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Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8

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SUMMARY

The human herpesvirus 8 (HHV-8) is a human γ2-herpesvirus that is implicated in the development of Kaposi's sarcoma (KS), primary effusion lymphoma and Castelman's disease. Since the responses of cytotoxic T lymphocytes (CTL) play a key role in the control of herpesvirus infection, it is important to identify and to characterize the CTL target epitopes of HHV-8 viral antigens. In this study, using peptide-binding motifs, we selected potential human leucocyte antigen (HLA)-A2-binding peptides from kaposin A and glycoprotein H (gH), that are latent and lytic HHV-8 antigens, respectively. HLA-A2-binding peptides were tested for their capacity to induce CTL responses in HHV-8-negative healthy donors. By this approach, we found that the majority of individuals responded to two HHV-8-derived CTL epitopes, namely, VLLNGWRWRL (amino acids 16–25), which derives from kaposin A, and FLNWQNLLNV (amino acids 59–68), which derives from gH. In addition, memory CTL responses to these epitopes were detected in disease-free individuals infected by HHV-8 demonstrating that the two epitopes are relevant targets of CTL-mediated immunity *in vivo*. The identified epitopes may be investigated for the development of immunotherapeutic strategies against HHV-8-associated malignancies.

INTRODUCTION

Human herpesvirus 8 (HHV-8) is a member of the γ2-herpesvirus family.[1] Infection by HHV-8 precedes and is associated with Kaposi's sarcoma (KS),[1] the most common tumour of human immunodeficiency virus (HIV)-infected individuals, as well as with primary effusion lymphomas (PEL) and the immunoblastic variant of Castelman's disease.[2,3] In KS lesion, the virus is mostly present in a latent form in KS spindle cells, but yields a lytic infection in lymphocytes and monocytes infiltrating the lesions.[4,5]

Cytotoxic T lymphocytes (CTL) play an important role in the control of viral infections, particularly as effectors of long-term immune surveillance against viruses persisting in the infected host. This is reflected by the frequency at which reactivation of persistent infection is observed in patients whose CTL responses are lost or suppressed. In addition, immunodeficient or immunosuppressed individuals such as patients with acquired immune-deficiency syndrome (AIDS) and organ transplant recipients, are more frequently prone to the development of virus-associated tumours such as Epstein–Barr virus (EBV)-associated lymphomas and HHV-8-associated KS or PEL. In the case of EBV it has been demonstrated that the lack of efficient CTL activities directed against the virus may be overcome by the passive transfer of EBV-specific CTLs to the immunosuppressed patients. This approach has been successfully employed in the management of some EBV-associated diseases, including immunoblastic B-cell lymphomas[6–10] and severe chronic active EBV infection.[11]

The molecular targets of CTL are peptide fragments that are derived from the processing of antigenic proteins, which are selectively presented at the surface of infected target cells by their ability to bind to the antigen-presenting groove of major histocompatibility complex (MHC) class I

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molecules.[12–14] In particular, dendritic cells (DC) are the most efficient antigen-presenting cells, that are also capable of inducing primary CTL responses *in vitro* and *in vivo* upon stimulation with CTL epitope peptides.[15–17] These cells can therefore be used to stimulate CTL responses *in vitro* to identify specific CTL epitopes. On the other hand, the precise characterization of the peptide antigens is important, since they can be used to develop immunotherapeutic strategies to control virus infection or disease development.[18–21]

At present, very little is known on the immune control of HHV-8 in infected individuals, however, there is evidence indicating the presence of CTL responses specific for latent and lytic antigens.[22–24] Since HHV-8-derived epitopes may be regarded as a potential target of specific immunotherapies for the treatment/prevention of HHV-8-associated malignancies, we sought to characterize CTL peptide antigens derived from HHV-8 antigens.

To this goal, we focused our studies on human leucocyte antigen (HLA)-A2-restricted epitopes within the lytic antigen glycoprotein H (gH) and the latent antigen kaposin A, that were previously shown as targets of CTL responses.[22,23] HLA-A2 was chosen since it is the most common HLA class I molecule in humans, and therefore represents an important restriction element for CTL-based immunotherapy. In addition, a large amount of information on the requirements for HLA-A2–peptide interactions is available, including the crystallographic structure of MHC–peptide complexes[25] and naturally processed peptide sequence motifs.[14] In particular, the HLA-A2-binding peptide motif has two anchor residues, namely leucine (L), which is the dominant residue at position 2, and valine (V), which prevails at the C terminus, although other amino acids may be found at these positions. Non-anchor residues are also crucial in determining binding to HLA-A2 molecules.[26,27] This information is relevant for the identification of potential immunogenic peptides within proteins of known sequence.[27]

In this work, potential HLA-A2-binding CTL epitopes were identified within gH and kaposin A. These HLA-A2-binding peptides were used to prime *in vitro* cytotoxic T-cell responses against HHV-8 by using autologous DC from HHV-8 seronegative donors, and to assess in HHV-8-positive individuals the presence of CTL able to kill peptide-loaded cells or PEL cells that express HHV-8 antigens endogenously.[2,3] By these approaches, we identified and characterized CTL epitopes expressed upon HHV-8 latent and productive infection useful for the development of immunotherapeutic strategies.

MATERIALS AND METHODS

Cell lines

The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T-cell line CEM.[28] Cells were maintained in RPMI-1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT).

Phytohaemagglutinin (PHA)-activated blasts were obtained by stimulation of peripheral blood lymphocytes (PBLs) with 1 μg/ml purified PHA (Wellcome Diagnostics, Dartford, UK) for 3 days and expanded in medium supplemented with human recombinant interleukin-2 (rIL-2; Proleukin; Chiron, Milan, Italy) as described.[29] BCBL-1 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were cultured in RPMI-1640 containing 10% FCS, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. BC-1 cells (obtained from P. Moore, Columbia University, New York, NY) and BC-3 cells (obtained from G. Gaidano, University of Torino, Italy) were cultured in RPMI-1640 containing 10% FCS.

Subjects

PBLs from six HHV-8-seronegative, HLA-A2-positive, healthy donors (D1–D6) obtained from the Blood Bank of S. Anna Hospital of Ferrara were used to evaluate primary CTL responses against HHV-8 peptides.

PBLs from four HHV-8-seropositive HLA-A2-positive subjects without KS or other HHV-8-associated diseases were used to evaluate memory CTL responses to HHV-8. Three of them (P1, P2 and P4) were also HIV-infected. HIV plasma loads and CD4 cell counts were available for subject P1 (2800 viral RNA copies/ml and 250 cells/μl, respectively) and P3 (65 000 viral RNA copies/ml and 920 cells/μl, respectively). Only subject P1 was receiving antiretroviral treatment at the time of bleeding.

Synthetic peptides

All peptides were synthesized by the solid-phase method using a continuous-flow instrument with on-line UV monitoring. The stepwise syntheses were carried out by Fmoc-chemistry (fluorenylmethoxy-carbonyl) as previously described.[30] Crude deprotected peptides were purified by high-performance liquid chromatography; purity was $>98\%$. Structure verification was achieved by elemental and amino acid analyses and mass spectrometry. Peptide stocks were prepared in dimethylsulphoxide at a concentration of 10^{-2} M, kept at -20°, and diluted in phopshate-buffered saline before use.

Detection of peptide binding to HLA-A2 molecules by immunofluorescence

Aliquots of 1×10^6 T2 cells were treated overnight at 26° in 1 ml of AIM-V medium containing increasing concentrations of synthetic peptides (from 10^{-9} to 10^{-5} M) and incubated at 37° for an additional 4 hr. After washing, surface expression of HLA class I molecules was detected by indirect immunofluorescence using MA2.1 monoclonal antibody, that is specific for HLA-A2 molecules. Mean logarithm fluorescence intensity was determined by fluorescence-activated cell sorter (FACS) analysis (Bryte HS, BioRad, Milan, Italy).[31] The percentage increase in HLA class I expression was calculated over the expression found in untreated T2 cells.

Detection of antibodies against HHV-8 latent or lytic antigens

Serum antibody titres to latent and lytic antigens of HHV-8 were determined by using two immunofluorescence assays (IFA) based on BCBL-1 cells, as described previously.[32,33] Briefly, for IFA to antilytic antigens, tetradecanoyl phorbol ester acetate (TPA)-induced BCBL-1 cells were used, whereas IFA to antilatent antigens was performed with isolated nuclei of untreated BCBL-1 cells.[32,33] TPA-induced cells or isolated nuclei were smeared on slides, fixed with a methanol/acetone (1 : 1; v/v) solution and preblocked by incubation with phosphate-buffered saline supplemented with 3% FCS, followed by two incubation steps of 45 min each at 37° with 1 : 10 diluted patient serum and goat fluorescein isothiocyanate-conjugated anti-human antibodies, respectively. Titrations were performed by fourfold serial dilutions. All microscopic examinations were evaluated in a blinded fashion.

Preparation of antigen-presenting cells

DCs from HLA-A2-positive donors or T2 cells were used as antigen-presenting cells. DCs were selectively expanded from total PBMC as described.[34] Briefly, PBMC were resuspended in RPMI-1640/10% FCS at 5×10^6 cells/ml and seeded into six-well plates. After 2 hr at 37° non-adherent cells were removed, and the adherent population was cultured in RPMI-1640/10% FCS supplemented with 800 U/ml granulocyte–macrophage colony-stimulating factor (recombinant HuGM-CSF, Schering-Plough, Milan, Italy) and 50 ng/ml IL-4 (PeproTech EC, London, UK). On days 2 and 4 half of the medium was replaced with fresh medium as described above; on day 7 non-adherent cells were harvested and used as a source of DCs. The quality of the DC population was checked in each case by immunofluorescence staining for the surface markers CD40, CD80, CD83, CD86, CD58, CD11c, CD14, CD1a, HLA-A, -B, -C and HLA-DR using specific monoclonal antibodies. DC were treated with mitomycin C to avoid cell proliferation, and pulsed with 10^{-5} M of the different peptides for 3 hr at 37° in AIM-V medium. After extensive washing the cells were used as stimulators.

T2 cells (5×10^6) were cultured overnight at 26° in 1 ml serum-free AIM-V medium. Cells were then washed, treated with mitomycin C to avoid cell proliferation, and pulsed with 10^{-5} M of the different peptides for 3 hr at 37° in AIM-V medium. After extensive washing, the cells were used as stimulators.[35]

Generation of primary CTL cultures

Monocyte-depleted PBLs from HLA-A2-positive, HHV-8 negative and HIV-negative healthy donors, were plated at 3×10^6 cells per well in 24-well plates in RPMI-1640/10% FCS (Hyclone) and stimulated with the selected peptides. The first stimulation was performed with autologous DC pulsed with 10^{-5} M of different peptides for 3 hr, at a stimulator : responder ratio of 1 : 20. The CTLs were then restimulated every 7 days with T2 cells incubated overnight at 26° and pulsed with 10^{-5} M synthetic peptides for 3 hr at 37°, at a stimulator : responder ratio of 1 : 20. The medium was supplemented from day 8 with 10 U/ml rIL-2.[35]

Generation of memory CTL cultures

Monocyte-depleted PBLs from HLA-A2-positive, HHV-8-positive subjects were plated at 3×10^6 cells per well in 24-well plates in RPMI-1640/10% FCS (Hyclone) and stimulated with the selected peptides. The first stimulation was performed with peptide-pulsed T2 cells at a stimulator : responder ratio of 1 : 20. In this assay, DC were not used as antigen-presenting cells due to their capability of inducing primary CTL responses. Cultures were restimulated after 7 days and the medium was supplemented from day 8 with 10 U/ml rIL-2. On day 14 T-cell cultures were tested for CTL activity.[36]

Cytotoxicity tests

Cytotoxic activity was tested by a standard 5-hr ^{51}Cr-release assay, as previously described.[37] Briefly, target cells were labelled with 0·1 μCi/10^6 cells of $Na_2{}^{51}CrO_4$ for 90 min at 37° and, where indicated, pulsed for 45 min with 10^{-7} M of different peptides at 37°.[29] Cells were then washed, and 4×10^3 cells were used as target of each CTL, at an E : T ratio of 20 : 1. Percentage specific lysis was calculated as 100 × (c.p.m. of sample − c.p.m. of medium)/(c.p.m. of Triton X-100 − c.p.m. of medium), where c.p.m. represents counts per min. Spontaneous release was always less than 20%. None of the tested peptides affected the spontaneous release. CTL responses were considered positive when ≥ 10% as compared with untreated target cells.

Induction of PEL cells

Exponentially growing BCBL-1, BC-1 and BC-3 cells (about 10^6 cells/ml) were collected and suspended in growth medium (5×10^5 cells/ml); BCBL-1 cells were grown for an additional 24 hr. To reactivate HHV-8 infection, the BCBL-1 and BC-3 cells were stimulated with 20 ng of TPA (Sigma, Milan, Italy), for 2 days, while BC-1 cells were induced with 3 mM sodium *n*-butyrate (Sigma) for the same period of time. Cells were then washed and used as targets by a standard 5-hr ^{51}Cr-release assay.

RESULTS

Identification of HLA-A2-binding peptides derived from glycoprotein H and kaposin of HHV-8

To identify potential HLA-A2-restricted epitopes within gH (ORF22), and kaposin A (ORF K12), the amino acid sequences of these proteins were analysed by a computer program designed to predict HLA-A2-binding peptides.[27] Potential epitopes, nine or ten amino acids long, are ranked based on an estimation of the half-life of HLA-A2–peptide complexes (Table 1). The affinity of a peptide for class I molecules plays an important role in determining CTL responsiveness, and it has been shown that the immunogenicity of peptide antigens depends on a low dissociation rate of MHC–peptide complexes.[36–39]

Table 1. Identification of potential HLA-A2-binding peptides within HHV-8 proteins*

Peptide sequence	Code	Protein	Residue	Score†
KLMLDIHTV	KLM	gH	440–448	2072
KLLRTYLMFT	KLL	gH	463–472	1221
FLVFQMLV	FLV	gH	310–318	1216
FLNWQNLLNV	FLN	gH	59–68	736
FQMLVAHFLV	FQM	gH	314–323	696
ALFLILSFI	ALF	gH	705–713	488
YLWLRDNGTV	YLW	gH	682–691	345
SILEGIANV	SIL	gH	412–420	334
SLFFPNKTV	SLF	gH	96–104	257
FLYRLFSIL	FLY	gH	721–729	245
VVYEVSEIFL	VVY	gH	587–596	229
GLPDSLPSL	GLP	gH	224–232	201
NLHIHYLWL	NLH	gH	677–685	176
VLLNGWRWRL	VLL	kap. A	16–25	1008
LLNGWRWRL	LNN	kap. A	17–25	272

kap. A, kaposin A.

*To identify the potential HLA-A2-binding peptides within gH and kaposin A a computer-based program was employed as described previously.[27] This program can be directly accessed through the world-wide web site: http://bimas.dcrt.nih.gov/molbio/hla-bind/index.htlm.

†Estimated half-time of dissociation from HLA-A2 moleclues (min).

Therefore, potential epitopes were chosen based on estimated half-time dissociation score ≥ 160. This represents the score of the subdominant CLGGLLTMV (CLG) epitope, an HLA-A2-restricted EBV-derived antigen that induces weak CTL responses.[40] Of note, other peptides, not selected by this approach, may also represent CTL epitopes.

Two sequences from kaposin A and 13 sequences from gH were found. The peptides derived from kaposin A were a 10-amino-acid-long peptide (amino acids 16–25) and its shorter version (amino acids 17–25). Since the decamer peptide is reported to have a higher estimated half-time dissociation score and contains both potential epitopes, it was selected for further studies. Potential binders were then selected for peptide synthesis and the products were tested for binding to HLA-A2 molecules (Table 1). Kaposin B and C[41] were also analysed but no additional HLA-A2-binding peptides were found.

HLA-A2–peptide association was then assessed by the induction of surface HLA-A2 expression in the mutant T2 cell line. T2 cells were treated at 26° for 18 hr in serum-free medium in the presence of increasing concentrations of peptides. Cells were then kept at 37° for 4 hr and extensively washed to remove unbound peptides. In all experiments the GILGFVFTL (GIL) peptide was used as the positive control. This peptide derives from the influenza virus matrix antigen, and has high binding affinity for HLA-A2 molecules.[42] The surface expression of HLA class I complexes was evaluated by immunofluorescence using the monoclonal antibody MA2.1 that recognizes HLA-A2 molecules. Representative results are shown in Fig. 1. All peptides, except NLH, bound to HLA-A2. This confirms the efficiency of the computer program designed to predict

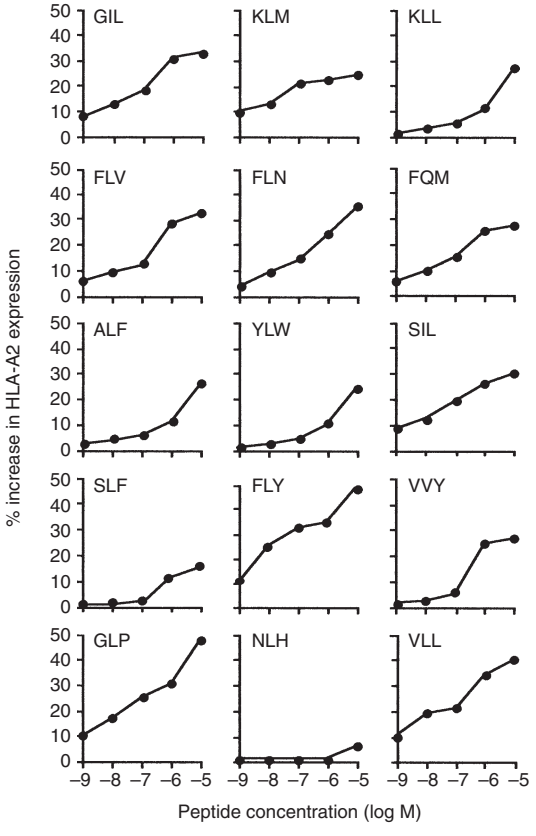

Figure 1. HLA-A2–peptide complexes at the cell surface. T2 cells were preincubated overnight at 26° in 1 ml serum-free medium containing the indicated concentrations of synthetic peptides, and kept at 37° for 4 hr. Cells were washed and the surface expression of HLA class I molecules was detected by indirect immunofluorescence using MA2.1 monoclonal antibody. Results are expressed as the percentage increase of HLA class I expression calculated with respect to that found in untreated T2 cells. The mean of three different experiments is shown.

HLA-A2-binding peptides[27] although stringent correlations between binding affinity and scores were not detected. Indeed, only the KLM, FLV, FLN, FQM, SIL, FLY, GLP, and VLL peptides bound to HLA-A2 molecules at concentrations lower than 10^{-7} M, whereas KLL, ALF, YLW, SLF, and VVY bound HLA-A2 molecules only at higher concentrations (10^{-6}–10^{-5} M).

Induction of primary CTL responses by selected HHV-8-derived peptides

Since the majority of immunogenic peptides display a high affinity for class I molecules, only peptides that showed binding at concentrations lower than 10^{-7} M (KLM, FLV, FLN, FQM, SIL, FLY, GLP, and VLL) were used for the immunogenicity studies.

To determine whether the eight potential epitopes represent real targets of HHV-8-specific CTL responses, the peptides were screened for their capacity to generate

peptide-specific CTL cultures. To this purpose peptide-pulsed dendritic cells (DC) were used as antigen-presenting cells for CTL stimulations, since DC allow induction of primary CTL responses.[15]

PBLs from HLA-A2-positive healthy donors were stimulated first with autologous DC pulsed with 10^{-5} M of the selected peptides, whereas subsequent stimulations were performed with peptide-pulsed T2 cells. To control the efficiency of both memory and primary activation of CTLs, parallel stimulations of memory precursors specific for the subdominant CLG epitope,[40] and of primary CTLs specific for the HLA-A2-restricted HIV-derived ILKEPVHGV (ILK) epitope[43] were performed. Six HLA-A2-positive healthy donors were analysed by this assay. Representative results of all CTL cultures obtained after four stimulations are shown in Fig. 2. All donors responded to CLG and ILK peptides, indicating that the protocol of stimulation efficiently induces both memory and primary CTL responses, as already observed by others.[15]

All donors responded to at least one of the HHV-8 epitopes. In particular, all subjects except donor 2 (D2) responded to the gH-derived FLN epitope, and four donors (D3, D4, D5 and D6) responded to the kaposin A-derived VLL epitope. The responses to FLV, FQM, SIL, and FLY epitopes were less frequent and weak, as indicated by the low level of specific lysis observed with CTL cultures obtained after stimulation with these peptides. The GLP epitope never induced CTL responses, although it showed a very high affinity for HLA-A2 molecules (Fig. 1).

The responses we detected in the six individuals represent primary CTL activations, since the six donors were all HHV-8-seronegative (data not shown). The kinetics of CTL responses indicated that responses to HHV-8 epitopes can be detected only after the third or fourth stimulation, as observed for CTL responses directed against the HIV-derived epitope ILK. A representative kinetic obtained with donor 3 is shown in Fig. 3. It has to be noted that, in contrast to the primary response specific for the ILK epitope, memory CTL responses directed at the EBV-derived CLG epitope can already be detected after the second stimulation.

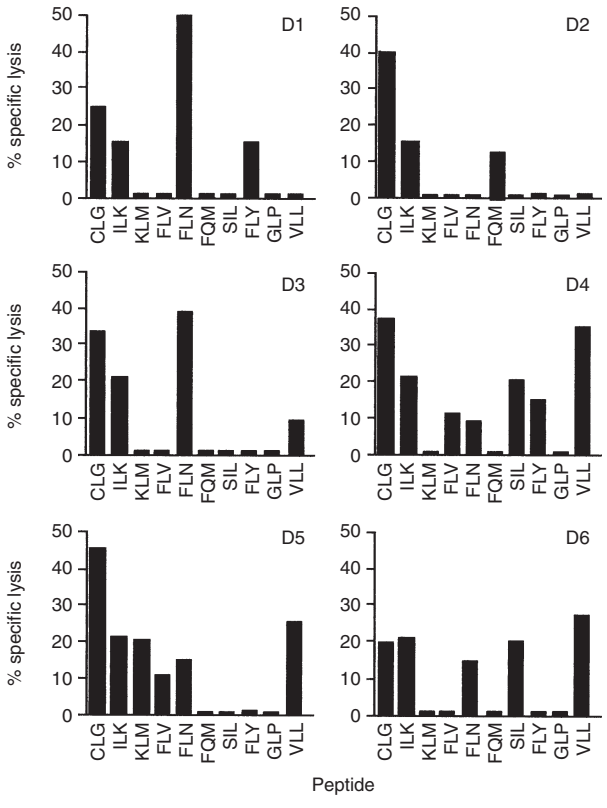

Figure 2. Cytotoxic activity of CTL cultures obtained by stimulation with synthetic HHV-8-derived peptides. Freshly isolated PBL from six HHV8-seronegative, HLA-A2-positive healthy donors were stimulated with the indicated peptides. The first stimulation was performed with autologous DC pulsed with 10^{-5} M peptides while subsequent stimulations were performed with peptide-pulsed T2 cells. Cytotoxic activity was assayed after four consecutive stimulations against HLA-A2 single matched PHA blasts treated with 10^{-7} M of the different peptides for 1 hr prior to the assay. Results are expressed as the percentage specific lysis recorded at an E : T ratio of 20 : 1. None of the cultures killed untreated PHA blasts. One representative experiment out of two to four different experiments is shown. Donor 3 gave consent for only one bleeding and was, therefore, analysed once.

FLN- and VLL-specific CTLs recognize endogenously presented HHV-8 target epitopes

A frequent feature of stimulations with synthetic peptides or with peptide-pulsed antigen-presenting cells is the induction of low-affinity CTLs that lyse target cells in the presence of relatively high concentrations of exogenous peptide, and that do not recognize epitopes from endogenously expressed antigens.[44,45] Therefore, to detect CTL activity against target cells that endogenously express viral antigens FLN- and VLL-specific CTL cultures were tested against cells expressing HHV-8. To this purpose, allogeneic HLA-A2-matched and HLA-A2-mismatched, PEL-derived cell lines were used as target in cytotoxicity experiments with FLN-specific and VLL-specific CTL cultures (Fig. 4).

Since gH is highly expressed in cells induced to lytic virus infection, the FLN-specific culture was tested against target cells induced or not with TPA or butyrate. The FLN-specific CTL culture lysed exclusively HLA-A2-matched BC-1 and BC-3 cells induced for lytic virus cycle, but not untreated HLA-A2-matched cells, nor untreated or treated HLA-A2-mismatched BCBL-1 cells. The VLL-specific CTL culture lysed HLA-A2-matched cells, but not the HLA-A2-mismatched BCBL-1 cell line. This demonstrates that FLN- and VLL-specific cultures obtained by peptide stimulation specifically recognize endogenously expressed HHV-8 antigens.

Detection of FLN- and VLL-specific CTL responses in HHV-8-seropositive individuals

We then evaluated the presence of CTL specific for FLN and VLL epitopes in four HHV-8-seropositive individuals

at risk of KS, three of which (P1, P3 and P4) were also HIV-positive and one (P2) was an elderly person of south Mediterranean origin. Memory CTL reactivation was performed by stimulation of PBLs with peptide-pulsed T2 cells, a stimulation protocol that was previously shown to be very effective in inducing only memory CTL responses[46] and that did not induce FLN- and VLL-specific responses in HHV-8-negative individuals (data not shown). CTL cultures obtained from the HHV-8-positive individuals after two consecutive stimulations were tested against HLA-A2-positive PHA-blasts pulsed or not with the relevant peptides. As shown in Fig. 5, all subjects responded to the LMP2-derived CLG, three subjects responded to the gH-derived FLN epitope (P1, P3 and P4) and two subjects responded to the kaposin A-derived VLL epitope (P1 and P2). In addition, FLN- and VLL-specific cultures obtained from HHV-8-positive donors by peptide-pulsed T2 cells lysed HLA-A2-matched, HHV-8-infected cells and not HLA-A2-mismatched, HHV-8-positive target cells (data not shown).

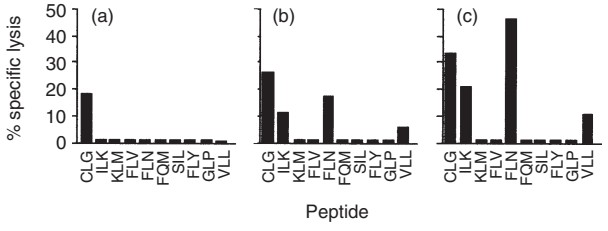

Figure 3. Kinetics of CTL reactivation induced by stimulation with synthetic peptides. Freshly isolated PBLs from the HHV8-seronegative donor D3 were stimulated with the indicated peptides. The first stimulation was performed with autologous DC pulsed with 10^{-5} M peptides, while subsequent stimulations were performed with peptide-pulsed T2 cells. Cytotoxic activity was assayed after the second (a), third (b) and fourth (c) stimulations against HLA-A2 single matched PHA blasts treated with 10^{-7} M of the different peptides for 1 hr prior to the assay. Results are expressed as the percentage specific lysis recorded at an E : T ratio of 20 : 1. None of the cultures killed untreated PHA blasts.

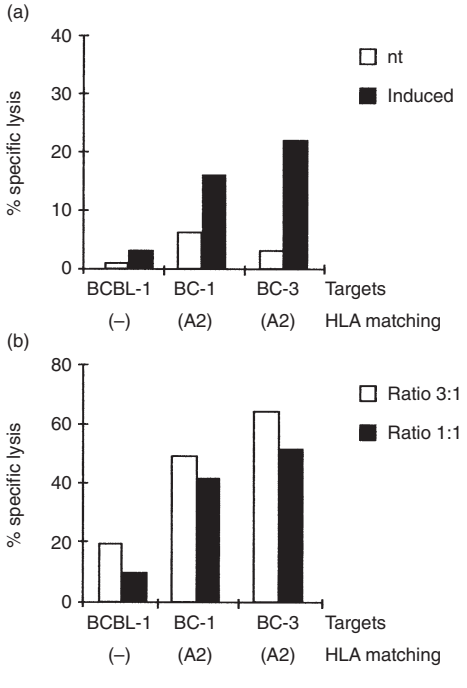

Figure 4. Cytotoxic activity of CTL cultures obtained by stimulation with FLN and VLL peptides. (a) FLN-specific CTL culture obtained from donor D1 was tested in cytotoxicity assays against the indicated target cells, induced or not to lytic infection as described in the Materials and Methods section. Results are expressed as the percentage specific lysis recorded at an E : T ratio of 10 : 1. (b) VLL-specific CTL culture obtained from donor D4 was tested in cytotoxicity assays against the indicated target cells. Results are expressed as the percentage specific lysis recorded at E : T ratios of 3 : 1 and 1 : 1. One representative experiment out of three is shown.

DISCUSSION

In this study we identified HLA-A2-presented CTL epitopes derived from kaposin A and gH, that are latent and lytic HHV-8 antigens, respectively. Primary CTL stimulation studies showed that the majority of healthy individuals respond to two HHV-8-derived CTL epitopes, namely, VLLNGWRWRL (amino acids 16–25, VLL), which derives from kaposin A, and FLNWQNLLNV (amino acids 59–68, FLN) which derives from gH.

CTL responses to FLN and VLL were also detected in individuals infected by HHV-8. CTL cultures raised against FLN and VLL peptides efficiently killed HLA-A2-matched,

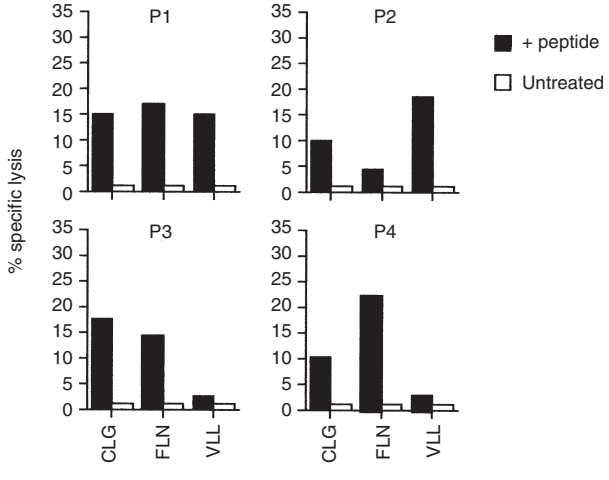

Figure 5. Reactivation of memory CTL responses in HHV-8 seropositive individuals. Freshly isolated PBL from four HLA-A2-positive, HHV-8-infected donors were stimulated twice with T2 cells pulsed with the control EBV-derived CLG epitope, or the HHV-8-derived FLN or VLL epitopes. Cytotoxic activity was assayed against HLA-A2 single-matched PHA blasts treated or not with 10^{-7} M of the indicated peptides for 1 hr prior to the assay. Results are expressed as the per cent specific lysis recorded at an E : T ratio of 10 : 1. One representative experiment out of three is shown.

but not HLA-mismatched, PEL cells. Moreover, killing of HLA-A2-matched PEL cells was inhibited upon treatment of target cells with anti-HLA class I antibodies (data not shown). Therefore, the data indicate that these two epitopes are relevant targets of CTL-mediated immunity *in vivo*, although we cannot exclude that kaposin or gH contain other immunogenic CTL peptides that do not span the HLA-A2 binding motif, or that were not identified by the CTL stimulation protocol used in this study.

A shorter version, peptide 17–25, of the peptide derived from kaposin A has been also recently identified by others as a target epitope of HHV-8-specific CTL responses in HHV-8-infected individuals.[47] However, we did not detect any killing of target cells treated with this nonamer (not shown). This suggests that peptide 16–25 is the minimal CTL epitope, as also indicated by very poor HLA-A2-binding capacity of peptide 17–25 (data not shown) and by the lower half-life of HLA-A2–peptide complexes predicted by the Parker program for the nonamer peptide as compared to the decamer (272 and 1008, respectively).

The peptide prediction algorithm that we used, though very useful for the determination of new CTL epitopes derived from known antigen sequences, did not show a stringent correlation between binding affinity and immunogenicity. However, it should be pointed out that the immunogenicity of peptide antigens depends also on other parameters including the presence of CTL precursors, the efficiency of peptide generation by proteasomes and the affinity with peptide transporters. Indeed, a new approach has been recently proposed for the identification of new naturally presented CTL epitopes. This approach combines prediction of peptide binding followed by binding assays with the analysis of *in vitro* proteasome-mediated digestions of polypeptides containing potential epitopes.[48]

In conclusion, we identified two CTL epitopes, derived from kaposin and gH, inducing CTL responses in the majority of seronegative individuals and memory CTL responses in HHV-8-seropositive individuals. CTL cultures specific for the two epitopes recognized cells that naturally presented the epitopes suggesting that CTL with these specificities may have a role in controlling HHV-8-infected cells. Further studies are needed to determine whether FLN- and VLL-specific HHV-8 CTL responses may have a role in preventing HHV-8-related diseases. This is relevant since the identified epitopes may be regarded as targets of specific immunotherapies for the treatment/prevention of HHV-8-associated malignancies. The passive transfer of *in vitro* amplified HHV-8-specific CTLs may represent a valid therapeutical approach relevant for treating HHV-8-infected, HIV-infected individuals that develop KS. It is tempting to speculate that reconstitution of HHV-8-specific CTL responses would control/treat KS. Indeed, it has been demonstrated that the recovery of immune responses, after highly active antiretroviral therapy, led to a decline in the incidence of KS in AIDS patients.[49] In addition, we have shown that peptide-pulsed DCs were able to induce primary CTL responses *in vitro*, suggesting their potential use for peptide vaccination.[16,17] A DC-based vaccine may have both a prophylactic and a therapeutic potential for the control of HHV-8-associated diseases in both HHV-8-seropositive or seronegative individuals with KS or PEL or at risk of HHV-8-related malignancies, including transplanted patients, elderly people, and HIV-positive individuals.

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