ESTABLISHMENT AND CHARACTERIZATION OF EPSTEIN-BARR VIRUS-SPECIFIC HUMAN CD4+ T LYMPHOCYTE CLONES

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Received August 3, 1998; accepted September 4, 1998

Summary. – We developed a simple method for establishing Epstein-Barr virus (EBV)-specific, human CD4+ T cell clones. The method originates from our experience that the regression of cell growth in *in vitro* EBV transformation of B cells occurs when round lymphoid cells appear in the culture. Peripheral blood mononuclear cells (PBMCs) were cultured with EBV, and IL-2 (20 U/ml) was added to the culture on day 17 after the virus addition. The phenotype of the growing cells was CD3+, CD4+, and CD8–. The cells were cytotoxic for autologous lymphoblastoid B cell line (LCL) and EBV-superinfected autologous LCL. The cytotoxic T lymphocytes (CTLs) were confirmed to be CD4+ T cells but not CD8+ T cells in the culture. CTL clones were established by a limiting dilution method. All the CTL clones had the phenotype of CD3+, CD4+ and CD8–, and proliferated in response to autologous LCL. They produced interferon (IFN)-gamma, interleukin 2 (IL-2) and tumour necrosis factor (TNF)-beta but not IL-4. All but one clone responded to both autologous, EBV-superinfected and non-superinfected LCLs. Proliferative and cytotoxic responses to allogenic LCLs were heterogeneous. These results suggest that this method induces heterogeneous, EBV-specific CD4+ CTL clones and is useful for analysis of CD4+ T cells in EBV infections.

Key words: Epstein-Barr virus; CD4+ T lymphocyte; cytotoxic T lymphocyte clone; limiting dilution method

Introduction

EBV is a gamma herpesvirus (subfamily *Gammaherpesvirinae,* genus *Lymphocryptovirus*) which causes various human diseases: infectious mononucleosis, nasopharyngeal carcinoma and Burkitt's lymphoma (Rickinson and Kieff, 1996). Most humans are infected with EBV during the first few years of life. In primary infection, EBV is transmitted orally and replicates locally in oropharyngeal epithelium where a full array of replication genes is expressed (Sixbey *et al.*, 1984). EBV also infects B cells, and the infected B cells selectively express the latent cycle genes. These latently infected B cells constitute a reservoir, and a life-long carrier state is established (Rickinson and Kieff, 1996). Primary infections with EBV are, however, asymptomatic in most cases. Periodic reactivation of latently infected B cells releases EBV. The released EBV then infects epithelial cells and low amounts of infectious EBV are shed from mucosal surface.

It is generally accepted that the host immune system contributes to limiting the primary infection and controlling the EBV carrier state. Among immune responses, the T cell

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Abbreviations: AIM-V-FCS = AIM-V medium with 10% FCS; BSA = bovine serum albumin; CTL = cytotoxic T lymphocyte; DMSO = dimethyl sulfoxide; EBV = Epstein-Barr virus; ELISA = enzyme-linked immunosorbent assay; E/T = effector/target; FCS = foetal calf serum; IFN = interferon; IL = interleukin; LCL = lymphoblastoid B cell line; MoAb = monoclonal antibody; PBS = phosphate-buffered saline; PBMC = peripheral blood mononuclear cell; p.i. = post infection; rIL-2 = recombinant IL-2; RPMI-FCS = RPMI 1640 medium with 10% FCS; TNF = tumour necrosis factor

immune responses are thought to play a major role in the control of EBV (e.g. Rickinson and Moss, 1997). There are papers which report on EBV-specific CD8+ CTLs and discuss their importance (e.g. Rickinson *et al.*, 1996). However, there is only a limited number of reports on EBV-specific CD4+ human T cells, and very little is known about the role of CD4+ T cells in the control of EBV. This is probably due to difficulties in establishing EBV-specific CD4+ human T cell clones rather than a lower frequency of specific CD4+ T cells compared to CD8+ T cells.

We often experienced a regression of the growth of LCLs during the *in vitro* transformation of B cells with EBV. We usually recognized the presence of round lymphoid cells in those cultures. On the basis of these observations, we have hypothesized that these lymphoid cells might be CTLs inducing the regression of the growth of the transformed B cells by cytotoxic activity. These observations have led us to the present study.

In the present paper, we have attempted to develop a simple method of inducing EBV-specific, human CD4+ T cells and establishing EBV-specific, human CD4+ T cell clones for analysis of human CD4+ T cell responses to EBV. The analysis of these human CD4+ T cell clones provided an important information about the human immune responses in the control of EBV infections.

Materials and Methods

PBMCs. Peripheral blood specimens were obtained from three healthy Japanese adults, donor 1, donor TT and donor AH. PBMCs were purified by Ficoll-Hypaque density gradient centrifugation (Boyam, 1968). When necessary, the cells were resuspended at 10^7/ml in RPMI 1640 Medium supplemented with 10% foetal calf serum (FCS) (RPMI-FCS) and 10% dimethyl sulfoxide (DMSO), and cryopreserved until use. The HLA types of the donor 1 were A24, A33; B44; DQ1, DQ3; DR2, DR5. The HLA types of the donor TT were A24, A33; B52, B35; CW3; DRB1*0405, DRB1*1502; DQB1*0401, DQB1*0601; DPB1*0201, and DPB1*0901 which were considered to be DR4, DR15; DQ4, DQ6; DPw2. The HLA types of the donor AH were A2, A24; B38, B59; CW1, CW7; DRB1*0405, DRB1*0803; DQB1*0301, DQB1*0401; DPB1*0402, and DPB1*1401 which were considered to be DR4, DR8; DQ3, DQ4; DPw4. The serum sample of the donor 1 was examined for anti-EBV antibodies. The titres of anti-VCA, anti-EBNA and anti-EADR IgGs were 1:20, 1:10, and <1:10, respectively.

Establishment and cultivation of LCLs. PBMCs ($1 - 5 \times 10^6$) were cultured in RPMI-FCS containing penicillin and streptomycin in the presence of diluted (1:3) supernatants of B95-8 cells (Kurane *et al.*, 1989), which were kindly provided by Dr. T. Sairenji, Tottori University School of Medicine, Yonago, Japan. Raji cells were obtained from F.A. Ennis, University of Massachussets Medical Center, Worcester, USA, and cultured in RPMI-FCS.

Cloning of EBV-specific T cell clones was done by a limiting dilution method (Kurane *et al.*, 1991; Livingston *et al.*, 1997). T cell blasts obtained from stimulated donor 1's PBMCs were cloned by placing 1 and 10 cells into each well of 96-well round-bottom microtitre plates in 0.2 ml of AIM-V Medium (Gibco BRL) containing 10% FCS (AIM-V-FCS) and 20 U/ml recombinant IL-2 (rIL-2). Mitomycin C (0.05 mg/ml)-treated autologous EBV-LCL cells (5×10^4) were added to the culture as feeder cells. Every 3 – 4 days, 0.1 ml of medium was removed from each well and same amount of AIM-V-FCS with 20 U/ml rIL-2 was added. On day14, the cells were tested for cytotoxic activity against autologous LCLs. Clones with positive cytotoxic activity were subjected to further studies. The clones were restimulated with 5×10^4 mitomycin C-treated, superinfected autologous LCL cells in 1.0 ml of AIM-V-FCS containing 20 U/ml rIL-2 in 48-well plates (Iwaki Glass, Japan).

Immunofluorescence staining. Cells (10^5) were stained with FITC-labelled anti-CD3, anti-CD4 and anti-CD8 antibodies (Dako, Denmark) for 30 mins on ice and washed with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) (Kurane *et al.*, 1991). The cells were resuspended in 50% glycerol in PBS and were examined under a fluorescent microscope.

Proliferation assay of T cell clones. T cell clones ($1 - 2 \times 10^4$ cells/well) were incubated with 0.05 mg/ml mitomycin C-treated autologous LCL cells (5×10^4/well) in 96-well V-bottom microtitre plates. After 48 hrs of culture, ^{3}H-thymidine (1 μCi/well) was added and its uptake was assayed 18 hrs later by scintillation counting. In determining HLA restriction, monoclonal antibodies (MoAbs) to HLA-DQ or HLA-DR (Cosumo Bio Co., Ltd, Japan) or control mouse IgG were added in optimal concentration to the culture.

Preparation of target cells. A total of $1 - 1.5 \times 10^5$ EBV-transformed LCL or Raji cells were washed once in RPMI-FCS. LCL cells were labelled by incubation with 0.25 mCi of ^{51}Cr in 0.1 ml of RPMI-FCS for 1 hr at 37°C. After labelling, the cells were washed 4 times with RPMI-FCS to remove the unincorporated label. The cells were counted and diluted to 1×10^4/ml for a cytotoxicity assay. In some experiments, LCL cells were superinfected with EBV and used as target cells 3 – 5 days post infection (p.i.).

Cytotoxity assay was performed in 96-well V-bottom plates as previously described (Zivny *et al.*, 1995). Effector cells in 0.1 ml of RPMI-FCS were added to 1000 ^{51}Cr-labelled target cells at effector/target (E/T) ratios of 10:1 to 20:1. The plates were incubated at 37°C for 5 hrs, their supernatant fluids were harvested and assayed for the ^{51}Cr content in an automatic gamma counter (Auto Well Gamma System ARC-300, Aloka Japan). The percentage of chromium release was calculated according to the formula

$$\frac{\text{cpm (experimental release)} - \text{cpm (spontaneous release)}}{\text{cpm (maximum release)} - \text{cpm (spontaneous release)}} \times 100$$

Each assay was done in triplicate and the results represented the averages.

Depletion of CTLs by MoAb and complement. OKT3 (anti-CD3), OKT4 (anti-CD4) and OKT8 (anti-CD8) MoAbs (Ortho Diagnostic Systems, USA) and anti-Leu11b (anti-CD16) MoAb (Becton Dickinson & Co., USA) were used in the complement-mediated

cell depletion assay. The cell depletion was performed as previously reported (Mathew *et al.*, 1996). EBV-stimulated effector cells (10^6) were resuspended in 0.5 ml of RPMI-FCS and 5 µl of a MoAb was added. After 30 mins of incubation at 4°C, the cells were washed twice and resuspended in 0.5 ml of RPMI-FCS to which 0.1 ml of rabbit complement (Cedarlane Laboratories, Canada) was added. After 60 mins of incubation at 37°C, the cells were washed three times, resuspended in RPMI-FCS and then used as effector cells in the cytotoxic assay. RPMI-FCS was used for each cell washing.

Assay of cytokines in culture supernatants. T clone cells (1 – 2 x 10^4/well) were incubated with 0.05 mg/ml mitomycin C-treated autologous LCL cells (5 x 10^4/well) in 96-well V-bottom microtitre plates. After 48 hrs of culture, the supernatant fluids were collected and assayed for IFN-gamma, IL-2, TNF-beta and IL-4 by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. The ELISA kits for IFN-gamma, IL-2 and IL-4 were purchased from Toyobo Co., Ltd., Japan, and that for TNF-beta was purchased from Amersham, UK.

Results

Phenotype of the cells which grew after stimulation with EBV

PBMCs of the donor 1 were stimulated with EBV in RPMI-FCS, and IL-2 at various concentrations was added to the culture on day 17 after the virus addition. Numbers and surface markers of the growing cells were examined on day 31. The addition of IL-2 at 20 – 200 U/ml increased the number of cells compared to the culture without addition of IL-2 (Table 1). Approximately 90% of the cells were CD3-positive, and the percentage of CD4+ cells was much higher than that of CD8+ cells regardless of the concentration of IL-2.

In the next experiments, 20 U/ml IL-2 was added to the culture on various days after the start of the culture (Table 2). When IL-2 was added on days 11 and 17 (exp. No. 1), or on days 14 and 21 (exp. No. 2), the number of cells increased compared to the culture without IL-2. Approximately 90% of the cells were CD3-positive, and the percentage of CD4+ cells was also much higher than that of CD8+ cells irrespective of the day of IL-2 addition.

Cytotoxic activity of the cells stimulated with EBV in the presence of IL-2

We examined the cytotoxic activity of the cells which were stimulated with EBV and were grown in the presence of IL-2 at various concentrations (Table 3). Autologous LCL cells, Raji cells and those superinfected with EBV 3 days earlier were used as target cells. The PBMCs which had been stimulated with EBV in the presence of IL-2 lysed autologous LCL cells and EBV-superinfected autologous LCL cells to higher levels than normal and EBV-superinfected Raji cells.

Table 1. Characterization of growing cells after stimulation of PBMCs with EBV in the presence of various concentrations of IL-2

IL-2 (U/ml)	No. of cells	Positive cells (%)		
		CD3	CD4	CD8
0	3.6 x 10^6	89.3	78.9	4.0
2	4.8 x 10^6	84.3	68.3	21.0
20	5.6 x 10^6	87.8	79.5	11 2
200	1.0 x 10^7	90.2	86.2	10.2
2000	5.2 x 10^6	86.5	83.1	8.0

PBMCs (5 x 10^6) were cultured with B95-8 cell culture supernatant at the final dilution of 1:2 in 1 ml of RPMI-FCS on day 0. One ml of RPMI-FCS was added to the culture on day 1. Half of the medium was replaced every 3 – 4 days. IL-2 was added to the culture to make indicated final concentrations first on day 17 and then at each medium replacement.The percentages of the cells positive for CD3, CD4 and CD8 were estimated on day 31.

Table 2. Characterization of growing cells after stimulation of PBMCs with EBV in the presence of IL-2 added on various days

Days of first addition of IL-2	No. of cells	Positive cells (%)		
		CD3	CD4	CD8
Exp. No. 1				
No IL-2	5.6 x 10^6	92.2	84.2	20.3
1	5.6 x 10^6	86.8	76.5	12.2
6	8.0 x 10^6	84.9	81.7	4.4
11	1.0 x 10^7	84.3	78.6	6.9
17	1.2 x 10^7	87.1	81.2	19.0
Exp. No. 2				
No IL-2	5.0 x 10^6	83.2	82.8	17.2
7	8.4 x 10^6	85.3	79.2	19.5
14	1.2 x 10^6	86.6	77.4	23.2
21	1.0 x 10^7	84.8	78.9	18.5
28	6.0 x 10^6	88.9	85.3	22.3

PBMCs (5 x 10^6) were cultured with B95-8 cell culture supernatant at the final dilution of 1:2 in 1 ml of RPMI-FCS on day 0. One ml of RPMI-FCS was added to the culture on day 1. Half of the medium was replaced every 3 – 4 days. IL-2 (20 U/ml) was added first time to the cultures on various days indicated and then at each medium replacement. The percentages of the cells positive for CD3, CD4 and CD8 were estimated on day 31.

The PBMCs which had been stimulated with EBV in the absence of IL-2 did not lyse these target cells. The levels of lysis of autologous and superinfected LCL cells were similar when PBMCs were cultured with 2 – 2000 U/ml IL-2.

We also examined the cytotoxic activity of the growing cells in culture to which 20 U/ml IL-2 was added on various days. A similar cytotoxic activity against autologous LCL cells was obtained when IL-2 was added on days 7, 21 or 28 (data not shown).

Identification of the cytotoxic cells as CD4+ T lymphocytes

We then attempted to characterize cytotoxic cells by use of MoAbs to CD3, CD4, CD8 and CD16. The PBMCs, which

Table 3. Lysis of autologous EBV-transformed LCL by PBMCs stimulated with EBV in the presence of various concentrations of IL-2

IL-2 (U/ml)	E/T ratio	Specific ^{51}Cr release (%)			
		Autologous LCL	Superinfected autologous LCL	Normal Raji cells	Superinfected Raji cells
0	100	3.2	4 3	0	2.6
	50	2.6	3.1	0	0
	25	2.4	2.2	0	0
2	100	47.7	71 9	16 5	21 2
	50	38.0	49.6	11.7	13.8
	25	34.2	46.3	7.6	8.2
20	100	33.0	44.0	14 6	19.8
	50	31.6	41.7	11.2	11.8
	25	24.1	33 9	6.3	5.3
200	100	21.6	45 0	5 7	7 6
	50	11.8	27.0	2.1	3.0
	25	8.3	22.0	1.2	2.8
2000	100	49.3	60.9	9.1	8.8
	50	37.9	55 1	6.1	5 6
	25	26.1	40.3	3.8	2.9

^{51}Cr release assay was performed on day 31. A thousand target cells were used in the assay.
PBMCs (5 x 10^6) were cultured with B95-8 cell culture supernatant at the final dilution of 1:2 in 1 ml of RPMI-FCS on day 0. One ml of RPMI-FCS was added to the culture on day 1. Half of the medium was replaced every 3 – 4 days. IL-2 was added to the culture to make indicated final concentrations first on day 17 and then at each medium replacement.

Table 4. Identification of the cytotoxic cells as CD4+ T lymphocytyes

Treatment of cells	Specific ^{51}Cr release (%)	
	Superinfected autologous LCL	Raji cells
C' alone	36.0	10.3
Anti-CD3 MoAb + C'	4.9	0
Anti-CD4 MoAb + C'	0	0
Anti-CD8 MoAb + C'	30.3	0 9
Anti-CD16 MoAb + C'	30.4	7.1

C' = complement. The E/T ratio was 20:1. Numbers of the cells after treatment were 1.2 x 10^6 for C' alone, 2.4 x 10^5 for anti-CD3 MoAb + C', 6.0 x 10^5 for anti-CD4 MoAb + C', 1.0 x 10^6 for anti-CD8 MoAb + C', and 1.2 x 10^6 for anti-CD16 MoAb + C'.

had been stimulated with EBV in the presence of 20 U/ml IL-2 for 31 days, were treated with complement and MoAbs to CD3, CD4 ,CD8 or CD16, and were used as effector cells. A pretreatment of PBMCs with complement and MoAbs to CD3 and CD4 abrogated their ability to lyse superinfected autologous LCL cells, but a pretreatment with complement and MoAbs to CD8 or CD16 did not (Table 4). These results indicate that the cytotoxic cells responsible for the lysis of autologous LCL cells were CD4+ CD8– T lymphocytes.

Establishment of CD4+ T cell clones which responded to autologous LCL cells

We established T cell clones with the aim to characterize further the CTLs induced by stimulation with EBV in the presence of IL-2. PBMCs were stimulated with EBV and 20 U/ml IL-2 was added on day 17 after the virus. The cells lysed superinfected and non-superinfected autologous LCL cells to 45.3% and 23.8%, respectively, at E:T ratio of 50:1 on day 30. The living cells were plated at 1 and 10 cells/well and stimulated for 14 days.

The cells were screened for cytotoxic activity against superinfected autologous and allogenic LCL cells on day 14. Cells which had lysed superinfected autologous LCL cells to the levels greater than 15% were selected for further expansion. Five clones were selected from the wells which originally contained 1 cell/well, and 20 clones were selected from the wells which originally contained 10 cells/well. The growing cells were stained with MoAbs to CD3, CD4 and CD8. All the cell lines had a phenotype of CD3+CD4+CD8– (data not shown).

Proliferation of CD4+ T cell clones in response to superinfected autologous LCL cells

The CD4+ T cell clones were stimulated with superinfected autologous LCL cells or PHA-stimulated autologous PBMCs. The clones proliferated in response to superinfected autologous LCL cells, but they did not proliferate in response to PHA-stimulated autologous PBMCs (Table 5).

Cytotoxic activity and cytokine production by CD4+ T cell clones

Most of these CD4+ T cell clones lysed superinfected autologous LCL cells to low but significant levels (Table 6). The clones, however, did not lyse non-superinfected autologous LCL or Raji cells.

All the tested clones produced IFN-gamma after stimulation with superinfected autologous LCL cells (Table 7). Five of the 7 tested clones produced IL-2, while 3 clones produced TNF-beta. None of the CD4+ clones produced IL-4. These results indicate that the EBV-specific CD4+ T cell clones were of the Th1 type.

Proliferative responses of EBV-specific CD4+ T cell clones to EBV-transformed allogenic LCL cells

We examined HLA restriction of the EBV-specific CD4+ T cell clones by use of EBV-transformed allogenic LCL as

Table 5. Proliferation of CD4+ T cell clones by stimulation with EBV-superinfected autologous LCL

Clone No.	^{3}H-thymidine incorporation (cpm)		
	Superinfected autologous LCL	PHA-stimulated autologous PBMCs	No feeder cells
Exp. No. 1			
21	3,488 (324)	49 (12)	41 (27)
38	9,543 (1,071)	37 (22)	25 (7)
Exp. No. 2			
41	1,683 (250)	0 (0)	43 (22)
43	1,425 (110)	0 (0)	35 (6)
51	5,085 (1,344)	0 (0)	79 (59)
64	19,411 (1,494)	1,512 (119)	60 (21)
89	8,488 (758)	78 (127)	95 (14)

^{3}H-thymidine incorporation by superinfected autologous LCLs alone and PHA-stimulated autologous PBMCs alone were deducted from ^{3}H-thymidine incorporation by the co-culture of T clone cells and feeder cells.
The assays were done in triplicate. The results represent averages with SD in parentheses.

Table 6. Lysis of superinfected EBV-transformed LCL by CD4+ T cell clones

Clone No. LCL	Specific ^{51}Cr release (%)		
	Superinfected autologous LCL	Autologous	Raji cells
21	20.6	4.7	0
38	13.7	2.2	0
39	9.0	1.4	0
41	11.7	1.2	0
43	10.1	2.1	0
51	7.4	1.6	0
64	14.1	2.7	0
89	3.7	0	0

The E/T ratio was 20:1.

Table 7. Production of lymphokines by EBV-specific CD4+ T cell clones

Clone No.	Cytokine concentration (pg/ml)			
	IFN-gamma	IL-2	TNF-beta	IL-4
21	741	0	0	0
38	>1000	112	141	0
41	517	80	0	0
43	49	0	0	0
51	65	41	0	0
64	>1000	342	128	0
89	59	80	19	0

The amounts of cytokines produced by superinfected LCL alone were deducted from the amounts of cytokines in the co-culture of CD4+ T cell clones and superinfected LCL.

stimulator cells. The T cell clones Nos. 21, 39 and 40 proliferated in response to superinfected TT-LCL and superinfected AH-LCL, but they did not proliferate in response to superinfected Raji cells (Table 8). The clones Nos. 38, 51 and 64 did not proliferate in response to superinfected TT-LCL, superinfected AH-LCL or superinfected Raji cells.

The MoAb to HLA-DR inhibited the proliferation of the clone No. 89, but it did not inhibit the proliferation of the clones Nos. 21, 39 and 64 (Table 9). The clones Nos. 38 and 64 were examined for cytotoxic activity against superinfected Raji cells and two allogenic LCLs (TT-LCL and AH-LCL). These clones that lysed superinfected autologous LCL did not lyse superinfected Raji cells, TT-LCL and AH-LCL (data not shown). These results indicate that the clone No. 89 was HLA-DR2- or DR5-restricted. The HLA-restriction of other clones (Nos. 21, 38, 39, 40, 51 and 64) could not be determined in these experiments.

Discussion

In the first part of the present paper we reported on a simple method of establishing EBV-specific CTL clones. This method originates from our experience that the regression of cell growth during *in vitro* transformation of B cells with EBV

Table 8. Presence or absence of responses of CD4+ T cell clones to allogenic LCLs

Clone No.	^{3}H-thymidine incorporation (cpm)					
	Sup. auto. LCL	Auto. LCL	Sup. Raji cells	Sup. TT-LCL	Sup. AH-LCL	T cells alone
HLA-DR	2, 5	2, 5		4, 15	4, 8	
HLA-DQ	1, 3	1, 3		4, 6	3, 4	
Exp. No. 1						
21	4,996	5,378	294	5,861	11,173	36
	(1,058)	(894)	(21)	(571)	(4,576)	(4)
38	4,698	4,700	221	754	921	45
	(639)	(510)	(75)	(76)	(202)	(10)
39	958	4,065	231	1,788	2,864	27
	(289)	(333)	(85)	(56)	(278)	(15)
40	1,592	2,607	389	1,606	2,294	351
	(194)	(281)	(35)	(288)	(725)	(60)
Exp. No. 2						
51	1,059	787	365	229	37	287
	(63)	(191)	(17)	(87)	(12)	(22)
64	543	2,215	0	966	979	19
	(411)	(411)	(0)	(107)	(281)	(3)

The assays were done in triplicate. The results represent averages with SD in parentheses.
^{3}H-thymidine incorporation by LCL alone was deduced from ^{3}H-thymidine incorporation in the co-culture of T cell clone and feeder cells.
Sup. = superinfected. Auto. = autologous.

Table 9. Inhibition of proliferation of CD4+ T cell clones by MoAb to HLA-DR

Clone No.	^{3}H-thymidine incorporation (cpm)		
	No MoAb	Anti-HLA-DR MoAb	Anti-HLA-DQ MoAb
21	1,371 (118)	1,077 (185)	1,620 (770)
39	3,127 (281)	2,364 (118)	3,214 (1,015)
64	5,834 (313)	3,845 (232)	5,460 (824)
89	1,193 (331)	107 (212)	1,313 (89)

The assays were done in triplicate. The results represent averages with SD parentheses.

occurs when round lymphoid cells appear in the culture. We assumed that these round lymphoid cells were EBV-specific CTLs which overcame the growth of EBV-transformed LCL by cytotoxic activity. Thus, we added IL-2 to the culture with the aim to induce further growth of CTLs at an appropriate time.We found that addition of IL-2 at 20 U/ml around day 17 after the start of the culture consistently induced strong killer cells against EBV-transformed autologous LCL.

We then characterized the phenotype of the CTLs. It was of great interest that their cytotoxic activity was abrogated by the treatment with complement and MoAbs to CD3 and CD4. The treatment with complement and MoAb to CD8 or CD16 (human natural killer cells) did not abrogate the cytotoxic activity. These results clearly indicated that the phenotype of the CTLs was CD3+CD4+CD8-. All the CTL clones established in the present experiments were of CD4+ type, as expected and revealed by the bulk culture analysis.

There were papers reporting on EBV-specific CD4+ CTLs (Khanna *et al.*, 1995; Khanna *et al.*, 1997). Almost all the EBV-specific CTL clones reported in the past were, however, of the CD8+ type (Wallace *et al.*, 1982; Rickinson and Kieff, 1996), and there was only a limited number of papers dealing with CD4+ EBV-specific CTL clones. It is not clear why CD4+ but not CD8+ EBV-specific CTLs were selected in our experimental conditions. Many investigators have first established EBV-transformed autologous LCLs and then have used them as a stimulator for PBMCs after gamma irradiation or mitomycin C treatment. In our experimental conditions, B cells were acutely infected with EBV and T cells were stimulated with these acutely infected LCL cells. Thus, the EBV proteins which stimulated T cells may have been more heterogeneous compared to those from the experiments where already established LCLs were used as stimulator cells. The predominant growth of CD4+ T cells could be also caused by the use of rIL-2 rather than T cell growth factor.

The role of EBV-specific CD4+ CTLs in controlling EBV infection is not completely understood. We have previously characterized CD4+ CTLs specific for dengue (Gagnon *et al.*, 1996; Green *et al.*, 1993; Kurane *et al.*, 1991; Zeng *et al.*, 1996) and Japanese encephalitis viruses (Aihara *et al.*, 1998). Other investigators have reported on CD4+ CTLs specific for HSV-1 (Yasukawa and Zarling, 1984), measles virus (Jacobson *et al.*, 1983), influenza virus (Kaplan *et al.*, 1984), cytomegalovirus (Borysiewicz *et al.*, 1983) and HIV-1 (Littaua *et al.*, 1992). These CD4+ CTLs probably play important role in controlling virus infections through helper functions in antibody production and cytotoxic activity. The role of CD4+ CTLs in EBV infection is an important objective to be studied in the future.

It has been reported that the epitopes recognized by EBV-specific CD8+ CTLs were mainly located on EBNA 3A, 3B or 3C proteins (Rickinson and Kieff, 1996). In some cases, epitopes were determined on LMP 2, less frequenlly on EBNA 2 or EBNA-LP and very rarely on EBNA 1. Only a few epitopes recognized by EBV-specific CD4+ T cells were defined, and these epitopes were located on EBNA1, EBNA 2 and BHRF1 (Kahnna *et al.*, 1995; Rickinson and Kieff, 1996; White *et al.*, 1996). We examined if some of our CD4+ T cell clones would recognize BHRF1 and LMP1 antigens in the proliferation assay using transfected cell lysates. The tested clones did not recognize these antigens. We intend to define the epitopes recognized by our CD4+ T cell clones. Concerning cytokine production, our CD4+ clones produced IFN-gamma, IL-2 and TNF-beta but not IL-4. Thus, the EBV-specific CD4+ T cell clones established by us were of Th1 type.

In conclusion,we developed a relatively simple method of establishing EBV-specific CD4+CTL clones. Our method leads to induction of more heterogeneous CTLs than do those of other groups, because the stimulator cells used are acutely infected with EBV and express multiple proteins. It is, therefore, likely that T cells which respond to more heterogeneous proteins are induced to grow in our assay compared to the assays where already transformed LCLs are used as stimulators. Although we were so far not able to determine the proteins recognized by EBV-specific CD4+ CTL clones, our method will certainly contribute to the better understanding of EBV-specific CD4+ CTL responses.

Acknowledgements. This work has been supported by a grant from the Programme for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan, grants-in-aid for Scientific Research Nos. 08457100 and 09044344 from the Ministry of Education, Science, Sports and Culture of Japan, and a grant from the Research on Emerging and Re-emerging Infectious Diseases from Ministry of Health and Welfare of Japan.

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