Clin. exp. Immunol. (1991) **84**, 501–507 ADONIS 000991049100178Q

Limiting-dilution analysis of the HLA restriction of anti-Epstein–Barr virus-specific cytolytic T lymphocytes

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(Accepted for publication 19 December 1990)

SUMMARY

Human Epstein–Barr virus (EBV) specific cytotoxic T lymphocytes (CTL) play an important role in maintaining the virus/host equilibrium during persistent infections. We analysed precursors of anti-EBV CTL by the limiting-dilution technique. Seven healthy EBV-seropositive and two EBV-seronegative donors were tested. All the donors seropositive for EBV gave clear-cut positive results, and it was remarkable that the frequency of CTL precursors (CTLp) observed was much higher than that reported for other viruses. In contrast, in the seronegative donors the frequency of CTLp was undetectable. The CTLp were derived from the $CD4^-CD8^+$ population only, although EBV-specific $CD4^+$ cytolytic T cell clones have been described. A study of the HLA restriction showed that some HLA-A or HLA-B antigens can function as preferential restricting molecules, but that CTLp restricted by the other HLA-A or HLA-B molecules also exist. However, the dominant population of CTL present in primary responses is sometimes different from that of long term cell lines established from the same donor.

Keywords Epstein–Barr virus liniting-dilution analysis cytotoxic T lymphocytes

INTRODUCTION

Following the primary infection with Epstein–Barr virus (EBV), whether clinically silent or manifest as infectious mononucleosis, EBV-specific memory T cells with cytotoxic potential persist in individuals. We have studied the number of cytotoxic T lymphocyte precursors (CTLp) in healthy seropositive donors by limiting-dilution analysis (LDA). This approach has two major interests. It allows quantification of the CTL response and characterization of the immune repertory of each donor with much more accuracy than any other technique; and its use makes possible the study of different subpopulations of CTL with a common target antigen and/or common restricting molecule and the following of their evolution without cloning. To our knowledge, the quantification of CTLp by LDA in circulating peripheral blood mononuclear cells (PBMC) has been applied to five human viruses: mumps virus (Enssle, Wagner & Fleischer, 1987) varicella-zoster virus (VZV) (Hickling, Borysiewicz & Sissons, 1987), human cytomegalovirus (CMV) (Borysiewicz *et al.*, 1988), herpes simplex virus (HSV) (Schmid, 1988), and influenza A virus (Bourgault *et al.*, 1989), but never to EBV, although recently the frequency of anti-EBV CTL in bulk culture after two *in vitro* stimulations was determined by Chen *et al.* (1989). The EBV system is remarkable for at least two reasons: it corresponds to a chronic viral infection persisting throughout life; and anti-EBV CTL are almost found after a secondary *in vitro* stimulation by autologous EBV-transformed lymphoblastoid cell lines (LCL), whereas only a small proportion of healthy donors produce CTL in the other viral systems. In this study, we aimed to determine whether: (i) all seropositive donors tested provided clear cut positive results in LDA; (ii) the number of CTLp was in the same range as that in other viral systems; (iii) the response was specific of EBV; (iv) the role of different HLA class I or class II molecules was the same in the presentation of EBV-associated antigens; and (v) the same populations of CTLp were present in primary response and in the course of the *in vitro* stimulations.

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MATERIALS AND METHODS

Human blood cells

PBMC were isolated by density gradient centrifugation on lymphocyte separation medium (MSL, Eurobio, France), and were used either immediately or stored at −180°C in liquid nitrogen. Seven healthy seropositive and two seronegative donors were tested. The HLA typing of the donors was performed either at the Centre National de Transfusion Sanguine (Paris and Poitiers) or at the Saint-Louis and Cochin Hospitals (Paris), using a standardized complement-mediated microcytotoxicity assay.

In vitro *induction of anti-EBV cell lines*

Human EBV LCL were obtained as previously described (Toubert *et al.*, 1984) with EBV produced by the B-95-8-E cell line. For the induction of anti-EBV CTL, primary cultures were performed by incubation of 2×10^6 PBMC with 5×10^4 irradiated (100 Gy) autologous EBV LCL in 2 ml of culture medium (RPMI 1640) supplemented with penicillin, streptomycin, glutamine, sodium pyruvate, non-essential amino acids, HEPES buffer (Flow Laboratories) and 10% pooled heat-inactivated human AB serum (SAB). The cultures were incubated in 24-well plates for 10 days. A 7-day secondary culture was performed in the same conditions using a 5:1 responder-to-stimulator cell ratio. Thereafter, continuously growing interleukin-2 (IL-2) dependent cell lines were established by weekly re-stimulations of 5×10^5/ml responder cells by 5×10^5/ml stimulator cells in culture medium supplemented with IL-2-containing supernatants (IL-2-SN) (5% final dilution) prepared as previously described (Healy *et al.*, 1988).

Cells fractionation

PBMC were incubated at a concentration of 10^7 cells/ml with either anti-CD3 monoclonal antibody (MoAb) (1/50) or anti-CD8 MoAb (1/50) (OKT3 or OKT8, Ortho Diagnostics) for 30 min at 4°C. Rabbit complement was then added to a final dilution of 1/7. After incubation for 30 min at 37°C, the cells were washed. T cells were purified by rosette formation with neuraminidase-treated sheep erythrocytes and subsequent serial centrifugations over MSL.

Cultures for limiting-dilution analysis (LDA)

Limiting dilution was performed as described by Langhorne & Fischer-Lindahl (1981) in the murine system, with some modifications for the human system. PBMC were seeded in limiting numbers in round-bottomed microwells (Titertek, Flow Laboratories) containing $2{\cdot}5 \times 10^4$ irradiated (100 Gy) autologous EBV LCL as stimulator cells, and 10^3 irradiated (40 Gy) autologous PBMC as feeder cells in 0·2 ml of culture medium. Twenty-four replicates were set up for each responder concentration. Subsequently, 5% IL-2-SN was added to each microwell at days 3 and 7. In preliminary experiments recombinant IL-2 was also used, but IL-2-SN was generally preferred since the results appeared either identical or slightly better with IL-2-SN.

Chromium release test

EBV LCL were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (CEA, France) for 1 h, washed twice and then used as target cells. In some experiments, Burkitt cell lines (a gift from Dr G. Lenoir) were also used as target cells. The cytolytic activity of LDA cultures was assayed after 10–14 days of culture, when proliferation (determined by optic microscope) was judged satisfactory. The cells were resuspended with a micropipette, then divided in two 100-μl aliquots and transferred to V-bottomed wells of microtitre trays. ^{51}Cr-labelled target cells (5×10^5) were then added (the final volume per well was 200 μl). Spontaneous release was determined in 12 control wells where the target cells were incubated with medium alone. Plates were incubated for 4 h at 37°C, after which 100 μl of supernatant were harvested from each well and analysed by a gamma counter. Results were expressed as specific chromium release (ct/min): 100 × (Experimental − Spontaneous release)/(Maximum − Spontaneous release). Cultures in which ^{51}Cr release exceeded the mean spontaneous release, usually less than 15% of total ^{51}Cr incorporated, by 8–10 % (i.e. always above 3 s.d.) were considered positive for cytolytic activity.

Determination of the frequency or cytolytic T lymphocyte precursors

Estimates of precursor frequency were obtained by the weighted mean method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of non-responding (negative) culture as described by Taswell (1981). The 95% confidence limit for each frequency was calculated. We verified, using the χ^2 test, that the points were aligned and that the kinetics were in line with the single hit model. The straight lines were traced only when the calculated χ^2 was inferior to the theorical χ^2 value for each degree of freedom. To determine whether two frequencies were significantly different, we used the χ^2 test.

RESULTS

Frequency and nature of anti-EBV CTLp in PBMC

Figure 1 illustrates the results of a typical LDA of anti-EBV CTLp, showing a frequency of 1/1000 cells. In all the seven donors tested, it appeared that the cytolytic activity was restricted by HLA molecules, since heterologous HLA-incompatible target cells were not lysed (Fig. 1a). Furthermore, the T cell nature of the effectors was confirmed by three additional observations: (i) the cytolytic activity was increased (1/220) when E^+ cells purified by rosetting were used as effectors (Fig. 1b); (ii) treatment of PBMC with anti-CD3 plus complement suppressed the response (Fig. 1c); and (iii) anti-CD8 plus complement treatment of PBMC greatly reduced the number of CTLp, indicating that the majority of these CTLp were $CD8^+$ cells (Fig 1d). The total number of anti-EBV CTLp found in the donors was approximately the same and always very high, from 1/400 to 1/3000 with unseparated PBMC.

Frequencies of CTLp in PBMC restricted by different HLA molecules

To separate CTLp subsets restricted by different HLA molecules, experiments were performed with effector and target cells compatible for only one class I or one class II molecule. It is noteworthy that in all experiments, the activity seemed to be due mostly to a single subset of CTL restricted by a single HLA specificity. This major restricting element (MRE) was responsible for 90–100% of the response. As shown in Table 1 for donor HC12 (HLA-A2/3, B7/60, Cw2/7, DR4/8), HLA-A3-restricted anti-EBV CTLp, identified by the use of target cells sharing only HLA-A3 with the CTL, represented 1/550 cells. This was almost identical to the frequency observed with autologous target cells.

However, the MRE varied from donor to donor, as shown in Table 1 with donor A69 (HLA-A3/24, B7/27, Cw2/7, DR4/5), where HLA-B7 acted as a MRE ($f = 1/840$), despite the presence of HLA-A3. In that case, HLA-A3 functioned at a very low level ($f < 1/8000$), as a minor restricting element (mRE), and HLA-A24 did not function at all. Other experiments (not shown) verified that A69 share the same HLA-A3 specificity as HC12 and the HLA-A3 target cells. With some donors, mRE appeared at intermediate levels, such as HLA-B27 for donor A69, since

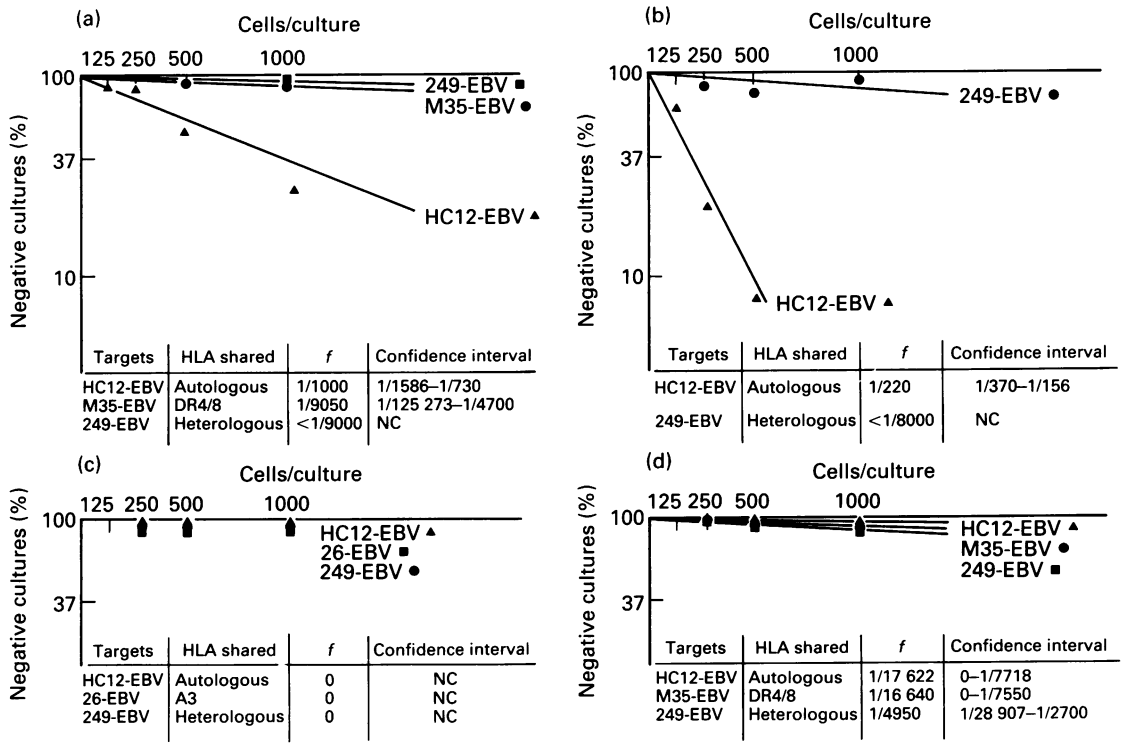

Fig. 1. Frequency analysis of anti-EBV cytotoxic T lymphocyte precursors from donor HC12. Responder cells were: (a) unfractionated peripheral blood mononuclear cells (PBMC); (b) E^+ cells purified by rosetting; (c) PBMC depleted of $CD3^+$ cells by treatment with an anti-CD3 MoAb plus complement at the beginning of the culture; (d) PBMC depleted of $CD8^+$ cells by treatment with an anti-CD8 MoAb plus complement at the beginning of the culture. Target cells were autologous, HLA-A3 compatible, or HLA-DR4/8 compatible or heterologous EBV lymphoblastoid cell lines.

Table 1. HLA-restriction of anti-EBV CTL induced in limiting dilution cultures

Effector cells*	Target cells	Shared HLA	Frequency	Confidence interval
HC12 anti-EBV	HC12-EBV	Autologous	1/540	1/790–1/407
	A65-EBV	A3	1/550	1/800–1/413
	D1-EBV	A2	1/3325	1/8910–1/2045
	9-EBV	B60	1/3930	1/12 768–1/2325
	D81-EBV	B7	1/4436	1/14 330–1/2624
	M13-EBV	Cw7	1/6930	1/67 830–1/3650
	M35-EBV	DR4/8	1/11580	0–1/5430
	249-EBV	Heterologous	1/5360	1/21 730–1/3060
A69 anti-EBV	A69-EBV	Autologous	1/1040	1/1922–1/711
	D666-EBV	B7	1/840	1/1320–1/615
	A19-EBV	B27	1/2217	1/4190–1/1507
	LES-EBV	A3	1/8619	1/93 275–1/4518
	21-EBV	A24	0	NC

* Effector cells were derived from peripheral blood mononuclear cells of donor HC12 (HLA-A2/3, B7/60, Cw5/7, DR4/8) or A69 (HLA-A3/24, B7/27, Cw2/7, DR4/5) after stimulation with autologous EBV LCL as described in Materials and Methods.

EBV, Epstein–Bart virus; CTL, cytotoxic T lymphocytes; NC, not calculable.

target cells sharing only HLA-B27 were lysed with a frequency of CTLp of 1/2200. The frequency of anti-EBV CTLp restricted by HLA-A or HLA-B mRE was often too slight to be calculated and HLA-Cw-restricted CTLp were never detected in these seven donors.

In the experiment illustrated in Table 1 with donor HC12, the number of class I-restricted CTLp was 1/550 for the MRE HLA-A3, and there appeared to be no class II-restricted CTLp. Similar results were found in repeated experiments using different donors, suggesting that class II-restricted CTLp are

Table 2. Role of fetal calf serum (FCS) in frequency analysis anti-EBV cytotoxic T lymphocyte precursors in donor HC12

Stimulator cells*	Target cells	Shared HLA	Frequency	Confidence interval
HC12-EBV-SAB	HC12-EBV-SAB	Autologous	1/1185	1/1915–1/858
	HC12-EBV-FCS	Autologous	1/1170	1/1952–1/836
	26-EBV-FCS	A3	1/653	1/1060–1/472
	249-EBV-FCS	Heterologous	0	NC
HC12-EBV-FCS	HC12-EBV-SAB	Autologous	1/830	1/1400–1/590
	HC12-EBV-FCS	Autologous	1/830	1/1400–1/590
	26-EBV-FCS	A3	1/760	1/1160–1/560
	249-EBV-FCS	Heterologous	1/11 750	0–1/3970

* Effector cells were derived from peripheral blood mononuclear cells of donor HC12 (HLA-A2/3, B7/60), Cw5/7, DR4/8) after stimulation with autologous EBV lymphoblastoid cell lines cultivated in human AB serum (SAB) or in FCS. Chromium release test was performed with target cells cultivated in SAB or in FCS.
EBV, Epstein–Barr virus; NC, not calculable.

Table 3. Specificity of anti-EBV cytotoxic T lymphocyte precursors in donor HC12

Effector cells*	Target cells†	Shared HLA	Frequency	Confidence interval
HC12-anti-EBV	HC12-EBV	Autologous	1/2933	1/7447–1/1826
	A65-EBV	A3	1/3624	1/9338–1/2248
	LCL-49	A3	1/1998	1/3626–1/1379
	BL-49	A3	0	NC
	02-AU-EBV	A2, B7	0	NC
	249-EBV	Heterologous	0	NC

* Effector cells were derived from peripheral blood mononuclear cells of donor HC12 (HLA-A2/3, B7/60, Cw5/7, DR4/8).

† Target cells were EBV lymphoblastoid cell lines or Burkitt tumour cell lines which share only HLA-A3 with donor HC12.

EBV, Epstein–Barr virus; NC, not calculable.

not present in PBMC, or that they are too scarce to be detectable. Furthermore, when $CD8^+$ cells were eliminated at the beginning of LDA in an attempt to overcome a possible down-regulation of $CD4^+$ CTL, detection of class II-restricted CTLp was still impossible in any of the experiments, as shown in Fig. 1d.

Specificity of anti-EBV CTLp

One of the major problems in the EBV system is that EBV non-infected LCL are not available as a control. It has been suggested that non-viral antigens, especially components of the FCS used in the culture medium could be involved in the CTL response (Misko, Kane & Hope, 1982; Torsteinsdottin *et al.*, 1986a). Experiments were therefore performed in which the stimulator and target cells were established and maintained in human SAB before beginning the LDA also in the absence of FCS. As shown in Table 2, the number of anti-EBV CTLp calculated in these conditions was 1/830 with stimulator cells maintained in FCS, and about 1/1180 with stimulator cells maintained in SAB. No statistically significant difference was therefore observed, whatever the serum used, for the maintenance of the target cells. It must be emphasized that in both cases the cytolytic activity was restricted by the same HLA class I molecule (HLA-A3), supporting the idea that the same antigen was involved.

Another possibility could be that part of the CTL population is directed against B cell-specific markers (Torsteinsdottin *et al.*, 1986b). In order to study further the specificity of the supposed anti-EBV-CTL, experiments were therefore carried out using autologous pokeweed mitogen-induced blasts as control target cells: LDA never revealed significant levels of CTLp with these targets (not shown). Furthermore, when EBV^+ LCL and autologous EBV^- Burkitt tumour cell lines compatible with donor HC12 only through HLA-A3 were tested as targets, a strong activity (1/2000) was found against LCL (LCL-49) but not against the Burkitt cells of the same patients (BL-49) (Table 3). The same results were found with donor A19 and HLA-A11 compatible target cells (not shown). These experiments therefore suggest that the target antigens of CTLp are not B-specific antigens or any other autologous antigen expressed in cultured B lymphocytes.

Furthermore, the comparison between donors seropositive and seronegative for EBV revealed very clear-cut differences since the two EBV seronegative donors tested were totally devoid of any CTLp anti-EBV reactivity (not shown).

Table 4. Evolution of HLA-restriction of anti EBV cytotoxic T lymphocyte precursors with donor A69

Number of *in vitro* stimulation	Target cells	Shared HLA	Frequency	Confidence interval
1	A69-EBV	Autologous	1/1040	1/1922–1/711
	D666-EBV	B7	1/840	1/1320–1/615
	A19-EBV	B27	1/2217	1/4190–1/1507
	LES-EBV	A3	1/8619	1/93 275–1/4518
	21-EBV	A24	0	NC
4	A69-EBV	Autologous	1/30	1/58–1/20
	D666-EBV	B7	1/81	1/114–1/63
	A19-EBV	B27	1/127	1/241–1/90
	21-EBV	A24	0	NC
6	A69-EBV	Autologus	1/86	1/137–1/62
	D666-EBV	B7	1/9030	0–1/14 466
	A19-EBV	B27	1/184	1/308–1/131
	21-EBV	A24	0	NC

* Limiting-dilution analyses were performed with peripheral blood mononuclear cells (1) of donor A69 (HLA-A3/24, B7/27, Cw2/7, DR4/5) or from cell line of donor A69 after three new stimulations (4) or after five new stimulations (6).

EBV, Epstein–Barr virus; NC, not calculable.

In vitro *evolution of anti-EBV CTL subsets*

A systematic study of the number of anti-EBV CTLp at different stages of the *in vitro* culture was carried out. In the experiments, a rapid enrichment of the number of CTLp was always found, reaching a plateau around the fourth or fifth *in vitro* restimulation using autologous LCL. For example, 1/2330 CTLp were found in the PBMC of donor A70, as compared with 1/16 CTLp at the fourth and 1/11 CTLp at the fifth restimulation. Concomitant with this evolution, the proportion of $CD8^+$ cells increased in the cultures so they represented at least 90% of the cells from the third *in vitro* stimulation onwards. Furthermore, the cytolytic activity of these bulk cultures as measured directly (without LDA) was always class I-restricted, as reported previously (Gomez *et al.*, 1989). It was interesting to verify whether the anti-EBV CTL selected *in vitro* were representative of the CTLp already dominant in the PBMC of the same donor. In many cases, the MRE found in PBMC remained dominant after the establishment of permanent *in vitro* cell lines and represented practically the total activity of the culture, as was the case with HLA-A3 and donor HC12 (not shown). Moreover, class II-restricted CTLp have never been found in any of the bulk cultures. However, in two other donors with an identical HLA typing, a different phenomenon was found: donor A69 had a primary *in vitro* anti-EBV response which was mediated by HLA-B7-restricted CTL (1/840), with a smaller HLA-B27-restricted subset (1/2217), and a very weak A3-restricted one (1/8619) (Table 4). After the third *in vitro* stimulation, comparable (not statistically different) activities were detected for HLA-B7 (1/81 CTLp) and HLA-B27-restricted CTL (1/127 CTLp), the frequency against autologous targets being 1/30. After five stimulations, the initial MRE (HLA-B7) practically disappeared (1/9030) whereas the HLA-B27-restricted subset constituted the MRE (1/184 CTLp). Similar results were found from several samples of donors A69 and also with donor A70 (the sister of donor A69), indicating that the MRE detected *in vitro* is not necessarily indicative of the *in vivo* situation. In the same experiments, the HLA-A3-restricted subset remained a MRE throughout the culture with no significant variations of activity, and this despite the high number of HLA-A3 target cells tested (not shown).

DISCUSSION

In our study, all the seven EBV seropositive donors tested evidenced high anti-EBV CTLp activities, whereas the only two available EBV seronegative donors were completely negative. It must be noted that with one of these EBV seronegative donors, a normal level of CTLp against influenza A virus was detected (1/18 000) (not shown). The major problem in the EBV system is the exact nature of the antigen(s) which induce a CTL response in healthy seropositive donors since uninfected LCL are not available. It has been suggested that the so-called anti-EBV CTL may in fact be directed against non-viral structures expressed on the LCL target cells. However, our experiments clearly ruled out the possible role of the FCS components since the same responses were found in the presence of SAB throughout the period of culture and during the CRT. The role of other than EBV antigens present on cultured B cells appears unlikely, since neither B cells blasts, nor autologous EBV^- Burkitt tumour cells from the same donor were lysed. Nevertheless, this last argument is not absolute to demonstrate the EBV specificity because some EBV^- Burkitt tumour cells may be not lysed in the absence of cell surface adhesion molecule LFA3 (Gregory *et al.*, 1988). In fact, the best argument is drawn from the comparison between seropositive and seronegative donors, whose frequency of CTLp was undetectable. Another problem may be the presence of autoreactive activity, since this is regularly observed in normal donors (Rosenkrantz, Dupont & Flomenberg, 1985). However, the role of autoreactive CTLp (1/3000 to 1/8000) appears negligible in our system, because clearly the number of

autoreactive CTLp is lower than the number of anti-EBV CTLp. Altogether, we can conclude that the measures of CTLp were EBV specific but we do not know what specific antigen is involved. In other experiments from our laboratory a peptide derived from the EBV-encoded latent membrane protein (LMP) of the virus, reported to be able to restimulate anti-EBV CTL (Thorley-Lawson & Israelsohn, 1987), did not function as a target antigen for the CTL (not shown). However, murine CTL have been found able to recognize LMP after transfection in murine cells (Reiss *et al.*, 1987) and this protein could be involved in the human system (A.B. Rickinson, personal communication). Furthermore, a peptide of LMP was recently identified as target of human CTL which show cross-recognition of the same peptide in the context of a mouse H-2K^d molecule (Murray *et al.*, 1990a). The role of a product of the EBNA-2 gene (Moss *et al.*, 1988) as well as that of a new gene created by circularization of the linear viral genome (Laux, Perricaudet & Farrell, 1988) must be also considered. Recently, a peptide from EBNA-3 protein (Murray *et al.*, 1990b, Burrows *et al.*, 1990a) and one from EBNA-6 protein (Burrows *et al.*, 1990b) were identified as target epitopes of anti-EBV T cell clones.

Previously data have shown that CTLp represent 1/540–1/8300 E$^+$ cells in response to mumps virus infection (Enssle *et al.*, 1987); 0–1/30 000 in the VZV system (Hickling *et al.*, 1987); 1/5000–1/20 000 in HCMV system (Borysiewicz *et al.*, 1988); and 1/3500–1/20 000 in the influenza A virus system (Bourgault *et al.*, 1989; and our unpublished data). The same situation exists with HSV-1 for which lower numbers of CTLp, (1/8000) have been found (Schmid, 1988). However, these experiments with HSV were performed using inactivated virus as the source of antigen, which could account for the lower responses and for the fact that CTLp detected were only CD4$^+$ class II-restricted CTLp. The differential stimulation of CD4$^+$ or CD8$^+$ subsets, depending on whether the virus was inactivated or not, has been previously demonstrated with VZV (Hickling *et al.*, 1987). Similar numbers of CTLp were found in BALB/c mice for anti-influenza virus responses (about 1/13 000 CTLp) (Wysocka & Bennink, 1988). Higher levels of CTLp were however described in mice for acute infection with herpes simplex (1/170–1/500 in CBA mice) (Hengel *et al.*, 1987) or influenza A virus (1/1200 in BALB/c mice) (Owen *et al.*, 1988), but in these two reports the LDA were performed with spleen or lymph node cells harvested a very short time (5 days) after hyper-immunization.

The strong anti-EBV CTL response from PBMC that we observed in LDA (1/550–1/2000) is therefore much greater than that reported in any other viral system in humans. This result was also obtained by Rickinson *et al.* (1981) using a different method (regression assay), and agrees with the observation that virtually 100% of donors gave a clear EBV-specific response, whereas no more than 25–30% of donors responded to influenza virus. We cannot exclude the role of different stimulating cells, since LCL produce by themselves many growth factors. Nevertheless, Chen *et al.* (1989), working with anti-EBV cell lines, have recently detected a frequency of CTL identical as that found in our experiments (1/25–1/120). The most likely hypothesis to explain the high frequency of anti-EBV CTLp in seropositive donors would be related to the chronic stimulation by viral antigens since, in contrast with mumps or influenza A virus, EBV persists in most people, with frequent endogenous reactivations. Similar *in vivo* stimulation could exist for anti-HIV1 CTLp. However, in this system LDA activity was not determine in PBMC but only at the level of cultured effector cells and the frequency was in the range from 1/150 to 1/1500 (Hoffenbach *et al.* 1988).

It must be emphasized that our effector cells are CD8$^+$. We did not find any class II-restricted anti-EBV CTL in these LDA experiments. This is in agreement with other experiments, where we have shown that class II-restricted antiviral CTL appear *in vitro* only after several antigenic stimulations and can be detected only after the elimination of CD8$^+$ cells (Gomez *et al.*, 1989). Furthermore, in most if not all cases, a single HLA class I molecule acts as a MRE in the LDA. Among MRE, we found only HLA-A and HLA-B molecules. Nevertheless, Chen *et al.* (1989) have recently suggested that CTL could be restricted by HLA-C molecules in LDA of bulk culture from a single donor. This confirms the results obtained from other methods in bulk cultures and suggests that only one, or at most a small number of antigens, could be involved in our experiments since it is clear that various peptide antigens are most often presented to T cells by different HLA molecules. Similar results using LDA from PBMC have been reported in the mumps virus system (Enssle *et al.*, 1987). However, subsets restricted by the mRE still persisted after several restimulations, demonstrating that CTL cell lines remained polyclonal *in vitro*. Interestingly, in two of our cases the dominant subset in an established cell line was different from that found in the primary *in vitro* response, suggesting that the *in vivo* dominant subset was not selected in culture. It would therefore be unwise to conclude from the observed dominant subset in permanent cell lines that the same subset is also dominant *in vivo*. Nevertheless, it must be emphasized that CTLp restricted by mRE can be evidenced among PBMC by LDA and not bulk culture. They are present *in vivo* and can be probably selected *in vitro* after several stimulations by the appropriate peptide, as demonstrated recently in the influenza system (Martinon *et al.*, 1990).

In conclusion, LDA is a valuable method to assess the whole *in vivo* immune repertory of each individual, and the evolution *in vitro* of CTL subsets restricted by different HLA molecules, as well as the respective strengths of the responses directed against various viral peptide antigens. It may also be of value as a test of efficiency of vaccination procedures.

ACKNOWLEDGMENTS

We would like to thank Dr Jean-Yves Muller and Dr Virginia Lepage for the HLA typing of donors, Dr Gilbert Lenoir for the gift of the Burkitt cell lines, Dr Franck Healy for his help and comments during the editing of the manuscript, Miss Bernadette Begue for expert technical assistance, and Miss Sandrine Vitteaud for typing the manuscript.

REFERENCES

Borysiewicz, L.K., Graham, S., Hickling, J.K., Mason, P.D. & Sissons, J.G.P. (1988) Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. *Eur. J. Immunol.* **18,** 269.

Bourgault, I., Gomez, A., Gomard, E., Picard, F. & Levy, J.P. (1989) A virus-specific CD4$^+$ cell-mediated cytolytic activity revealed by CD8$^+$ cell elimination regularly develops in uncloned human anti-viral cell lines. *J. Immunol.* **142,** 252.

Burrows, S.R., Sculley, T.B., Misko, I.S., Schmidt, C. & Moss, D.J. (1990a) An Epstein–Barr virus-specific cytotoxic T cell epitope in EBV nuclear antigen 3 (EBNA 3). *J. exp. Med.* **171,** 345.

BURROWS, S.R., MISKO, I.S., SCULLEY, T.B., SCHMIDT, C. & MOSS, D.J. (1990b) An Epstein–Barr virus-specific cytotoxic T-cell present on A- and B-type transformants. *J. Virol.* **64,** 3974.

CHEN, B.P., LAM , V., KRAUS, E.E., DEMARS, R & SONDEL, P.M. (1989) Restriction of Epstein–Barr virus-specific cytotoxic T cells by HLA-A, -B and -C molecules. *Hum. Immunol.* **26,** 137.

ENSSLE, K.H., WAGNER, H. & FLEISCHER, B. (1987) Human mumps virus-specific cytotoxic T lymphocytes: quantitative analysis of HLA restriction. *Hum. Immunol.* **18,** 135.

GOMEZ, A., BOURGAULT, I., GOMARD, E., PICARD, F. & LEVY, J.P. (1989) Role of different lymphocyte subsets in human antiviral T cell cultures. *Cell Immunol.* **118,** 312.

GREGORY, C.D., MURRAY, R.J., EDWARDS, C.F. & RICKINSON, A.B. (1988), Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein–Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. *J. exp. Med.* **167,** 1811.

HEALY, F., SIRE, J., GOMARD, E., YSSEL, H., JORDAN, B.R. & LEVY, J.P. (1988) A study of functionally active amino acids involved in the interaction of HLA-A2 or HLA-A3 molecules with cytolytic T lymphocytes. *J. Immunol.* **141,** 2487.

HENGEL, H., LINDNER, M., WAGNER, H. & HEEG, K. (1987) Frequency of herpes simplex virus-specific murine cytotoxic T lymphocyte precursors in mitogen- and antigen-driven primary in vitro T cell responses. *J. Immunol.* **139,** 4196.

HICKLING, J.K., BORYSIEWICZ, L.K. & SISSONS, J.G.P. (1987) Varicella-Zoster virus-specific cytotoxic T lymphocytes (Tc): detection and frequency analysis of HLA class I-restricted Tc in human peripheral blood. *J. Virol.* **61,** 3463.

HOFFENBACH, A., LANGLADE-DEMOYEN, P., DADAGLIO, G., VILMER, E., MICHEL, F., MAYAUD, C. AUTRAN, B. & PLATA, F. (1989) Unusually high frequencies of HIV-specific cytotoxic T lymphocytes in humans. *J. Immunol.* **142,** 452.

LANGHORNE, J. & FISHER-LINDAHL, K. (1981) Limiting dilution analysis of precursors of cytotoxic T lymphocytes. *J. immunol. Methods.* **ii,** 221.

LAUX, G., PERRICAUDET, M. & FARRELL, P.J. (1988) A spliced Epstein–Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. *EMBO J.* **7,** 769.

MARTINON, F., GOMARD, E., HANNOUN, C. & LEVY, J.P. (1990) In vitro human T cell responses against influenza A virus can be induced and selected by synthetic peptides. *Eur. J. Immunol.* **20,** 2171.

MISKO, I.S., KANE, R.G. & HOPE, J.H. (1982) Generation in vitro of HLA-restricted EB virus-specific cytotoxic human T cells by autologous lymphoblastoid cell lines: the roles of previous EB virus infection and foetal calf serum. *Int. J. Cancer* **29,** 41.

MOSS, D.J., MISKO, I.S., BURROWS, S.R., BURMAN, K., MCCARTHY, R. & SCULLEY,, T.B. (1988) Cytotoxic T-cell clones discriminate between A- and B-type Epstein–Barr virus transformants. *Nature*, **331,** 719.

MURRAY, R.J., BROOKS, J.M., RICKINSON, A.B. & ROWE, M. (1990a). Cross-recognition of a mouse H-2-peptide complex by human HLA-restricted cytotoxic T cells. *Eur. J. Immunol.* **20,** 659.

MURRAY, R.J., KURILLA, M.G., GRIFFIN, H.M., BROOKS, J.M., MACKETT, M., ARRAND, J.R., ROWE, M., BURROWS, S.R., MOSS, D.J., KIEFF, E. & RICKINSON, A.B. (1990b) Human cytotoxic T-cell responses against Epstein–Barr virus nuclear antigens demonstrated by using recombinant vaccinia viruses. *Proc. natl. Acad. Sci USA*, **87,** 2906.

OWEN, J.A., DUDZIK, K.I., KLEIN, L. & DORER, D.R. (1988) The kinetics of generation of influenza-specific cytotoxic T-lymphocyte precursor cells. *Cell Immunol.* **111,** 247.

REISS, C.S., WANG, D., GHOSH, D., GAPOSCHKIN, C. & KIEFF, E. (1987) Recognition of EBV plasma membrane protein expressed on murine cells after gene transfer. *J. Immunol.* **139,** 711.

RICKINSON, A.B., MOSS, D.J., WALLACE, L.E. ROWE, M., MISKO, I.S., EPSTEIN, M.A. & POPE, J.H. (1981) Long-term T-cell-mediated immunity to Epstein–Barr virus. *Cancer Res.* **41,** 4216.

ROSENKRANTZ, K., DUPONT, B. & FLOMENBERG, N. (1985) Generation and regulation of autocytotoxicity in mixed lymphocyte cultures: evidence for active suppression of autocytotoxic cells. *Proc. natl. Acad. Sci. USA*, **82,** 4508.

SCHMID, D.S. (1988) The human MHC-restricted cellular response to herpes simplex virus type 1 is mediated by $CD4^+$, $CD8^-$ T cells and is restricted to the DR region of the MHC complex. *J. Immunol.* **140,** 3610.

TASWELL, C. (1981) Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126,** 1614.

THORLEY-LAWSON, D.A. & ISRAELSOHN, E.S. (1987) Generation of specific cytotoxic T cells with a fragment of the Epstein–Barr virus-encoded p63/latent membrane protein. *Proc. natl Acad. Sci. USA*, **84,** 5384.

TORSTEINSDOTTIR, S., MASUCCI, M.G., BRAUTBAR, C., LENOIR, G., KLEIN, G. & KLEIN, E. (1986a) Differential recognition of tumor-derived and in vitro Epstein–Barr virus-transformed B cells lines by fetal calf serum-specific T4-positive cytotoxic T-lymphocyte clones. *Cell Immunol.* **98,** 453.

TORSTEINSDOTTIR, S., MASUCCI, M.G., EHLIN-HENRIKSON, B., BRAUTBAR, G., BEN BASSAT, H., KLEIN, G. & KLEIN, E. (1986b) Differentiation-dependent sensitivity of human B-cell-derived lines to major histocompatibility complex restricted T-cell cytotoxicity. *Proc. Natl Acad. Sci. USA*, **83,** 5620.

TOUBERT, A., GOMARD, E., GRUMET, F.C., AMOR, B., MULLER, J.Y. & LEVY, J.P. (1984) Identification of several functional subgroups of HLA-B27 by restriction of the activity of antiviral T killer lymphocytes. *Immunogenetics*, **20,** 513.

WYSOCKA, M. & BENNINK, J.R. (1988) Limiting dilution analysis of memory cytotoxic T lymphocytes specific for individual influenza virus gene products. *Cell Immunol.* **112,** 425.