JOURNAL OF VIROLOGY, June 1993, p. 3461–3469
0022-538X/93/063461-09$02.00/0

Vol. 67, No. 6

Selective Interference with Class I Major Histocompatibility Complex Presentation of the Major Immediate-Early Protein following Infection with Human Cytomegalovirus

MARK J. GILBERT,* STANLEY R. RIDDELL, CHENG-RONG LI, AND PHILIP D. GREENBERG

Fred Hutchinson Cancer Research Center and Departments of Medicine and Immunology, University of Washington, Seattle, Washington 98104

Received 21 December 1992/Accepted 15 March 1993

Responses of cytotoxic T-cells (Tc) to human cytomegalovirus (CMV) represent the predominant mechanism by which hosts resist CMV infection. The CMV major immediate-early protein (IE) is present throughout the virus replicative cycle. Studies were performed to determine whether Tc specific for IE effectively lyse CMV-infected targets and are thus capable of providing protective immunity against infection. After in vitro stimulation of peripheral blood mononuclear cells with CMV-infected autologous fibroblasts, Tc specific for IE were not readily detectable in CMV-reactive polyclonal Tc lines. However, after stimulation of peripheral blood mononuclear cells with cells selectively expressing IE, weak but detectable IE-specific Tc responses were observed. The frequency of IE-specific Tc clones derived from cultures stimulated with IE-expressing cells was 50 to 100 times lower than the frequency of Tc clones specific for other CMV proteins isolated from cultures stimulated with CMV-infected cells. All of the IE-specific Tc clones, which efficiently lysed targets selectively expressing IE, demonstrated minimal lysis of CMV-infected fibroblasts, despite abundant IE expression in these target cells. In contrast to these results with IE, other viral proteins were efficiently presented during all phases of CMV infection. These data suggest that CMV has evolved a unique mechanism for selectively limiting the presentation of the potentially immunogenic IE protein, which may preclude IE-specific Tc from providing protective immunity to CMV infection.

Cytotoxic T lymphocytes (Tc) specific for human cytomegalovirus (CMV) can be isolated from the peripheral blood of healthy seropositive individuals. The loss of such Tc in immunocompromised hosts is associated with a high risk of CMV disease (30, 31). CMV has the largest genome of any DNA virus causing disease in humans, encoding more than 200 potentially immunogenic proteins (22). Several CMV proteins have been shown to serve as target antigens for the class I-restricted $CD8^+$ Tc responses to CMV, including the major immediate-early protein (IE) (5), glycoprotein B (gB) (5), and nonenvelope structural virion proteins such as the matrix protein pp65 (24). However, the specificity of Tc capable of mediating protective immunity to CMV and the identities of the viral proteins that might be most efficiently used to stimulate therapeutic or protective Tc responses to CMV have not been completely defined.

The CMV major IE represents a potentially important target antigen for Tc responsible for controlling and/or eliminating CMV infection. First, IE is among the initial viral proteins expressed and is present in abundance within 4 h of infection (3, 11, 17, 25, 36–38), long before new virions can be assembled (22). Second, the nucleotide sequence of the IE gene is highly conserved among many strains of CMV (1, 17), suggesting that strains containing a mutated IE gene which may evade recognition by an IE-specific cellular immune response are unlikely to arise. Third, IE gene expression is necessary for inducing the expression of other CMV genes and initiating the viral life cycle in latently infected cells (16, 23, 39). Finally, immunization of mice with a recombinant vaccinia virus containing the functional homolog of CMV IE in murine cytomegalovirus (MCMV), pp89, induces $CD8^+$ Tc responses that mediate protective immunity from challenge with MCMV (19).

The presence of a Tc response to IE as part of the host response to human CMV infection has been variably reported in different studies (4, 5, 34). Our laboratory has developed methods to reproducibly generate and expand Tc reactive with CMV (6, 21, 33, 34), and the target antigen specificity of these responses can be analyzed with the use of vectors selectively expressing products of individual CMV genes. In this study, we have used this approach to evaluate the relative frequency of IE-specific Tc compared with Tc recognizing other CMV proteins and to determine the relative efficiency of IE-specific Tc in lysing CMV-infected target cells over the course of replicative infection. Our results suggest that CMV has evolved a mechanism by which other proteins encoded by the virus can selectively interfere with the presentation of IE-derived peptides in association with class I major histocompatibility complex (MHC) molecules and that Tc which recognize IE may not be well suited to provide protective responses against CMV infection.

MATERIALS AND METHODS

Virus and cell lines. Dermal fibroblast lines were generated from 3-mm skin biopsies obtained from human CMV seropositive volunteers as described previously (34). Fibroblasts were used between passages 3 and 14 for all experiments. Human foreskin fibroblast cultures were established and propagated in Dulbecco's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Fibroblast lines constitutively expressing the CMV IE gene were established by transduction with a retrovirus (LNC IE) kindly provided by E. Gilboa (Memorial Sloan Kettering Cancer Center), in which the neomycin resistance

* Corresponding author.

gene is under control of the Moloney virus long terminal repeat and the CMV IE1 gene is under the transcriptional control of the CMV alpha promoter. The retrovirus was added to fibroblast cultures at 50% confluence in the presence of 4 μg of Polybrene per ml, and transduced cells were selected by the addition of 1.5 mg of G418 (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) per ml. The IE-expressing fibroblasts, denoted $FIBRO_{IE}$, were maintained with Waymouth's medium supplemented with 16% fetal calf serum, penicillin, streptomycin, and G418.

Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines were generated as described previously (32).

Human CMV strain AD169 was obtained from the American Type Culture Collection, Rockville, Md., and propagated by three serial passages as described previously (34). Stocks of AD169 were stored frozen at a titer of 5×10^6 PFU/ml.

Wild-type vaccinia virus, strain Var, and the vaccinia recombinant virus (vacIE) encoding the 72-kDa IE protein of CMV under the control of the vaccinia virus p7.5 promoter were kindly provided by E. Paoletti (Virogenetics Corp.).

Isolation of Tc lines and clones. Short-term Tc lines and clones specific for CMV were isolated from seropositive donors MR (HLA phenotype, A24,25/B18,35) and TM (A3,24/B8,51), and Tc clones specific for gB were isolated from donor MR as previously described (34). Short-term IE-specific Tc lines and $CD8^+$ Tc clones were isolated from donors MR and TM by a modification of methods previously described (34). Briefly, after isolation from heparinized whole blood, peripheral blood mononuclear cells (PBMC) were cocultured at a responder-to-stimulator ratio of 30:1 with either $FIBRO_{IE}$ stimulators or vacIE-infected autologous fibroblasts in RPMI-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) medium supplemented with 10% human AB^+ serum, penicillin, streptomycin, 2-mercaptoethanol, and glutamine. After 12 h of infection, vacIE-infected fibroblasts were exposed to a UV germicidal lamp to inactivate any viable virus. Stimulator cells identically derived were used for each restimulation and for the generation of IE-specific Tc clones.

Tc specific for pp65 were isolated from seropositive donor CM (A1,2/B35,57) by a recently described method (24). Briefly, cultures were stimulated with autologous fibroblasts pulsed for 1 h at 37°C with 100-μg/ml fractions of pp65 peptides purified by high-performance liquid chromatography after CNBr digestion of purified pp65 protein generated by recombinant DNA techniques. The peptide fractions of pp65 were provided by Steven Forman, City of Hope Medical Center (28), and resuspended in medium for use.

Chromium release assay. Cytolytic activity was measured by a standard 5-h chromium release assay with CMV- or vaccinia virus-infected targets as described previously (34). In selected experiments, fibroblasts were pretreated by culture in the presence of 100 U of gamma interferon (IFN-γ) (Boehringer-Mannheim, Indianapolis, Ind.) per ml for 48 h. The fibroblast cultures were then washed to remove the IFN-γ prior to virus inoculation. Spontaneous release for all assays was less than 20% of maximum values.

Radioimmunoprecipitation. Radioimmunoprecipitation was performed by modifications of methods previously described (34). Monolayers of human foreskin fibroblasts were infected with CMV at a multiplicity of infection (MOI) of 5 with or without 48-h pretreatment with IFN-γ and were radiolabelled for 30 min at 37°C with 0.2 mCi of [^{35}S]methionine (Amersham, Downers Grove, Ill.) per ml during the final half hour of infection prior to their harvest. Cells were lysed with lysis buffer (1% [vol/vol] Nonidet P-40, 0.5% [vol/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate (SDS), 1 μg of aprotinin per ml, 100 μg of phenylmethylsulfonyl fluoride per ml, 0.02% [wt/vol] sodium azide, 1% [wt/vol] bovine hemoglobin in phosphate-buffered saline, pH 8.5) and precleared with *Staphylococcus* protein A cells (Immuno-Precipitin; Bethesda Research Laboratories). The 72-kDa IE protein was immunoprecipitated from lysates with monoclonal antibody (MAb) 6E3 (38) kindly provided by S. Barr (Syva Corporation, San Jose, Calif.) and *Staphylococcus* protein A cells and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Gels were incubated with Autofluor (National Diagnostics) for 1 h prior to desiccation and autoradiography.

Surface phenotyping and MHC quantification. An analysis of the surface phenotypes of isolated Tc clones was performed as previously described (34) with the following murine MAbs: anti-Leu 1 to CD16, anti-Leu 2a to CD8, anti-Leu 3a to CD4, anti-Leu 4 to CD3, and anti-TCR1 to the α/β Tc receptor (Becton-Dickinson, Mountain View, Calif.) or anti-pan-delta to the γ/δ Tc receptor (T Cell Science, Cambridge, Mass.). Fibroblasts with or without a 48-h preincubation with IFN-γ were mock infected or infected with CMV at an MOI of 5 for 6, 24, or 48 h. Murine MAb w6/32, which recognizes a public HLA class I epitope, was kindly provided by D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, Wash.).

RESULTS

Isolation and characterization of IE-specific Tc clones. Previous studies in our laboratory demonstrated that Tc from PBMC of CMV-seropositive donors isolated in vitro after stimulation with CMV-infected autologous fibroblasts predominantly recognize CMV antigens derived from the nonenvelope structural proteins of the virion (34). To determine whether these bulk cultures contained IE-specific Tc, polyclonal Tc lines generated by two cycles of stimulation were analyzed for MHC-restricted lysis of target cells infected for 6 h with CMV or for 12 h with vacIE. Target cells were lysed equally well by Tc from 6 to 24 h after CMV infection and from 12 to 24 h after vacIE infection; targets infected for 6 h with CMV or for 12 h with vacIE were selected for analysis to avoid any contribution of cytopathic effect by these viruses on the cytolytic results. Bulk cultures of Tc isolated from each of 10 seropositive individuals representative of a broad number of MHC alleles were highly lytic for CMV-infected targets but demonstrated no significant lytic activity for autologous compared with allogeneic vacIE-infected targets (Table 1). Two of these Tc lines were subsequently studied in more detail (Table 2). These lines demonstrated specific lyses of CMV-infected targets of 30 and 23% at an effector/target ratio of 20:1 and <2% lysis of vacIE-expressing targets.

The failure to detect IE reactivity in these virus-specific Tc lines might result from preferential activation of Tc other than those recognizing IE during in vitro culture with CMV-infected stimulator cells. Therefore, Tc from two seropositive individuals (MR and TM) were isolated by in vitro stimulation of PBMC with autologous fibroblasts which selectively expressed IE after either transduction with a retrovirus (denoted $FIBRO_{IE}$) or infection with vacIE (Table 2). After two to three weekly stimulation cycles, the polyclonal Tc cultures were analyzed for lysis of autologous and HLA-mismatched fibroblasts selectively expressing IE. Only very weak cytolytic activity was observed with IE-

TABLE 1. CMV- versus IE-specific Tc responses in Tc cultures stimulated with CMV-infected cells[a]

Polyclonal Tc line	HLA type	E/T	% Specific lysis of					
			Autologous targets			Allogeneic targets		
			CMV	Mock	vacIE	CMV	Mock	vacIE
BH	A1/B8	15:1	60	3	5	13	14	12
CG	A2,32/B27,35	20:1	47	0	10	5	2	11
CM	A1,2/B35,57	12.5:1	45	3	3	0	0	3
CO	A28,33/B14	20:1	49	1	6	0	0	5
DH	A1,31/B38,44	20:1	39	1	0	2	0	2
JB	A1,2/B8,44	20:1	23	2	3	ND	ND	ND
MR	A24,25/B18,35	20:1	53	3	1	3	3	3
PJS	A1,24/B37,49	15:1	35	4	7	ND	ND	ND
SS	A1,2/B13,51	20:1	52	7	11	8	9	9
TM	A3,24/B8,51	20:1	38	3	2	2	1	3

[a] Comparison of CMV- versus IE-specific Tc responses in polyclonal Tc cultures from 10 individuals. Autologous and allogeneic fibroblast targets were mock infected (Mock), infected with CMV strain AD169 at an MOI of 5, or infected with vacIE at an MOI of 10. Polyclonal Tc lines were generated in vitro by stimulation with CMV-infected autologous fibroblasts. Standard 5-h chromium release assays were performed at the effector/target ratios (E/T) indicated. HLA alleles are also displayed. ND, not determined.

expressing targets, and these Tc exhibited no detectable lytic activity with CMV-infected targets. The low level of IE-specific lytic activity detected in these cultures suggested that IE-reactive Tc either are present in low frequency or lyse IE-expressing targets inefficiently.

To better analyze the nature and function of Tc specific for the IE protein, Tc clones were generated from Tc cultures initiated by stimulation of PBMC with autologous fibroblasts either constitutively expressing IE or infected with CMV. After two stimulation cycles, Tc clones were isolated from the polyclonal cultures by plating Tc at a limiting dilution of 0.3 Tc per well with autologous stimulator and filler cells, and the frequency of IE-reactive Tc was compared with that of Tc reactive with other CMV proteins. Wells which demonstrated growth were analyzed for phenotype by flow cytometry for CD3, CD8, and α/β Tc receptor expression and for function by chromium release assay for MHC-restricted cytotoxicity of CMV- or vacIE-infected targets. From 3,840 wells plated at 0.3 cells per well from donors MR and TM, two MR and four TM IE-specific Tc clones were isolated, resulting in frequencies of 0.001 and 0.003, respectively (Table 2). In contrast to the weak lytic activity observed with the polyclonal IE-reactive Tc lines, all of the clones exhibited strong lytic activity for targets selectively expressing IE but again failed to lyse CMV-infected targets. By comparison, 62 MR CMV-specific Tc clones and 45 TM

TABLE 2. Isolation frequencies, HLA restricting elements, and specificities of target cell lysis of CMV- versus IE-specific Tc clones

Tc clone	Isolation frequency[a]	HLA restricting element[b]	% Specific lysis				
			Autologous targets				Allogeneic targets
			CMV	$FIBRO_{IE}$	vacIE	vac	vacIE
CMV specific							
MR polyclonal Tc lines			30[c]		1[c]	2[c]	3[c]
20D11	62/288 (0.215)	B18	46	0	0	0	1
2D10		B18	55	0	0	2	1
3F6		B18	44	0	0	1	1
8C8		B18	53	2	1	3	2
TM polyclonal Tc lines			23[c]		2[c]	2[c]	3[c]
6C3	45/288 (0.156)	A24	53	0	0	1	4
4C3		A24	34	1	0	0	2
2E10		A24	40	3	0	2	1
2B1		A24	49	1	1	2	1
IE specific							
MR polyclonal Tc lines			1[c]	10[c]	6[c]	2[c]	1[c]
10H5	2/1,152 (0.001)	B18	0	79	35	0	1
4C9		B18	0	70	42	1	1
TM polyclonal Tc lines			2[c]	15[c]	9[c]	1[c]	0[c]
1B8	4/1,152 (0.003)	A24	0	62	43	0	1
2A2		A24	0	65	39	2	1
2A3		A24	0	60	38	2	1
2A6		B8	0	92	32	0	2

[a] The isolation frequency (in parentheses) was calculated by dividing the number of clones isolated (numerator) by the number of wells seeded times 0.3 (denominator) (the 0.3 multiplier corrects for the number of wells expected to not contain cells since cells were seeded at 0.3 cells per well).

[b] HLA restriction elements were determined for each clone by chromium release assay by analyzing the lytic responses of infected allogeneic fibroblasts which express a single class I HLA allele common to both targets and effectors. All chromium release assays were performed at an effector/target ratio of 10.

[c] Specific lysis of designated targets by original polyclonal Tc culture.

CMV-specific Tc clones were isolated from 960 wells plated at 0.3 cells per well with CMV-infected targets as stimulators, resulting in frequencies of 0.215 and 0.156, respectively (Table 2). These clones efficiently lysed CMV-infected targets but failed to lyse targets selectively expressing IE. Thus, IE-specific Tc are ineffective at lysing CMV-infected targets and are present at a frequency approximately 50- to 100-fold less than that of Tc reactive to other CMV proteins.

The HLA-restricting element utilized by representative Tc clones was analyzed with allogeneic fibroblasts matched for a single MHC allele infected with either CMV or vacIE (Table 2). Tc elicited in response to CMV demonstrated an immunodominant response, as previously described (34), and all MR clones and TM clones tested were restricted to B18 and A24, respectively. IE-specific Tc were restricted to the same allele as the immunodominant CMV-specific response for each individual but also utilized other alleles, as evidenced by TM clone 2A6, which was restricted by B8.

Lysis of CMV-infected cells pretreated with IFN-γ. The failure of IE-specific Tc to lyse CMV-infected target cells could result from quantitatively inefficient processing and presentation of the abundant viral protein or from an absolute blockade in presentation of IE in CMV-infected cells. The sensitivity of CMV-infected fibroblasts to lysis by Tc has been previously demonstrated to be enhanced by pretreatment of targets with IFN-γ (21). Therefore, the effect of IFN-γ on the presentation of IE epitopes and the subsequent lysis of these targets by IE-specific Tc were evaluated. Class I gene expression on fibroblasts incubated for 48 h with 100 U of IFN-γ per ml was examined by labelling cells with fluorescein-conjugated MAb w6/32, which binds to a class I public epitope (29). IFN-γ-pretreated fibroblasts demonstrated significantly higher levels of class I cell surface expression throughout the course of infection from 6 to 48 h relative to fibroblasts not treated with IFN-γ (Fig. 1). To determine the effect of IFN-γ specifically on IE protein expression, CMV-infected fibroblasts treated or not treated with IFN-γ were compared for the level of [^{35}S]methionine-labelled IE immunoprecipitated from cell lysates by an IE-specific MAb at 6 and 24 h after infection with CMV at an MOI of 5. A 72-kDa protein band was identified at similar densities in lysates of cells independent of IFN-γ pretreatment (Fig. 2). Thus, IE is abundantly expressed in CMV-infected cells, as previously reported (3, 11, 17, 25, 36–38), but, unlike expression of class I genes which have an IFN-responsive regulator element (7), expression of IE is not significantly altered by IFN-γ pretreatment.

To determine the effect of enhanced class I expression on lysis of CMV-infected cells by IE-specific Tc, autologous and HLA-mismatched fibroblasts were incubated for 48 h with or without 100 U of IFN-γ per ml, infected for 24 h with CMV at an MOI of 5, and then analyzed for lysis by IE-specific Tc clones TM 1B8, MR 5H10, TM 2A3, MR 4C9, and TM 2A6. These Tc clones demonstrated between 16 and 28% specific lysis of IFN-γ-pretreated autologous targets and less than 2% lysis of untreated infected targets (Fig. 3). These data suggest that the presentation of IE in the context of class I MHC molecules is quantitatively limited in cells naturally infected with CMV.

Presentation of IE by target cells throughout the period of lytic CMV infection. The above-described studies with IFN-γ-treated target cells document that IE-derived peptides can be processed and presented for recognition by Tc. CMV genes are expressed in a temporal cascade after acute infection (16, 23, 39), and thus the inefficient lysis by IE-specific Tc observed with target cells infected for 24 h with CMV might be predicted if effective presentation of IE occurs only at selected time points after viral infection. The availability of newly synthesized IE protein for presentation in infected cells was assessed by labelling targets with [^{35}S]methionine, lysing the cells at multiple time points postinfection, and immunoprecipitating labelled IE with a MAb. Bands corresponding to the 72-kDa IE protein were readily detectable in lysates of cells from 4 to 48 h postinfection (Fig. 4). Thus, as predicted (36, 38), the IE protein is expressed in the immediate-early period postinfection and is present in the cell throughout the lytic cycle.

The presentation of IE was assessed by infecting autologous and HLA-mismatched fibroblasts with CMV for selected durations between 2 and 48 h and monitoring susceptibility to lysis by IE-specific Tc clones. All eight IE-specific Tc clones demonstrated less than 2% specific lysis of targets throughout the period of infection (representative data from clone TM 1B8 is shown in Fig. 5A). For comparison, we evaluated the lysis of these targets by Tc clones specific for two other CMV proteins, pp65 and gB, which were isolated by stimulation with cells selectively expressing these proteins. The matrix protein pp65 is a prototype of the nonstructural virion genes which we have previously described to be introduced into the cytoplasm after viral penetration and uncoating (34), and gB is a viral envelope protein which is synthesized during the late phase of infection (37). A pp65-specific Tc clone, CM 10G1, demonstrated between 11 and 51% specific lysis of autologous targets throughout the 48-h period of infection (Fig. 5B), with peak efficiency at approximately 12 h. By contrast, the gB-specific Tc clone MR 1E1 demonstrated most efficient lysis of autologous targets at 48 h postinfection (Fig. 5C). Thus, in contrast to the IE protein, CMV-infected cells efficiently present other CMV proteins during the early and late phases of infection.

DISCUSSION

The IE protein of human CMV represents a theoretically attractive target for a protective Tc response, since it is synthesized in large amounts immediately upon infection of cells and is present prior to the assembly of new virions. However, our findings demonstrate that human $CD8^+$ Tc specific for the CMV IE protein are ineffectively activated by cells infected with CMV and are recovered from CMV-seropositive individuals at a low frequency relative to the total $CD8^+$ Tc response to other CMV proteins. Moreover, the IE-specific Tc that can be isolated do not efficiently recognize or lyse autologous CMV-infected target cells throughout the immediate-early, early, and late phases of infection, despite abundant IE expression in the target cells during these time periods. The low frequency of IE-specific Tc and poor lytic activity of these Tc for CMV-infected target cells suggest that IE epitopes are inadequately presented in cells infected with this virus. By contrast, Tc specific for other CMV proteins, such as pp65 or gB, can efficiently lyse CMV-infected target cells. Thus, the interference demonstrated by CMV-infected cells both in the presentation of IE-derived peptides and in the susceptibility to lysis by IE-reactive Tc is selective and does not result from the virus inducing a general blockage of antigen presentation or rendering cells resistant to lysis.

The basis for this inefficient presentation of IE-derived peptides requires further investigation, but several possible explanations emerge from these studies. First, the IE protein may have intrinsic qualities that interfere with processing and/or binding to MHC molecules. However, this seems

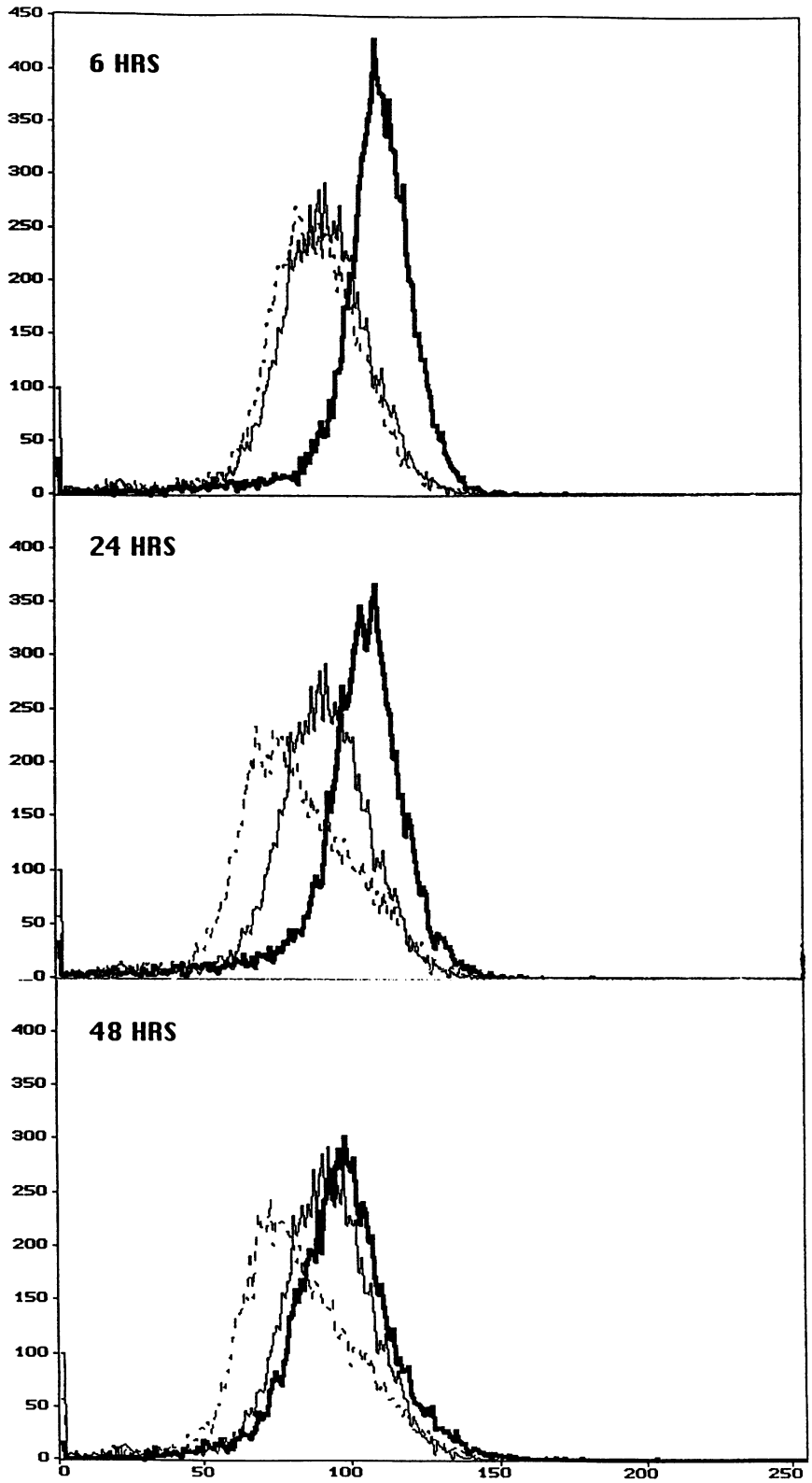

FIG. 1. Effect of IFN-γ pretreatment on the cell surface expression of class I with fibroblasts infected with CMV (MOI = 5. Fibroblasts were mock infected (lightface solid line) or infected with CMV with a 48-h IFN-γ pretreatment (boldface solid line) or without IFN-γ pretreatment (lightface dashed line) and then labeled sequentially with MAb w6/32 and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. Histograms represent the cell surface densities for the 10,000 cells analyzed from each fibroblast culture at each time point postinfection. The mean background fluorescence for cells labeled with an unrelated antibody was 55.

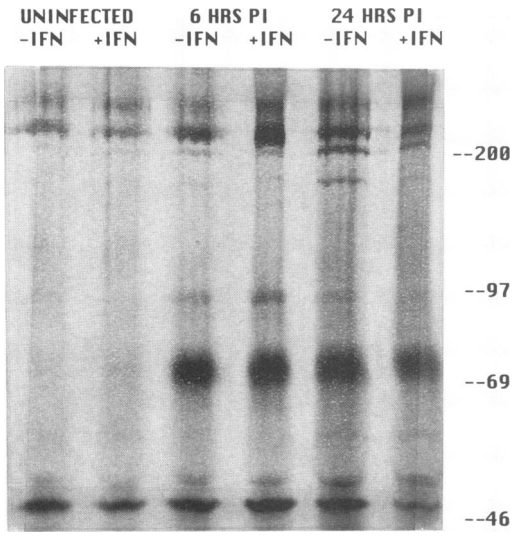

FIG. 2. Effect of IFN-γ pretreatment on IE expression in CMV-infected fibroblasts. Fibroblasts, with or without a 48-h incubation with IFN-γ, were mock infected or infected with CMV (MOI = 5) for 6 or 24 h and then metabolically labelled with [^{35}S]methionine for the final 30 min of infection. Cells were lysed and then subjected to immunoprecipitation with MAb 6E3 and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. PI, postinfection.

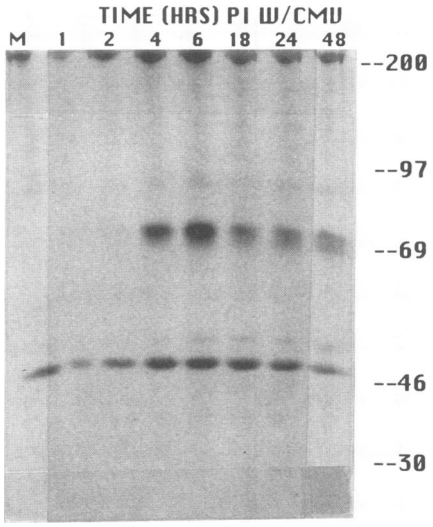

FIG. 4. Level of new synthesis of IE in CMV-infected fibroblasts over the course of acute CMV infection. Fibroblasts were either mock infected or infected with CMV (MOI = 5) for 1, 2, 4, 6, 18, 24, or 48 h and analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 2. PI, postinfection.

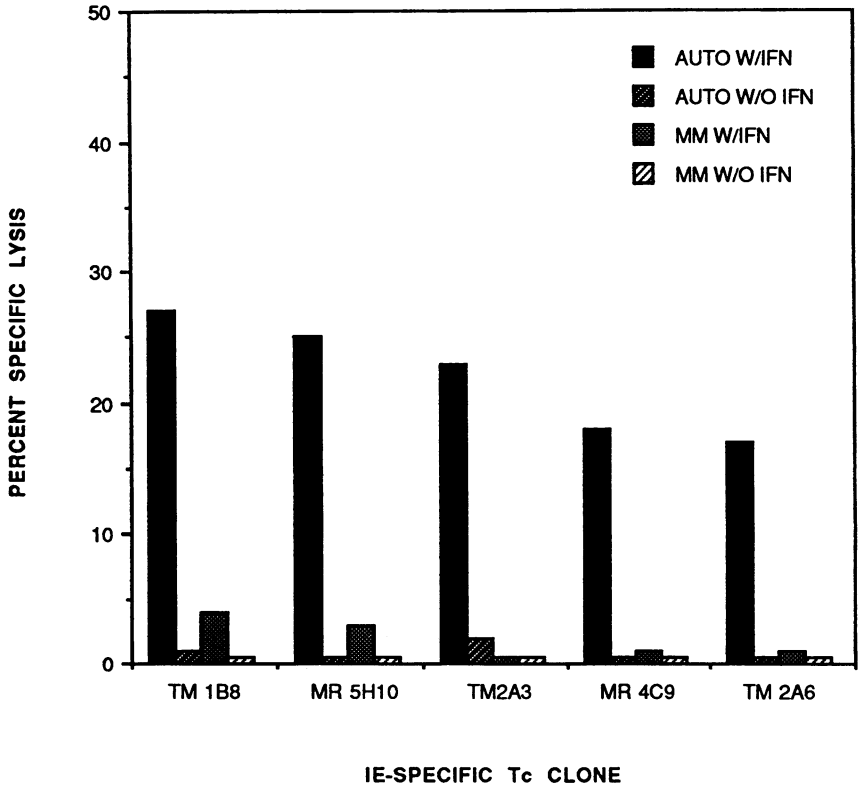

FIG. 3. Effect of IFN-γ pretreatment on lysis of CMV-infected fibroblasts by IE-specific Tc. Autologous (AUTO) and HLA-mismatched (MM) fibroblasts were incubated with or without IFN-γ for 48 h and then infected with CMV (MOI = 5) for 24 h. Target cells were radiolabelled with 10 μCi of ^{51}Cr overnight and then analyzed for lysis by each IE-specific Tc clone at an effector/target ratio of 10. The data represent the percent specific lysis of autologous fibroblasts treated or not treated with IFN-γ and similarly treated and infected allogeneic targets by each clone.

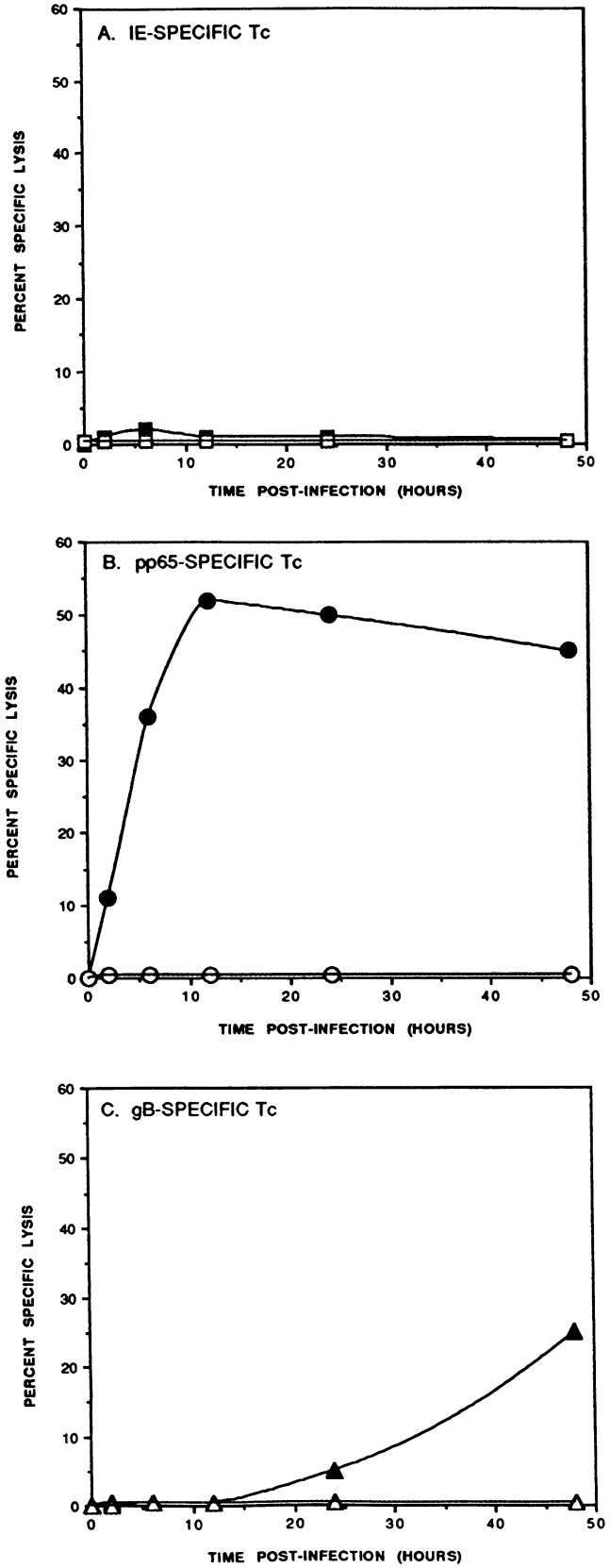

FIG. 5. Comparison of HLA-restricted lysis of fibroblasts during acute infection with CMV by Tc specific for IE, lower matrix protein pp65, or envelope protein gB. Autologous and allogeneic fibroblasts were either mock infected or infected with CMV (MOI = 5) for 2, 6, 12, 24, or 48 h. Target cells were analyzed by chromium release assay as described in the legend to Fig. 3 with one of the following effectors (effector/target ratio of 10): IE-specific Tc clone TM 1B8 (A), pp65-specific Tc clone CM 10G1 (B), gB-specific Tc clone 1E1 (C). The data represent the percent specific lysis of both targets by each clone versus time after infection with CMV.

unlikely since target cells expressing IE selectively do efficiently present IE peptides. Second, the peptides derived from IE may compete poorly with peptides derived from other CMV proteins. However, for the haplotypes studied in detail in this work, IE contains peptide sequences that match the consensus motifs previously reported to bind with high affinity to HLA A24 (13) and HLA B8 (10, 27). Moreover, our analysis of 10 patients with diverse HLA haplotypes has failed to reveal a single individual in which IE efficiently elicits a response. A third alternative explanation is that cells infected with CMV selectively interfere with processing steps for the IE protein that precede entry into the endoplasmic reticulum and thus limit the availability of IE peptides for binding to the class I heterodimer. If this is the case, inefficient IE presentation would be expected to be independent of the HLA alleles expressed on infected cells. Current studies are exploring this issue.

IFN-γ-induced upregulation of MHC-linked genes has previously been shown to increase the lysis of CMV-infected cells by virus-specific Tc (21). Even though we observed a more modest decline in class I expression after CMV infection than has been reported after infection at a much higher MOI (2), pretreatment of CMV-infected cells with IFN-γ upregulated class I and rendered the cells susceptible to lysis by IE-specific Tc. This effect of IFN-γ may be important for the in vivo generation of the weak IE-specific response detected in CMV-infected individuals. Tc responding to CMV infection would be expected to secrete IFN-γ, resulting in the enhancement of IE presentation in neighboring CMV-infected cells. However, the low frequency of IE-specific Tc detected in this study suggests that the IFN-γ production is inadequate to render the IE protein an efficient immunogen in CMV-infected targets.

Our data suggest that IE-specific Tc may have limited biological importance in the cellular immune response against CMV. Borysiewicz et al. have previously suggested, on the basis of having identified in CMV-seropositive individuals significant levels of cellular cytotoxic reactivity to cells selectively expressing IE, that human IE-specific Tc may provide protective immunity (5). However, in their study, the majority of CMV-specific Tc responses were in fact against CMV proteins other than IE, and the lytic activity measured with IE-expressing targets likely includes contributions by natural killer effector cells, suggesting that the frequency of IE-specific Tc responses had been overestimated. Prior work in our laboratory has demonstrated that CMV-specific Tc can recognize structural virion proteins as target antigens and can lyse CMV-infected cells within 2 h of virion entry (34). The importance of these Tc in protective immunity against CMV is supported by recent studies from our laboratory with adoptive transfer of CMV-specific Tc clones to human bone marrow transplant recipients (35). The transfer of Tc clones which recognize structural virion proteins appears to provide protection from CMV disease in these immunosuppressed individuals, who lack spontaneous recovery of CMV-specific Tc responses after transplant.

Viruses have evolved many distinct mechanisms to limit or evade immune detection (18), presumably to promote viral replication and/or facilitate survival of latently infected cells that express viral genes. Some herpesviruses, such as EBV and MCMV, demonstrate restricted immunogenic presentation of their transcription factors for recognition by effector Tc. Tc responses specific for EBNA1 have not been isolated in vitro despite the use of stimulators infected with EBV (26) or selectively expressing EBNA1 (20). Similar to results in our studies with the IE protein of human CMV, inefficient presentation is limited to EBNA1 and other EBV proteins are highly immunogenic (20, 26). MCMV-infected target cells have been shown to be resistant to lysis by pp89-specific Tc clones during the early phase of infection (15), although this occurs by a more general mechanism than observed with the IE protein from human CMV, since other antigens such as simian virus 40 T antigen and β-galactosidase are also poorly presented (12, 14). Moreover, pp89-specific Tc can recognize MCMV-infected target cells at the immediate-early and late phases of acute infection. Other viruses, such as adenoviruses, encode proteins that down-regulate class I expression, thereby generally interfering with the presentation of all viral proteins (8, 9). Thus, the expression of novel viral proteins in target cells cannot be assumed to predict the effective presentation of peptides derived from that protein or the potential for efficient induction of a protective Tc response reactive with that protein. In this study, we have demonstrated selective interference with the presentation of the IE protein after CMV infection, despite the fact that this protein can be selectively presented in cells expressing IE and no other CMV proteins. Further studies to elucidate the mechanism by which CMV interferes with IE presentation may provide insights into more general means by which other proteins, such as those encoded by other viruses or found in transformed cells, fail to induce host protective immune responses.

ACKNOWLEDGMENTS

We thank Eli Gilboa for the gift of retrovirus encoding IE, Enzo Paoletti for the gift of the vaccinia virus recombinant encoding IE, Adam Geballe and Ed Mocarski for helpful discussions, and Joanne Factor for assistance in the preparation of the manuscript.

This project was supported in part by grants CA18029 from the NCI and P30-AI27757-05 from the NIH. M.J.G. also received support through grant T32 CA09515-07 from the NIH. S.R.R. is a Special Fellow of the Leukemia Society of America.

REFERENCES

1. **Akrigg, A., G. Wilkinson, and J. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. **2**:107–121.
2. **Barnes, P., and J. Grundy.** 1992. Down-regulation of the class I HLA heterodimer and beta-2-microglobulin on the surface of cells infected with cytomegalovirus. J. Gen. Virol. **73**:2395–2403.
3. **Blanton, R., and M. Tevethia.** 1981. Immunoprecipitation of virus-specific immediate-early and early polypeptides from cells lytically infected with human cytomegalovirus strain AD169. Virology **112**:262–273.
4. **Borysiewicz, L., S. Graham, J. Hickling, and J. Sissons.** 1988. Precursor frequency and stage specificity of human cytomegalovirus specific cytotoxic T cells. Eur. J. Immunol. **18**:269–275.
5. **Borysiewicz, L., V. Hickling, S. Graham, J. Sinclair, M. Cranage, G. Smith, and J. Sissons.** 1988. Human cytomegalovirus specific cytotoxic T cells relative treatment of stage specific CTL recognizing the 72kD immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. J. Exp. Med. **168**:919–931.
6. **Borysiewicz, L., S. Morris, J. Page, and J. Sissons.** 1983. Human cytomegalovirus-specific cytotoxic T lymphocytes: requirements for *in vitro* generation and specificity. Eur. J. Immunol. **13**:804–809.
7. **Bukowski, J., and R. Welsh.** 1985. Interferon enhances the susceptibility of virus-infected fibroblasts to cytotoxic T cells. J. Exp. Med. **161**:257–262.
8. **Burgert, H., and S. Kvist.** 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell **41**:987–997.
9. **Burgert, H., J. Maryanski, and S. Kvist.** 1987. E3/19K protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proc. Natl. Acad. Sci. USA **84**:1356–1360.
10. **Burrows, S., S. Rodda, A. Suhrbeir, M. Geysen, and D. Moss.** 1992. The specificity of recognition of a cytotoxic T lymphocyte epitope. Eur. J. Immunol. **22**:191–195.
11. **Cameron, J., and C. Preston.** 1981. Comparison of the immediate early polypeptides of human cytomegalovirus isolates. J. Gen. Virol. **54**:421–424.
12. **Campbell, A. E., J. S. Slater, V. J. Cavanaugh, and R. M. Stenberg.** 1992. An early event in murine cytomegalovirus replication inhibits presentation of cellular antigens to cytotoxic T lymphocytes. J. Virol. **66**:3011–3017.
13. **Dai, L. C., K. West, R. Littaua, K. Takahashi, and F. A. Ennis.** 1992. Mutation of human immunodeficiency virus type 1 at amino acid 585 on gp41 results in loss of killing by $CD8^+$ A24-restricted cytotoxic T lymphocytes. J. Virol. **66**:3151–3154.
14. **del Val, M., H. Hengel, H. Häcker, U. Hartlaub, T. Ruppert, P. Lucin, and U. Koszinowski.** 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J. Exp. Med. **176**:729–738.
15. **del Val, M., K. Munch, M. Reddehasse, and U. Koszinowski.** 1989. Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. Cell **58**:305–315.
16. **DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan.** 1980. Pattern of transcription of human cytomegalovirus in permissively infected cells. J. Virol. **35**:277–286.
17. **Gibson, W.** 1981. Immediate-early protein of human cytomegalovirus strains AD169, Davis and Towne differ in electrophoretic mobility. Virology **112**:350–354.
18. **Gooding, L.** 1992. Virus proteins that counteract host immune defenses. Cell **71**:5–7.
19. **Jonjić, S., M. del Val, G. M. Keil, M. J. Reddehasse, and U. H. Koszinowski.** 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. J. Virol. **62**:1653–1658.
20. **Khanna, R., S. Burrows, M. Kurilla, C. Jacob, I. Misko, T. Sculley, E. Kieff, and D. Moss.** 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. **176**:169–176.
21. **Laubscher, A., H. Bluestein, S. Spector, and N. Zvaifler.** 1988. Generation of human cytomegalovirus-specific cytotoxic T lymphocytes in a short-term culture. J. Immunol. Methods **110**:69–77.
22. **Mathews, R.** 1979. Classification and nomenclature of viruses. Third International Committee on Taxonomy of Viruses. Intervirology **12**:132–296.
23. **McDonough, S., and D. Spector.** 1983. Transcription in human fibroblasts infected by human cytomegalovirus strain AD169. Virology **125**:31–46.
24. **McLaughin, E., B. Tanamache, H. Pande, C. Li, J. Zaia, S. Forman, P. Greenberg, and S. Riddell.** Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for $CD8^+$ virus-specific CTL. Submitted for publication.
25. **Michelson, S., F. Horodniceanu, M. Kress, and M. Tardy-Panit.** 1979. Human cytomegalovirus-induced immediate early antigens: analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis after immunoprecipitation. J. Virol. **32**:259–267.

26. **Murray, P., M. Kurilla, W. Thomas, M. Rowe, E. Kieff, and A. Rickinson.** 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus: implication for the immune control of EBV-positive malignancies. J. Exp. Med. **176:**157–168.
27. **Nixon, D., and A. McMichael.** 1991. Cytotoxic T-cell recognition of HIV proteins and peptides. AIDS **5:**1049–1059.
28. **Pande, H., S. Baak, A. Riggs, B. Clark, J. Shirey, and J. Zaia.** 1984. Cloning and physical mapping of a gene fragment coding for a 64 kilodalton major late antigen of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81:**4965–4967.
29. **Parham, P., C. Barnstable, and W. Bodmer.** 1979. Use of monoclonal antibody (w6/32) in structural studies of HLA-A,B,C antigens. J. Immunol. **123:**342–349.
30. **Quinnan, G., N. Kirimani, A. Rook, J. Manischewitz, L. Jackson, G. Moreschi, G. Santos, R. Saral, and W. Burns.** 1982. Cytotoxic T cells in cytomegalovirus infection: HLA-restricted T lymphocytes and non-T lymphocytes cytotoxic responses correlate with recovery from cytomegalovirus infection in bone marrow transplantation. N. Engl. J. Med. **307:**7–13.
31. **Reusser, P., S. Riddell, J. Meyers, and P. Greenberg.** 1991. Cytotoxic T lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood **78:**1373–1380.
32. **Rickinson, A., M. Rowe, I. Hart, Q. Yao, L. Henderson, H. Rabin, and M. Epstein.** 1984. T cell-mediated regression of "spontaneous" and of Epstein-Barr virus-mediated B cell transformation *in vitro*: studies with cyclosporine A. Cell. Immunol. **87:**646–650.
33. **Riddell, S., and P. Greenberg.** 1990. The use of anti CD3 and anti CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J. Immunol. Methods **128:**189–201.
34. **Riddell, S., M. Rabin, A. Gaballe, W. Britt, and P. Greenberg.** 1991. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. J. Immunol. **146:** 2795–2804.
35. **Riddell, S., K. Watanabe, J. Goodrich, C. Li, M. Agha, and P. Greenberg.** 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science **257:**238–241.
36. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63:**2699–2708.
37. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26:**686–701.
38. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. **46:**1–14.
39. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. J. Virol. **38:**446–459.