

## HUMAN CYTOMEGALOVIRUS-SPECIFIC CYTOTOXIC T CELLS

Relative Frequency of Stage-specific CTL Recognizing the 72-kD  
Immediate Early Protein and Glycoprotein B Expressed by  
Recombinant Vaccinia Viruses

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Human cytomegalovirus (HCMV)<sup>1</sup> is a human herpesvirus that establishes lifelong persistent infection, after an often asymptomatic primary infection. Persistent infection may reactivate and become clinically evident after immunosuppression, resulting in significant morbidity and mortality (1). Reactivation in the context of immunosuppression suggests that virus-specific T cells, including HCMV-specific CTL, play a major role in maintaining the normal virus-host equilibrium. We have previously reported that MHC class I-restricted, CD8<sup>+</sup>, HCMV-specific CTL precursors (CTLp) are present in PBL of asymptomatic, persistently HCMV-infected individuals (2). These CTL are present at a precursor frequency of 1/5,000–20,000 PBL, higher than that of varicella zoster virus (VZV)-specific CTL (3). Furthermore, HCMV-specific CTL killed virus-infected cells before viral DNA replication, suggesting that they recognized predominantly nonstructural viral proteins (2–4). However, by limiting dilution analysis a minor population of CTL were identified that only killed HCMV-infected target cells expressing late (structural) viral proteins (3). In murine cytomegalovirus (MCMV) infection, which is similar in some respects to HCMV infection, MCMV-specific CTL may protect against virus infection (5). These protective CTL are specific for viral immediate early (IE) proteins, the first, nonstructural viral proteins expressed in the infected cell (6).

After HCMV infection of diploid human fibroblasts, the only cells fully permissive for HCMV replication *in vitro*, there is a regulated expression of virus gene products (1). Within 4–6 h of infection, viral IE genes are expressed. They code for four to five predominantly intranuclear proteins, the most abundant of which

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<sup>1</sup> *Abbreviations used in this paper:* CTLp, precursor CTL; E, early; gB, glycoprotein B; HCMV, human cytomegalovirus; IE, immediate early; L, late; MCMV, murine cytomegalovirus; PPF, phosphonoformate; VZV, varicella zoster virus.

is a 72-kD protein (7, 8). These proteins positively trans-regulate expression of the early (E) viral genes (7-9), and negatively autoregulate their own expression (10). The E genes encode a number of proteins, including the viral DNA polymerase, essential for HCMV DNA replication that occurs ~18 h after infection; only then are the 30-33 late (L) genes, coding mainly for the structural proteins of the virion, expressed.

The size of the HCMV genome (235 kbp) allows for a large potential coding capacity (1), and thus there are obvious difficulties in determining whether individual viral proteins are immunodominant with respect to the CTL response. The specificity of CTL for particular stages of virus gene expression can be examined using metabolic inhibitors, such as phosphonoformate (PPF) (3, 4), to prevent expression of L genes. However, positive identification of viral determinants recognized by CTL can best be achieved by using expression vectors that permit expression of single HCMV genes.

In the case of human persistent virus infections, there is little information on the relative preponderance of CTL specific for individual or stage-specific virus gene products. In these studies we have examined HCMV-specific CTL using recombinant vaccinia viruses that encode the HCMV structural glycoprotein B (vac.gB) (11) and the 72-kD IE protein (vac.IE). This approach was combined with limiting dilution culture, which permitted both a quantitative and clonal analysis of the CTL response to these viral proteins. Both vac.gB- and vac.IE-infected cells were recognized by HCMV-specific CTL. The frequency of IE protein-specific CTL was between 18 and 58% of HCMV-specific CTLp in the two subjects studied ( $n = 138/21$  and 77, respectively), compared with 0-2% and 6% for gB. These results suggest that the 72-kD IE protein is recognized by a large proportion of HCMV-specific CTL in the asymptomatic persistently HCMV-infected individual.

### Materials and Methods

*Cells and Viruses.* HCMV strain AD169 (No. VR-538; American Type Culture Collection, Rockville, MD) was grown in F5000 (Flow Laboratories, Inc., McLean, VA) diploid human fibroblasts, inoculated at a multiplicity of infection (MOI) of 0.1:1. Supernatant virus was harvested, plaqued on F5000 cells, and virus stocks with a titer  $>10^7$  plaque-forming units (PFU)/ml were stored at  $-70^{\circ}\text{C}$  until use. Vaccinia WR strain (vac.WR) was grown in Vero cells and supernatant virus was harvested, plaqued (titer  $10^9$ ), and stored in 1-ml aliquots at  $-70^{\circ}\text{C}$ . VZV and HSV were propagated as previously described (3).

Primary human fibroblast lines were established by explant cultures as previously described (2), from individuals of known HLA type. These cells and F5000 cells were maintained in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100,000 IU/liter penicillin, 10 mg/liter streptomycin, and 1% nonessential amino acids (Gibco Laboratories, Grand Island, NY). Fibroblasts, free of mycoplasma contamination by DNA staining and culture, and between passage 10-20, were used in these experiments.

*Recombinant Vaccinia Viruses.* Vac.gB encoding HCMV gB was constructed as previously described (11). Vero cells, diploid human fibroblasts, and EBV-transformed B cells infected with vac.gB expressed gB, as detected by a mAb (provided by H. Hart, Inveresk Research International, Edinburgh, Scotland [11]), in a cytoplasmic distribution, with some weak nuclear membrane fluorescence, again as previously described (11).

Vac.IE was constructed from the cDNA clone (pJDO83) of the IE gene encoding the 72-kD IE protein of HCMV strain AD169, inserted into the Pst I site of pUC9 (provided by J. Oram, Public Health Laboratory Service, Centre for Applied Microbiology & Research, Porton Down, UK) (12) (Fig. 1). A 1.7-kb Eco RI-Hind III fragment containing the com-

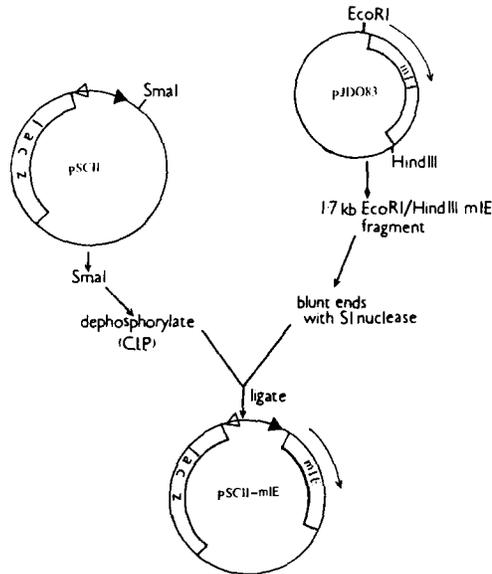


FIGURE 1. Construction of the vac.IE recombinant from the cDNA of the HCMV AD169 72-kD IE protein in pJD083 (see text for details).

plete major IE coding region was gel purified and blunt ended with Klenow fragment. The fragment was ligated into the unique Sma I site of the coinfection vector pSC11 (13), downstream of the vaccinia early promoter (p7.5). Correct insertion and orientation of the reading frame were confirmed by diagnostic restriction enzyme digestion and agarose gel electrophoresis.

Transfection and coinfection with vac.WR of CV1 cells was performed according to established methods (14). TK<sup>-</sup> progeny virus was selected using BUdR (50 µg/ml). TK<sup>-</sup> recombinants were distinguished from spontaneous TK<sup>-</sup> mutants, by overlaying with 1% agarose containing 0.1% neutral red and 300 µg/ml Xgal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) to screen for β-galactosidase activity (13). Plaques giving an intense blue colour after 4–6h were selected, further plaque purified and recombinant virus stocks grown in CV1 cells (14).

The recombinant virus genome was analyzed by restriction endonuclease digestion and Southern blotting (data not shown) which confirmed that the HCMV major IE gene had been inserted into the vaccinia TK gene with no other genomic alterations.

**Generation of HCMV-specific and gB-specific CTL.** PBL from HCMV-seropositive subjects were stimulated by cocultivation with autologous fibroblasts (responder/stimulator = 50:1), infected with either HCMV for 48 h or recombinant vaccinia virus for 12 h, then UV inactivated in 24-well plates (Linbro; Flow Laboratories, Inc.), for 5 d in RPMI supplemented as for MEM but with 10% HCMV-seronegative AB serum. The medium was then changed and 2/IU/ml rIL-2 (Boehringer Mannheim Biochemicals, Indianapolis, IN) added. Stimulator cells with irradiated autologous PBL (2,500 rad) were added at 7 d, and the cells were allowed to grow for a further 7 d in rIL-2. Viable cells were harvested by Ficoll/Hypaque density centrifugation and assayed in <sup>51</sup>Cr-release assays (2) as indicated in the text.

**Limiting Dilution Cultures.** Limiting dilution cultures to estimate the precursor frequency and analyze the clonal specificity of HCMV-specific CTL were performed as described (3). Briefly, PBL or E<sup>+</sup> cells isolated from PBL by rosetting with AET-treated SRBC (15) were used. Between 100 and 10,000 lymphocytes were placed in 96-well round-bottomed plates (Linbro; Flow Laboratories, Inc.) in 30–120 replicate cultures at each input cell concentration. These were divided between several plates to overcome plate-to-plate variation. 2 × 10<sup>3</sup> 48-h HCMV-infected autologous fibroblasts, together with autologous irradiated PBL

to a final cell concentration of  $10^5$  cells/well, were added and the cultures were incubated in RPMI with 10% AB serum supplemented with 2 IU/ml rIL-2.

The cultures were refed at 3-d intervals for 11-14 d. Identical cultures were also established with irradiated (2,500 rad) PBL as feeder cell controls.

The cultures were then equally divided and cytotoxicity was determined against four to six different target cells as described in individual experiments. For each target cell, maximum and spontaneous release values between plates were compared for interplate variation by one-way analysis of variance using the Scheffe multiple comparison test (16). Each test well was compared with the "feeder cell only" controls and significant  $^{51}\text{Cr}$  release was determined if the counts were  $>3$  SD above the release observed in the control wells (3). Each well was scored by the different target cells lysed. Virus-specific, MHC-restricted lysis was observed when the effector cells from an individual well lysed the autologous virus-infected cells and target cells in the presence of lectin, but not HLA-mismatched virus-infected cells, or autologous cells infected with an irrelevant virus.

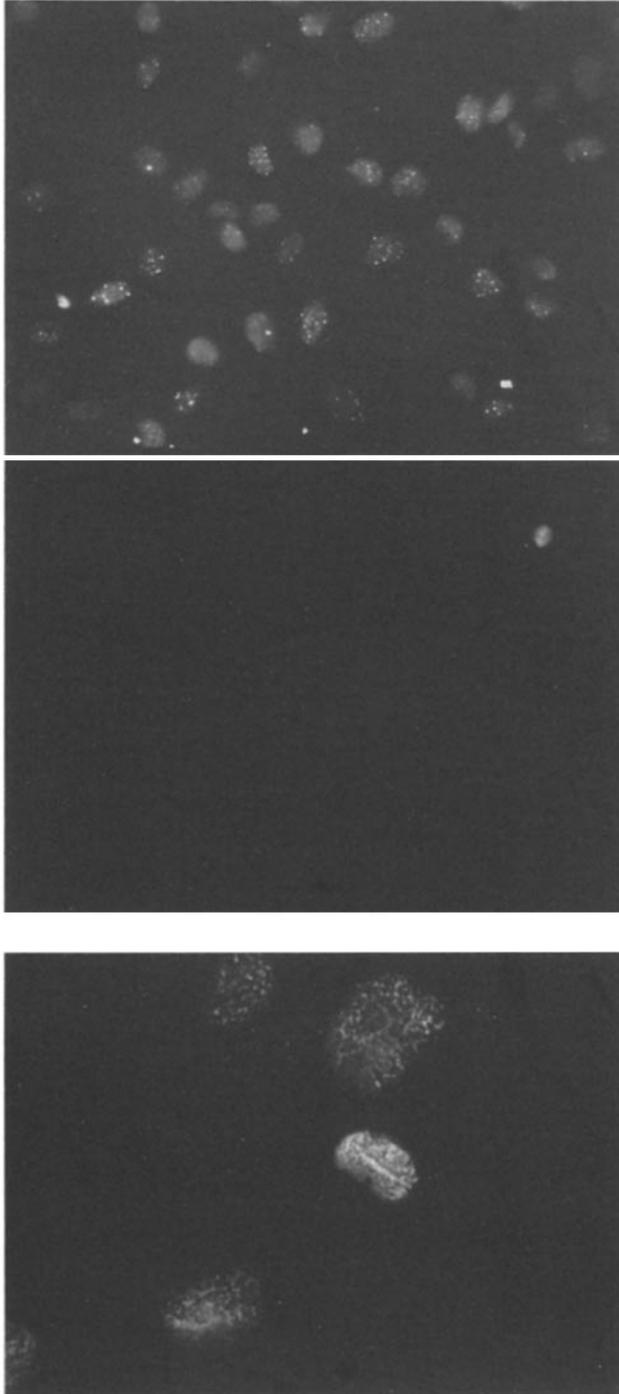
The best straight line fit comparing the  $\log_{10}$  of the proportion of negative wells against the input cell number was determined by the method of least squares, and the linearity was further examined by regression analysis (3, 16, 17). From these estimates the frequency of specific CTLp could be estimated as the input cell number that would produce 37% negative cultures against a particular target(s) (17). All statistical analysis was performed using SPSS/PC+ (SPSS Inc., Chicago, IL) (16).

*Other Reagents.* mAbs L14 (kindly provided by J. A. Nelson and M. B. A. Oldstone, Scripps Clinic and Research Institute, La Jolla, CA) (18) and C11 (provided by Dr. G. The, University of Gronigen, The Netherlands) against the 72 kD major IE protein of HCMV were used for indirect immunofluorescence of fixed and permeabilized infected monolayers of cells (19).

## Results

*Expression of 72-kD Major IE Protein in Cells Infected with vac.IE.* Two mAbs specific for the HCMV 72-kD IE protein, L14 and C11, were used to confirm its expression by indirect immunofluorescence. Monolayers of Vero cells and human fibroblasts infected for 12 h at 10 PFU/cell with vac.IE or vac.WR, were fixed and permeabilized (19), and then stained with L14 or C11 and (Fab) $_2$ , rabbit anti-mouse Ig-FITC (Dako) (Fig. 2). Vac.IE-infected cells had positive nuclear fluorescence, detectable as early as 4 h after infection, increasing up to 12 h. There was no fluorescence in vac.WR-infected cells. On Western blot analysis a protein of 71-73 kD was detected in HCMV- or vac.IE-infected cell lysates, using C11, although not with L14 (data not shown).

*HCMV-specific CTL Recognize the 72-kD IE Protein and gB.* To determine if the HCMV 72-kD IE protein and gB were recognized by HCMV-specific CTL, PBL from seropositive and seronegative subjects were cocultured with 48-h HCMV-infected autologous fibroblasts under limiting dilution conditions, as described in Materials and Methods. After 11 d individual wells were assayed against HCMV-infected autologous and HLA-mismatched fibroblasts to determine the frequency of all HCMV-specific CTL (3). HCMV-specific CTL in subject CH were present at a frequency of 1/11,000 (95% confidence limits: 1/5,000-1/17,000) (Fig. 3). In addition, each individual well was assayed against autologous and HLA-mismatched B cell lines infected with the vaccinia recombinants. HCMV 72-kD IE protein-specific CTL were present at 1/76,000 PBL (1/36,000-116,000). In the same experiment, HCMV gB-specific CTL were present at the limits of the assay at 1/181,000 PBL. There was no specific lysis of vac.WR-infected target cells. No HCMV, HCMV 72-kD IE protein, or HCMV gB-specific CTL were present in seronegative subjects.



**FIGURE 2.** Human primary fibroblasts infected with vac.IE expressed the 72-kD IE protein as detected by mAbs. Fibroblasts were infected for 12 h (MOI 10:1) with vac.IE (*top*) ( $\times 200$ ), infected with vac.WR (*middle*) ( $\times 200$ ), and HCMV for 24 h (*bottom*) ( $\times 400$ ). The cells were fixed in methanol/acetone, permeabilized, and stained by indirect immunofluorescence using mAb C11. vac.IE-infected cells expressed the HCMV 72-kD IE protein in nuclear distribution.

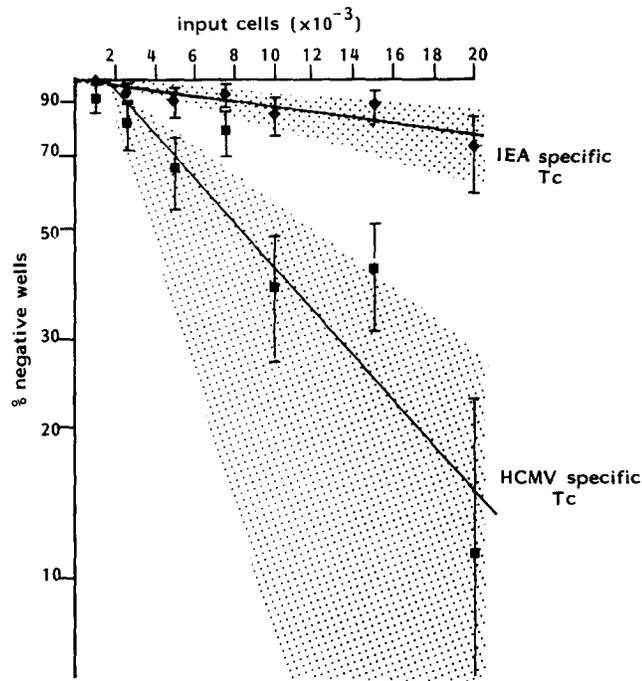


FIGURE 3. Frequency analysis of HCMV- and vac.IE-specific CTLp. PBL from a seropositive subject (CH) were grown in limiting dilution (120 replicate cultures at each input cell number), with autologous HCMV-infected fibroblasts, irradiated PBL and rIL-2. After 11 d the cultures were assayed against 48-h HCMV-infected autologous and HLA-mismatched fibroblasts, to determine the presence of HCMV-specific CTL (■), and against vac.IE- and vac.gB-infected autologous and vac.IE-infected, HLA-mismatched EBV-transformed B cell lines, to determine 72-kD IE-specific CTL (◆). Bars indicate 95% confidence limits for each input cell number; hatched area indicates 95% confidence interval for the regression lines.

*Relative Frequencies of HCMV 72-kD and gB-specific CTL Clones.* The relative frequencies of clonally derived (3, 17), vac.IE- and vac.gB-specific CTL were compared, together with the ability of the same clones to mediate lysis of HCMV infected autologous fibroblasts. The data are shown in Table I. In three separate experiments in two seropositive subjects (CH and AR), the frequency of vac.IE-specific CTL

TABLE I  
*Relative Numbers of vac.gB- and vac.IE-specific CTL Clones in Asymptomatic HCMV-seropositive Subjects*

Exp.	Subject	Number of wells (percent of row total)			Total
		vac.IE	vac.gB	Other	
1	CH				
	HCMV lysed	25 (18)	4 (2)	109 (80)	138
	HCMV not lysed	4 (2)	0 (0)	138 (98)	142
	Total	29	4	247	280
2	CH				
	HCMV lysed	9 (43)	0 (0)	12 (57)	21
	HCMV not lysed	0 (0)	0 (0)	100 (100)	100
	Total	9	0	112	121
3	AR				
	HCMV lysed	45 (58)	5 (6)	27 (36)	77
	HCMV not lysed	16 (29)	0 (0)	40 (71)	56
	Total	61	5	67	133

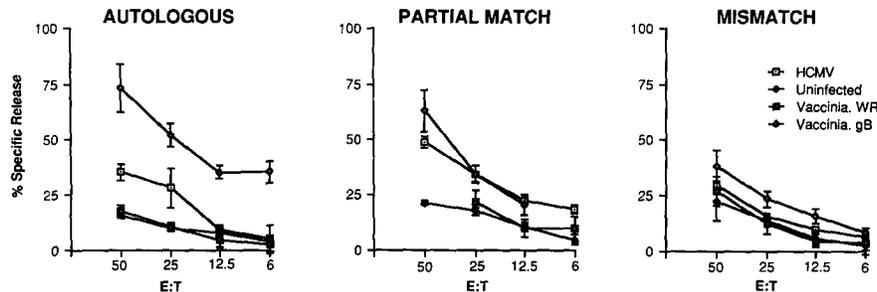


FIGURE 4. Vac.gB-infected fibroblasts activated HCMV-specific CTL in vitro. PBL from a seropositive subject (AR), were cocultured with vac.gB-infected, UV-inactivated, autologous fibroblasts as described in Materials and Methods. After 14 d, viable lymphocytes were assayed in a  $^{51}\text{Cr}$ -release assay against autologous, partially HLA A and B-matched and HLA-mismatched fibroblasts that were uninfected (●) or infected with HCMV (□), vac.gB (○), or vac.WR (■). Bars indicate  $\pm 1$  SD.

clones was greater than vac.gB-specific clones. In the first and third studies, vac.IE-specific CTL clones derived from the different donors lysed HCMV-infected autologous fibroblasts and represented 18.1 and 58.4%, respectively, of all wells mediating HCMV-specific CTL lysis. However, in these two experiments, although the vac.IE-specific CTL were clonally derived, the cultures lysing HCMV-infected fibroblasts were not by the standard criteria for limiting dilution analysis described by Lefkowitz and Waldeman (17).

When the experiment was repeated with cells from donor CH (Table I, Exp. 2), although fewer clones were studied, both cultures with HCMV-specific and vac.IE-specific CTL activity were clonally derived and 43% of all HCMV-specific CTL clones also lysed autologous vac.IE-infected cells.

There was no instance of an individual well killing both vac.IE- and vac.gB-infected cells. However, a small number of vac.IE-specific CTL clones did *not* lyse autologous HCMV-infected fibroblasts.

**Generation of HCMV-specific CTL with vac.IE.** To determine whether HCMV-specific CTL could be generated using vac.IE, PBL were cocultured with UV-inactivated vac.IE-infected autologous fibroblasts or EBV-transformed B cell lines. In five experiments from three seropositive subjects no HCMV-specific CTL were generated, although in two of the five studies, nonspecific cytotoxicity was observed.

**Generation of HCMV-specific CTL with vac.gB.** To determine whether HCMV memory CTL could be activated in vitro by a single structural HCMV protein (gB) expressed in vaccinia-infected cells, PBL were cocultured with cells infected with vac.gB. Autologous fibroblasts were infected with vac.gB for 12 h, UV treated to inactive residual and released vaccinia virus, and cocultured with PBL from HCMV-seropositive and seronegative subjects. The cultures were restimulated with vac.gB-infected cells after 7 d and the lymphocytes were allowed to grow in the presence of rIL-2 for a further week. Viable cells were then assayed against autologous, partially HLA-matched and -mismatched fibroblasts, uninfected or infected with HCMV, vac.WR, and vac.gB. Only autologous and partially HLA-matched cells infected with the recombinant or HCMV were lysed by the effector cells from a seropositive subject (Fig. 4); there was no significant lysis by PBL from a seronegative subject.

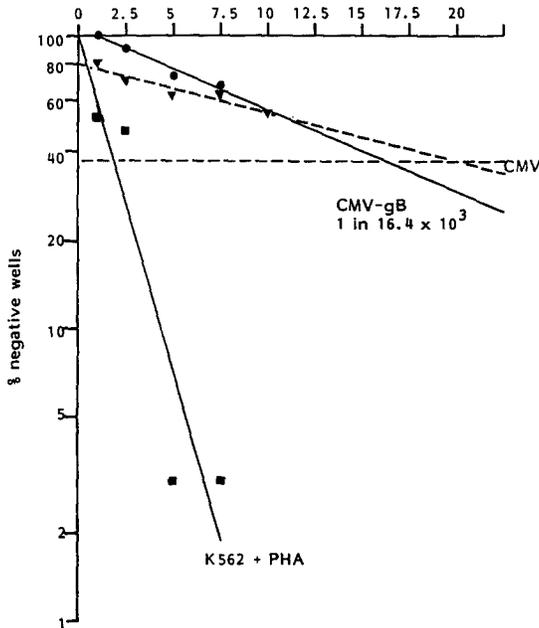


FIGURE 5. Limiting dilution analysis of vac.gB-activated, HCMV-specific CTL. 1,000 to 20,000 E<sup>+</sup> PBL (subject AR) were grown in 30 replicate cultures at each input cell concentration, with 2,000 vac.gB-infected, UV-inactivated autologous fibroblasts, irradiated (2,500 rad) PBL and IL-2 for 12 d. The individual wells were assayed against HCMV-infected (48 h) (▼), vac.gB-infected (12 h) (●) autologous fibroblasts, and K562 cells with PHA (■). No significant lysis of uninfected or VZV-infected autologous fibroblasts was detected (data not shown). Precursor frequencies from limiting dilution plot: HCMV-specific CTL, 1/20,000; vac.gB-specific CTL, 1/16,000.

To analyze the frequency of such CTLp the experiment was repeated under limiting dilution conditions (Fig. 5). A similar frequency of CTL lysing vac.gB- and HCMV-infected cells would be expected under these conditions, as only a single HCMV protein was used to stimulate the response. 1/20,000 E<sup>+</sup> PBL lysed HCMV-infected autologous fibroblasts and 1/16,000 lysed vac.gB-infected fibroblasts. Furthermore, there was almost complete concordance when the lysis of HCMV- and vac.gB-infected cells by individual wells containing the progeny of a single cell, as predicted from the limiting dilution plots, was compared (data not shown). In parallel experiments the HCMV-specific CTLp frequency, using autologous HCMV-infected fibroblasts as stimulator cells in limiting dilution cultures, was 1/5000 E<sup>+</sup> PBL (3).

### Discussion

In this study we have shown, using vaccinia recombinants encoding the HCMV 72-kD IE protein and gB, that HCMV-specific CTL from individuals persistently infected with HCMV recognized these viral proteins. The relative number of HCMV 72-kD IE protein-specific CTL in PBL was much greater than gB-specific CTL in the subjects studied. HCMV-specific memory CTL were activated by coculture with vac.gB-, but not vac.IE-infected fibroblasts. These results suggest that whereas some CTL specific for HCMV structural proteins, such as gB, are present in PBL, a considerably greater proportion of HCMV-specific CTL recognize a nonstructural protein, the 72-kD IE protein.

We have previously shown that HCMV-specific CTLp were present in PBL of normal clinically asymptomatic HCMV seropositive individuals (2) and at a relatively high frequency (3). In addition, the majority of HCMV-specific CTL clones

recognized target cells that expressed only the nonstructural IE and E proteins. However, these results also showed that a small number of CTL clones lysed HCMV-infected target cells expressing L proteins only (3). To further identify which HCMV proteins were recognized by CTL, we decided to express individual HCMV genes in target cells for use in cytotoxicity assays. Initial attempts were made to achieve this by direct transfection with a plasmid (pES) encoding the HCMV AD169 72-kD IE protein (20), but <10% of cells expressed the HCMV protein and then only transiently (<1% of cells after 48 h). We therefore used vaccinia recombinants encoding single HCMV genes to increase the number of target cells expressing the inserted HCMV gene.

Vaccinia recombinants have recently been used to identify a number of viral determinants recognized by human CTL, including influenza nucleoprotein (21), RSV nucleoprotein (22), HSV glycoproteins (23), and HIV glycoprotein (24). Most of the viral antigens recognized by virus-specific CTL, which have been identified to date, are intranuclear/intracellular viral proteins, but viral glycoprotein-specific CTL have also been described (23–25). There has been no previous study to determine the relative frequency of MHC class I-restricted CTL specific for different virus gene products during persistent herpesvirus infection in man.

Many HCMV-specific CTL present in PBL recognized vac.IE-infected autologous B cell lines and fibroblasts. Two subjects were investigated in detail; extrapolation from this information to an outbred heterogenous population must be guarded, particularly as the specificity of many of the CTL clones (subject CH) remains to be identified and it is possible that specificity may vary with HLA haplotype, as in the case of influenza (21). However, the high frequency of CTLp in peripheral blood directed against a single viral protein suggests that the 72-kD IE protein is an important recognition element for HCMV-specific CTL during persistent infection. The CTL response against HCMV and other human herpesviruses may be important in maintaining the virus/host equilibrium. The HCMV IE gene encoding the 491-amino acid, 72-kD protein consists of four exons, and although its exact role is unknown, it is among the first HCMV genes transcribed. Its expression is essential for virus replication (1, 7–10, 12), and thus the ability of CTL to lyse cells expressing this intranuclear viral protein may permit the early recognition and limitation of local HCMV reactivation, even before virus replication. It should also be noted that certainly *in vitro*, and possibly *in vivo*, some cell types only express IE genes, with an apparent block to later HCMV gene expression. It is reported that IE mRNA can be detected in small fraction (<0.01%) of human PBMC during persistent infection (26). It may be that such expression of IE genes provides selective restimulation *in vivo* and contributes to the relatively high frequency of 72-kD IE-specific CTLp.

From previous studies we identified a subpopulation of MHC class I-restricted, HCMV-specific CTL that recognized structural or L viral proteins (3). Vac.gB (11) was used to determine if memory CTL could be activated by and recognize vac.gB-infected cells. The gene for gB was originally identified within the HCMV AD169 genome, in the Hind III fragment, and it encodes a 906-amino acid protein that is present in HCMV-infected cells in two major glycosylated forms of 144 and 55 kD. It shares homology with HSV gB, EBV gB, and VZV gpII, is expressed in infected cells and HCMV virions, and antibodies to gB neutralize HCMV infectivity

(11). We found that HCMV gB-specific CTL were present in PBL and could be activated in vitro using the vaccinia recombinant to lyse HLA-matched HCMV-infected fibroblasts. Although HCMV gB-specific CTL were activated using this protocol, no vaccinia-specific CTL were generated, even when PBL from revaccinated subjects were used, either in bulk or limiting dilution culture (Graham, S., and L. K. Borysiewicz, submitted for publication).

Although gB-specific CTL were activated in vitro by vac.gB, no 72-kD IE protein-specific CTL have been generated using vac.IE-infected autologous fibroblasts or EBV-transformed B cell lines as stimulator cells. The majority of PBL proliferating in such cultures were CD4<sup>+</sup> and, only non-MHC-restricted lysis was observed. There are several possible explanations for this discrepancy, but generation of CTL by vac.gB in vitro seems to be an exception to the general rule that it is difficult to use recombinant vaccinia viruses for secondary in vitro stimulation, not least because of the need to prevent spread of vaccinia in the culture.

A further point requiring explanation is the observation that when B cell lines infected with vac.IE recombinants were used as target cells in limiting dilution assays, a small proportion of the vac.IE-specific CTLp lysed these but not autologous HCMV-infected fibroblasts. One possible explanation for this would be the presence of HCMV-specific MHC class II-restricted CTL (27) in these cultures, preferentially killing the B cell targets.

Evidence for any protective role for HCMV-specific CTL in man must of necessity be indirect, drawn from clinical correlations (28) and representative animal models. In this context, MCMV infection, although MCMV is distinct from HCMV by DNA hybridization, displays some similarities with the biology of HCMV infection. Reddehase et al. (29, 30) have examined the CTL response in BALB/c mice after MCMV infection. After primary footpad inoculation, two stage-specific subpopulations of CTL are present in draining lymph nodes, recognizing MCMV IEA and "viral late membrane proteins" (inferred to be glycoproteins as the target cells were prepared by infection at high multiplicity in vitro, with UV-irradiated virus that would not permit *de novo* viral protein synthesis). In persistently infected mice both subpopulations are detected, but only after secondary in vitro stimulation. A MCMV 89-kD IE-specific CTL clone has been established and maintained in vitro (31, 32), and although this clone is not itself protective, mixed populations of Lyt-2<sup>+</sup>, IEA-specific CTL protect against lethal infection in irradiated mice (5, 6). Using deletions of the MCMV 89-kD IE gene in vaccinia recombinants, the region of the protein recognized by the CTL clone was identified and protection after vaccination with the deleted recombinant was demonstrated, although in surviving animals persistent MCMV infection was not prevented (33).

Our observations concerning the high frequency of HCMV nonstructural protein, and more particularly, 72-kD IE protein-specific CTLp, accord well with the studies of MCMV-specific CTL (5, 6, 29-33), and it is possible that CTL of this specificity may provide an important host defence mechanism against HCMV reactivation. However, it remains difficult to directly extrapolate from MCMV studies to man; for instance, the possibility exists that as with other virus infections, CTL may also be implicated in immunopathological injury (34, 35).

It has been shown that human and murine influenza-specific CTL recognize processed linear peptides of predominantly, but not exclusively, intracellular viral

proteins associated with MHC class I molecules (36), and that different peptide or viral protein specificities may predominate, depending on the MHC haplotype (21). We intend to use vaccinia recombinants encoding different HCMV proteins to identify other possible CTL determinants and to use deletions within the gene for the 72-kD IE protein to define the CTL epitope(s) important for recognition. A further advantage of vaccinia recombinants as regards HCMV is that their wider host cell range in vitro enables cells other than fibroblasts, notably B cell lines, to be used as target cells. This should permit investigation of the role of MHC class II-restricted HCMV-specific CTL, and CTL responses in larger numbers of subjects particularly those at risk of HCMV disease, something that has previously been difficult because of the requirement for primary fibroblast lines.

In this study we have identified the virus gene products recognized by a proportion of the human, MHC class I-restricted HCMV-specific CTL during persistent virus infection. This has been achieved by coupling the use of vaccinia recombinants encoding a major IE gene and an important structural viral glycoprotein that has neutralizing epitopes, with limiting dilution analysis of the CTL response. Further studies will be aimed at better defining the specificity and role of CTL in HCMV infection. Such information would contribute to a logical approach to HCMV prophylaxis and enhancing specific CTL in individuals at high risk of HCMV reactivation. In particular, the use of a combination subunit vaccine encoding both a virus glycoprotein and IE protein(s), with the aim of stimulating the generation of neutralizing antibody and protective virus-specific CTL, might warrant consideration.

### Summary

CTL are held to be an important host defense mechanism in persistent herpesvirus infections. We have therefore studied the nature and specificity of human cytomegalovirus (HCMV)-specific CTL in normal persistently infected individuals. This was achieved by using vaccinia recombinants encoding viral genes expressed at different stages of the virus replicative cycle, a structural glycoprotein gB (vac.gB) and the major 72-kD immediate early nonstructural protein (vac.IE) of HCMV, combined with limiting dilution analysis of the CTL response. In two subjects, 43 and 58% of HCMV CTL precursors (CTLp) lysed vac.IE-infected cells, in contrast to <6% lysing gB-infected cells. HCMV-specific CTL could also be generated by secondary in vitro stimulation with vac.gB- but not vac.IE-infected autologous fibroblasts. The high frequency of 72-kD IE protein-specific CTL suggests that this is at least a major recognition element for the HCMV-specific CTL response in asymptomatic persistently infected individuals, and CTL with this specificity may be important in maintaining the normal virus/host equilibrium.

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