JOURNAL OF VIROLOGY, Sept. 1991, p. 4812–4820
0022-538X/91/094812-09$02.00/0

Vol. 65, No. 9

Fine Specificity of Cellular Immune Responses in Humans to Human Cytomegalovirus Immediate-Early 1 Protein

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Received 12 December 1990/Accepted 6 June 1991

Cell-mediated immunity is important in maintaining the virus-host equilibrium in persistent human cytomegalovirus (HCMV) infection. The HCMV 72-kDa major immediate early 1 protein (IE1) is a target for $CD8^+$ cytotoxic T cells in humans, as is the equivalent 89-kDa protein in mouse. Less is known about responses against this protein by $CD4^+$ T cells, which may be important as direct effector cells or helper cells for antibody and $CD8^+$ responses. Proliferative-T-cell responses to HCMV IE1 were studied in normal seropositive subjects. Peripheral blood mononuclear cells from 85% of seropositive subjects proliferated in response to HCMV from infected fibroblasts, and of these, 73% responded to recombinant baculovirus IE1. Responding cells were predominantly $CD3^+$ $CD4^+$. IE1 antigen preparations, including baculovirus recombinant protein, transfected rat cell nuclei, and synthetic peptides, induced IE1-specific T-cell lines which cross-reacted between the preparations. The fine specificity of these IE1-specific T-cell lines was studied by using overlapping synthetic peptides encompassing the entire sequence of the IE1 protein. The regions of the IE1 molecule recognized were identified and these varied between individuals, possibly reflecting differences in major histocompatibility complex (MHC) class II haplotype. In one subject, the peptide specificities of proliferative and MHC class I-restricted cytotoxic determinants on IE1 were spatially distinct. Thus, no single immunodominant T-cell determinant within HCMV IE1 was identified, suggesting that multiple peptides or a region of the 72-kDa IE1 protein would be required to induce specific T-cell responses in humans.

Human cytomegalovirus (HCMV) is a widely distributed species-specific betaherpesvirus. It establishes a subclinical persistent infection in healthy individuals but is an important pathogen in the immunocompromised patient. The virus genome encodes up to 180 proteins (9) expressed in a temporally regulated sequence. Immediate-early proteins (IE proteins) (30) are expressed 2 to 6 h after infection, and then early antigens are expressed from 6 to 18 h postinfection. Both IE and early proteins are prerequisites for viral DNA replication, which occurs after 18 h with subsequent expression of structural or late proteins.

The most abundant mRNA (88% of total IE mRNA) at immediate-early times after infection is a 1.95-kb transcript encoding the major IE1 protein (22). IE1 is a 72-kDa phosphoprotein of 491 amino acids located in the nucleus of infected cells (Fig. 1). It is highly conserved between isolates: there are only two amino acid differences between HCMV AD169 and Towne strains (2, 41). IE1 transactivates other viral genes (40) and cellular genes (13) but is also a target for the immune response.

The association of HCMV disease with immunosuppression has focused attention on the role of cell-mediated immunity, in particular $CD8^+$ cytotoxic T lymphocytes (CTL), in maintaining the virus-host equilibrium during persistent infection. Following primary murine cytomegalovirus (MCMV) infection in BALB/c mice, $CD8^+$ CTL recognized the 89-kDa IE protein (pp89) of MCMV (26, 27) and protected against lethal virus challenge after adoptive transfer (36). These CTL recognized a single immunodominant epitope from pp89 (12, 37), and when mice were immunized with a recombinant vaccinia virus encoding this determinant, they were again protected from lethal virus challenge but not from establishment of persistent infection (23).

In humans we have identified HCMV-specific $CD8^+$ CTL (7) in the peripheral blood of healthy seropositive subjects. The CTL precursors were present at high frequencies and recognized predominantly nonstructural viral antigens (6). In two subjects, the CTL precursors were predominantly directed against the IE1 gene product (5).

Thus, $CD8^+$ CTL specific for the IE1 antigen may regulate HCMV infection in the persistently infected host. However, there is less information on the role of $CD4^+$ T cells in HCMV infection or on their antigen specificities. Such cells may serve a variety of functions: direct major histocompatibility complex (MHC) class II-restricted cytolytic activity, cytokine release, or augmentation of both CTL and humoral responses. Anti-HCMV antibodies may protect seronegative subjects by passive or maternofetal transfer (29, 39), but this may be mediated largely by neutralizing antibodies directed against structural components of the virus. Antibodies against nonstructural IE proteins are present in serum (3, 30, 45), but their roles are unclear; in BALB/c mice, protection is mediated by $CD8^+$ CTL in the absence of antibodies against pp89 (21). Little is known about $CD4^+$ T cells directed against IE and early proteins. Therefore, prior to doing functional studies of the possible role of these cells in humans, we investigated whether $CD4^+$ T cells specific for the IE1 protein were present in normal seropositive individuals and whether an immunodominant proliferative T-cell determinant in IE1 could be identified. Such cells were present in the majority of subjects tested, and $CD4^+$ T cells recognized primary rather than conformational (32) determinants of this protein. Linear regions of the IE1 molecule

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which were recognized varied between individuals, reflecting their diverse MHC class II haplotypes, and studies of one subject suggest that proliferative and cytotoxic determinants on IE1 are spatially distinct.

MATERIALS AND METHODS

Cells and reagents. Cell cultures were grown in RPMI 1640 medium (Flow Laboratories Ltd., Irvine, Scotland) supplemented with 10% (vol/vol) Myoclone Plus fetal calf serum (GIBCO, Grand Island, N.Y.) or pooled human AB serum, 2.0 mM L-glutamine, 1.0×10^5 IU of penicillin per liter, and 100 mg of streptomycin (Flow Laboratories) per liter. Peripheral blood mononuclear cells (PBMC) were prepared from fresh heparinized venous blood by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient separation (21). B-lymphoblastoid cell lines (BCL) were established from PBMC by Epstein-Barr virus transformation as previously described (47).

Viruses. HCMV AD169 (American Type Culture Collection catalog no. VR-538) was grown in F2002 fibroblasts (Flow Laboratories) infected at a multiplicity of infection of 0.1:1. Supernatant fluid from infected cultures containing approximately 1.0×10^6 50% tissue culture infective doses of virus ml^{-1} was heated at 60°C for 45 min and used to stimulate PBMC at a final and optimal (data not shown) dilution of 1:4 (referred to as HCMV). Recombinant vaccinia virus encoding IE1 (vac-IE) (5) and gB (11) were grown in Vero cells (infected at a multiplicity of infection of 0.1:1). After 48 h, infected cells were harvested, and the supernatant was stored at −70°C. Virus stocks were used at 1.0×10^7 50% tissue culture infective doses ml^{-1}.

Recombinant baculovirus IE1 antigen. HCMV IE1 protein was produced by using a baculovirus expression system. A cDNA clone for the HCMV 72-kDa IE1 protein was ligated into the *Pst*I site of plasmid pUC9. A 1.7-kb *Bam*HI fragment containing this sequence was inserted into the baculovirus polyhedrin gene in plasmid pAc36C. This vector was cotransfected with wild-type *Autographica californica* nuclear polyhedrosis virus DNA into *Spodoptera frugiperda* cells. Recombinant virus (bac-IE1) was isolated by plaque purification, and stocks were cultured. To generate IE1 antigen, *S. frugiperda* cells were infected at a multiplicity of infection of 5 and cultured for 48 h at 27°C. Infected cells were harvested and stored in phosphate-buffered saline at −70°C in the presence of protease inhibitor. Control antigen (bac-0) was prepared by similarly infecting and processing *S. frugiperda* cells with a nonexpressing baculovirus recombinant. Frozen samples of 2.5×10^5 cells were thawed and sonicated for 60 s at power level 7 in a W-375 sonicator (Heat Systems Ultrasonics Inc., Luton, United Kingdom) and used to stimulate PBMC at a final dilution of 1:10. Immunoblot analysis of the sonic extract with anti-IE1 antibody 1D4 (B. Rodgers, Wellcome Biotech, Beckenham, United Kingdom; data not shown) confirmed the presence of the 72-kDa protein in large quantities, although products of lower molecular weights were also identified, suggesting that epitopes recognized by 1D4 were conserved despite partial degradation.

Rat-alpha-1 nuclei. Nuclei stably expressing HCMV IE1 were prepared from Rat-9G cells (kind gift from R. Boom). These cells were obtained by transfection of Rat-1-TK$^-$ cells with a plasmid, paIEA-1, constructed from exons 1 to 4 of the HCMV IE1 coding region, including 465 bp of the IE1 upstream regulatory sequence (4). Nuclear antigen expression was observed in approximately 1% of cells by immunofluorescence. Transcriptional activation of the stably transfected gene was induced by heat shock or arsenite treatment (14). Under these conditions, IE1 nuclear fluorescence was observed in up to 80% of cells, and synthesis of the major 72-kDa IE1 protein was demonstrated by immunoprecipitation. Frozen nuclei were thawed and used to stimulate PBMC at a nucleus-to-cell ratio of 1:10.

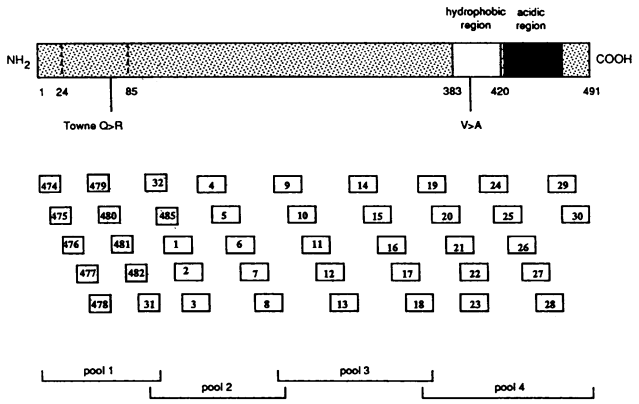

FIG. 1. Representation of the primary structure of HCMV 72-kDa IE1 protein (AD169 strain). The two amino acid differences (Q→R and V→A) between AD169 and Towne strains are shown. IE1 is translated from a spliced transcript of 4 exons, of which residues 1 to 24 from exon 2, 24 to 85 from exon 3, and 85 to 491 from exon 4 are translated. Synthetic peptides (overlapping by 6 residues) and peptide pools are indicated. Peptides 22 and 23 are identical except for the AD169 and Towne strain differences (position 394, Val↦Ala).

Peptides. A series of peptides (15-mers to 20-mers overlapping by 6 residues) encompassing the entire sequence of IE1 was constructed (Fig. 1). Peptides were synthesized as described by Houghten (18). Briefly, standard *t*-butoxycarbonyl amino acid resin (100 mg) was sealed in polypropylene mesh bags for simultaneous multiple peptide synthesis. Standard deprotection, neutralization, coupling, and wash protocols were employed as described by Houghten et al. (19, 20). To prevent acylation and racemization of histidine residues during coupling, 2,4-dinitrophenol-protected histidine was used. The 2,4-dinitrophenol was removed at the end of synthesis by treatment with 1% (vol/vol) thiophenol in dimethyl formamide. Twenty peptides were cleaved simultaneously from the resin by using a conventional HF procedure (19). Cleaved peptides were extracted from the bags in 5 to 30% acetic acid, lyophilized, and dissolved in either phosphate-buffered saline or dimethyl sulfoxide (DMSO) at 5.0 mg ml^{-1}, according to their solubility. In parallel experiments (data not shown), DMSO was found to be toxic to proliferating PBMC at concentrations of >1% (vol/vol). Therefore, DMSO-soluble peptides were used in proliferation assays with a final DMSO concentration of <1%. Purity of peptides was assessed by reverse-phase high-pressure liquid chromatography, using a Novapax C18 column (Waters Chromatography, Watford, United Kingdom) running in a gradient from water to 50% acetonitrile (in 0.02% triethylamine; buffered in acetic acid [pH 7.0]). Peptides with a purity of greater than 70% were used in experiments.

Establishment and maintenance of T-cell lines. Fresh PBMC were cultured at 37°C in 24-well flat-bottom plates (2.0×10^6 cells per well) (Linbro; Flow Laboratories) in 2.0 ml of RPMI with 10% human AB serum. Antigen (IE1 peptides grouped together, Rat-alpha-1 nuclei, soluble

HCMV antigen, bac-IE1, or bac-0) was prepared for PBMC stimulation as described above. On days 5 and 8, 5 IU of recombinant interleukin 2 (rIL-2) (Boehringer Mannheim, Lewes, United Kingdom) per ml was added, and the cells were subcultured 1:3. On day 12, the specificity of the cells was tested in a standard thymidine incorporation assay. T-cell lines were subsequently maintained by the weekly addition of irradiated (3.0 kilorads) PBMC with 5 IU of rIL-2 per ml.

Proliferation assays. PBMC were cultured at 2.0×10^5 cells per well in 96-well round-bottom plates (Linbro; Flow Laboratories) in triplicate with appropriate antigens for different periods as described in Results. Experiments were performed to optimize concentrations of the various antigens (data not shown). T-cell lines were stimulated with antigen-presenting cells prepared by incubating autologous irradiated (3.5 kilorads) PBMC with bac-IE1 or bac-0 (1:10 dilution) or with peptides (10 $\mu g\ ml^{-1}$, optimal concentration) for 12 h and then washing them to remove nonadherent cells. T cells (1.0×10^5 per well) were then cultured with the antigen-presenting cells for 2 days. Proliferation was determined by adding 1.0 μCi of [*methyl*-^{3}H]thymidine (Amersham International plc., Aylesbury, United Kingdom) in 50 μl of RPMI to each well and incubating the wells for 12 to 20 h. Cells were harvested onto filter paper (Titertek; Flow Laboratories), dried, and loaded into 0.5 ml of scintillation fluid (OptiScint Hisafe; LKB, FSA Laboratory Supplies, Loughborough, United Kingdom). Means and standard errors of triplicate samples were calculated, and the data were expressed as counts per minute or, for PBMC, as stimulation indices calculated as counts per minute of antigen/counts per minute of control. Values greater than 3.0 were considered significant (46).

Cytotoxicity assays. For the initial peptide screening experiment, PBMC (effector cells) were cultured at 2.0×10^5 cells per well in 96-well round-bottom plates (Linbro; Flow Laboratories) in triplicate with each individual peptide (10 $\mu g\ ml^{-1}$, optimal concentration) for 14 days. rIL-2 (5 IU ml^{-1}) was added to the cultures on days 5 and 10. After 14 days, effector cell cultures were dispersed in their individual wells and equal volumes were transferred to three fresh 96-well round-bottom plates to test for specific MHC-restricted cytotoxicity. Targets were (i) autologous BCL infected 6 h prior to the assay with either vac-IE or vac-gB (control) at 5.0 50% tissue culture infective doses per cell and (ii) allogeneic BCL similarly infected with vac-IE. Maximal expression of recombinant IE1 and gB in infected BCL was between 6 and 12 h postinfection (55 to 62% of cells were infected), as assessed by flow cytometry. Target cells were labeled in 100 μl of suspension for 45 min with 100 μCi of ^{51}Cr (Amersham) and washed three times in RPMI 10. Each target type (5,000 cells per well) was then cocultured for 6 h with the equally distributed effector cells. On each plate, six wells were incubated with aqueous 2% (vol/vol) Triton X-100 (BDH, Poole, United Kingdom) to determine maximum ^{51}Cr release, and six wells were incubated with RPMI alone to determine spontaneous release. The percent specific lysis ± standard deviation was calculated as follows: (test release − spontaneous release)/(maximum release − spontaneous release) × 100%.

HCMV-specific CTL were generated in bulk culture by stimulating PBMC (1.0×10^6 cells per well) with irradiated autologous fibroblasts infected for 48 h with HCMV (stimulator/responder ratio, 1:10) in 24-well flat-bottom plates. rIL-2 (5 IU ml^{-1}) was added on days 5 and 10. The cultures were harvested on day 14 and tested for cytotoxicity against autologous or allogeneic peptide-labeled BCL. Target cells were pulsed with peptide (100 $\mu g\ ml^{-1}$) for 1 h at 37°C, labeled with ^{51}Cr, and used in a standard cytotoxicity assay, as described above, with effector/target ratios from 6:1 to 1:1.

HCMV serology. HCMV antibodies in sera were assayed by a standard competitive enzyme immunosorbent assay (Northumbria Biologicals Ltd, Cramlington, United Kingdom) as described by Wreghitt et al. (48). A positive result was recorded when the optical density (OD) of the sample was less than half the OD of the negative control. A negative result was recorded when the OD of the sample was >66% of the OD of the negative control.

Cell surface phenotype. Phenotypes of cultured PBMC were determined by flow cytometry (EPICS; Coulter Electronics, Luton, United Kingdom). PBMC samples were stained with 50 μl of the appropriate monoclonal antibody (anti-CD3, anti-CD4, and anti-CD8 [American Type Culture Collection]) and a 1:100 dilution of goat anti-mouse immunoglobulin fluorescein isothiocyanate conjugate (Sigma Chemicals, St. Louis, Mo.).

CD4$^+$ and CD8$^+$ T-cell depletion. PBMC were stained for 30 min with a 1:50 dilution of mouse anti-CD4 (OKT4) or anti-CD8 (OKT8) monoclonal antibody, washed twice in phosphate-buffered saline, and incubated with immunomagnetic beads (bead/cell ratio, 10:1) coated with sheep anti-mouse immunoglobulin G (Dynal; Wirral, United Kingdom) for 30 min at 4°C. Bead-coated cells were removed magnetically, and the procedure was repeated twice. A sample from the treated cells was examined for depletion efficiency by flow cytometry.

RESULTS

Proliferation of PBMC from subjects seropositive to bac-IE1 antigen. To determine whether T cells responded by proliferation to HCMV antigens, and to IE1 in particular, PBMC were cultured for 6 days as described in Materials and Methods with either soluble HCMV antigen, bac-IE1, bac-0, or RPMI alone as a control and then tested for thymidine incorporation. Antigens were used at a range of concentrations to determine the optimal concentration for each subject. PBMC from one of four seronegative subjects proliferated in response to soluble HCMV antigen, and none responded to the bac-IE1 product (Table 1). Of 13 seropositive subjects, 11 (85%) responded to soluble HCMV antigens. Of these 11, 8 (73%) also had specific responses to the bac-IE1 product. In addition, one of the two subjects who did not respond to HCMV antigen had a small but significant response to bac-IE1.

IE1-specific T cells. Three different antigen preparations were used to establish short-term T-cell lines from PBMC of several seropositive subjects: the bac-IE1 preparation (1:10 dilution), the entire group of IE1 synthetic peptides (10 $\mu g\ ml^{-1}$), and Rat-alpha-1 nuclei stably expressing IE1 (1:10 nucleus/cell ratio). T cells were tested after 12 days for specific proliferation in response to bac-IE1 (and bac-0 control) as described in Materials and Methods.

Four seropositive subjects of diverse human leukocyte antigen (HLA) types (Table 2) were studied. T-cell lines from all four subjects when induced by peptides or Rat-alpha-1 responded specifically to bac-IE1 (two-tailed t test, $P < 0.05$). The reproducibility of the responses (two separate experiments) to IE1 for subject 1 are shown (Table 3). When T-cell lines were generated by stimulation with the entire group of 40 peptides or with Rat-alpha-1 cell nuclei, there

TABLE 1. Proliferative response of PBMC from normal HCMV-seropositive and -seronegative subjects to HCMV, bac-IE1, and bac-0 antigens

Subject	HCMV serologic result	Stimulation index + SE		
		HCMV	bac-IE	bac-0
6	+	9.4 + 0.6	14.4 + 1.1	4.6 + 1.0
7	+	30.5 + 11.9	109.2 + 12.7	25.2 + 3.9
8	+	16.3 + 0.6	13.0 + 2.9	3.9 + 0.8
9	+	43.5 + 2.4	226.7 + 9.9	24.2 + 6.9
10	+	76.5 + 4.8	6.3 + 0.7	2.7 + 0.6
11	+	134.2 + 7.4	49.6 + 4.3	11.1 + 3.6
3	+	58.1 + 2.8	35.6 + 2.9	10.9 + 0.9
12	+	35.8 + 1.1	41.2 + 1.9	20.3 + 6.1
13	+	15.1 + 1.8	1.2 + 0.1	0.8 + 0.1
14	+	10.8 + 0.6	13.8 + 2.2	14.7 + 0.3
15	+	31.9 + 4.0	17.0 + 7.8	40.3 + 9.8
16	+	1.2 + 0.1	4.2 + 0.9	1.0 + 0.1
2	+	0.8 + 0.1	2.6 + 0.5	1.0 + 0.4
17	−	0.3 + 0.1	1.6 + 0.1	1.6 + 0.2
18	−	0.1 + 0.1	0.2 + 0.1	0.2 + 0.1
19	−	4.6 + 1.7	1.5 + 0.2	2.0 + 0.2
4	−	0.9 + 0.2	0.7 + 0.3	1.2 + 0.2

was a specific proliferation in response to secondary stimulation by bac-IE1. T-cell lines generated by stimulation with bac-0 or bac-IE1 responded as expected to both bac-0 and bac-IE1, probably because of other antigens shared in these preparations. Thus, IE1 stably transfected into cells or an IE1 peptide pool could induce IE1-specific T-cell lines recognizing the same protein in the product generated from baculovirus recombinants. This suggests that the determinants recognized were conserved, particularly since peptide fragments could induce responses against the whole protein, and were not conformationally dependent.

The phenotypes of these IE1-specific T-cell lines from subjects 2 and 3 were >90% $CD3^+$ and 50 to 70% $CD4^+$.

Selective depletion experiments were performed to confirm that proliferation was mediated by $CD4^+$ cells. $CD4^+$ (>95%)-depleted T-cell lines did not proliferate in response to bac-IE1 in the presence or absence of exogenous rIL-2 (5 IU ml^{-1}) or in the presence of autologous irradiated feeder cells. When the same T-cell lines were $CD8^+$ depleted, significant specific proliferation to bac-IE1 was observed (data not shown).

Fine specificity of proliferative responses to IE1. Synthetic peptides were used to investigate the fine specificity of T-cell proliferative responses to individual determinants on IE1. T-cell lines from seropositive subjects 1, 2, and 3 were induced with bac-IE1 and then tested for proliferation to peptides, either individually or in our pools (Fig. 1), as described in Materials and Methods. A bac-IE1-specific T-cell line from subject 3 responded to peptides in pool 2 (Fig. 2A). When the line was tested against the individual peptides constituting this pool, a significant response to peptide 6 was observed (Fig. 2B). The bac-IE1-specific T-cell line from subject 1 was assayed similarly: the line responded to peptides 485, 1, and 2 from peptide pool 2 (Fig. 2C and D). This line also responded to peptide pool 4, but the particular peptides recognized have not been reproducibly determined. The T-cell line from subject 2 (95% $CD3^+$ and 62% $CD4^+$) responded to just two peptides, 22 and 23, though much more strongly to peptide 22 (Fig. 2E). This response was particularly interesting since peptides 22 and 23 were identical except for a Val-to-Ala change (position 394 in the AD169 strain primary sequence), which is one of the two differences between the IE1 sequences of HCMV AD169 and Towne strains (2, 41). This observation implied that the Val residue is important in the recognition of this determinant by $CD4^+$ T cells from subject 2. To determine whether these $CD4^+$ T cells were able to mediate MHC class II-restricted CTL activity, the T-cell line was assayed for its ability to lyse ^{51}Cr-labeled autologous or HLA-mismatched BCL infected with the vac-IE recombinant. BCL, rather than fibroblasts infected with HCMV, were selected as targets because BCL express MHC class II molecules. No MHC-restricted or nonspecific lysis by this T-cell line was observed (data not shown).

To determine whether individual peptides could resolve T-cell specificities directly from peripheral blood, PBMC from subjects 4 and 5 were cultured in the presence of each

TABLE 3. Proliferative responses of T-cell lines established to respond to different preparations of HCMV IE1

Expt	Primary antigen	Proliferative response + SD (cpm) to secondary antigen:	
		bac-IE1	bac-0
1	bac-0	103,000 + 14,100	93,000 + 10,300
	bac-IE1	112,000 + 5,700	90,000 + 11,000
	Rat-alpha-1	34,000 + 1,600	10,000 + 3,000
	Peptides	31,000 + 4,300	8,000 + 2,740
	Medium control	9,000 + 8,900	5,000 + 1,800
2	bac-0	67,000 + 5,400	57,000 + 8,700
	bac-IE	91,000 + 11,600	50,000 + 34,000
	Rat-alpha-1	18,000 + 1,700	8,200 + 520
	Peptides	8,400 + 640	1,400 + 800
	Medium control	3,200 + 4,200	200 + 120

TABLE 2. Establishment of T-cell lines to total HCMV and HCMV IE1 antigen

Subject	HLA type	CMV serologic result	Specific proliferative T-cell responses to:			
			HCMV	bac-IE1	Rat-alpha-1[a]	IE1 peptides
1	A2 B8 B12/44 Cw5 Cw7 DR3 DR4 DRw52 DRw53 DQw2 DQw3	+	+	+	+	+
2	A28 A32 B16 DR4 DR6	+	−	+	+	+
3	A1 B7 B17 Cw4 Cw5 DR1 DR3	+	+	+	ND	+
4	A19 A28 A30 B13 B18 Cw8 DR5 DR6/7	−	−	−	ND	−
5	A3 A9/24 B5 B40 Bw4 Bw6 DR1 DR2	+	+	+	ND	+

[a] ND, not determined.

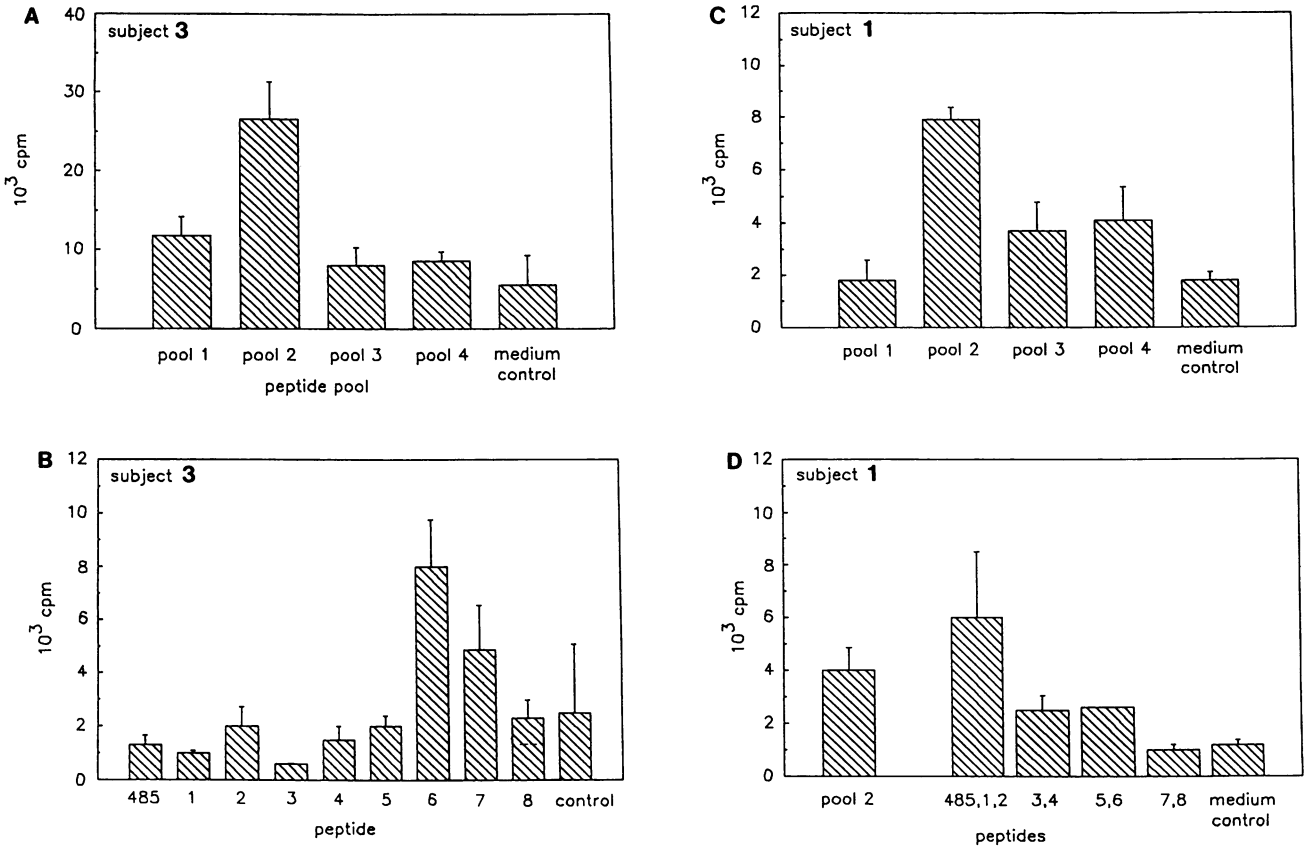

FIG. 2. Analysis of the fine specificity of T-cell lines established by coculture with bac-IE1 by using synthetic peptides. T-cell lines from subjects 3 and 1 were initially assayed against pooled peptide fragments (pool 1, 474 to 482, and 31; pool 2, 32, and 485, 1 to 8; pool 3, 9 to 18; pool 4, 19 to 32) presented by autologous irradiated adherent cells pulsed with peptide pools (10 μg ml^{-1}) (A and C). Both subjects responded preferentially to peptides in pool 2 and the specificity was further analyzed with individual peptides presented as just described (B and D). T cells from subject 3 responded preferentially to peptide 6 (175-H D V S K G D D N K L G G A L Q A K A-193) and less well to adjacent and overlapping peptide 7 (188-A L Q A K A R D K K D E L R R K M M Y-206), whereas the response of subject 1 was localized to peptides 485 (101-K E H M L K K Y T Q T E E K F-115), 1 (110-Q T E E K F T G A F N M M G G C L Q N-129), and 2 (122-M G G C L Q N A L D I L D K V H E P F E-141). The T-cell line from subject 2 was assayed against all the individual peptides (10 μg ml^{-1}) in the presence of irradiated autologous adherent cells (as described above) (E). Specific proliferation was observed in response only to peptides 22 and 23, which are identical except for a single amino acid substitution (peptide 22, 383-A I V A Y T L A T A G V S S S D S L V-401; peptide 23, 383-A I V A Y T L Δ T A G A S S S D S L V-401). The dashed line in panel E represents proliferation to medium alone.

peptide at 10 μg ml^{-1} for 6 days and then tested for proliferation, as described in Materials and Methods. Seronegative control subject 4 did not respond to any of the peptides (Fig. 3B). Seropositive subject 5 responded to peptides 4, 6, 9, and 14 and more strongly to peptide 8 (Fig. 3A), and this response was confirmed in two similar experiments (data not shown). The response to peptide 8 was dose dependent, and a smaller response to overlapping peptide 9 but not to peptide 7 or 10 was observed (Fig. 4).

Fine specificity of cytotoxic T-cell responses to IE1. In other investigations on subject 2, there was a relatively high frequency of IE1-specific CTL precursors (1:65,000 PBMC) (unpublished data). To map the fine specificity of CTL determinants on IE1, PBMC from subject 2 were stimulated in an initial screening experiment with individual peptides (10 μg ml^{-1}) and tested after 14 days of culture for specific cytotoxicity against vac-IE-infected autologous BCL, as described in Materials and Methods. Because of the large number of peptides tested, effector/target ratios were not determined. Autologous BCL infected with vac-gB controlled for specificity of responses; allogeneic BCL (HLA type A1 A2 B14 B15 B62 DR2 DR7) infected with vac-IE controlled for MHC-restricted cytotoxicity. Only peptides 5 and 13 induced significant (two-tailed t test, $P < 0.05$) IE1-specific and MHC-restricted CTL (Table 4). Other pep-

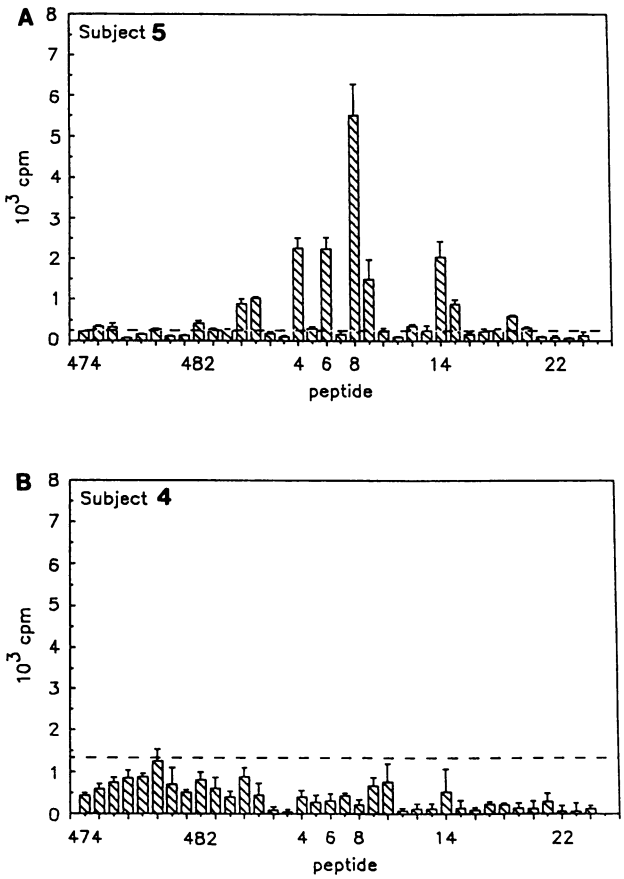

FIG. 3. Individual peptides of HCMV IE1 generated a proliferative T-cell response from an HCMV-seropositive (subject 5) (A) but not a seronegative (subject 4) (B) subject. PBMC were cultured for 6 days with 10 μg of each peptide per ml and then tested for proliferation as described in Materials and Methods. Subject 5 responded to peptide 8 (201-R R K M M Y M C Y R N I E F F T K N S-219) and marginally to adjacent peptide 9 (214-F F T K N S A F P K T T N G C S Q A M-232). Smaller responses to peptides 4 (149-T M Q S M Y E N Y I V P E D K R E M W-167), 6 (175-H D V S K G D D N K L G G A L Q A K A-193), and 14 (278-C V E T M C N E Y K V T S D A C M M T-297) overlapping with peptide 15 (292-D A C M M T M Y G G A S L L S E F C R-310) were observed. The dashed line represents proliferation to medium alone.

tides induced either nonspecific (peptides 18 and 32) or non-MHC-restricted (peptide 16) cytotoxicity. Peptides 5 and 13 and irrelevant peptide 479 were then tested for their abilities to sensitize BCL targets for MHC-restricted lysis by an HCMV-specific CTL line, as described in Materials and Methods. Only peptide 5 sensitized targets for MHC-restricted lysis (Fig. 5): targets labeled with peptide 13 or 479 or left unlabeled were not recognized. Thus, one CTL determinant identified on IE1 for subject 2 was spatially distinct from the determinant recognized by the $CD4^+$ noncytotoxic T-cell line from the same subject.

DISCUSSION

We have previously shown that human $CD8^+$ CTL recognize the 72-kDa HCMV IE protein (5). This study was performed to determine (i) whether there is also a significant $CD4^+$ T-cell response to this important regulatory protein of

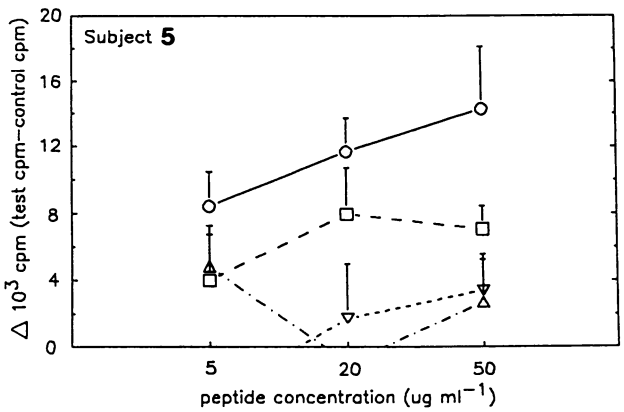

FIG. 4. Dose response of PBMC proliferative response in subject 5 to peptides 8 (○) and 9 (□) but not to adjacent peptides 7 (△) and 10 (▽) (227-G C S Q A M A A L Q N L P Q C S P D E-245).

HCMV and (ii) whether a single immunodominant MHC class I- or class II-restricted determinant is present. PBMC from the majority of HCMV-seropositive but not -seronegative subjects responded to the IE1 protein, and this response occurred irrespective of whether a baculovirus recombinant, pooled peptides, or stably transfected mammalian cells were used as the source of antigen. No MHC-restricted cytolytic activity was detected in the responding lymphocytes. The fine specificity of such T cells was mapped with synthetic peptides, indicating that linear rather than conformational determinants were recognized, but no single immunodominant region with respect to this response was detected. Furthermore, in one subject we mapped both CD8 and CD4 determinants, and these were spatially distinct in the primary structure of HCMV IE1.

The role of $CD4^+$ T-cell responses against such nonstructural proteins of HCMV and other persistent viruses is unknown. In other immune responses, $CD4^+$ T cells play an important regulatory role in antibody generation, in augmenting $CD8^+$ CTL responses by cytokine release, or directly as MHC class II-restricted cytolytic T cells. The importance of anti-IE1 antibody responses, other than as a marker of primary infection (30) (4a), is unknown, although the observations that $CD8^+$ CTL alone may protect against primary MCMV infection suggests that antibody has a relatively minor role (23). The ability of these cells to augment a $CD8^+$ CTL response is under investigation. Evidence that $CD4^+$ T cells may effect MHC class II-restricted cytolysis of HCMV-infected cells was suggested by Lindsley et al. (28), but the lymphocytes generated in these experiments failed to lyse autologous BCL infected with vac-IE under conditions that permitted MHC class II-restricted lysis (5). However, $CD4^+$ lymphocytes partially protected BALB/c mice against MCMV challenge when mononuclear cells from CD8-depleted mice were adoptively transferred. Purified $CD4^+$ cells from these animals did not protect, but they were required to generate and maintain the protective response (24). Thus, further investigation of the function of HCMV IE1-specific $CD4^+$ T cells will be important in defining their precise role in acute infection and in maintenance of the virus-host equilibrium during persistence.

As a prerequisite to this, we wished to establish the fine specificity of these responses to determine whether conformational epitopes, as identified in influenza virus hemagglu-

TABLE 4. Lysis of autologous or HLA-mismatched BCL infected with vac-IE or vac-gB by T cells cocultured with individual peptides of HCMV IE1 (10 μg ml^{-1}) from subject 2

Peptide	% Specific lysis of targets ± SD		
	Autologous vac-IE	Autologous vac-gB	Allogeneic vac-IE
474	3.1 ± 0.8	1.0 ± 0.5	0.4 ± 0.2
475	0.5 ± 0.4	1.3 ± 0.7	1.0 ± 0.6
476	3.7 ± 0.9	2.7 ± 0.8	1.7 ± 0.9
477	4.9 ± 1.0	4.2 ± 1.2	2.3 ± 1.2
478	8.0 ± 3.6	3.3 ± 2.1	2.5 ± 0.9
479	5.6 ± 2.1	2.9 ± 1.7	1.8 ± 0.9
480	7.4 ± 1.5	5.7 ± 1.6	2.7 ± 1.2
481	13.8 ± 3.4	9.5 ± 2.7	1.3 ± 0.7
482	2.4 ± 0.4	2.3 ± 0.4	3.3 ± 0.3
31	3.4 ± 0.3	3.2 ± 0.5	1.6 ± 0.6
32	11.2 ± 3.4	10.6 ± 2.7	2.6 ± 0.7
485	6.4 ± 2.3	8.8 ± 2.5	3.6 ± 1.2
1	1.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
2	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0
3	1.7 ± 0.4	2.0 ± 0.4	2.7 ± 0.5
4	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.3
5[a]	10.4 ± 1.2[b,c]	2.0 ± 0.7[b]	2.9 ± 0.3[c]
6	0.0 ± 0.0	1.7 ± 0.5	2.7 ± 0.8
7	8.9 ± 4.4	3.7 ± 2.1	1.6 ± 1.2
8	8.5 ± 3.9	4.3 ± 2.7	1.4 ± 0.9
9	1.9 ± 0.7	5.3 ± 0.9	1.9 ± 0.6
10	4.3 ± 1.2	3.8 ± 1.7	3.0 ± 1.3
11	1.1 ± 0.4	3.6 ± 0.9	4.4 ± 1.5
12	2.6 ± 0.4	2.7 ± 0.3	1.5 ± 0.3
13[d]	15.2 ± 1.7[e,f]	5.4 ± 0.9[e]	1.9 ± 0.5[f]
14	3.1 ± 1.2	4.8 ± 3.1	3.7 ± 2.1
15	4.7 ± 2.1	4.8 ± 2.3	4.7 ± 1.5
16	10.5 ± 4.2	3.7 ± 2.7	5.6 ± 3.4
17	4.0 ± 2.1	7.6 ± 2.2	3.8 ± 1.6
18	20.2 ± 5.6	11.5 ± 3.7	3.4 ± 2.5
19	2.6 ± 1.3	4.9 ± 2.1	5.5 ± 2.6
20	2.0 ± 1.0	6.1 ± 2.2	4.4 ± 1.4
21	3.0 ± 1.2	5.1 ± 1.8	2.7 ± 1.7
22	4.1 ± 2.1	6.4 ± 2.2	4.8 ± 1.1
23	2.5 ± 1.3	4.0 ± 2.1	2.5 ± 0.3
24	1.7 ± 0.9	3.0 ± 1.4	1.3 ± 1.9

[a] Peptide 5, 162-D K R E M W M D C I K E L H D V S K G-180.
[b] Two-tailed Student's t test, $P < 0.05$.
[c] Two-tailed Student's t test, $P < 0.05$.
[d] Peptide 13, 265-T H I D H I F M D I L T T C V E T M C-283.
[e] Two-tailed Student's t test, $P < 0.05$.
[f] Two-tailed Student's t test, $P < 0.02$.

tinin (32), were recognized or to define whether a single immunodominant region within the primary structure of IE1 is important. This would have obvious implications for the design of immunogens to induce cell-mediated immunity directed against this protein. In a number of subjects studied by using a pool of synthetic peptides, a stably transfected mammalian cell line expressing IE1 (selected because of its probable similarity to the IE1 expressed in infections but without the need for extensive purification from other HCMV proteins which may destroy its conformation), or a baculovirus recombinant, a proliferative T-cell response which cross-reacted between the preparations was observed. Furthermore, it was possible to map the epitope specificity of bac-IE1-generated T cells by using a series of linear peptides. This implies that the proliferative T-cell response against IE1 is predominantly directed against linear rather than conformational determinants, although a low frequency of T cells recognizing the latter cannot be excluded until clonal analysis is performed. Although the purity of the peptides was greater than 70%, spurious responses may have been induced by contaminants in the peptide solutions.

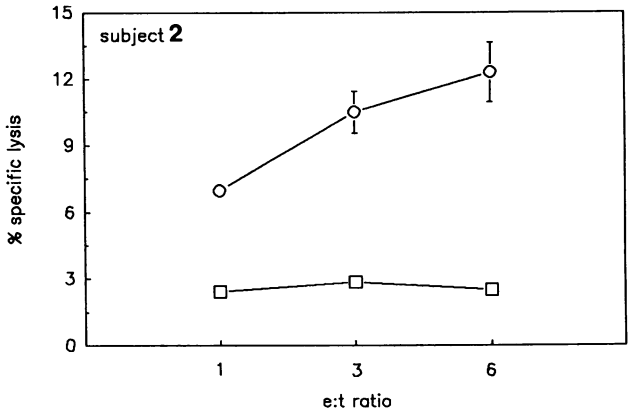

FIG. 5. Cytotoxicity of peptide-labeled BCL targets by an HCMV-specific CTL line from subject 2. There was lysis of autologous (○) but not allogeneic (□) targets labeled with peptide 5, indicating MHC-restricted cytotoxicity. Specific lysis of autologous or allogeneic targets labeled with peptides 13 or 479 or left unlabeled was between 0 and −5%. e:t ratio, effector cell/target cell ratio.

Subject 5 was studied by examining the proliferative response of PBMC to peptides without prior stimulation with bac-IE1. Responses to a number of peptides were observed, probably reflecting the number of MHC class II alleles expressed (Table 2), each potentially restricting a different peptide of IE1. When T cells are expanded in the presence of antigen, selection of the most prevalent precursor CD4$^+$ cells would occur, thereby reducing the number of minor peptides recognized. In spite of this, subject 3 shared HLA DR1 with subject 5, and it is interesting that peptide 6 was recognized by both individuals. However, this was the only peptide recognized by proliferative T cells between subjects (Fig. 6), suggesting that no single immunodominant class II-restricted determinant is present in the primary structure of HCMV IE1.

A response of particular interest was that observed in subject 2. IE1-specific T cells from this subject responded much more strongly to peptide 22 than to peptide 23. These two peptides differ in only one amino acid (Val→Ala; residue 394), which is one of the two differences between HCMV

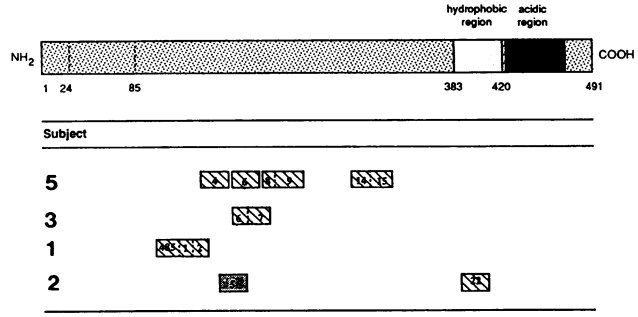

FIG. 6. Summary of proliferative (CD4) (▧) and cytotoxic (CD8) (▩) T-cell determinants identified within the primary structure of HCMV IE1 by analysis with synthetic peptides.

AD169 and Towne (2, 41). This implies that for subject 2, the Val-394 residue is critical in the formation of the MHC–peptide–T-cell receptor complex. Studies are in progress to define this specificity further. Thus, among the subjects studied, no single immunodominant region in HCMV IE1 protein was recognized by proliferative (predominantly $CD4^+$) T cells.

However, the question remained whether in a given individual proliferative and MHC class I-restricted CTL recognized the same determinants. We have used two approaches to examine the MHC class I-restricted determinant. First, peptides were used to induce in vitro CTL which were assayed on target cells expressing the complete IE1 protein. This approach has already been successfully employed to generate human immunodeficiency virus-specific CTL in limiting dilution systems (15). Second, CTL generated by coculture with HCMV-infected fibroblasts were assayed on peptide-sensitized target cells. In subject 2, both techniques identified peptide 5 as a class I-restricted CTL determinant which was spatially distinct from that recognized by the $CD4^+$ T-cell line. In addition, peptide 13 induced a CTL response but failed to sensitize targets for lysis by HCMV-specific CTL. One explanation for this dichotomy may be that the relative CTL precursor frequencies for peptide 5 are higher than for peptide 13, and this may be addressed by limiting dilution analysis.

The current model of antigen processing and presentation proposes two discrete pathways: (i) exogenous proteins are endocytosed and processed in the endosome-lysosome network, and they then associate with class II MHC molecules (1, 16, 17, 33); (ii) endogenous antigens are processed by a poorly defined mechanism and associate with class I MHC molecules (44). Both class I and class II molecules containing peptide antigen are transported to and expressed on the cell surface for recognition by appropriately restricted T cells. The major 72-kDa IE1 protein is located in the nucleus of infected cells and is not present as intact protein on the cell surface, in tissue fluid, or in the virion. How then might endogenous IE1 be processed and presented via the class II pathway to $CD4^+$ T cells? First, evidence that the two pathways may not be so distinct is accumulating. Some endogenous proteins have been shown to be processed via the class II pathway (10, 34), while exogenous protein in immune stimulatory complexes can be recognized by $CD8^+$ class I-restricted CTL (35, 42). Endogenous IE1 may also be processed and presented in the context of class II molecules. Alternatively, IE1 may become available to antigen-presenting cells in vivo by release of virus antigen from disrupted HCMV-infected cells, possibly by the action of IE1-specific $CD8^+$ CTL. Antigen-presenting cells might then present exogenous IE1 to $CD4^+$ class II-restricted T cells in the classic fashion. Studies are in progress to distinguish between the two possibilities.

This work has shown that PBMC of most normal seropositive subjects respond by proliferation to HCMV IE1 protein, and the responding cells are predominantly $CD3^+$ $CD4^+$. However, the linear regions of the IE1 molecule recognized vary between individuals, reflecting the MHC class II haplotype. Similar results have been observed in a number of other human and murine systems. Studies of T-cell proliferation in response to human immunodeficiency virus peptides in humans (38) showed that responses varied with the peptides and the MHC class II haplotype of the subjects. In a murine model of hepatitis B virus infection, T cells from different mouse strains proliferated in response to different regions of the hepatitis B surface antigen-P25 particle (31). In addition, peptides representing murine CTL determinants of the simian virus 40 T antigen were restricted through different MHC class I loci (43). Although the incorporation of CTL determinants into recombinant vaccines protected mice from lethal virus challenge in MCMV (23) and lymphocytic choriomeningitis virus (25) models, the observed protection was limited to the inbred strain from which the CTL determinant was identified. These results have implications for the design of subunit human vaccines. If, as has been suggested (8), specific MHC class II-restricted cells are required for the efficient generation of a protective $CD8^+$ CTL response, then the diffuse location of such determinants observed in HCMV IE1 would make a multivalent peptide vaccine for protection of an outbred population difficult to design. Thus, any attempts to generate or augment HCMV IE1-specific $CD8^+$ CTL in vivo may require the presence of the whole or part of the IE1 protein for their induction.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council and the Wellcome Trust. N. J. Alp is a Wellcome Trust Prize Student.

We thank J. Schmidt for help and advice in synthesizing the IE1 peptides.

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