JOURNAL OF VIROLOGY, May 1992, p. 2773–2779
0022-538X/92/052773-07$02.00/0

Vol. 66, No. 5

Establishment and Functional Characterization of Human Herpesvirus 6-Specific CD4$^+$ Human T-Cell Clones

YOSHIHIRO YAKUSHIJIN,* MASAKI YASUKAWA, AND YUZURU KOBAYASHI

First Department of Internal Medicine, Ehime University School of Medicine, Shigenobu, Ehime 791-02, Japan

Received 11 November 1991/Accepted 24 January 1992

In order to clarify the protective immune responses against a newly identified herpesvirus, human herpesvirus 6 (HHV-6), we established HHV-6-specific human T-cell clones and examined their functional properties. Five CD3$^+$CD4$^+$CD8$^-$ T-cell clones, which proliferated in response to stimulation with two different strains of HHV-6 in the presence of autologous antigen-presenting cells but not with herpes simplex virus type 1 or human cytomegalovirus, were established from peripheral blood lymphocytes of a healthy individual. The proliferative response of all T-cell clones to HHV-6 antigen was inhibited by addition of anti-HLA-DR monoclonal antibody, indicating that these clones were human leukocyte antigen (HLA) class II DR restricted. Of the five clones, two lysed HHV-6-infected autologous lymphoblasts, but not HHV-6-infected allogeneic cells or natural killer-sensitive K562 cells (group 1); one showed cytotoxicity against HHV-6-infected autologous lymphoblasts as well as HHV-6-infected allogeneic cells and K562 cells (group 2); and the remaining two showed no cytotoxic activity (group 3). The cytotoxic activity of group 1 was inhibited by addition of anti-HLA-DR monoclonal antibody to the culture, whereas this monoclonal antibody had no effect on the cytotoxicity of group 2 and did not induce the cytotoxicity of group 3. Perforin, which is one of the mediators of cytotoxicity, was abundantly expressed in group 1 and 2 clones. Moreover, all groups of clones produced gamma interferon after culture with antigen-presenting cells followed by HHV-6 antigen stimulation. These results suggest that HHV-6-specific CD4$^+$ T cells have heterogeneous functions.

Human herpesvirus 6 (HHV-6) is a novel herpesvirus originally isolated from patients with AIDS and lymphoproliferative disorders (9, 35, 42). A study by Yamanishi et al. (44) has revealed that HHV-6 is a causative agent of exanthem subitum, and seroepidemiological studies have shown that most individuals become seropositive for this virus by the age of 2 years (3, 4, 21, 24, 50).

It has been reported that various clinical symptoms, such as lymphadenitis (11), hepatitis (10), mononucleosis (40), and pneumonitis (5) may result from the reactivation of HHV-6 in immunocompromised hosts. In addition, HHV-6 is frequently isolated from patients with a deficiency of cell-mediated immunity, such as that associated with AIDS or organ transplantation (1, 9, 35, 42). This evidence strongly suggests that cellular immunity is most important in protection against and recovery from HHV-6 infection and that investigation of the T-cell immune response against HHV-6 infection is required. However, although a few reports describing the effects of HHV-6 on cytokine production by monocytes and non-T cells have been published (12, 20), very little is known about the T-cell response to HHV-6 infection.

Recently, we found that memory T cells showing a specific response to stimulation with HHV-6 are generally present in healthy adult populations (43). In the present study, we established HHV-6-specific T-cell clones from peripheral blood lymphocytes of a healthy individual and characterized their functional properties to clarify the mechanisms of protection against HHV-6 infection. Our results revealed that human CD4$^+$ HHV-6-specific T cells produce gamma interferon (IFN-γ) in response to stimulation with HHV-6 and that they show heterogeneous cytotoxic reactivity. On the basis of the present data, the roles of CD4$^+$ T cells in protection and recovery from infection with HHV-6 are discussed.

MATERIALS AND METHODS

Viruses. The prototype GS strain of HHV-6 was isolated from a patient with lymphoproliferative disorder (35), and the Hashimoto (HST) strain was isolated from a patient with exanthem subitum (44). They were grown in cord blood mononuclear cells (CBMC), which were stimulated with phytohemagglutinin P (PHA-P; Difco Laboratories, Detroit, Mich.), at a final dilution of 1:1,000, and cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO) and 0.5 U of human recombinant interleukin-2 (IL-2) (Takeda Chemical Industries, Osaka, Japan) per ml as described previously (44). The KOS strain of herpes simplex virus type 1 (HSV-1) was grown in HEp-2 cells. The AD169 strain of human cytomegalovirus (CMV) was grown in Flow 7000 human embryonic fibroblasts.

Preparation of virus antigens. HHV-6-infected CBMC were washed with phosphate-buffered saline (pH 7.2) and rapidly frozen and thawed. The cells were then sonicated for 30 s in an ultrasonic disruptor (TOMY, Tokyo, Japan). After centrifugation at 600 × g for 10 min, the supernatant was exposed to UV light from a 15-W UV bulb for 10 min at a distance of 10 cm to inactivate the virus. No cytopathic effect on CBMC was detected after UV irradiation. Mock-infected CBMC were also treated as described above and prepared for the control antigen. HSV-1 and CMV antigens were prepared from HSV-1-infected HEp-2 cells and CMV-infected fibroblasts, respectively, as described above. Optimal dilutions of HHV-6, HSV-1, and CMV antigens for stimulation of T cells determined in previous experiments were 1:20, 1:200, and 1:200, respectively.

Generation of T-cell clones. Peripheral blood mononuclear

* Corresponding author.

cells (PBMC) from an HHV-6-, HSV-1-, and CMV-seropositive healthy adult were separated by Ficoll-Conray gradient centrifugation and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human AB serum (this medium will be referred to as culture medium). HHV-6(GS) antigen was then added to the cells, which were seeded in a 96-well round-bottom microtiter plate at a concentration of 1×10^5 cells per well and cultured at 37°C in a 5% CO_2 incubator. After 7 days, the blasts were seeded at a concentration of 1 cell per well in round-bottom microtiter wells containing 0.2 ml of culture medium supplemented with 0.5 U of IL-2 per ml and 1×10^5 mitomycin C (MMC)-treated autologous PBMC. The growing cells were then transferred to 16-mm wells and expanded. MMC-treated autologous PBMC and HHV-6(GS) antigen were added to the wells every 1 or 2 weeks. T-cell clones reactive with HSV-1 and CMV were generated from PBMC stimulated with HSV-1 antigen and CMV antigen, respectively, as described above.

Monoclonal antibodies. Culture supernatants of hybridomas, HU-4 directed against human leukocyte antigen DR (HLA-DR) (16), HU-11 directed against HLA-DQ (22), and w6/32 directed against HLA-A, HLA-B, and HLA-C (2) were used for blocking experiments. OKT3 (CD3) (Ortho Pharmaceutical Corp., Raritan, N.J.) was used for depletion of T cells. OKT8 (CD8; Ortho), Leu11b (CD16; Becton Dickinson, Mountain View, Calif.), and NKH1A (CD56; Coulter, Hialeah, Fla.) were used for depletion of $CD8^+$ T cells and natural killer (NK) cells. Phenotype analysis was performed by direct immunofluorescence by using an Epics profile flow cytometer (Coulter) with Leu4 (CD3), Leu3a (CD4), and Leu2a (CD8) monoclonal antibodies (Becton Dickinson).

Proliferative response of T-cell clones to virus antigens. One thousand clone cells and 1×10^5 MMC-treated autologous PBMC, as a source of antigen-presenting cells, in 0.2 ml of culture medium, were seeded in round-bottom microtiter wells to which 0.02 ml of virus antigen was added at the optimal dilution. The culture plates were incubated at 37°C in a 5% CO_2 incubator for 72 h. For the final 16 h of incubation, 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; New England Nuclear, Boston, Mass.) was added to each well, and the cells were harvested onto fiberglass filter paper by using a semiautomatic multiple-cell harvester. The incorporation of [^{3}H]TdR was determined by liquid scintillation counting. To examine HLA restriction of the clones in the proliferative response, we added monoclonal antibodies HU-4, HU-11, and w6/32 to the culture medium at a final dilution of 1:500. The incorporation of [^{3}H]TdR was determined as described above. These monoclonal antibodies had been proven previously to have no toxic effect at the concentration used in this experiment (15, 16, 22).

Cytotoxicity assays. ^{51}Cr-release assays were performed as described previously (45). HHV-6-infected target cells were prepared as follows. Autologous and allogeneic PBMC were treated with 20 μl of each of the OKT8, Leu11b, and NKH1A monoclonal antibodies and 1 ml of nontoxic rabbit complement (Cedarlane, Hornby, Ontario, Canada) at a final dilution of 1:3 to deplete $CD8^+$ T cells and NK cells. The cells treated with monoclonal antibodies were stimulated with PHA and cultured for 4 days in RPMI 1640 medium supplemented with 10% FCS and 0.5 U of IL-2 per ml. The PHA-stimulated blasts were then infected with the GS strain of HHV-6 and cultured in RPMI 1640 medium supplemented with 10% FCS and IL-2. When the cytopathic effect was detected, the cells were collected and used as the target cells after elimination of dead cells. The target cells were incubated for 1 h at 37°C with 200 μCi of ^{51}Cr (Na_2CrO_4; New England Nuclear) and then washed three times with cold RPMI 1640 medium supplemented with 10% FCS. Ten thousand ^{51}Cr-labeled target cells and various numbers of effector cells were incubated together in 0.2 ml of RPMI 1640 medium supplemented with 10% FCS in round-bottom microtiter wells. Target cells were also added to wells containing medium alone and to wells containing 1% Triton X-100 (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) to determine the spontaneous and maximal release, respectively. After 5 h, 0.1 ml of supernatant was removed from each well and transferred to tubes for counting in a gamma counter. The percentage of specific ^{51}Cr release was calculated as follows: 100 × (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release). The spontaneous release from the target cells never exceeded 20% of the maximal release. To examine the ability of monoclonal antibodies to block the lysis of target cells, we added HU-4, HU-11, and w6/32 to each well at a final dilution of 1:50, as described previously (45). ^{51}Cr-release assays were performed as described above.

Production of IFN-γ by clone cells. Cloned T cells were extensively washed, and then 5×10^5 clone cells and 1×10^6 cells that did not form rosettes with sheep erythrocytes (E^- cells), which had been completely depleted of T cells by treatment with OKT3 and complement, were suspended in 2 ml of RPMI 1640 medium supplemented with 10% FCS and cultured in 16-mm wells with HHV-6 antigen. The cells were cultured for 72 h, and then supernatants were collected for IFN-γ assay, which was performed by radioimmunoassay with anti-human IFN-γ monoclonal antibodies (Centocor, Malvern, Pa.).

Northern (RNA) blot hybridization. Cloned T cells were stimulated with HHV-6 antigen in the presence of autologous antigen-presenting cells and cultured in culture medium for 72 h. Next, the cells were harvested, and total cellular RNAs were extracted as described previously (8). RNAs (10-μg portions) were denatured with formaldehyde and size fractionated by electrophoresis on a 1% agarose gel. The RNAs were then transferred to a nitrocellulose filter and hybridized with ^{32}P-labeled probes ([^{32}P]dCTP; ICN Radiochemicals, Irvine, Calif.). The DNA fragments used as probes were 1.4-kb human IFN-γ cDNA (13) and 2.1-kb human perforin cDNA (38).

RESULTS

Proliferative response of clone cells to HHV-6 antigens. Ten T-cell clones were established by seeding blasts that had been activated by stimulation with HHV-6(GS) antigen, at 1 cell per well in a total of 1,056 wells. Of these, five clones, designated UTGS-1.3, UTGS-1.5, UTGS-2.1, UTGS-2.2, and UTGS-2.3, were selected and used for further experiments. Immunofluorescence with anti-HHV-6 antibody and polymerase chain reaction amplification of HHV-6 DNA revealed that these T-cell clones were not infected with HHV-6 (data not shown). We first examined their ability to respond to stimulation with two strains of HHV-6 (GS and HST) and HSV-1 and CMV antigens in the presence of autologous antigen-presenting cells. As shown in Table 1, T-cell clones generated from PBMC stimulated with HHV-6(GS) were all found to proliferate upon stimulation with HHV-6(GS) antigen. These clones also showed a proliferative response to stimulation with the other strain of HHV-6, HST, but failed to respond to HSV-1, CMV, and mock-

TABLE 1. Proliferative response of clones[a]

Clone	Incorporation of [^{3}H]TdR (cpm)[b]				
	HHV-6(GS)	HHV-6(HST)	HSV-1	CMV	Mock
UTGS-1.3	4,394 (356)	4,681 (377)	953 (210)	1,142 (182)	944 (52)
UTGS-1.5	11,786 (978)	14,008 (491)	1,079 (21)	924 (58)	545 (18)
UTGS-2.1	4,902 (268)	5,557 (138)	594 (116)	951 (189)	846 (72)
UTGS-2.2	3,872 (570)	3,527 (166)	503 (152)	1,020 (207)	765 (216)
UTGS-2.3	3,511 (49)	4,572 (413)	999 (125)	1,026 (101)	921 (169)
UTKOS-3	314 (31)	ND[c]	10,143 (697)	313 (49)	292 (29)
UTKOS-6	339 (33)	ND	14,884 (2,272)	785 (20)	416 (56)
UTAD169-3	729 (67)	ND	543 (104)	8,471 (1,016)	841 (73)
UTAD169-4	523 (12)	ND	479 (48)	11,735 (1,290)	631 (25)
MMC-PBMC	221 (14)	188 (25)	191 (14)	369 (10)	277 (50)

[a] Incorporation of [^{3}H]TdR into 10^4 clone cells in the presence of 10^5 MMC-treated autologous PBMC with HHV-6(GS), HHV-6(HST), HSV-1, CMV, or mock-infected control antigen was determined during the final 16 h of a 72-h incubation.
[b] The values represent the mean counts per minute (standard deviation in parentheses) of triplicate wells.
[c] ND, not done.

infected control antigens. Conversely, T-cell clones which proliferated in response to stimulation with HSV-1 and CMV were generated from PBMC of the same donor stimulated with HSV-1 and CMV antigens, respectively. These data indicated that the five clones all had a HHV-6-specific proliferative response. Their cell surface phenotype determined by flow cytometry appeared to be $CD3^+CD4^+CD8^-$ in all cases (data not shown).

HLA restriction in proliferative response. HLA restriction in the proliferative response of these HHV-6-specific $CD4^+$ T-cell clones was examined by performing blocking experiments with monoclonal antibodies against various HLA framework determinants. The results (Table 2) revealed that the proliferative response to HHV-6 antigen in the presence of autologous antigen-presenting cells was inhibited by the addition of monoclonal antibody directed against the class II HLA-DR framework, but not by those against class I HLA-A, HLA-B, HLA-C or class II HLA-DQ. These data indicated that the restriction element of the interaction between these T-cell clones and antigen-presenting cells in the proliferative response to HHV-6 is HLA-DR.

Cytotoxic reactivity of clones. We next examined the ability of clones to lyse HHV-6-infected cells. We prepared Epstein-Barr virus-transformed B-lymphoblastoid cell lines as target cells for ^{51}Cr release assays, but we found that B-lymphoblastoid cell lines were hardly infected with HHV-6, only a few virus particles being evident by electron microscopy. Therefore, because of the finding by Takahashi et al. (41) that HHV-6 preferentially infects on $CD4^+$ T cells, PHA-stimulated PBMC which had been depleted of $CD8^+$ T cells and NK cells (mostly $CD4^+$ T cells) were infected with HHV-6 and used as target cells for HHV-6-specific T-cell clones. Flow cytometry revealed that both HLA class I and class II antigens were abundantly expressed on the cell surface, and many virus particles were found in HHV-6-infected T lymphoblasts by electron microscopy (data not shown), suggesting that PHA-stimulated $CD4^+$ T lymphoblasts were useful as HHV-6-infected target cells in cytotoxicity assays.

As shown in Table 3, the cytotoxic reactivity of HHV-6-specific $CD4^+$ T-cell clones was classified into three categories. UTGS-1.3 and UTGS-2.3 showed cytotoxic activity against HHV-6-infected autologous cells, but not against HHV-6-infected allogeneic cells or NK-sensitive K562 cells (group 1). UTGS-2.1 showed cytotoxicity against HHV-6-infected autologous cells as well as HHV-6-infected allogeneic cells and K562 cells (group 2). UTGS-1.5 and UTGS-2.2 did not show any cytotoxic activity (group 3). Cytotoxicity assays were performed three times, and other HHV-6-infected allogeneic cells, which shared no HLA antigens with the clone cells, were also used for experiments. Consequently, the same cytotoxicity patterns were obtained

TABLE 2. HLA-DR-restricted proliferative response of clones to HHV-6[a]

Clone	Incorporation of [^{3}H]TdR (cpm)[b]			
	No antibody	w6/32	HU-4	HU-11
UTGS-1.3	4,390 (356)	3,946 (151)	518 (45)	3,108 (367)
UTGS-1.5	11,786 (978)	10,892 (260)	949 (124)	7,677 (879)
UTGS-2.1	4,902 (268)	5,027 (409)	267 (21)	4,118 (693)
UTGS-2.2	3,872 (570)	4,225 (817)	396 (65)	2,520 (388)
UTGS-2.3	3,511 (49)	2,949 (202)	244 (60)	2,698 (319)

[a] Incorporation of [^{3}H]TdR into 10^4 clone cells in the presence of 10^5 MMC-treated autologous PBMC and HHV-6(GS) antigen with monoclonal antibodies w6/32 (anti-HLA-A, anti-HLA-B, anti-HLA-C), HU-4 (anti-HLA-DR), or HU-11 (anti-HLA-DQ) or without antibody was determined during the final 16 h of a 72-h incubation.
[b] The values represent the mean counts per minute (standard deviation in parentheses) of triplicate wells.

TABLE 3. Cytotoxic reactivity of clones[a]

Clone	% Specific ^{51}Cr release from target cells			
	Auto(HHV-6$^+$)	Auto(−)[b]	Allo(HHV-6$^+$)	K562
UTGS-1.3	36.1	2.6	3.5	4.0
UTGS-2.3	21.4	0	2.9	4.2
UTGS-2.1	27.9	0.6	24.0	74.8
UTGS-1.5	0	0	0	1.2
UTGS-2.2	0	0	2.3	0.7

[a] Clones generated as described in Materials and Methods were tested for their ability to lyse various target cells in 5-h ^{51}Cr release assays at an effector-to-target ratio of 10:1.
[b] Uninfected autologous cells.

TABLE 4. Inhibition of cytotoxicity by monoclonal antibodies[a]

Clone	% Specific ^{51}Cr release from HHV-6-infected autologous cells			
	No antibody	w6/32	HU-4	HU-11
UTGS-1.3	36.1	26.6	0.8	33.9
UTGS-2.3	21.4	16.0	0	17.6
UTGS-2.1	27.9	26.0	25.9	29.2
UTGS-1.5	0	0	0	0.1
UTGS-2.2	0	0	0	0

[a] Lysis of HHV-6-infected autologous cells by clone cells was examined at an effector-to-target ratio of 10:1 in the presence and absence of monoclonal antibodies w6/32 (anti-HLA-A, anti-HLA-B, anti-HLA-C), HU-4 (anti-HLA-DR), or HU-11 (anti-HLA-DQ).

(data not shown). These data indicated that HHV-6-specific $CD4^+$ human T cells have functionally heterogeneous cytotoxic reactivity.

Effect of monoclonal antibodies on cytotoxicity of clones. To determine HLA restriction in the cytotoxicity of clones, we performed blocking experiments with monoclonal antibodies directed against HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ. As shown in Table 4, the cytotoxicity of group 1, which lysed only HHV-6-infected autologous cells, was inhibited by a monoclonal antibody directed against the HLA-DR framework. On the other hand, the cytotoxicity of group 2, which lysed HHV-6-infected autologous cells as well as HHV-6-infected allogeneic cells and K562 cells, was not inhibited by monoclonal antibodies directed against HLA-A, HLA-B, HLA-C, HLA-DR, or HLA-DQ. The cytotoxicity of group 3 clones was not induced by addition of monoclonal antibodies directed against HLA molecules on target cells. These data suggested that group 1 consisted of HLA-DR-restricted HHV-6-specific $CD4^+$ cytotoxic T cells (CTL), group 2 consisted of HHV-6-specific $CD4^+$ T cells with antigen-nonspecific cytotoxicity, and group 3 consisted of HHV-6-specific noncytotoxic $CD4^+$ T cells.

Production of IFN-γ by clones. The HHV-6-specific $CD4^+$ T-cell clones were examined for IFN-γ production after stimulation with HHV-6 antigen. Some clones produced a small amount of IFN-γ spontaneously, and all clones produced a large amount of IFN-γ after stimulation with HHV-6 antigen (Table 5). Production of IFN-γ by HHV-6-specific $CD4^+$ T-cell clones after stimulation with HHV-6 antigen was confirmed by Northern blot analysis (Fig. 1).

Expression of perforin by clones. Finally, we investigated whether the ability of clones to lyse target cells is correlated with the expression of perforin, which is one possible candidate for a cytotoxic mediator. As shown in Fig. 2, perforin mRNA expression was detected in all HHV-6-specific $CD4^+$ T cells, although the level of perforin expression was higher in HHV-6-specific and nonspecific CTL clones than in noncytotoxic T-cell clones. Densitometric analysis performed after short-term and long-term exposures showed that the level of perforin expression in the cytotoxic clones was about three times higher than that in the noncytotoxic clones (data not shown).

TABLE 5. Production of IFN-γ by clones[a]

Cells cultured	IFN-γ production (U/ml) with:	
	No antigen	HHV-6 antigen
E^-	<10	<10
UTGS-1.3 + E^-	32.2	97.2
UTGS-1.5 + E^-	<10	100.3
UTGS-2.1 + E^-	<10	100.9
UTGS-2.2 + E^-	<10	54.4
UTGS-2.3 + E^-	31.6	95.7

[a] Clone cells (5×10^5) and autologous E^- cells (1×10^6) were cultured with or without HHV-6(GS) antigen for 72 h, and IFN-γ production in culture supernatants was assayed as detailed in Materials and Methods.

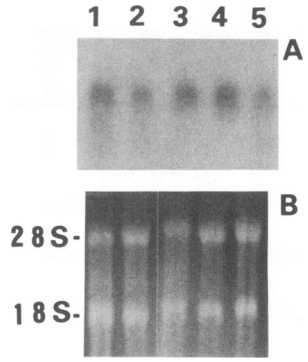

FIG. 1. Northern blot analysis of IFN-γ mRNA in HHV-6-specific $CD4^+$ human T-cell clones. RNA was prepared from each of the following clones: UTGS-1.3 (lane 1), UTGS-1.5 (lane 2), UTGS-2.1 (lane 3), UTGS-2.2 (lane 4), and UTGS-2.3 (lane 5). (A) Samples (10 μg) of total cellular RNA were hybridized with a ^{32}P-labeled IFN-γ cDNA probe. (B) Photograph of the corresponding total RNAs stained with ethidium bromide. The size markers are 18S and 28S rRNAs.

DISCUSSION

We have recently demonstrated that HHV-6-reactive memory T cells are present in the vast majority of healthy adults (43). In the present study, to further clarify the T-cell-mediated immune response against HHV-6 infection we established HHV-6-specific human T-cell clones and examined their various biological functions. Important findings obtained were as follows: (i) HHV-6-specific $CD4^+$ T-cell clones, which were restricted by HLA-DR in their

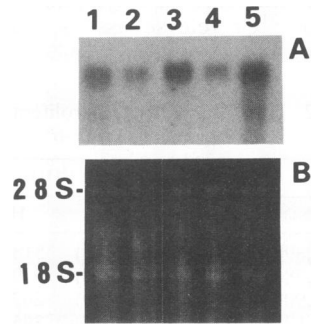

FIG. 2. Northern blot analysis of perforin mRNA in HHV-6-specific $CD4^+$ human T-cell clones. RNA was prepared from each of the following clones: UTGS-1.3 (lane 1), UTGS-1.5 (lane 2), UTGS-2.1 (lane 3), UTGS-2.2 (lane 4), and UTGS-2.3 (lane 5). (A) Samples (10 μg) of total cellular RNA were hybridized with a ^{32}P-labeled perforin cDNA probe. (B) Photograph of the corresponding total RNAs stained with ethidium bromide. The size markers are 18S and 28S rRNAs.

proliferative response, showed heterogeneous cytotoxic reactivity; (ii) all HHV-6-specific $CD4^+$ T-cell clones, irrespective of cytotoxic activity, secreted IFN-γ in response to stimulation with HHV-6; (iii) all HHV-6-specific $CD4^+$ T-cell clones expressed perforin mRNA, and the level of perforin expression was higher in cytotoxic than in noncytotoxic clones.

Functionally, $CD4^+$ T cells had been considered to be helper/inducer T cells (34). However, we first demonstrated the presence of HSV-specific $CD4^+$ human CTL, which were restricted by HLA class II antigens in both their cytotoxicity and proliferative response (48, 49). Since our reports, various virus-specific $CD4^+$ major histocompatibility complex class II-restricted CTL have been found in humans as well as in mice (14, 17, 18, 25, 27, 28). The group 1 clones reported here seem to correspond to such virus-specific and major histocompatibility complex class II-restricted $CD4^+$ CTL. The antigenic specificities of group 1 clones for cytotoxicity and the proliferative response were both HHV-6 specific, and these two functions were both inhibited by addition of a monoclonal antibody directed against HLA-DR. Therefore, this type of $CD4^+$ T cell seems to exhibit these two different functions, cytotoxicity and proliferative response with lymphokine production, simultaneously via a single antigen-recognizing receptor, the CD3–T-cell receptor complex. On the other hand, the group 2 clone showed HHV-6-specific proliferative response, but its cytotoxic reactivity was not specific for HHV-6. In addition, although the proliferative response was inhibited by addition of a monoclonal antibody directed against HLA-DR, the cytotoxic reactivity was HLA unrestricted. These findings strongly suggest that the T-cell receptor on the group 2 clone is undoubtedly specific for the epitope of HHV-6, although its cytotoxicity is mediated via a structure other than the CD3–T-cell receptor complex.

Murine $CD4^+$ T cells are reported to show divergence into two functionally distinct subpopulations (7, 29). That is, $CD4^+$ T-cell clones designated Th1 synthesize IL-2, IFN-γ, and tumor necrosis factor beta, whereas Th2 clones produce IL-4 and IL-5. In addition, it has been shown that Th1 clones are cytolytic for appropriate target cells and can mediate delayed-type hypersensitivity responses, whereas Th2 clones induce B-cell activation and are particularly important in mediating immunoglobulin G1 and immunoglobulin E production, but fail to mediate cytotoxicity (6, 30). In contrast with the definite dichotomy of murine $CD4^+$ T cells, many human $CD4^+$ T-cell clones have been reported to secrete both Th1 and Th2 lymphokines simultaneously (26, 31, 33, 36). All HHV-6-specific $CD4^+$ human T-cell clones generated in the present study secreted IFN-γ in response to stimulation with HHV-6 antigen, but were classified into distinct groups according to the presence and specificity of cytotoxic activity. These data are compatible with the results of our recent study, which showed that the majority of HSV-specific $CD4^+$ human T-cell clones were able to produce IL-2, IL-4, and IFN-γ simultaneously, but were classified into HLA class II-restricted CTL, HLA-unrestricted CTL, and noncytotoxic clones (47). These results suggest that human $CD4^+$ T cells certainly constitute heterogeneous populations, although the classification of human $CD4^+$ T-cell subpopulations seems to differ somewhat from that of murine $CD4^+$ T-cell subpopulations, Th1 and Th2.

The mechanisms of cytolysis mediated by CTL and NK cells have recently been studied in detail, and several cytolytic mediators of lymphocytes have been characterized. One of these, perforin, has been suggested to be a candidate for an effector molecule responsible for cell-mediated cytotoxicity (32, 51). Recent studies have demonstrated that the expression of perforin is restricted in some $CD8^+$ T cells and NK cells (19, 39). In view of these findings, we investigated whether $CD4^+$ T cells possessing cytotoxic ability express perforin mRNA. Contrary to our expectation from previous studies, although the expression level was higher in cytotoxic than noncytotoxic clones, perforin mRNA was detected in all HHV-6-specific $CD4^+$ T-cell clones, irrespective of the cytotoxic reactivity. Recent studies by us and other investigators have demonstrated that the frequency of $CD4^+$ T cells capable of lysing appropriate target cells is higher than was previously thought, especially for herpesvirus infection (37, 46). In addition, it was found that even $CD4^+$ T cells without spontaneous cytotoxic activity can lyse target cells under certain circumstances, such as culture with lectin (47). Indeed, the group 3 clones, UTGS-1.5 and UTGS-2.2, which showed no spontaneous cytotoxicity, lysed HHV-6-infected cells and K562 cells in the presence of PHA (data not shown). Therefore, from the results presented here, it can be suggested that perforin is an important mediator for cytolysis in $CD4^+$ T cells as well as $CD8^+$ T cells and NK cells.

Five of the HHV-6-specific T-cell clones generated in the present study had a $CD4^+CD8^-$ surface phenotype. Although the number of established clones is limited, this result suggests that $CD4^+$ memory T cells may be activated easily by stimulation with HHV-6 antigen. When considering the evidence that reactivation of HHV-6 is frequently observed in patients with $CD4^+$ T-cell immunodeficiency, such as that associated with AIDS and human T-lymphotropic virus infection (1, 9, 35, 42), the role of $CD4^+$ T cells in protection against HHV-6 infection in vivo is of considerable interest. The role of $CD4^+$ T cells in protection against viral infections has been discussed, focusing mainly on their helper activity. However, the present results shed more light from different viewpoints on the mechanisms of immune responses against viral infections. Specifically, it can be suggested that $CD4^+$ T cells may directly and indirectly limit the spread of HHV-6 infection by their cytotoxicity, as well as their helper function for antibody production by B cells. In addition, IFN-γ produced by $CD4^+$ T cells may inhibit the replication of HHV-6 and augment the NK activity which is important for resistance against viral infections. Moreover, IFN-γ, which is known to be an inducer of major histocompatibility complex class II antigens, may augment the expression of HLA class II antigen on HHV-6-infected cells and may consequently make virus-infected cells more sensitive to lysis by $CD4^+$ CTL. A recent study demonstrating that HHV-6 latently infects mainly monocytes, which express HLA class II antigens on the cell surface (23), also suggests the importance of $CD4^+$ T cells in protection against reactivation of HHV-6 in vivo. Taking these results together, it can be postulated that $CD4^+$ T cells play a key role in protection against and recovery from HHV-6 infection.

ACKNOWLEDGMENTS

We thank R. Mori, Kyushu University, K. Yamanishi, Osaka University, and Y. Minamishima, Miyazaki Medical College, for providing viruses; K. Arai, Tokyo University, and K. Okumura, Juntendo University, for providing cDNA probes; and A. Wakisaka, Hokkaido University, and N. Ohta, Okayama University, for providing monoclonal antibodies. We also thank S. Fujita, Ehime University, for helpful discussions and for reviewing the manuscript.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Agut, H., D. Guetard, H. Collandre, C. Dauguet, L. Montagnier, J.-M. Miclea, H. Baurmann, and A. Gessain.** 1988. Concomitant infection by human herpesvirus 6, HTLV-I, and HIV-2. Lancet **i:**712.
2. **Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler.** 1978. Production of monoclonal antibodies to Group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. Cell **14:**9–20.
3. **Briggs, M., J. Fox, and R. S. Tedder.** 1988. Age prevalence of antibody to human herpesvirus 6. Lancet **i:**1058–1059.
4. **Brown, N. A., C. V. Sumaya, C.-R. Liu, Y. Ench, A. Kovacs, M. Coronesi, and M. H. Kaplan.** 1988. Fall in human herpesvirus 6 seropositivity with age. Lancet **ii:**396.
5. **Carrigan, D. R., W. R. Drobyski, S. K. Russler, M. A. Tapper, K. K. Knox, and R. C. Ash.** 1991. Interstitial pneumonitis associated with human herpesvirus-6 infection after marrow transplantation. Lancet **338:**147–149.
6. **Cher, D. J., and T. R. Mosmann.** 1987. Two types of murine helper T cell clones. II. Delayed-type hypersensitivity is mediated by Th1 clones. J. Immunol. **138:**3688–3694.
7. **Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann.** 1987. Two types of mouse helper T cell clones. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. **166:**1229–1244.
8. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
9. **Downing, R. G., N. Sewankambo, D. Serwadda, R. Honess, D. Crawford, R. Jarrett, and B. E. Griffin.** 1987. Isolation of human lymphotropic herpesviruses from Uganda. Lancet **ii:**390.
10. **Dubedat, S., and N. Kappagoda.** 1989. Hepatitis due to human herpesvirus-6. Lancet **ii:**1463–1464.
11. **Eizuru, Y., T. Minematsu, Y. Minamishima, M. Kikuchi, K. Yamanishi, M. Takahashi, and T. Kurata.** 1989. Human herpesvirus 6 in lymph nodes. Lancet **ii:**1391.
12. **Flamand, L., J. Gosselin, M. D'Addario, J. Hiscott, D. V. Ablashi, R. C. Gallo, and J. Menezes.** 1991. Human herpesvirus 6 induces interleukin-1β and tumor necrosis factor alpha, but not interleukin-6, in peripheral blood mononuclear cell cultures. J. Virol. **65:**5105–5110.
13. **Gray, P. W., D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, C. C. Simonsen, R. Derynck, P. J. Sherwood, D. M. Wallace, S. L. Berger, A. D. Levinson, and D. V. Goeddel.** 1982. Expression of human immune interferon cDNA in *E. coli* and monkey cells. Nature (London) **295:**503–508.
14. **Hayward, A. R., O. Pontesilli, M. Herberger, M. Laszlo, and M. Levin.** 1986. Specific lysis of varicella-zoster virus-infected B lymphoblasts by human T cells. J. Virol. **58:**179–184.
15. **Hirayama, K., S. Matsushita, I. Kikuchi, M. Iuchi, N. Ohta, and T. Sasazuki.** 1987. HLA-DQ is epistatic to HLA-DR in controlling the immune response to schistosomal antigen in humans. Nature (London) **327:**426–430.
16. **Ikeda, H., M. Kasahara, K. Ogasawara, T. Takenouchi, T. Okuyama, N. Ishikawa, A. Wakisaka, Y. Kikuchi, and M. Aizawa.** 1984. Evidence for polymorphism of MB3 antigens among three HLA-D clusters associated with HLA-DR4. Immunogenetics **19:**381–390.
17. **Jacobson, S., J. R. Richert, W. E. Biddison, A. Satinsky, R. J. Hartzman, and H. F. McFarland.** 1984. Measles virus-specific $T4^+$ human cytotoxic T cell clones are restricted by class II HLA antigens. J. Immunol. **133:**754–757.
18. **Kaplan, D. R., R. Griffith, V. L. Braciale, and T. J. Braciale.** 1984. Influenza virus-specific human cytotoxic T cell clones: heterogeneity in antigenic specificity and restriction by class II MHC products. Cell. Immunol. **88:**193–206.
19. **Kawasaki, A., Y. Shinkai, Y. Kuwana, A. Furuya, Y. Iigo, N. Hanai, S. Itoh, H. Yagita, and K. Okumura.** 1990. Perforin, a pore-forming protein detectable by monoclonal antibodies, is a functional marker for killer cells. Int. Immunol. **2:**677–684.
20. **Kikuta, H., A. Nakane, H. Lu, Y. Taguchi, T. Minagawa, and S. Matsumoto.** 1990. Interferon induction by human herpesvirus 6 in human mononuclear cells. J. Infect. Dis. **162:**35–38.
21. **Knowles, W. A., and S. D. Gardner.** 1988. High prevalence of antibody to human herpesvirus-6 and seroconversion associated with rash in two infants. Lancet **ii:**912–913.
22. **Kojima, H., Y. Fukasawa, N. Ishikawa, Y. Tajima, A. Wakisaka, and M. Aizawa.** 1988. Detection of a novel HLA-DQ specificity. II. cellular analysis by cytotoxic T-cell clones. Immunogenetics **27:**145–147.
23. **Kondo, K., T. Kondo, T. Okuno, M. Takahashi, and K. Yamanishi.** 1991. Latent human herpesvirus 6 infection of human monocytes/macrophages. J. Gen. Virol. **72:**1401–1408.
24. **Levy, J. A., F. Ferro, D. Greenspan, and E. T. Lennette.** 1990. Frequent isolation of HHV-6 from saliva and high seroprevalence of the virus in the population. Lancet **335:**1047–1050.
25. **Lindsley, M. D., D. J. Torpey III, and C. R. Rinaldo, Jr.** 1986. HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by Leu-3-positive T cells. J. Immunol. **136:**3045–3051.
26. **Maggi, E., G. Del Prete, D. Macchia, P. Parronchi, A. Tiri, I. Chretien, M. Ricci, and S. Romagnani.** 1988. Profiles of lymphokine activities and helper function for IgE in human T cell clones. Eur. J. Immunol. **18:**1045–1050.
27. **Misco, I. S., J. H. Pope, R. Hutter, T. D. Soszynski, and R. G. Kane.** 1984. HLA-DR antigen-associated restriction of EBV-specific cytotoxic T-cell colonies. Int. J. Cancer **33:**239–243.
28. **Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale.** 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. **163:**903–921.
29. **Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman.** 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136:**2348–2357.
30. **Mosmann, T. R., and R. L. Coffman.** 1987. Two types of mouse helper T-cell clone. Immunol. Today **8:**223–227.
31. **Paliard, X., R. de Waal Malefijt, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J. de Vries, and H. Spits.** 1988. Simultaneous production of IL-2, IL-4, and IFN-γ by activated human $CD4^+$ and $CD8^+$ T cell clones. J. Immunol. **141:**849–855.
32. **Podack, E. R., H. Hengartner, and M. G. Lichtenheld.** 1991. A central role of perforin in cytolysis? Annu. Rev. Immunol. **9:**129–157.
33. **Quint, D. J., E. J. Bolton, L. A. McNamee, R. Solari, P. H. Hissey, B. R. Champion, A. R. Mackenzie, and E. D. Zanders.** 1989. Functional and phenotypic analysis of human T-cell clones which stimulate IgE production in vitro. Immunology **67:**68–74.
34. **Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman.** 1979. Separation of functional subsets of human T cells by a monoclonal antibody. Proc. Natl. Acad. Sci. USA **76:**4061–4065.
35. **Salahuddin, S. Z., D. V. Ablashi, P. D. Markham, S. F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R. C. Gallo.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science **234:**596–601.
36. **Salmon, M., G. D. Kitas, and P. A. Bacon.** 1989. Production of lymphokine mRNA by $CD45R^+$ and $CD45R^-$ helper T cells from human peripheral blood and by human $CD4^+$ T cell clones. J. Immunol. **143:**907–912.
37. **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:**3610–3616.
38. **Shinkai, Y., M. C. Yoshida, K. Maeda, T. Kobata, K. Maruyama, J. Yodoi, H. Yagita, and K. Okumura.** 1989. Molecular

cloning and chromosomal assignment of a human perforin (PFP) gene. Immunogenetics **30:**452–457.

39. **Smyth, M. J., J. R. Ortaldo, Y. Shinkai, H. Yagita, M. Nakata, K. Okumura, and H. A. Young.** 1990. Interleukin 2 induction of pore-forming protein gene expression in human peripheral blood $CD8^+$ T cells. J. Exp. Med. **171:**1269–1281.
40. **Steeper, T. A., C. A. Horwitz, D. V. Ablashi, S. Z. Salahuddin, C. Saxinger, R. Saltzman, and B. Schwartz.** 1990. The spectrum of clinical and laboratory findings resulting from human herpesvirus-6 (HHV-6) in patients with mononucleosis-like illnesses not resulting from Epstein-Barr virus or cytomegalovirus. Am. J. Clin. Pathol. **93:**776–783.
41. **Takahashi, K., S. Sonoda, K. Higashi, T. Kondo, H. Takahashi, M. Takahashi, and K. Yamanishi.** 1989. Predominant CD4 T-lymphocyte tropism of human herpesvirus 6-related virus. J. Virol. **63:**3161–3163.
42. **Tedder, R. S., M. Briggs, C. H. Cameron, R. Honess, D. Robertson, and H. Whittle.** 1987. A novel lymphotropic herpesvirus. Lancet **ii:**390–392.
43. **Yakushijin, Y., M. Yasukawa, and Y. Kobayashi.** 1991. T-cell immune response to human herpesvirus-6 in healthy adults. Microbiol. Immunol. **35:**655–660.
44. **Yamanishi, K., T. Okuno, K. Shiraki, M. Takahashi, T. Kondo, Y. Asano, and T. Kurata.** 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet **i:**1065–1067.
45. **Yasukawa, M., A. Inatsuki, and Y. Kobayashi.** 1988. Helper activity in antigen-specific antibody production mediated by $CD4^+$ human cytotoxic T cell clones directed against herpes simplex virus. J. Immunol. **140:**3419–3425.
46. **Yasukawa, M., A. Inatsuki, and Y. Kobayashi.** 1989. Differential in vitro activation of $CD4^+CD8^-$ and $CD8^+CD4^-$ herpes simplex virus-specific human cytotoxic T cells. J. Immunol. **143:**2051–2057.
47. **Yasukawa, M., A. Inatsuki, and Y. Kobayashi.** 1991. Functional heterogeneity among herpes simplex virus-specific human $CD4^+$ T cells. J. Immunol. **146:**1341–1347.
48. **Yasukawa, M., and J. M. Zarling.** 1984. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class II MB and DR antigens. J. Immunol. **133:**422–427.
49. **Yasukawa, M., and J. M. Zarling.** 1984. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. II. Bifunctional clones with cytotoxic and virus-induced proliferative activities exhibit herpes simplex virus type 1 and 2 specific or type common reactivities. J. Immunol. **133:**2736–2742.
50. **Yoshikawa, T., S. Suga, Y. Asano, T. Yazaki, H. Kodama, and T. Ozaki.** 1989. Distribution of antibodies to a causative agent of exanthem subitum (human herpesvirus-6) in healthy individuals. Pediatrics **84:**675–677.
51. **Young, J. D.-E., Z. A. Cohn, and E. R. Podack.** 1986. The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological and functional similarities. Science **233:**184–190.