsed with gp120(121–129) peptide for 4 h either immediately after 90 min adherence or after 24 h *in vitro* culture. Thereafter, they were re-stimulated and assayed for cytotoxicity against peptide-pulsed (hatched bars) or unpulsed (solid bars) target cells, as described, at an E:T ratio 25:1. The percentage of the specific CTL lines generated with the two conditions was reported. A CTL line was defined specific when it expressed a specific lysisof >15%.

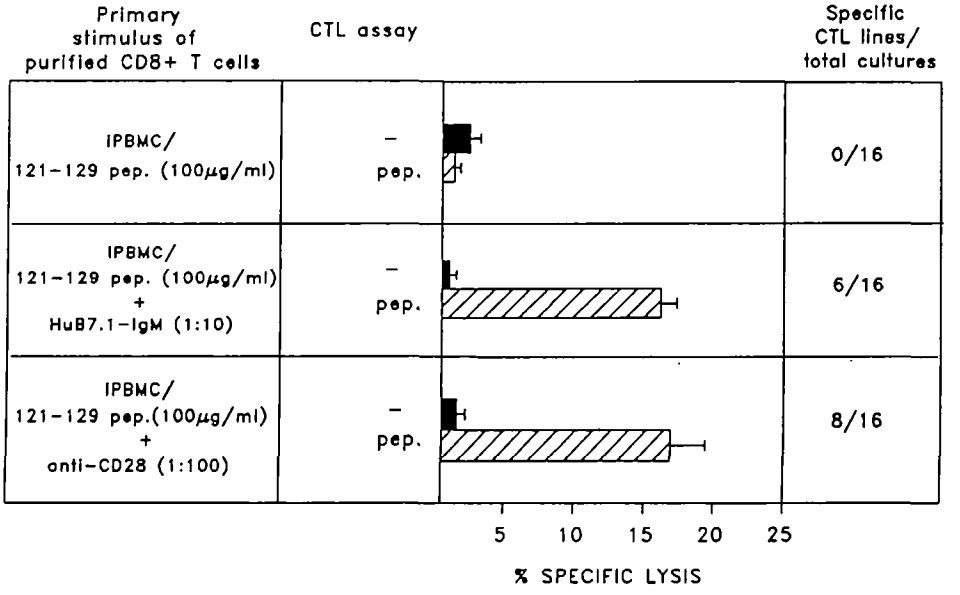


Fig. 5. Co-stimulation by soluble B7.1 and anti-CD28 allows the response of unseparated peptide-pulsed PBMC. Peptide-pulsed unseparated PBMC, used as a source of APC, were cultured with purified $CD8^+$ T cells in the absence or presence of either chimeric huB7.1–IgM molecules or mouse anti-CD28 IgM antibodies. After 7 days, the cells were re-stimulated, as previously described, and after a further 7 days, were tested in a cytotoxicity assay against peptide-pulsed (hatched bars) or unpulsed (solid bars) target cells, as described, at an E:T ratio 25:1. Results represent the percentage mean of specific lysis expressed by all the specific CTL lines.

deliver the co-stimulatory signal to T cells might reverse this effect. When a huB7.1–IgM chimeric protein or an IgM anti-CD28 mAb was added to unseparated PBMC and peptide, a clear CTL response was detected (Fig. 5). These results demonstrate that co-stimulation via CD28 is sufficient for the induction of the CTL response and suggest that peptide presentation on frequent non-professional APC present among PBMC (resting T and B cells expressing class I but not co-stimulatory molecules) may induce peptide-specific anergy in CTL precursors.

To further study the co-stimulatory requirements for the induction of a primary anti-peptide response, we tested the blocking effect of anti-B7.1 mAb or soluble CTLA-4, that can bind with high affinity to both B7.1 and B7.2. Interestingly, neither anti-B7.1 nor soluble CTLA-4 was able to significantly inhibit the induction of a primary peptide-specific CTL response by activated monocytes (Fig. 6). Similar results were obtained using autologous activated T cells as APC (not shown). Control experiments showed that both anti-B7.1 mAb and soluble CTLA-4, both added at 5 μg/ml at the initiation of culture, significantly blocked the proliferative response by resting T cells in a primary mixed lymphocyte reaction

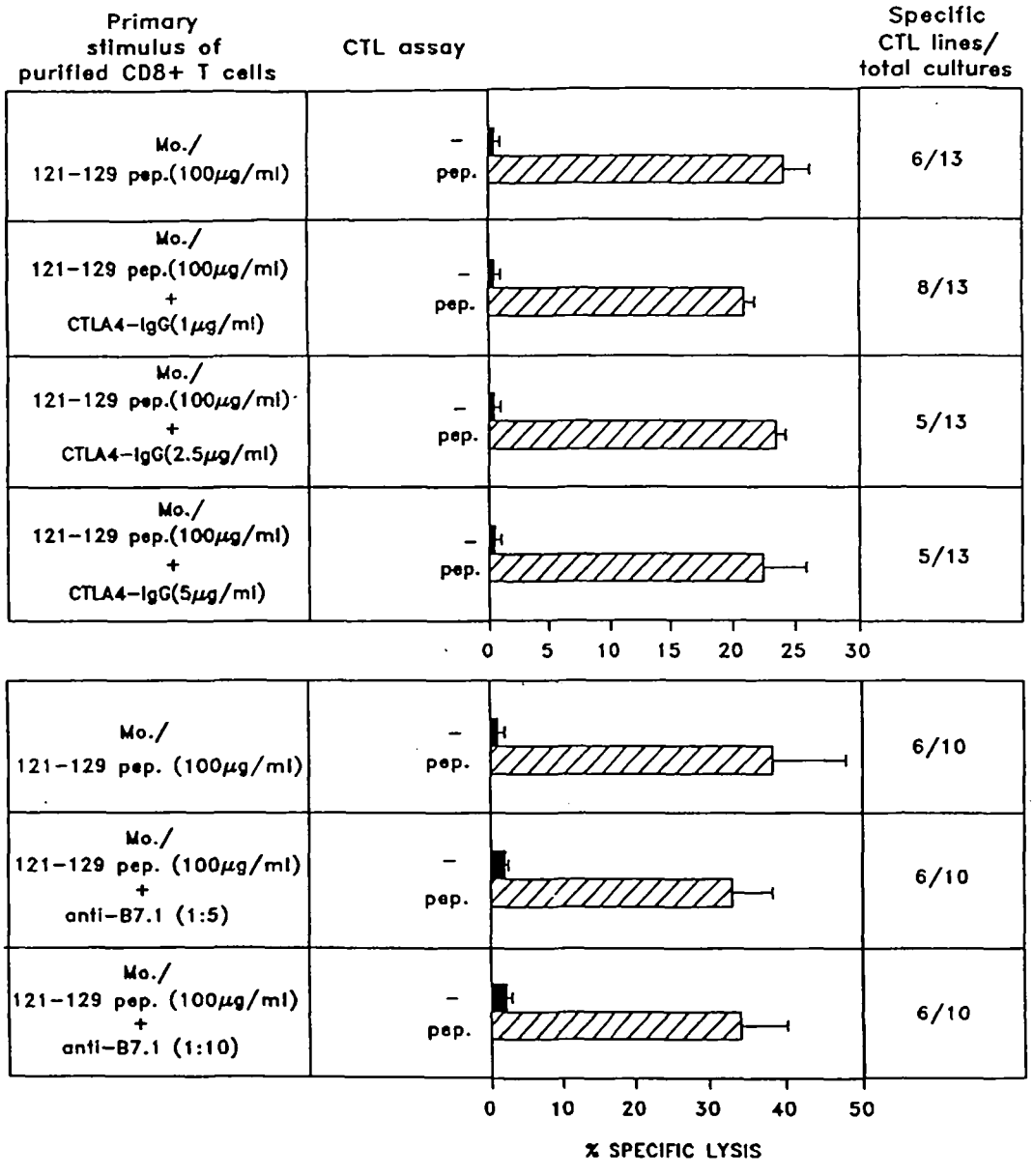


Fig. 6. The induction of a specific CTL response by activated monocytes is not blocked by anti-B7.1 mAb nor by soluble huCTLA-4–hulgG. Peptide-pulsed monocytes were used as APC to prime a specific CTL response in the presence or absence of different concentrations of either anti-B7.1 mAb or soluble huCTLA-4–IgG. Results represent the percentage mean of specific lysis expressed by the specific CTL lines against peptide-pulsed (hatched bars) or unpulsed (solid bars) target cells.

stimulated by either T cell clones or monocytes or dendritic cells (not shown).

These results suggest that, in addition to B7.1/B7.2 molecules, professional APC may possess additional co-stimulatory ligands that are sufficient for the induction of a CTL response. Thus, it appears that each of the two pathways is in itself sufficient, although not strictly necessary for CTL activation.

Characterization of the responding T cells

To investigate whether the CTL response was due to *in vitro* priming of virgin CTL precursors or to the reactivation of memory cells that had been primed *in vivo* by the same or cross-reacting peptides, we compared the response of total $CD8^+$ T cells with that of $CD8^+$ T cells depleted of $CD45RO^+$ putative memory T cells. As shown in Table 2, the CTL response was comparable in both populations, indicating that the contribution to the response of $CD45RO^+$ cells (usually only 10–20% of total $CD8^+$) is indeed negligible. We cannot exclude, however, that the responding cells may belong to a population of resting $CD45RA^+$ cells that had reverted from $CD45RO^+$ cells.

CTL obtained by in vitro priming with synthetic peptides recognize naturally processed peptides on infected cells

We tested whether the primary peptide-specific $CD8^+$ T cells were able to recognize naturally processed peptides derived

Table 2. Primary peptide-induced $CD8^+CD45RO^-$ T cells lyse peptide-pulsed target cells

Responding population[a]	Specific lysis (%)	
	Peptide-pulsed targets	Unpulsed targets
Unseparated $CD8^+$ T cells	27	0
$CD8^+CD45RO^-$ T cells	29	0

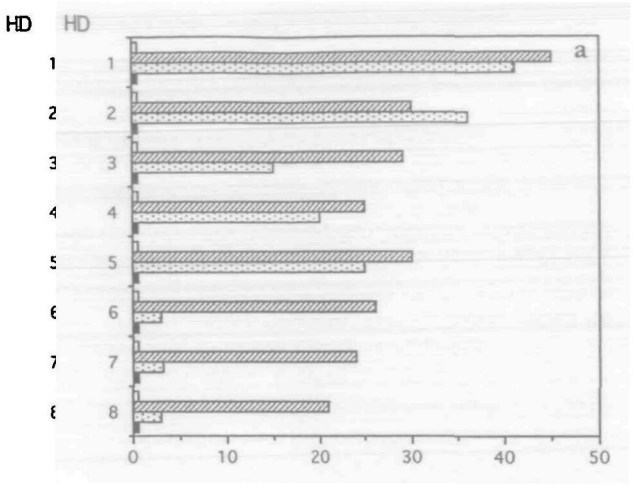
[a]Unseparated $CD8^+$ T cells or CD45RO depleted-$CD8^+$ T cells ($CD8^+CD45RO^-$ T cells), sorted from peripheral blood as described in Methods, were cultured with peptide-pulsed adherent monocytes for 7 days followed by re-stimulation with peptide-pulsed autologous T cell blasts. Afterwards they were tested for their capacity to kill ^{51}Cr-labelled EBV-B cells, pulsed or not with peptide. Percentage of specific lysis is expressed as mean of triplicate determinations.

from endogenously synthesized proteins. Out of eight HIVgp120(121–129) peptide-specific CTL lines tested, five efficiently cross-reacted on HLA-$A2^+$ EBV-B cells infected with rVV expressing HIVgp160 (Fig. 7). Figure 7 also shows that two representative $CD8^+$ T cell clones, generated from HIVgp120(121–129) peptide-primed T cells, killed both the peptide-sensitized $A2^+$ EBV-B cells and the same targets endogenously expressing HIVgp160. Similarly, $CD8^+$ T cell clones specific for HBenvAg(335–343) peptide recognized EBV-B cells infected with rVV expressing HBenvAg (not shown).

Discussion

In the perspective of an adoptive immunotherapy, it is important to generate *in vitro* primary CTL responses, i.e. responses to antigens to which the individual has not been primed *in vivo*. We used two nonamer HLA-A2 binding peptides from HIVgp120 and HBenvAg to define the conditions for the generation of such CTL responses by $CD8^+$ cells from seronegative donors. A critical factor appeared to be the mode of antigen presentation. While peptide pulsing of unseparated PBMC was invariably ineffective, pulsing of adherent monocytes or activated T cells with peptide induced a peptide-specific, HLA-A2-restricted CTL response. The responding $CD8^+$ T cells recognize naturally processed peptides on target cells infected with rVV expressing HIVgp160. This last is a fundamental requirement in the perspective of adoptive immunotherapy, in which specific CTL have to recognize peptides expressed in association with class I molecules on the surface of host tumor or infected cells, as a product of endogenous processing. Indeed, the majority of our peptide-specific CTL lines tested, as well as the CTL clones derived from them, efficiently killed infected target cells, confirming previous reports indicating that the immunodominant peptides always belong to the highest HLA-binding peptides (10,14,17).

Previous attempts to generate primary *in vitro* CTL responses in the mouse system involved the use of either dendritic cells, which possess high stimulatory capacity (40), or of the transporter defective mutant RMA-S, which expresses high levels of empty MHC molecules (12). Recently, human



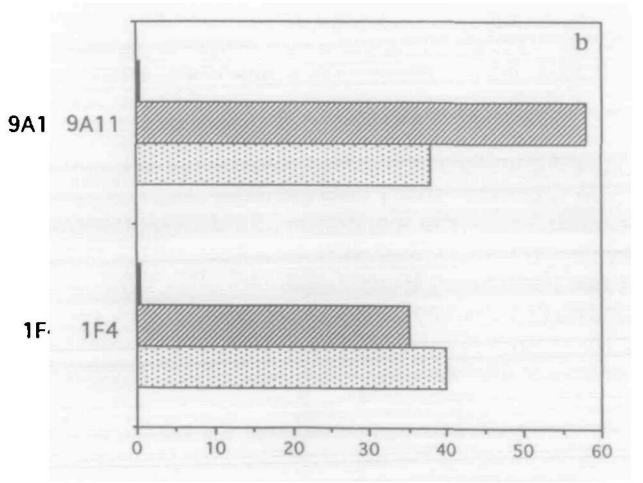


Fig. 7. Primary viral peptide-induced $CD8^+$ T cells recognize naturally processed peptide on infected cells. (a) Purified $CD8^+$ T cells, derived from healthy donors (HD), were primed with HIVgp120(121–129)-sensitized adherent monocytes, followed by re-stimulation with peptide-sensitized autologous T cell blasts. After, they were tested for their capacity to kill (at an E:T ratio 25:1): ^{51}Cr-labelled HLA-$A2^+$ EBV-B cells (JY line: HLA-A2.1, B7, C7) infected with wild-type VV (□), or same targets both infected with wild type VV and pulsed with peptide (▨), or same targets infected with rVV expressing HIVgp160 (▩), or ^{51}Cr-labelled HLA-$A2^-$ EBV-B cells (SA line: HLA-A24, B7, C7) infected with rVV expressing HIVgp160 (■). (b) Two representative $CD8^+$ T cell clones (9A11 and 1F4), generated from gp120(121–129)-primed T cells, were tested for their ability to kill both HLA-$A2^+$ EBV-B cells sensitized with HIVgp120(121–129)-peptide (▨), and same targets infected with either wild-type VV (■) or rVV expressing HIV gp160 (▩), at an E:T ratio 10:1. Percentage of specific lysis is expressed as mean of triplicate determinations.

dendritic cells have been demonstrated to be very efficient APC in priming both naive $CD4^+$ and $CD8^+$ T cells (41,42). Moreover, the report by Houbiers *et al.* describes the generation of primary peptide-specific CTL responses in humans

after *in vitro* stimulation with mutant T2 cells (43). This protocol was not successful in our hands. Indeed, in preliminary experiments we found that the human transporter defective mutant T2 cells, although able to bind high peptide levels (Fig. 1), was not suitable for *in vitro* priming, because of the very high level of background activation.

Out of all APC tested, only monocytes and T cell clones gave reproducible results. The fact that cultured monocytes are efficient for CTL priming implies that they can bind sufficient amounts of peptide and present it in the appropriate co-stimulatory context. This efficient presentation may be dependent upon the strong up-regulation of MHC class I, as well as adhesion and co-stimulatory molecules that appear spontaneously upon *in vitro* culture within the first 2–6 hours (Table 1) (44), as shown by the fact that adherent monocytes are much more effective if pulsed in the first few hours of culture. The peptide pulsing, done during the up-regulation of class I molecules by APC, may favour a more efficient peptide binding to that small percentage of newly synthesized empty class I molecules, available to bind exogenous peptides.

We also found that autologous activated T cell clones can function as APC for CTL priming with antigenic peptides. An advantage in the use of activated T cells is the lack of a very low non-specific background of stimulation in the absence of peptide. We and others have previously demonstrated that activated T cell clones are indeed professional APC and very effective in priming other T cells (38,39).

Recently, an alternative approach for inducing primary anti-tumor CTL in humans was successfully carried out using as APC non-transformed B cell blasts (45). This together with the finding that EBV-transformed B cells used as APC were not able, in our system, to prime antigen-specific CTL responses, suggests that the two B cell preparations have different APC capabilities.

It has been previously reported that the density of T cell epitopes required for the induction of primary CTL response is much higher than the concentration required for secondary responses (12). This difference is evident also in the induction of human CTL, since a 10-fold lower peptide concentration is required to sensitize target cells for killing.

There are three points that need further discussion. First, as reported in several experimental systems (12,32,46–49), our data demonstrate that, when antigen is presented by professional APC, the requirement for antigen-specific T_h cells in the induction of CTL may not be evident. This result gives rise to speculation that CTL precursors primed by professional APC do not require T_h cooperation, that is instead essential when antigen is presented by non-professional APC; in this last instance, activated T cells themselves, working as professional APC (39), could simultaneously present peptide and provide co-stimulation for an appropriate CTL priming.

The second point concerns the pathway of co-stimulation. Our results demonstrate that direct co-stimulation of responding $CD8^+$ T cells by soluble ligands via CD28 can revert the inability of peptide presentation by non-professional APC. In apparent contrast, however, we found that anti-B7.1 antibody as well as soluble CTLA-4 that blocks both B7.1 and B7.2 (24,25) fail to inhibit induction of a specific CTL response by activated monocytes. These results suggest that the B7–CD28 interaction, although by itself sufficient for priming, is not strictly necessary and that alternative co-stimulatory pathways may exist. Similar conclusions have been recently reached by Johnson and Jenkins in an anti-CD3-dependent T cell activation system (50).

Finally, our data favour the idea that a real CTL priming is carried out by virgin T cells in our system for two reasons: (i) the donors were healthy and seronegative and (ii) the depletion of $CD45RO^+$ cells did not affect the CTL response, indicating that most of the responding cells belong to the RA^+RO^- compartment, even though we cannot exclude that this last population may contain some $CD45RA^+$ resting T cells that have reverted from memory $CD45RO^+$ phenotype (51,52).

In conclusion, exploiting the strategies affected by the immune system for inducing a primary T cell response, we define a protocol of CTL priming, by using activated monocytes or T cell clones as professional APC, well defined HLA-binding peptides as immunogenic antigen, and the conditions favouring productive interactions between specific CTL precursors and professional APC.

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Abbreviations

β_2m	β_2-microglobulin
CTL	cytotoxic T lymphocyte
EBV-B	Epstein–Barr virus-transformed B cell
GAM	goat anti-mouse Ig
HBenvAg	hepatitis B envelope antigen
PBMC	peripheral blood mononuclear cell
PHA	phytohaemagglutinin
VV	vaccinia virus

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