Recent Clinical Isolates of Cytomegalovirus Suppress Human Cytomegalovirus-Specific Human Leukocyte Antigen-Restricted Cytotoxic T-Lymphocyte Activity†

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Peripheral blood mononuclear cells harvested from healthy adults seropositive for human cytomegalovirus (HCMV) and cultured with laboratory strain AD-169 demonstrated human leukocyte antigen-restricted and HCMV-specific killing on target cells infected with either HCMV laboratory strain AD-169 or recent low-passage HCMV isolates. These results indicated that the determinants recognized by cytotoxic T lymphocytes (CTLs) are shared among different strains of HCMV. However, when low-passage isolates, rather than high-passage AD-169 virions, were used to stimulate CTL activity, the lytic response was significantly lower against all targets. Mixing of AD-169 and low-passage HCMV isolates induced low CTL activity. Collectively, the findings suggest that low-passage HCMV isolates have dual effects—antigenic stimulation and immunosuppression—whereas laboratory strain AD-169 is primarily immunogenic. The study of several recent isolates indicated that they varied in their ratio of immunostimulation to suppression, that infectious virus was necessary to produce suppression, and that suppressive isolates did not have to be present at the initiation of culture to exert their suppressive effects.

Human cytomegalovirus (HCMV) infections are frequently associated with immunosuppression. As with other members of the herpesvirus family, the interaction between HCMV and the host immune system is complex. Following an initial infection, the virus exists in a latent state and occasionally reactivates (8, 9, 14, 25). Initial and recrudescent HCMV infections are of clinical significance mainly in neonates and such immunoincompetent individuals as transplant or acquired immune deficiency syndrome patients (11, 15, 24). Susceptibility to clinical HCMV infection correlates strongly with defects in cellular immunity. There is also evidence that transplant patients with clinical HCMV infection who develop cytotoxic T lymphocytes (CTLs) and show good natural killer cell activity have a better prognosis than those who fail to develop these immune responses (16, 21).

HCMV can also cause immunosuppression. HCMV mononucleosis patients show depressed lymphocyte proliferative responses (5, 18). Recently we observed that HCMV, particularly low-passage isolates, suppressed T-cell proliferation and natural killer cell activity (R. D. Schrier, G. P. A. Rice, and M. B. A. Oldstone, *J. Infect. Dis.*, in press). Additionally, these clinical isolates infected lymphocytes and produced the major 72-kilodalton immediate-early protein but did not produce late viral proteins (17). HCMV-specific CTLs may play an important role in the control of viral infection (16, 21) and may be suppressed during HCMV infection (3). Here we report our studies on the ability of different HCMV strains (recent clinical isolates and a high-passage laboratory strain [AD-169]) to generate and suppress CTL responses.

MATERIALS AND METHODS

Virus. HCMV strain AD-169 was obtained from the American Type Culture Collection, Rockville, Md. Recent clinical

(low-passage) isolate strains were collected from bone marrow and renal transplant patients or infected neonates. Low-passage isolate viruses were passaged no more than 12 times and were transferred as infected cells. In these experiments, virus was always added to peripheral blood mononuclear cells (PBM) in the form of infected fibroblasts (5×10^5 infected fibroblasts per 8×10^6 PBM). Borysciewicz et al. (3) reported that the presence of soluble virus selects for the generation of proliferative T cells and against the generation of CTLs. Titration of isolate and AD-169 stocks indicated that isolate-infected cells release 100-fold less virus than do AD-169-infected fibroblasts. When both viruses were added to CTL cultures, fibroblasts were infected separately (multiplicity of infection = 5) and mixed later to limit any interference of one with the other. Virus was not released in measurable quantities in the CTL cultures, and the infected fibroblasts were destroyed during the culture period.

Development and assay of CTL. CTLs specific for HCMV were generated as previously reported (3). Briefly, normal HCMV-seropositive donors were selected and skin biopsies were obtained to grow out fibroblast stimulators and targets. PBM were separated on Ficoll-Hypaque, washed, adjusted to a concentration of 2×10^6 /ml (in RPMI 1640 with 10% human AB⁺ HCMV⁻ serum), and cultured with autologous fibroblasts which had been infected with HCMV 2 h earlier. Seven days later, the cultures were supplemented with fresh media containing additional infected autologous fibroblasts, irradiated (1,500 rads) autologous filler cells, and 7% interleukin II (IL-II; Cellular Products, Buffalo, N.Y.). Viability was about 80% (as tested by trypan blue exclusion) in all cultures. Lytic activity of cultured lymphocytes was tested 5 to 7 days later by using 12-h infected (or uninfected) ⁵¹Cr-labeled fibroblast targets at a ratio of 20:1 (effector:target) in 96-well round-bottom plates for 5 h. Total lysis was based on counts released from 1% Nonidet P-40-treated targets, and all values are the average of counts in triplicate wells. Where lysis of uninfected targets is not shown, it has been subtracted to give virus-specific lysis. Percent lysis was calcu-

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TABLE 1. CTLs restimulated with AD-169 and a low-passage isolate (I-G) of HCMV

Expt	Virus strain used to restimulate CTLs ^a	% Cr release from targets infected with the following virus strain ^b :			
		AD-169	I-G	Un	AD-169 (allo)
A	AD-169	57	41	10	2
	I-G	16	16	0	0
B	AD-169 (C')	24		5	
	AD-169 (α CT2 + C')	6		4	
	AD-169 (α T4 + C')	26		15	
	AD-169 (α T8 + C')	6		4	

^a PBM from normal seropositive individuals were restimulated in vitro with virus-infected autologous fibroblasts for 2 weeks and with interleukin II for the second week. Different donors were used for experiments A and B. Mature CTLs were treated with rabbit complement (C')(1/2; Pel Freeze) and antibodies to sheep erythrocyte receptor molecules (α CT2)(1/100; Hybritech, La Jolla, Calif.), OKT4 (α T4), and OKT8 (α T8) (1/40; Ortho Diagnostics, Inc., Raritan, N.J.).

^b Targets were infected and ⁵¹Cr labeled for 12 h, mixed with effectors (effector/target ratio, 20:1) and incubated at 37°C for 6 h. Un, Untreated; allo, different AD-169 line.

lated as follows: [(counts per minute released from infected targets with CTL – uninfected targets without CTL)/(CPM released by Nonidet P-40 treatment of targets)] $\times$ 100.

RESULTS

Generation of CTLs by HCMV strain AD-169 and fresh HCMV isolates. PBM from HCMV-seropositive donors were restimulated with either laboratory strain HCMV AD-169 or low-passage isolates and tested for CTL activity. Cells from a donor restimulated with AD-169 showed high cytotoxicity on autologous targets infected with either AD-169 or isolate I-G but not on uninfected or human leukocyte antigen-mismatched targets (Table 1). Effectors from the same donor stimulated with isolate I-G showed less cytotoxicity on any of the HCMV-infected targets tested. This pattern was consistent in multiple experiments with five different low-passage HCMV isolates. Table 1 describes the surface phenotype of cells mediating cytotoxic activity. Treatment of cells with complement alone or anti-OKT4 and complement did not affect lytic activity, but treatment with anti-CT2 (an antibody to the sheep erythrocyte receptor) or anti-OKT8 and complement abrogated the response.

Suppression of CTL activity by low-passage isolates of HCMV. Since our initial data indicated that the recent HCMV isolates generated lower CTL activity, experiments were designed to determine whether the isolates were poor restimulators due to slow replication (with insufficient antigen presentation) or whether they were exerting an immunosuppressive influence. Figure 1 depicts experiments in which CTLs were generated with either AD-169, a low-passage isolate (I-S), or a mixture of AD-169 and I-S. Cells stimulated with AD-169 alone showed good cytotoxic activity on either AD-169- or I-S-infected autologous targets. However, cells cultured with either I-S alone or I-S and AD-169 together showed no specific lysis, indicating that the isolate induced suppression. Virus specificity is also demonstrated (Fig. 1), since HCMV-restimulated CTLs were unable to kill herpes simplex virus type 1-infected autologous targets.

Suppression varied with the low-passage isolate tested. To examine whether the suppression was common to all low-

passage isolates of HCMV, several different low-passage HCMV strains were tested for their ability to abrogate CTL activity. An experiment comparing the suppressive and immunostimulatory effects of three low-passage isolates (I-G, I-R, and I-L) and strain AD-169 is shown in Table 2. Cells from seropositive donors stimulated with HCMV AD-169 showed good lytic activity; however, I-G, either alone or in combination with AD-169, blocked development of CTL activity. I-R alone reduced CTL activity (compared to the AD-169 control), and the mixture of AD-169 and I-R further depressed the response. I-L alone generated CTL activity comparable to that of AD-169; however, the CTL activity was significantly reduced when the combination of I-L and AD-169 was used. In most experiments (8 of 10), low-passage isolates were more suppressive when mixed with AD-169.

Requirement for infectious virus for immunosuppression. Next, we determined whether infectious virus was necessary to produce suppression of CTL activity. CTLs were generated with a combination of AD-169 and low-passage I-G which was either infective or inactivated by UV treatment or heat. Although addition of infectious I-G suppressed CTL development, I-G, inactivated by either method, did not depress CTL activity (Table 3).

Kinetics of suppression. The kinetics of suppression were examined by studying the stage of CTL maturation blocked by the low-passage isolates. In the experiment shown in Table 4, AD-169, a low-passage isolate, or a mixture of the two was added to PBM on either the day of initial stimulation (day 0) or the day of secondary stimulation (day 6). Addition of the highly suppressive isolate I-G, alone or mixed with AD-169, on day 0 or 6 completely abrogated the response. Although AD-169 generated CTL-lysed I-G-infected cells on day 12 (Table 4), addition of I-G at day 12 to CTLs generated with AD-169 also diminished the response on day 16 (data not shown). The less suppressive isolate (I-L) showed some suppression only when it was used together with AD-169 at the initiation (day 0) of CTL development, but the suppression was not to the degree seen with I-G.

DISCUSSION

We have shown that CTLs specific to HCMV, human leukocyte antigen restricted and bearing the OKT8 marker, can be detected in normal seropositive donors by restimulation with laboratory strain AD-169 and several low-passage isolates of HCMV. These results reinforce and extend those of Borysciewicz et al. (3), who first developed this CTL assay which uses strain AD-169 and thereby showed human leukocyte antigen A and B restriction, virus specificity, and identified the CTL population as OKT8 positive. The experiments presented here demonstrate that (i) CTLs generated with any HCMV strains tested lysed targets infected with all the other strains, indicating that the viral determinants for CTL recognition are shared; and (ii) the CTL response was frequently lower when low-passage isolates rather than high-passage AD-169 were used for restimulation. This appeared to be a result of the immunosuppressive qualities of the low-passage strains. The magnitude of suppression varied with the isolate tested. Suppression was most pronounced when both laboratory strain AD-169 and a low-passage isolate were used together for restimulation of HCMV-specific CTLs. Also, a strongly suppressive isolate did not have to be present at the initiation of the restimulation to abrogate HCMV CTL activity. Addition of a suppressive isolate either at the middle (day 6) of the period of

FIG. 1. Low-passage isolates of HCMV suppressed AD-169 CTL activity. CTLs were generated as described in Materials and Methods by using AD-169 (□), I-S (■), or a combination of AD-169 and I-S (▨). Lysis was tested in a 5-h assay on AD-169, I-S, herpes simplex virus, or uninfected ^{51}Cr -loaded fibroblasts.

generation of CTLs or at day 12 to a fully lytic CTL reduced lytic activity. The reduction in CTL activity did not appear to be the result of viral toxicity, since viability was comparable (approximately 80%) in all cultures, regardless of lytic activity.

Often we found that a combination of a low-passage isolate and laboratory strain AD-169 was more suppressive than the isolate alone. Stimulation with a combined total of infected cells equal to that used for each isolate or AD-169 alone (5×10^3 HCMV fibroblasts per 8×10^6 PBM) or equal numbers combined (for double the total number of infected cells) did not affect this result. Our hypothesis is that AD-169 lost most of its immunosuppressive potential because of forced fibroblast passage or selection for production of large amounts of extracellular virus and hence serves primarily as a stimulator. In contrast, the low-passage isolate strains had both immunostimulatory (i.e., antigenic) and immunosuppressive properties. In other viral systems, such as measles (2, 6), blastogenesis makes lymphocytes permissive for viral replication. In the cultures of seropositive lymphocytes in

which both AD-169 and low-passage isolates are present, AD-169 induces blastogenesis which may allow more expression of isolate-mediated suppression. This possibility is currently under study.

As yet we have noted no correlation between the ability of a given isolate to suppress CTL activity and the severity or type of disease it caused in the host. Other experiments (G. P. A. Rice, R. Schrier, and M. B. A. Oldstone, manuscript in preparation; unpublished observations) have revealed that HCMV viruses (low- and high-passage strains) which failed to suppress CTL activity were also unable to infect and express the major immediate-early 72,000-molecular-weight protein (72K protein) in lymphocytes. The genetic basis for the differences between the suppressive isolates and AD-169 has yet to be defined and will be difficult to examine given the variability in DNA sequence among HCMV strains (10).

The mechanism by which HCMV causes immunosuppression is unknown. The virus may act through an accessory cell or on the CTL directly. Immunosuppression may reflect lysis or abrogation of differentiated cell function (1, 13). HCMV-infected monocytes have been shown to inhibit natural killer activity and to indirectly suppress T-cell proliferation (4, 19, 20). Currently, we do not know whether

TABLE 2. Suppression of CTL activity varied with low-passage isolate tested

Virus strain used to restimulate CTLs ^a	% Specific Cr release ^b from AD-169 targets
AD-169	60
I-G	0
AD-169 + I-G	0
AD-169	34
I-R	24
AD-169 + I-R	12
AD-169	60
I-L	58
AD-169 + I-L	38

^a See Table 1, footnote a.

^b See Table 1, footnote b.

TABLE 3. Low-passage-isolate-mediated suppression was prevented by UV or heat inactivation

Virus strain used to restimulate CTLs ^a	% Specific Cr release ^b
AD-169 + I-G	7
AD-169 + I-G (UV) ^c	93
AD-169 + I-G (HI) ^d	94

^a See Table 1, footnote a.

^b See Table 1, footnote b.

^c Virus (I-G)-infected cells were UV treated (20 min, 1,200 ergs/cm² per s at a distance of 4 in. [ca. 10.16 cm]). No infectious virus was detected.

^d I-G-infected cells were treated at 56°C for 30 min. No infectious virus was detected. HI, Heat inactivated.

TABLE 4. Suppression of HCMV CTLs by two different low-passage isolates at different times during CTL maturation

Expt	Virus strains used to restimulate CTLs ^a		% Specific Cr release ^b (day 12) from target:	
	Primary stimulation (day 0)	Secondary stimulation (day 6)	AD-169	I-G
1	AD-169	AD-169	60	48
	AD-169	I-G	17	29
	AD-169	AD-169 + I-G	0	4
	I-G	AD-169	0	
	I-G	I-G	0	
	I-G	AD-169 + I-G	0	
	AD-169 + I-G	AD-169	4	
	AD-169 + I-G	I-G	0	
	AD-169 + I-G	AD-169 + I-G	0	
2	AD-169	AD-169	60	
	AD-169	I-L	57	
	AD-169	AD-169 + I-L	39	
	I-L	AD-169	51	
	I-L	I-L	58	
	I-L	AD-169 + I-L	50	
	AD-169 + I-L	AD-169	30	
	AD-169 + I-L	I-L	30	
	AD-169 + I-L	AD-169 + I-L	38	

^a Cells from normal seropositive donors were restimulated with AD-169, an isolate strain (I-G in experiment 1, I-L in experiment 2), or both, on day 0 or day 6 as noted. Interleukin II was added on day 6, and cultures were tested for cytotoxicity on day 12.

^b See Table 1, footnote b.

HCMV-induced suppression is specific to HCMV CTLs or affects other viral CTL responses as well. However, in other experiments it has been shown that HCMV can suppress not only T-cell proliferation to HCMV (R. Schrier, unpublished observations), but also to herpes simplex virus and mitogens (17, 23).

HCMV can infect lymphocytes and monocytes, but only a fraction (average, 2%; range, 0 to 15%) of the population shows viral nucleic acid or protein (17, 23). Because of the small numbers of PBM involved, it has been difficult to determine how this fraction mediates suppression and whether these cells are killed by the virus. Other viruses have been shown to infect lymphocytes and to affect their function in different ways. For example, measles and influenza viruses infect lymphocytes and suppress *in vitro* responses, but do not kill lymphocytes (6). In contrast, the virus which causes acquired immune deficiency syndrome selectively kills most OKT4-positive cells (7), leaving remaining lymphocytes with a reduced ability to form clones, suggesting a chronic but eventually terminal infection (12). Clearly, the effects of viruses on lymphocytes are complex and vary with the virus, the PBM subset infected, and the stage of lymphocyte activation. Study of specific virus-lymphocyte interactions should enhance our understanding of both molecular events during infection and the means by which viruses inactivate the lymphocyte functions designed to limit them.

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