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CONCISE COMMUNICATION

CD8+ Cytotoxic T Lymphocyte Responses to Lytic Proteins of Human Herpes Virus 8 in Human Immunodeficiency Virus Type 1–Infected and –Uninfected Individuals

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T cell immunity to lytic proteins of herpesviruses is important in host control of infection. We have characterized the cytotoxic T lymphocyte (CTL) response to 5 human herpesvirus 8 (HHV-8) homologues of lytic proteins in HHV-8–seropositive individuals. HLA class I–restricted, CD8+ CTL responses to ⩾1 HHV-8 lytic protein were detected in all 14 HHV-8–seropositive study subjects tested, with or without human immunodeficiency virus type 1 (HIV-1) infection, but not in any of 5 HHV-8–seronegative individuals. Seven of these study subjects with both HHV-8 and HIV-1 infection had greater anti-CTL reactivity to glycoprotein H (open-reading frame 22) than did the 7 study subjects infected only with HHV-8. Moreover, there was a strong, inverse correlation between HIV-1 load and glycoprotein H–specific CTL lysis in the study subjects infected with both viruses. CTL reactivity to HHV-8 lytic proteins may be involved in host control of HHV-8–related diseases, such as Kaposi's sarcoma.

Human herpesvirus 8 (HHV-8) is the etiologic agent of Kaposi's sarcoma (KS) [1]. The incidence of KS is high in individuals who are immunosuppressed because of human immunodeficiency virus type 1 (HIV-1) infection. Because T cell immunity is important in host control of herpesvirus infections, such as Epstein-Barr virus (EBV) [2], loss of cytotoxic T lymphocyte (CTL) responses to HHV-8 infection due to HIV-1 infection may be related to the development of HIV-1–associated KS.

HHV-8 encodes lytic cycle proteins homologous to the human and murine γ-herpesviruses, EBV, and murine herpesvirus 68 (MHV-68) [3]. These lytic proteins may be important in eliciting CD8+ CTL responses that are operative in controlling HHV-8 infection. This type of immune control is analogous to the regulation of EBV and MHV-68 infections by CTL to structural and regulatory lytic cycle proteins of these viruses [4, 5]. Therefore, we constructed vaccinia virus vectors that express 5 lytic proteins for herpesvirus homologues of glycoprotein B (gB; open reading frame [ORF] 8), glycoprotein H (gH; ORF22), major capsid protein (MCP; ORF25), a minor capsid protein (MiCP; ORF26), and an immediate-early protein (IE; ORF57). We then characterized CTL responses to these HHV-8 lytic proteins in HHV-8–seropositive individuals who were HIV-1 infected or uninfected, as well as individuals who were both HIV-1 and HHV-8 seronegative.

Subjects and Methods

Study subjects. The study subjects were selected on the basis of seropositivity for HHV-8 from the Pittsburgh portion of the Multicenter AIDS Cohort Study (MACS) [6] and from heterosexual laboratory volunteers. Three groups of study subjects were identified on the basis of HIV-1 and HHV-8 antibody status (table 1). Group A included 7 individuals who were infected with HIV-1 and were HHV-8 seropositive, 5 of whom had a history of KS. Group B included 7 study subjects who were HIV-1 seronegative and HHV-8 seropositive; 6 were homosexual men from the MACS (including 1 [study subject 8] with biopsy-proven KS), and 1 was

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Informed consent was obtained for all participants in this study, and human experimentation guidelines of the US Department of Health and Human Services and those of the authors' institutions were followed in the conduct of this research.

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Table 1. Human herpesvirus (HHV) type 8–specific cytotoxic T lymphocyte (CTL) responses to lytic proteins.

Group, ID	HIV-1 Ab	HIV-1 virus load[a]	HHV-8 Ab	Duration of HHV-8 seropositivity[b]	KS (years)[c]	Treatment (years)[d]	CD4 count[e]	CTL activity (% of net-specific lysis) to[f]				
								gB	gH	MCP	MiCP	IE
A												
1	Pos	300	Pos	13	Yes (5)	Lamivudine, stavudine, and indinavir (2)	203	1	**13**	4	0	0
2	Pos	300	Pos	13	Yes (3.5)	Lamivudine, stavudine, and indinavir (1)	298	0	**15**	0	**20**	1
3	Pos	21,417	Pos	13	Yes (1)	Stavudine and indinavir (0.5)	780	5	**12**	7	0	2
4	Pos	931	Pos	13	No	Stavudine and indinavir (0.5)	713	**11**	**13**	2	2	5
5	Pos	1,309,495	Pos	7	Yes (1)	None	312	1	**11**	0	0	0
6	Pos	92,354	Pos	4	No	None	492	0	**10**	**18**	0	2
7	Pos	447,732	Pos	4	Yes (1)	None	368	5	2	1	1	**11**
B												
8	Neg	—	Pos	13	Yes (3)	—	502	**11**	**11**	6	**10**	5
9	Neg	—	Pos	12	No	—	1144	**22**	3	8	**31**	**21**
10	Neg	—	Pos	1	No	—	469	5	2	1	1	**11**
11	Neg	—	Pos	8	No	—	898	0	5	5	**10**	8
12	Neg	—	Pos	13	No	—	637	1	0	0	**18**	0
13	Neg	—	Pos	3	No		1007	**10**	3	**16**	**10**	**20**
14	Neg	—	Pos	ND	No		938	**10**	**29**	**10**	**18**	**10**
C												
15	Neg	—	Neg	—	—		1265	0	1	3	0	0
16	Neg	—	Neg	—	—		1334	0	0	0	4	4
17	Neg	—	Neg	—	—		ND	4	8	8	0	3
18	Neg	—	Neg	—	—		ND	0	6	1	1	1
19	Neg	—	Neg	—	—		ND	2	3	0	0	1

NOTE. ID, study subject identification number; Ab, antibody; HIV, human immunodeficiency virus; KS, Kaposi's sarcoma; gB, glycoprotein B; gH, glycoprotein H; MCP, major capsid protein; MiCP, minor capsid protein; IE, immediate-early protein; Pos, positive; Neg, negative; ND, no data.

[a] Copy no. of HIV-1 RNA per mL.

[b] Estimated minimal duration of HHV-8 seropositivity.

[c] Years between diagnosis of KS and when the PBMC were tested for anti–HHV-8 CTL.

[d] Years between initiation of treatment and when the PBMC were tested for anti–HHV-8 CTL.

[e] No. per mm^3.

[f] Data in boldface are positive CTL responses for each HHV-8 antigen.

a heterosexual volunteer. Group C included 5 individuals who were both HIV-1 and HHV-8 seronegative.

HIV-1 serology and viral load. HIV-1 antibody was measured by enzyme immunoassay (Genetic Systems, Redmond, WA) and immunoblot (Biorad Laboratories, Hercules, CA). Plasma samples were assayed for HIV-1 RNA by the quantitative reverse transcriptase–polymerase chain reaction (PCR) assay, with the lower limit of detection being 500 copies of RNA/mL of plasma (Amplicor; Roche Diagnostics, Nutley, NJ).

T cell phenotyping. T cell subsets were measured by flow cytometry (Profile II; Beckman Coulter, Fullerton, CA) after staining with monoclonal antibodies specific for CD3, CD4, and CD8 T cells (Becton-Dickinson, Mountain View, CA).

Indirect immunofluorescence assay for HHV-8 antibodies. HHV-8 antibodies against HHV-8 lytic antigens were determined by use of an indirect immunofluorescence assay using 10-Q-tetradecanoyl phorbol 13-acetate–induced body cavity B cell lymphoma (BCBL)–1 cells containing the HHV-8 genome [7]. All serum samples were tested twice in a blinded fashion and were assessed microscopically for the presence of whole cell immunofluorescence by the same reader. Positive samples were serially diluted to determine the antibody titers (reciprocal of the last positive dilution). The titer for a positive reactivity was ≥ 50.

Construction of recombinant vaccinia viruses that express HHV-8 genes. The ORF for gB was obtained as the plasmid gB-Blunt-pCR (P. Pellett, unpublished data). The ORFs for gH, MCP, MiCP, and the IE proteins were amplified by PCR using the HHV-8 genome contained in the BCBL-1 cell line as the template. The primer sets used for PCR amplification were (gH) 5′-CACCTAGAGGATCCGACATGCAGGGTC, 3′-TAAAAAATCTAAAGCTTTATTGACCG; (MCP) 5′-CTCGAGCGCTGGATCCATGGAGGCG, 3′-CACGATGAAAGCTTTCGAGCC; (MiCP) 5′-GGCTAGTCATATGGCACTCGAC, 3′-CACGATGAAAGCTTTCGAGCC; and (IE) 5′-CTCCCTGCGAATTCGCATGATAATTG, 3′-GTTACATGGAATTCACGGGAGACAC. The amplified ORFs were cloned into the vaccinia virus shuttle vector (pSC11) and the recombinant vaccinia viruses constructed by transfection of cells infected with wild-type vaccinia virus, as described elsewhere [8]. Each virus was plaque-purified 3 times. Rabbit polyclonal antibodies against MiCP and mouse polyclonal antibodies against MCP were generated by immunization of animals with purified bacterial fusion proteins. Mouse polyclonal antibodies against IE protein were generated by immunization of animals with a peptide corresponding to amino acids EYYRPGDVMGLLNVLV. Rabbit antiserum against gB and gH was generated against recombinant proteins. Identification of the HHV-8 proteins expressed by the recombinant vaccinia viruses was determined by Western blot analysis of infected cell lysates, as described elsewhere [8]. Protein bands

corresponding to the predicted sizes of the individual proteins were noted in vaccinia virus recombinant-infected cell lysates as 120 kDa (gB), 74 kDa (gH), 150 kDa (MCP), 32 kDa (MiCP), and 27 kDa (IE).

Anti–HHV-8 CTL bulk lysis assay. On day 0, autologous, EBV-transformed B lymphocyte cell lines (B-LCL) were infected overnight with recombinant vaccinia viruses at 4:1 multiplicity of infection in RPMI medium (Life Technologies, Gaithersburg, MD) supplemented with 15% fetal calf serum (FCS) at 37°C in 5% CO_2. On day 1, these cells were inactivated with psoralen (10 μg/mL; Sigma, St. Louis) and ultraviolet light (40 mW/cm^2) for 10 min, and then were resuspended at the concentration of 5×10^4 cells/mL in stimulation medium (RPMI 1640 + 15% FCS + recombinant interleukin 2 [100 U/mL; Chiron, Emeryville, CA]). Frozen peripheral blood mononuclear cells (PBMC; effectors) were thawed and resuspended at the concentration of 2.5×10^5 cells/mL in stimulation medium. The viability of thawed PBMC obtained by staining with 0.4% trypan blue dye ranged from 89% to 97%. Gamma-irradiated (3000 Rad) allogeneic fresh PBMC (feeders) were resuspended at 1×10^6 cells/mL in stimulation medium. Stimulators, feeders, and effectors were plated into round-bottom 96-well plates (Becton Dickinson) as 50 μL, 50μL, and 100 μL, respectively. The plates were incubated at 37°C in 5% CO_2 for 14 days, and the medium was changed every 3–4 days. On day 13, the targets were prepared by radiolabeling infected autologous B-LCL with ^{51}Cr (100 μCi/mL; Dupont NEN, Boston) for 16 h at 37°C in 5% CO_2. On day 14, the target cells were washed, and the effectors were harvested and plated at effector (E)–to–target (T) ratios of 20:1, 10:1, and 5:1 in triplicate. The expression of all of these recombinant vaccinia viruses in the B-LCL was ~80%–90% as shown by β-gal expression. The plates were then incubated for 4 h, harvested, and counted in a gamma counter. The percentage of specific cytotoxicity was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. The results were expressed as net specific lysis (i.e., the level of lysis of cells expressing the control vaccinia virus vector [VSC11] subtracted from the percentage of lysis of cells expressing HHV-8 antigens). The mean lysis ± SE for the VSC11 control was 8.5 ± 1.0% at the E-to-T ratio of 20:1. A level of ⩾10% HHV-8–specific net lysis at the E-to-T ratio of 20:1 was defined as positive CTL reactivity on the basis of the mean + 3 SD of virus-specific lysis by PBMC obtained from HHV-8–seronegative donors.

Statistical analysis. Nonparametric correlations of CTL lysis with viral load and CD4$^+$ T cell count were assessed by use of the 2-tailed Spearman's rank correlation test. Differences among the 3 groups in the cross-sectional study were evaluated by Fisher's exact test and the Mann-Whitney *U* test.

Results

The composite results show that HHV-8–specific CTL responses could be detected to ⩾1 of the 5 HHV-8 lytic proteins in all of the HHV-8–seropositive group A and B study subjects tested (table 1). There was good agreement between the CTL results obtained from repeat testing of the same, cryopreserved PBMC samples from the same study subjects (i.e., an average difference of only 2 ± 0.5% net specific lysis between tests [data not shown]). Using anti–human leukocyte antigen (HLA) class I antibody (clone W6/32), 97% of the HHV-8–specific CTL reactivity was blocked at E-to-T ratio of 20:1, whereas only 30% of such activity was blocked by anti-HLA class II antibody (clone I3; data not shown). Moreover, >99% of the anti–HHV-8 CTL reactivity was mediated by CD8$^+$ T cells enriched to 92% purity by negative selection with immunomagnetic beads and <1% by CD4$^+$ enriched T cells. Thus, these HHV-8–specific CTL responses were mediated by major histocompatibility complex–class I restricted CD8$^+$ T cells.

The 7 group A study subjects had CTL reactivity to ⩾1 lytic antigen but did not have CTL reactivity to >2 antigens (median CTL response, 1; range, 1–2; table 1); however, they had greater anti-CTL reactivity to gH (median, 12; range, 2–15) than did the group B study subjects (median, 3; range, 0–29; $P < .025$). Moreover, there was a strong, inverse correlation between HIV viral load and only gH-specific CTL lysis ($r = -0.88$; $P = .008$; figure 1), but there was no correlation between anti-gH CTL lysis and numbers of CD4$^+$ T cells ($r = 0.10$; $P = .82$) in the group A study subjects.

The 7 group B HHV-8–seropositive, HIV-1–seronegative study subjects had CTL reactivity to a median of 3 HHV-8 lytic proteins (range, 1–5), particularly MiCP and IE protein (table 1). The anti-MiCP (median, 11; range, 1–31) and anti-IE CTL lysis (median, 10; range, 0–21) in group B study subjects were significantly higher than in the group A study subjects ($P < .025$). There were no HHV-8–specific CTL responses detected in group C study subjects who were HIV-1 and HHV-8 seronegative (table 1).

Discussion

Our data show that HLA class–I restricted, CD8$^+$ CTL specific for ⩾1 HHV-8 lytic protein were detected in all HHV-8–seropositive persons, including HIV-1–infected individuals with

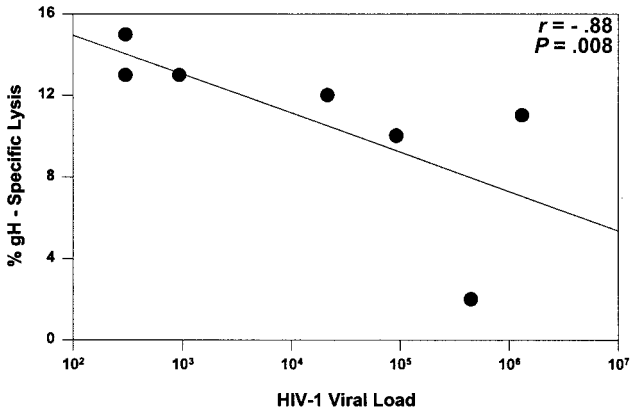

Figure 1. Correlation between human herpesvirus 8 glycoprotein H (gH)–specific cytotoxic T lymphocytic reactivity and human immunodeficiency virus (HIV) type 1 viral load in group A. Specific lysis (%) was net-specific lysis at an effector-to-target ratio of 20:1.

and without a history of KS. In contrast, a recent study has detected HHV-8–specific, T cell–proliferative responses in only 42% of HHV-8–seropositive persons who were HIV-1 uninfected and in 0% of HHV-8–seropositive persons who were HIV-1 seropositive [9]. Moreover, HLA class I–restricted CTL responses to HHV-8 have been reported in 100% and 33% of HHV-8 seropositive subjects who were HIV-1 infected with or without KS, respectively [10]. This lower prevalence of anti–HHV-8 T cell responses may be due to differences in the $CD4^+$ and $CD8^+$ T cell reactivity to HHV-8 antigens and to the types of antigens and techniques used in the CTL assays.

T cell reactivity was lower to all of the HHV-8 lytic proteins, except to gH in the HIV-1–infected cohort compared with the HIV-1-uninfected group. These results suggest that progressive HIV-1 infection causes a loss of memory CTL reactivity to HHV-8, similar to the loss of anti–HIV-1 CTL and CTL specific for other herpesviruses [11]. The implications of the predominant response to gH in our HIV-1-infected group is unclear. Interestingly, there was a strong inverse correlation between HIV-1 viral load and anti-gH CTL lysis. Possible reasons for the predominant CTL activity to gH during HIV-1 infection include cross-reactivity to non–HHV-8 antigens, such as the EBV homologue of gH. Indeed, EBV infection is active in a large proportion of HIV-1–infected individuals, as shown by persistent shedding of the virus and high titers of antibodies to EBV [12, 13]. However, this is unlikely to be the cause of the anti–HHV-8 CTL activity detected in our study, because we have shown in longitudinal studies that $CD8^+$ T cell responses to these HHV-8 lytic proteins are not due to cross-recognition by T cells (Q. J. Wang, F. J. Jenkins, L. P. Jacobson, L. A. Kingsley, R. D. Day, Z.-W. Zhang, Y.-X. Meng, P. E. Pellet, K. G. Kousoulas, A. Baghian, and C. R. Rinaldo, Jr., unpublished data).

Alternatively, this more robust anti-gH T cell reactivity could be related to selective production or more dominant immunogenicity of this lytic protein during persistent HIV-1 infection, with consequent differential T cell reactivity that is suppressed at higher HIV-1 loads. There was, however, broad specificity of these CTL for HHV-8 proteins in the HHV-8–seropositive, HIV-1–seronegative persons. This suggests that there is no predominant antigen specificity among these 5 proteins for CTL present during this presumably latent phase of HHV-8 infection.

This lack of a dominant CTL response to a specific HHV-8 protein concurs with our longitudinal study of CTL reactivity during primary HHV-8 infection (Q. J. Wang, F. J. Jenkins, L. P. Jacobson, L. A. Kingsley, R. D. Day, Z.-W. Zhang, Y.-X. Meng, P. E. Pellet, K. G. Kousoulas, A. Baghian, and C. R. Rinaldo, Jr., unpublished data). Using comparable in vitro systems with vaccinia virus recombinant vectors, as well as synthetic peptides, recent studies have shown that the IE regulatory protein BMLF1 and other lytic cycle proteins of EBV are targets for $CD8^+$ CTL during primary and latent infection [4]. Moreover, lytic cycle epitopes are predominant targets during MHV-68 infection [5]. Further research is needed to determine whether this immune function is operative in the control of HHV-8 infection and the prevention of HHV-8–related diseases.

The increased incidence of KS in HIV-1–infected, immunosuppressed patients suggests that cell-mediated immune responses are important in the control of HHV-8 infection and therefore the development of KS [1]. A recent study from the MACS showed that there is a shorter incubation period for development of KS after HHV-8 infection in homosexual men who are already HIV-1–infected, compared with men who are infected with HHV-8 before being infected with HIV-1 [14]. Primary infection with HHV-8 in immunosuppressed persons thus has a more severe outcome than reactivated HHV-8 infection, similar to primary EBV infection in organ and tissue transplant recipients [15]. In addition, the incidence of KS has declined dramatically in HIV-1–infected individuals after suppression of HIV-1 load by combination antiretroviral drug therapy [16], in which T cell numbers and function are partially restored [17]. These cumulative findings, together with our data and those of others [10], that anti–HHV-8 CTL responses are lower in HIV-1 infected individuals, support the role of $CD8^+$ T cell immunity in the control of HHV-8 and KS.

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