Clin. exp. Immunol. (1990) **79**, 409–415

Absence of Epstein–Barr virus-specific, HLA class II-restricted CD4$^+$ cytotoxic T lymphocytes in infectious mononucleosis

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(*Accepted for publication 18 October 1989*)

SUMMARY

Cytotoxic T lymphocytes (CTL) with the CD4$^+$ phenotype that recognize major histocompatibility complex (MHC) class II antigens are detectable very frequently in cultures of human alloreactive or virus-specific T cells. The significance of these CD4$^+$ CTL for an immune reaction *in vivo* is not clear. Since Epstein–Barr virus (EBV) transformed B cells express HLA-class I and class II antigens equally well both CD8$^+$ and CD4$^+$ CTL should be stimulated during an acute EBV infection. We analysed the MHC specificity and the phenotype of EBV-specific CTL from patients with infectious mononucleosis (IM). When tested directly without any previous culture, T cells from patients in the acute phase of IM showed specific MHC-restricted cytotoxicity against the autologous B cell line. Addition of a HLA class I specific monoclonal antibody (MoAb) but not of a HLA class II specific MoAb resulted in a complete blocking of the lytic activity. Cell sorting revealed that the entire cytotoxic activity was present in the CD8$^+$ fraction whereas no specific CTL were detectable in the CD4$^+$ fraction. The absence of cytotoxicity in CD4$^+$ cells was not due to a lack of activation of these cells since both CD8$^+$ and CD4$^+$ cells were activated *in situ*, showing spontaneous growth in interleukin-2 (IL-2) and expressing the activation marker TP103. Frequency estimation revealed that 1/300–1/600 CD8$^+$ but only 1/2000–1/4000 CD4$^+$ T cells gave rise to a specific CTL colony after 10 days. If CD4$^+$ colonies were tested repeatedly for cytotoxicity we found that CD4$^+$ CTL acquired their cytotoxicity during *in vitro* culture. In addition, we isolated EBV-specific CD4$^+$ T cell clones able to lyse their stimulator cells in the presence but not in the absence of lectin, even after a long period of culture. Taken together our results show that cytotoxicity mediated by CD4$^+$ T cells does not play a role in an anti-viral immune response.

Keywords CD4$^+$ T lymphocytes cytotoxic T lymphocytes Epstein–Barr virus infectious mononucleosis

INTRODUCTION

Restriction of T cells by MHC class I or class II molecules correlates in most cases well with the CD8 or CD4 phenotype. In contrast, there is no clear correlation between phenotype and function of a T cell. CD4$^+$ and MHC class II restricted cytotoxic T lymphocytes (CTL) were demonstrated in T cell lines (Krensky *et al.*, 1982b) and T cell clones (Moretta *et al.*, 1981; Biddison *et al.*, 1982; Krensky *et al.*, 1982a; Spits *et al.*, 1982). However, analysis of CTL after stimulation with alloantigens or viral antigens for 5–7 days shows a nearly exclusive occurrence of CTL with the CD8 phenotype that are restricted by MHC class I molecules. Such CTL were found, e.g. in the primary and secondary CTL response against mumps virus of patients with acute mumps virus infection (Kreth *et al.*, 1982), with secondary Epstein–Barr virus (EBV) specific CTL after bulk culture (Wallace *et al.*, 1982) or in a limiting dilution analysis of virus-specific CTL (Enssle, Wagner & Fleischer, 1987). In contrast, cloning studies of anti-viral *in vitro* stimulated CTL showed frequently or even exclusively CD4$^+$ MHC-class II restricted CTL (Jacobson *et al.*, 1984; Meuer *et al.*, 1983; Misko *et al.*, 1984; Yasukawa & Zarling 1984).

CD4$^+$ and class II restricted T cell clones with specificity for influenza A virus or alloantigens can acquire cytotoxic activity after *in vitro* culture (Fleischer, 1984). Thus it is possible that all the CD4$^+$ CTL found in anti-viral immune reactions might have acquired their cytotoxic activity *in vitro*. However, CD4$^+$ CTL may be important effector cells in a virus infection *in vivo* (Braakman *et al.*, 1987). In this report we tried to find evidence for the occurrence of CD4$^+$ CTL in an anti-viral immune reaction *in vivo*.

In patients with infectious mononucleosis (IM), EBV-transformed B cells serve as stimulator and target cells for EBV-specific T cells. EBV-transformed B cells have a relative

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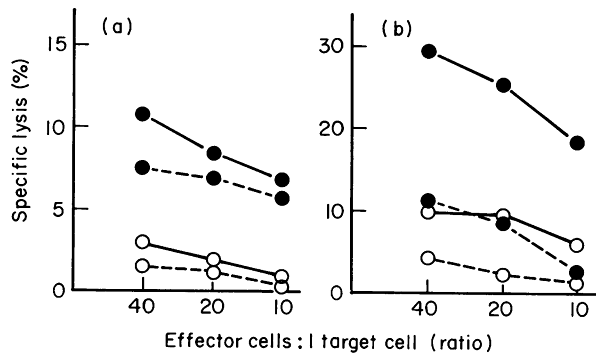

Fig. 1. Epstein-Barr virus (EBV) specific cytotoxic activity measured directly in tonsillar (——) and peripheral blood (- - - -) T cells of donor A and B. Cytotoxic activity against the autologous (●) and allogeneic (○) target cells is shown.

constant expression of HLA class II and class I molecules during cell cycle (Matsui *et al.*, 1986). In IM, therefore, class II restricted CD4+ CTL should be as easily activated as CD8+ T cells. We tested enriched T cell populations from IM patients for specific cytotoxicity before and after *in vitro* culture. We describe evidence that CD4+ T cells are not cytotoxic *in vivo* in the acute phase of infection but acquire lytic activity during culture. In addition, we isolated cytotoxic T cell clones in which triggering of proliferation and cytotoxicity could be dissociated: antigen recognition led to proliferation but not cytotoxicity, and specific cytotoxicity was not acquired even after a long period of culture.

MATERIALS AND METHODS

Patients

Tonsils were taken from two patients (A, B) with acute IM. Both patients had anti-VCA IgM antibodies in their serum and were negative for anti-EBNA antibodies. Tonsillectomy was performed because of obstruction and difficulties to swallow. Tonsils were also obtained from a patient (C) several weeks after IM. At the day of tonsillectomy this patient was negative for anti-VCA IgM but had anti-EBNA antibodies.

Cells

Tonsils were finely minced with scissors under sterile conditions and passed through a sieve in RPMI 1640 culture medium. Mononuclear cells (MNC) were isolated out of this single cell suspensions by centrifugation on Ficoll–Hypaque gradients and stored in liquid nitrogen. T cells were enriched by separating rosette-forming cells with neuraminidase-treated sheep erythrocytes. Transformation of B lymphocytes was performed by infection with B95-8 virus. HLA serotyping was kindly performed by Drs T. Eiermann and S. F. Goldmann (DRK-Blutspendezentrale, Ulm).

Cloning of T cells

T lymphocytes were cloned by seeding of 0·5 T cells per well in 60-well Terasaki plates (Nunc, Roskilde, Denmark) using RPMI 1640 supplemented with 25 U/ml recombinant interleukin-2 (IL-2) and 50% PHA-TCGF as previously described (Fleischer & Kreth, 1983).

Recombinant IL-2 was kindly provided by Dr D. Amerding, Sandoz Research Institute, Vienna, Austria. MNC (1×10^4) irradiated with 4000 rad from a caesium source served as feeder cells. After 8–10 days positive cultures were set in 96-well round-bottomed microtitre plates (Nunc) with the same culture medium and 4×10^5 irradiated feeder cells per well. Expanded cultures were tested for lytic activity by a PHA-mediated unspecific readout and phenotyped using immunoperoxidase staining. Immunoperoxidase testing was performed with cells fixed on Terasaki plates (Holzmann & Johnson, 1983) using monoclonal antibody (MoAb) BMA 041 (anti-CD4 MoAb) and BMA 081 (anti-CD8). Clones of interest were expanded for further testing in RPMI 1640 supplemented with 25 U/ml recombinant human IL-2 (Fleischer, 1988).

Cell fractionation

T cells were purified by E-rosetting and stained with fluorescein- or phycoerythrin-conjugated MoAbs for 30 min on ice. When CB.1 was used, cells were stained with fluorescein-conjugated goat anti-mouse antibodies as second reagent. After washing the cells twice they were fractionated in an EPICS V cell sorter (Coulter, Hialeah, FL). Purity of sorted fractions as assessed by reanalysis was usually 95%.

Antibodies

The following antibodies were used in this study: W6/32, and BBM.1, specific for HLA class I, and L243, specific for HLA-class II, were obtained from the American Tissue Type Culture Collection (Rockville, MD). Leu 2a, coupled with FITC, specific for CD8, and Leu3a, coupled with phycoerythrine, specific for CD4, from Becton Dickinson (Mountain View, CA); CB.1, specific for Tp103 (Fleischer, 1987); Tac-1, specific for human IL-2 receptor, from Dr T. Waldmann (National Institutes of Health, Bethesda, MD); NKH-1, specific for a human natural killer (NK) cell antigen (Coulter); Leu4, coupled with phycoerythrine, specific for CD3 (Becton Dickinson); Leu11a, coupled with FITC, specific for CD16 (human NK cell antigen) (Becton Dickinson); BMA 030, specific for CD3 (Behringwerke); BMA 031, specific for the human $\alpha\beta$ T cell receptor (Behringwerke); and goat anti-mouse $F(ab')_{2'}$ coupled with FITC (Tago, Burlingame, CA).

Limiting dilution culture

Culture under limiting dilution condition was performed as described (Enssle *et al*, 1987). Graded numbers of responder cells (purified T cells or sorted CD8+ or CD4+ cells) were cultured with 150 autologous irradiated (4000 rad) transformed B lymphocytes as stimulator cells. The same number of stimulator cells was added after 2 days of culture. Culture was performed in 0·2-ml volumes in 96-well round-bottomed microtitre plates (Nunc). Culture medium was RPMI 1640 containing antibodies, 5% heat-inactivated human male **AB** serum, and 25 U/ml recombinant human IL-2. Cultures were assayed for cytotoxicity after 10 days.

Cytotoxicity assay

A standard ^{51}Cr release assay was performed as described (Fleischer, 1984; Enssle *et al.*, 1987). If target cells are not indicated at the corresponding experiment, the EBV-transformed B cell line RB4 (effector cells A and C) or a EBV-line from patient A (effector cells from patient B) were used as

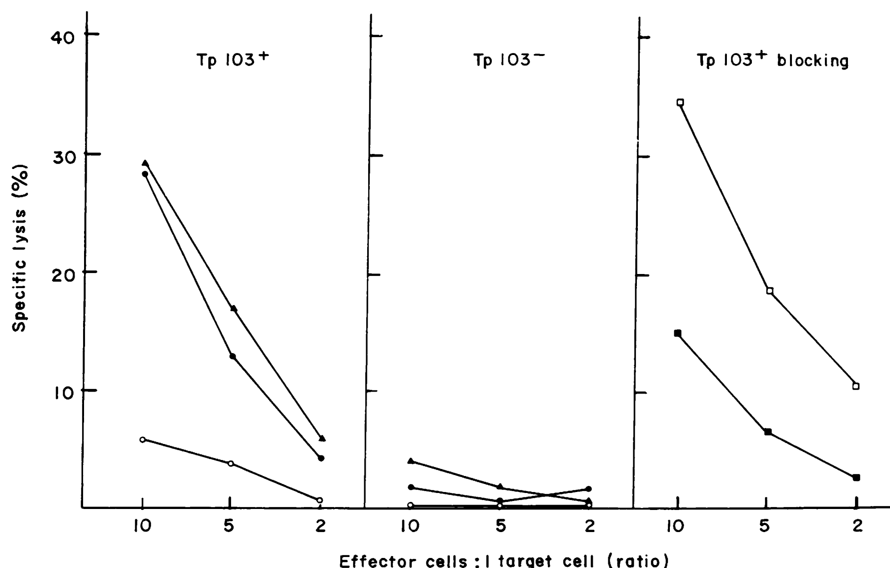

Fig. 2. Analysis of the specific cytotoxic activity in Tp103-positive and Tp103-negative cell fractions (donor B) after cell sorting without *in vitro* culture. The blocking experiments were performed by adding the monoclonal antibodies BBM.1 (■) (anti-class I) or L243 (□) (anti-class II) to the assay at a final dilution of ascites of 1:200. (▲), allogeneic+PHA; (●), autologous; (○), allogeneic.

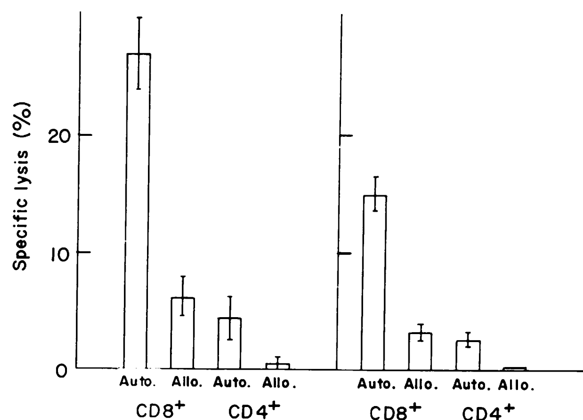

Fig. 3. Cytotoxic activity of $CD8^+$ and $CD4^+$ fractions. Enriched T cells from patients A (right) or B (left) were stained with Leu-3a (specific for CD4, phycoerythrin-conjugated) and Leu-2a (specific for CD8, FITC-conjugated), sorted in these fractions and tested without further *in vitro* culture. EBV-transformed B cell lines from donor A or B were used vice versa as autologous or allogeneic target cells.

allogeneic target cells. When effector cells from limiting dilution cultures were used, individual microcultures were resuspended at day 10 of culture and aliquots were distributed into V-shaped microtitre plates (Greiner, Nürtingen, FRG). Target cells (1000/well) were added to a final volume of 0·2 ml. Spontaneous release was measured by testing 16 or 24 wells that had been cultured only with stimulator cells. Maximum release was measured by vigorously resuspending several cultures before harvesting. Percent specific release was calculated as:

$$\frac{\text{Experimental–Spontaneous release}}{\text{Maximum–Spontaneous release}} \times 100.$$

Assay for proliferation

The assay was performed as previously described (Fleischer, 1983).

Statistical analysis

Mean spontaneous release +2 s.d. usually gave a 'cut' to separate positive from negative cultures around 3% specific lysis. To exclude non-specifically lysing cultures from the analysis, the cut was arbitrarily set at 5% specific lysis. Frequency analysis, probability for single hit conditions (p), and the definition of the 95% confidence interval were determined by means of a computer program based on statistical methods described by Taswell (1981). Probability of clonality was determined as described by Miller (1982).

RESULTS

Phenotypical analysis of tonsillar CTL

Tonsillar T cells showed a higher degree of specific lysis than did peripheral blood lymphocytes when tested against autologous and allogeneic EBV-transformed B cells of K562 cells as targets (Fig. 1). Apparently tonsillar tissue is enriched with anti-EBV reactivity as a primary site of virus infection. Therefore we used for further experiments T cells of tonsillar origin.

Cell sorter analysis of enriched tonsillar T cells revealed a CD4:CD8 ratio of 1·5 and 1·8 in patients A and B with acute IM and of 4·5 in patient C. In order to check for *in vivo* activated T cells we stained with the MoAb CB.1 specific for the activation marker Tp103 that is preferentially expressed on activated T cells (Fleischer, 1987). In contrast to tonsillar T cells of patient C (5% CB.1 positive) enriched T cells from the patients A and B had 27% and 30%, respectively, of *in vivo* activated Tp103-expressing T cells. The enrichment of $CD8^+$ T cells and the increase in activated T cells in tonsils of patients A and B correlated well with the serological findings of an acute IM in these two patients.

Table 1. Frequency analysis of tonsillar cytotoxic T cells (CTL)

Patient	Time of culture (day)	Addition on day 10	Phenotype of responder cells	Autologous target cells			Allogeneic target cells		
				1: frequency	95% confidence limits	P	1: frequency	95% confidence limits	P
A	10		CD8	566	448–769	0·07	ND	—	—
	10		CD4	2226	1751–3055	0·19	5397	3985–8362	0·25
	17	Medium	CD4	1108	881–1491	0·08	4571	3397–6982	0·98
B	10		CD8	333	239–549	0·24	ND	—	—
	10		CD4	3767	2614–6736	0·21	9799	6569–19277	0·65
	17	Medium	CD4	1190	957–1573	0·84	3890	2910–5864	0·35
	17	IFN-γ	CD4	1251	997–1679	0·72	6970	4909–12016	0·06
C	10		CD8	213	172–279	1·00	3148	2038–6912	0·79
	10		CD4	3141	2403–4532	0·32	6855	4889–11465	1·00
	17	Medium	CD4	1139	917–1503	0·14	ND	—	—
	17	IFN-γ	CD4	1038	835–1371	0·65	ND	—	—

After a simultaneous staining with monoclonal antibody directed against CD4 (Leu-3a, conjugated with phycoerythrine) and CD8 (Leu-2a, conjugated with FITC), enriched T cells were sorted in these subpopulations and cultured under limiting dilution conditions (50–3200 cells/well). The cultures were split at day 10 and tested against autologous and allogeneic target cells. Two aliquots from each microculture of the $CD4^+$ cells were restimulated with autologous B-LCL cells at day 10 and tested again at day 17. Recombinant gamma-interferon (IFN-γ) was added to one aliquot as indicated. ND, not determined—frequency too low to be calculated.

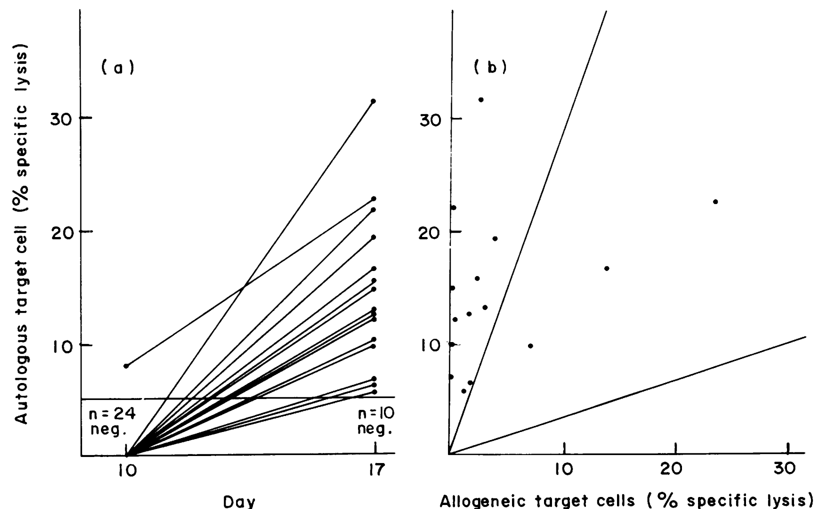

Fig. 4. Acquisition of specific cytotoxicity by $CD4^+$ tonsillar cells from donor A. The responder cells were expanded under limiting dilution conditions and tested after 10 days and 17 days of culture for specific cytotoxicity against autologous and a HLA mismatched allogeneic EBV-transformed target cells. (a), Specific lysis of autologous targets at day 10 and day 17; (b), specificity analysis of cytotoxicity at day 17.

Functional analysis of tonsillar CTL

Direct specific cytotoxicity of uncultured enriched T cells against autologous EBV-transformed target cells and not HLA-A, B, C allogeneic target cells was only found with tonsills A, B but not C (not shown). For a functional definition of the activated tonsillar T cells, enriched T cells from patient A and B were stained with CB.1 and sorted in Tp103-positive and Tp103-negative fractions. Only the Tp103-positive cell fraction contained blast-like cells (not shown).

The phenotypical analysis of the sorted Tp103-positive fraction of tonsil B revealed that this population consisted of 69% $CD8^+$, 30% $CD4^+$, 90% $Tp103^+$ and 90% Tac^+ cells. When Tp103-positive and -negative fractions were tested for cytotoxic activity it was found that the entire virus-specific cytotoxic activity was present in the Tp103-positive, but not Tp103-negative cells (Fig. 2). Lysis could only be blocked by addition of anti-class I MoAb (BBM.1) but not with anti-class II MoAb (L243). Using cells from patient A the same results were found. Even in the presence of PHA the Tp103-negative cells showed no cytotoxic activity. These results show that both $CD4^+$ and $CD8^+$ T cells are activated *in vivo* and that the lytic activity of the *in vivo* activated cells is correlated with the

Table 2. Proliferative and cytotoxic activity of $CD4^+$ clones from a patient (A) with acute infectious mononucleosis

	^{3}H-thymidine incorporation after stimulation of clones with			% lysis of target cells tested with	
Clone	Medium	Autologous B cell line	Allogenic B cell line	Autologous B cell line	Autologous B cell line+PHA
68	148±23	15472±3208	188±17	2±1	24±1
82	173±96	6707±497	90±3	2±1	26±3
84	97±13	8598±2712	105±24	1±1	13±1
1T14	246±86	10944±1847	142±19	5±1	48±4
CD8 line	4015±845	30975±2679	6711±345	22±1	36±1

The test was performed after 102 days of total culture time. Clones 81, 82 and 84 were cloned out of limiting dilution cultures, 1T14 was generated as described in Results. The $CD8^+$ line was propagated by a initial culture of enriched T cells of patient A in recombinant IL-2 containing culture medium, followed by repeated specific restimulation with the autologous B cell line. The test for specific proliferation was performed by stimulation of 1×10^4 responder cells with 3×10^4 mitomycin C treated autologous or allogeneic EBV-transformed B cell lines. ^{3}H-thymidine was added after 24 h and after 48 h total culture time the cells were harvested. Cytotoxic activity was determined at a ratio of effector-to-target cells of 5:1. PHA was added to a final concentration of 5 μg/ml. The allogeneic B-LCL (RB4) was completely HLA-mismatched.

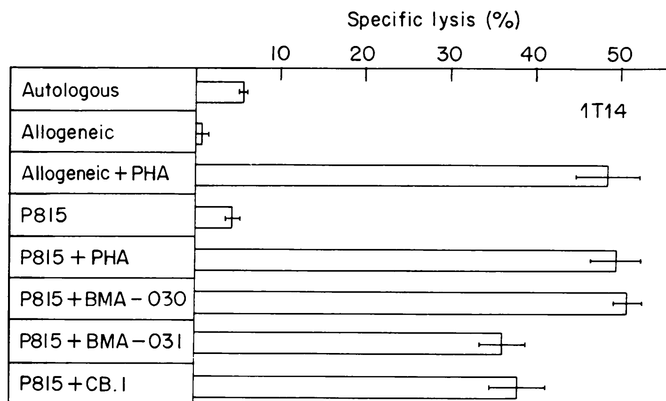

Fig. 5. Cytotoxic activity of clone 1T14 after culture of 102 days (origin donor A). Clone IT14 was CD4-positive and CD8-negative as analysed repeatedly during culture and shortly before the experiment. The test was performed with a ratio of 5:1 between effector and target cells. For non-specific triggering of cytotoxic activity, PHA was used with 10 μg/ml, BMA 030 (anti-CD3) with 0·5 μg/ml, BMA (anti-$\alpha\beta$ T cell receptor) with 10 μg/ml and CB.1 (anti-Tp103; ascites) with a 1:500 dilution (final dilution in the assay).

expression of the activation marker Tp103.

In order to analyse the cytotoxic activity in the $CD4^+$ and $CD8^-$ and $CD8^+CD4^-$ subpopulations, T cells of patients A and B were stained with MoAbs directed against CD8 (Leu2a, FITC-conjugated) and CD4 (Leu3a, phycoerythrin-conjugated). The stained T cells were then sorted in $CD8^+$ and $CD4^+$ populations and tested directly for specific cytotoxic activity without culture *in vitro*. As demonstrated in Fig. 3, the entire cytolytic activity was detected in the $CD8^+$ subpopulations. No significant cytotoxic activity of CD4 cells was visible. To exclude blocking effects on CD4 or CD8 molecules by residual antibodies bound to these molecules from staining, cells were incubated for about 20 h in culture medium with recombinant IL-2 and tested then, for cytolytic activity. No specific cytotoxicity was detectable in the $CD4^+$ fraction (data not shown).

Acquisition of specific cytotoxicity in the $CD4^+$ population after culture in vitro

To obtain evidence about the clonal distribution of $CD8^+$ and of $CD4^+$ CTL after culture *in vitro*, experiments under limiting dilution conditions were performed. Graded numbers of sorted $CD8^+$ $CD4^-$ and $CD4^+$ $CD8^-$ T cells were cultured with 100–150 irradiated autologous EBV-transformed B lymphocytes per microculture. Previous experiments had shown that these low numbers of stimulator cells were sufficient for optimal stimulation and necessary for a specific response. With higher numbers, an unspecific polyclonal response was observed (Kabelitz *et al.*, 1985). In order to quantify the EBV-specific cytotoxic response, CTL frequencies in sorted $CD4^+$ and $CD8^+$ subpopulations were estimated (Table 1). As expected, the experiments showed marked differences in the CTL response for CD8 (1/213–1/566) and $CD4^+$ cells (1/2226–1/3767).

The still relatively high frequencies of $CD4^+$ CTL after 10 days of culture were surprising, because we had not been able to detect any cytotoxic activity in the uncultured $CD4^+$ populations. In order to test the influence of culture time *in vitro* on the amount of $CD4^+$ $CD8^-$ CTL in the populations we performed split experiments. Microcultures were tested on day 10 of culture for cytotoxic activity. One aliquot of each microculture was restimulated with fresh irradiated autologous EBV-transformed B cells and tested again at day 17 for specific cytotoxicity. In Fig. 4 data from 25 microcultures (patient A, probability of clonality > 80%) are summarized. These cultures were controlled microscopically to contain similar cell numbers per microwell at day 10 and 17. Cells of some typical cultures were counted at day 10 and day 17; the effector-to-target cell ratio was approximately the same on both days, usually less than 40:1; 14 of 25 microcultures showed a clear acquisition of specific cytotoxic activity

from day 10 to day 17 of culture, in one culture with low cytotoxicity at day 10, cytotoxic activity increased. Twelve out of 15 cultures were highly specific for the autologous target cells (Fig. 4b). In Table 1 the frequencies of EBV-specific CTL for patients A and B at day 10 and day 17 are given. An increase in frequency of CD4+ CTL by a factor of 2–3 was found in all cases. Addition of interferon-gamma (IFN-γ; 100 U/ml, Thomae, Biberach, FRG) did not lead to a further increase in CTL frequency. The same acquisition of cytotoxicity was found with CD4+ colonies derived from patient C (Table 1). In contrast, all CD8+ colonies had a high cytotoxic activity already on day 10 (up to 35% specific lysis, data not shown). Taken together these data show that a large percentage of EBV-specific CD4+ colonies had acquired their specific lytic activity during day 10 and day 17 of culture.

CD4+ cytotoxic clones that fail to lyse their specific stimulator cells

To investigate whether every antigen-specific CD4+ T cell had in principle the ability to acquire specific cytotoxicity, T cell clones were established. Unstimulated T cells of patient A were cloned at 0·5 cells/well by unspecific stimulation with T cell conditioned medium (including PHA) in the presence of irradiated MNC. The clones were tested at day 18 or 19 of culture for lectin-mediated non-specific cytotoxicity and for their CD4 or CD8 phenotype. CD4-positive clones without any cytotoxic activity were further expanded and tested for specific proliferation against autologous and allogeneic EBV-transformed B cells. Table 2 shows the proliferative and cytotoxic activities of such CD4+ clones and a CD8+ T cell line as a control, tested 102 days after cloning. All clones had the ability of antigen-specific proliferation. They expressed cytotoxic activity since they were active in lectin-mediated cytotoxicity, but they were not able to lyse their stimulator cells specifically in the absence of lectin. These clones could be triggered to unspecific lysis by addition of PHA or MoAbs against CD3 (BMA 030), $\alpha\ \beta$ T cell receptor (BMA 031) or the T cell triggering molecule Tp103 (CB.1). Data of a representative clone are given in Fig. 5. To allow optimal triggering by the MoAbs Fc-receptor-positive P815 cells were used as target cells. These data show that a fraction of EBV-specific CD4+ T cells is able to acquire only non-specific but not antigen-specific cytotoxic activity.

DISCUSSION

The frequent description of CD4+ T cell clones with cytotoxic function has led to a discussion about the possible biological relevance of CD4+ CTL *in vivo* (Braakman *et al.*, 1987). We (Fleischer, 1984) and others (Chen et al., 1986) have described that *a priori* non-cytotoxic CD4+ T cells can acquire their cytotoxic activity during *in vitro* culture. Since the acquisition of cytotoxic function by non-cytotoxic CD4+ T lymphocytes is a very frequent event *in vitro* that can be observed with nearly every CD4+ T cell it should be possible to detect CD4+ CTL *in vivo*.

Only few experiments with primary, *in vivo* activated human T cells have been reported. In the peripheral blood of patients with acute mumps infection, virus-specific CTL can be found that are restricted by class I HLA antigens and not by HLA-DR antigens (Kreth *et al.*, 1982). Since PHA blasts were used as target cells in these experiments, class II antigen expression on the targets might not be sufficient to detect class II restricted CD4+ CTL. Furthermore, *in vivo*, most infected cells may not express class II antigens and therefore precursors of CD4+ CTL may not be appropriately expanded. In this report we therefore tried to detect CD4+ CTL in acute IM. The EBV system appears to be well suited since the target cells in EBV infections, the transformed B lymphocytes express abundant class II HLA antigens and therefore should stimulate class II restricted CD4+ CTL as well as class I restricted CD8+ CTL. Since in the tonsillar tissue specific cytotoxic activity was apparently enriched we studied the phenotype and specificity of T cells from tonsillar tissue of acutely infected patients. The limited number of target cells used was sufficient to document the autologous restriction of the cytotoxic reaction. It has been shown previously that CD4+ clones derived from the peripheral blood of seropositive individuals were nearly all cytotoxic (Meuer *et al.*, 1983; Misko *et al.*, 1984). Our results show that in IM the entire EBV-specific cytotoxic activity is present in the CD8+ fraction and can be inhibited by MoAb to HLA class I antigens. No cytotoxicity was detected in the CD4+ fraction of tonsillar T cells although these cells were *in vivo* activated to the same degree as the CD8+ cells and contained virus-specific T cells.

By limiting dilution analysis low numbers of EBV-specific CTL could be detected after a 10-day culture period in the CD4+ fraction. If these cells were further propagated we could detect the development of CD4+ CTL within individual colonies within 7 days. The high probability of clonality in these colonies argues that cytotoxicity had developed within CD4+ clones. Apparently, antigen and recombinant IL-2 were sufficient for the development of cytotoxic activity in a CD4+ cell. Other exogenous mediators such as IFN-γ (Chen *et al.*, 1986; Gromo *et al.*, 1987) appeared not to be necessary.

During the generation of CD4+ clones we identified clones that were capable of proliferating in response to the autologous LCL without killing the stimulator cells. In the presence of PHA, however, these clones efficiently lysed the specific LCL targets as well as any other third party target cell. In this 'intermediate' stage between a non-cytotoxic CD4+ cell and a CD4+ CTL the expression of cytotoxic activity is apparently differentially regulated depending on the quality of the triggering signal. These clones can be triggered to kill by MoAb to CD3 or to the T cell receptor or by lectin that probably also reacts with the T cell receptor. A likely explanation for these findings is that triggering of cytotoxicity has a higher activation threshold than triggering of proliferation. Triggering via MoAb or lectins stimulates high affinity binding and leads to immediate clustering of many T cell receptor molecules. Most of these clones did not acquire specific cytotoxicity within the observation period; some, however, did so. This could indicate that one mechanism contributing to the acquisition of cytotoxic activity by CD4+ cells is a lowering of the threshold for triggering of cytotoxicity.

Our results indicate that at least the majority of EBV-specific CD4+ CTL found after long-term culture or cloning has acquired their cytotoxic activity. Although it is striking that the longer CD4+ cells are cultivated the more easily cytotoxicity is detectable, it is impossible to assess if every CD4+ CTL has acquired its cytotoxicity or if CD4+ CTL can be preferentially expanded from infrequent CD4+ precursors. It is, however, obvious from our results that the capacity of CD4+ cells to acquire cytotoxic activity is not realized to a significant extent in an immune reaction *in vivo*. CD4+ T cells *in vivo* will thus

preferentially exert helper functions for the production of EBV-specific antibodies or the generation of $CD8^+$ CTL but will not contribute directly to the elimination of virus-infected cells.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 322).

We thank Ms S. Muth for her excellent secretarial assistance.

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