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The Human Cytotoxic T-Lymphocyte (CTL) Response to Cytomegalovirus Is Dominated by Structural Protein pp65: Frequency, Specificity, and T-Cell Receptor Usage of pp65-Specific CTL

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Cytotoxic T lymphocytes (CTL) appear to play an important role in the control of human cytomegalovirus (HCMV) in the normal virus carrier: previous studies have identified peripheral blood CD8$^+$ CTL specific for the HCMV major immediate-early gene product (IE1) and more recently, by bulk culture and cloning techniques, have identified CTL specific for a structural gene product, the lower matrix protein pp65. In order to determine the relative contributions of CTL which recognize the HCMV proteins IE1, pp65, and glycoprotein B (gB) to the total HCMV-specific CTL response, we have used a limiting-dilution analysis system to quantify HCMV-specific CTL precursors with different specificities, allowing the antigenic specificity of multiple short-term CTL clones to be assessed, in a group of six healthy seropositive donors. All donors showed high frequencies of HCMV-specific major histocompatibility complex-restricted CTL precursors. There was a very high frequency of CTL specific for pp65 (lower matrix protein); IE1-specific CTL were also detectable at lower frequencies in three of five donors, while CTL directed to gB were undetectable. A pp65 gene deletion mutant of HCMV was then used to estimate the contribution of pp65-specific CTL to the total HCMV-specific CTL response; this showed that between 70 and 90% of all CTL recognizing HCMV-infected cells were pp65 specific. Analysis of the peptide specificity of pp65-specific CTL showed that some donors have a highly focused response recognizing a single peptide; the T-cell receptor Vβ gene usage in these two donors was shown to be remarkably restricted, with over half of the responding CD8$^+$ T cells utilizing a single Vβ gene rearrangement. Other subjects recognized multiple pp65 peptides: nine new pp65 CTL peptide epitopes were defined, and for five of these the HLA-presenting allele has been identified. All four of the HLA A2 donors tested in this study recognized the same peptide. This apparent domination of the CTL response to HCMV during persistent infection by a single structural protein, irrespective of major histocompatibility complex haplotype, is not clearly described for other persistent virus infections, and the mechanism requires further investigation.

There is good experimental evidence from animal models, and inferential evidence from studies of immunosuppressed humans, that the control of disease due to human cytomegalovirus (HCMV) in the persistently infected host (virus carrier) depends on the cellular immune response; cytotoxic T lymphocytes (CTL) appear to be a particularly important component of this response (21, 22). HCMV persists in the host for life following primary infection, yet normal, healthy HCMV-seropositive individuals show no morbidity due to the presence of the virus. Immunosuppression of HCMV-seropositive subjects (as in bone marrow transplant recipients) can lead to HCMV reactivation and replication of the virus, resulting in significant morbidity and, in some cases, mortality. There is a direct correlation between recovery of HCMV-specific CTL responses in bone marrow transplant patients and protection from CMV disease (24). In an animal model, murine cytomegalovirus (MCMV)-infected BALB/c mice, a major fraction of CD8$^+$ CTL mediating protection has been shown to be specific for the MCMV immediate-early 1 (IE1) protein, although it was recognized that MCMV structural proteins could also act as antigenic targets for CTL (16). We have previously shown that HCMV IE1-specific CD8$^+$ CTL are detectable in some normal virus carriers (5), although it was clear from this initial work that other HCMV proteins must also be recognized by CTL. More recent studies by Riddell, Greenberg, and colleagues have identified a structural gene product, the lower matrix protein pp65, as a target for HCMV-specific CTL (13, 18). That work utilized bulk CTL culture and subsequent cloning rather than limiting-dilution analysis (LDA) and thus did not provide information on the relative frequency of CTL precursors (CTLp) directed against pp65. The same workers (25) showed that polyclonal HCMV-specific CTL were able to lyse HCMV-infected target cells which had been treated with the RNA synthesis inhibitor actinomycin D, and they also identified these CTL as being specific for matrix proteins pp65 and pp150. CTL clones of this specificity have been derived from donors prior to and then used in adoptive transfer experiments, in which they have been shown to persist and confer in vitro CTL responses in the recipients (30).

In the present study we have developed an improved LDA system and applied it to determine the relative frequencies of CTLp specific for a number of HCMV proteins. We have further quantified the contribution of pp65-specific CTL to the total HCMV-specific CTLp frequency by using a recently constructed pp65 gene deletion mutant of HCMV (RVAd65) (26).

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We also characterized the fine specificity of the pp65-specific CTL by using overlapping synthetic peptides covering the whole of the pp65 gene product. Only a single pp65 CTL peptide epitope has previously been identified (amino acids [aa] 123 to 131, HLA B35 restricted [12]); knowledge of common pp65 peptide-HLA class I associations could be valuable in establishing protocols to boost specific HCMV immunity in risk groups and could provide a clearer idea of which components should constitute a potential HCMV vaccine.

The results show that all infected donors tested had very high frequencies of pp65-specific CTL; seronegative donors had undetectable frequencies, as expected. CTL specific for the major immediate-early protein IE1 were also detected, but at lower frequencies. Analysis of the fine specificity of pp65-specific CTL showed that some donors have a highly focused response recognizing only a single peptide, while others recognize multiple peptides throughout the pp65 gene product. pp65-specific CTL restricted through HLA A2 were detected in four donors, and all recognized the same peptide fragment. In addition, an analysis of T-cell receptor (TcR) gene usage showed that many of the donors had a restricted usage of TcR Vβ genes in CTL recognizing specific pp65 peptides: frequently the Vβ gene used by HCMV-specific CTL corresponded to the largest single Vβ population in $CD8^+$ T cells in unstimulated peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Donors. Six normal, healthy HCMV-seropositive and two HCMV-seronegative laboratory donors were included in this study. HCMV serostatus was determined by an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (Public Health Laboratory Service, Addenbrookes Hospital, Cambridge, United Kingdom).

Viruses and cell lines. HCMV AD169 (ATCC VR-538) and HCMV RVAd65 (26) were grown in MRC-5 fibroblasts infected at a multiplicity of infection (MOI) of 0.1. Supernatants from infected cultures were harvested 5 days after 90 to 100% cytopathic effect was evident, and the virus-containing supernatant was stored at −70°C. The RVAd65 HCMV was constructed by replacing the pp65 gene with the bacterial neomycin phosphotransferase gene; the resulting virus grew to levels of infectivity comparable to that of the wild type. The kinetics of expression of the immediate-early genes UL122 and UL123, the early gene UL44 (p52), and the late gene UL32 (pp150) were also unaltered compared with those in the wild-type virus (26).

Recombinant vaccinia viruses encoding HCMV proteins included those expressing pp65 (a kind gift of S. Riddell, Fred Hutchinson Cancer Research Center, Seattle, Wash. [12, 18]), IE1 (2), glycoprotein B (gB) (5, 10), or a negative control bacteriophage T7 RNA polymerase (T7) and were grown in Vero cells infected at an MOI of 0.1. After 48 to 72 h, the infected cells were harvested and subjected to three rounds of freezing and thawing followed by sonication. The cell debris was removed by centrifugation, and supernatant containing virus was aliquoted and stored at −70°C.

Primary human fibroblast lines were established from skin biopsies for each of the HCMV-seropositive and -seronegative donors included in the study. Major histocompatibility complex (MHC) class I tissue types were also established for each of the donors (Tissue Typing Department, Addenbrookes Hospital). Primary fibroblasts were grown in Eagle minimum essential medium (Flow) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10^5 IU of penicillin per liter, and 100 mg of streptomycin per liter. Fibroblast lines were cryopreserved and stored in liquid nitrogen to provide long term stocks. B-lymphoblastoid cell lines (BCL) were established from PBMC by Epstein-Barr virus (EBV) (B95.8) transformation and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10^5 IU of penicillin per liter, and 100 mg of streptomycin per liter.

Generation of HCMV-specific CTL in LDA. The technique of LDA has previously been described in detail (8). PBMC were prepared from fresh heparinized venous blood samples by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient centrifugation. To remove natural killer (NK) cells, 1.5×10^7 PBMC were incubated with 20 μl of Leu-11b monoclonal antibody (MAb) (anti-CD16 IgM; Becton Dickinson) for 30 min at room temperature. Following one wash in phosphate-buffered saline (Oxoid), the antibody-labelled PBMC were mixed with a 1:2 dilution of baby rabbit complement in RPMI 1640 and incubated for 45 min at 37°C. After being washed in RPMI 1640, the NK cell-depleted PBMC were used as responder cells in HCMV-specific LDA. Replicate microcultures (n = 27) of the PBMC were established in 96-well round-bottom plates in which the number of PBMC per well was progressively reduced over an appropriate range of dilutions in RPMI 1640 supplemented with 10% human AB serum (HCMV seronegative; Blood Transfusion Service, Addenbrookes Hospital), 2 mM L-glutamine, 10^5 IU of penicillin per liter, and 100 mg of streptomycin per liter. Each well also received 5×10^4 irradiated (2,400 rads) autologous PBMC (as feeder cells) and 2×10^3 HCMV-infected (18 h postinfection at an MOI of 10) fibroblasts; the medium was further supplemented with human recombinant interleukin-2 (IL-2) to give a final concentration of 5 IU/ml. The cultures were incubated at 37°C in 5% CO_2 and refed with medium supplemented with 5 IU of IL-2 per ml on days 5 and 10. On day 14, the cells in each individual well were resuspended and divided into five aliquots that were assayed for cytotoxicity against radiolabelled target cells expressing different HCMV antigens in 4- or 8-h chromium-51 (^{51}Cr) release assays, depending on the target cell. Target cells were autologous or MHC-mismatched primary human fibroblasts (2×10^3 cells per well) infected for 18 h with HCMV (MOI = 10) and autologous or MHC-mismatched BCL (4×10^3 cells per well) infected for 18 h with recombinant vaccinia viruses (MOI = 10).

Production of overlapping peptides from the matrix protein pp65. Overlapping peptides were constructed to cover the whole primary amino acid sequence of pp65 (SwissProt accession number P06725). The peptides were designed to be 15 amino acids long and to overlap each other by 9 amino acids. The first 14 peptides were constructed by using 9-fluorenylmethoxycarbonyl chemistry on a Zinsner peptide synthesis machine. The remaining 78 peptides were made commercially (Affinity Research Products Ltd., Exeter, United Kingdom). The peptides were dissolved in RPMI 1640 at 200 μg/ml and frozen in small aliquots at −70°C. Initial screening for peptide-reactive CTL was performed with pools of peptides; each pool contained five consecutive peptides at a final concentration of 40 μg/ml each.

Preparation of peptide-expressing target cells. EBV-transformed BCL were labelled with ^{51}Cr (Amersham) for 45 min at 37°C and then pulsed with 200 μl of an individual peptide or pool of peptides (peptide concentration, 40 μg/ml) for 1 h longer. The chromium-labelled, peptide-pulsed target cells were washed three times in 20 ml of RPMI 1640 plus 10% fetal calf serum before use in chromium release assays.

LDA. Each individual well was regarded as positive for cytotoxicity when the ^{51}Cr release exceeded a defined threshold. The method of analysis is an extension of our previously published studies (8). The percent specific lysis was calculated for each well by using the following formula: (test release − spontaneous release)/(maximum release − spontaneous release) × 100. Because split-well analysis was used for each target cell type, the percent specific lysis against the MHC-mismatched control was also calculated and subtracted from the percent specific lysis against the autologous target. A well was considered to be positive for MHC-restricted cytotoxicity if this value exceeded 10%. In LDA with cytotoxicity assays, it is necessary to classify each microculture as being either positive or negative for cytotoxicity, using a defined threshold above which the well is considered positive. Previous studies reported in the literature have used a threshold calculated as the mean of the spontaneous chromium release from control microcultures plus two standard deviations (SD); other studies have used the mean plus three SD. We have previously applied this method of analysis to a large series of cytotoxicity assays and have found two important disadvantages. First, the threshold when calculated as the mean of spontaneous release plus two SD can be very low, with the result that microcultures are classified as positive when the absolute level of killing is so low that it is unlikely to be of biological significance; e.g., for target cells with a maximum release of 4,000 cpm, a spontaneous release of 400 cpm, and an SD of spontaneous release of 40 cpm, the calculated threshold (mean plus two SD) is 480 cpm, which is equivalent to only 2.2% specific lysis. Such low thresholds lead to very high CTLp frequency estimates. Second, because the spontaneous releases from different target cell populations often have different SDs, the threshold calculated as mean plus two SD can vary substantially for different target cell types within the same assay and between assays, e.g., thresholds equivalent to 3% specific lysis for one target cell and 12% for another target cell. The use of a stringent uniform threshold within and between experiments is essential for longitudinal analysis. We depleted NK cells from the PBMC prior to culture to minimize nonspecific cytotoxicity (usually less than 10% and always less than 20% specific lysis in all experiments). We specifically included MHC-mismatched control target cells for every microculture in each assay so that microcultures were classified as positive only if (after having been split into five aliquots, which reduces the final effector cell/target cell [E/T] ratio by a factor of five) they showed a biologically meaningful level of MHC-restricted killing, i.e., specific lysis 10% greater than that for the mismatched control. Limiting-dilution plots for MHC-restricted killing were produced by plotting the proportion of negative wells against the initial responder cell number per well on a semilogarithmic plot. From the single-hit Poisson model, the frequency of antigen-specific CTLp was estimated from the initial responder cell number at which 37% of the wells were negative for cytotoxicity (8).

MAbs and cell surface phenotyping. The cell surface phenotypes of unstimulated PBMC and of responder cell populations (pooled from multiple LDA cultures) were determined by using directly conjugated MAbs (Becton Dickinson) followed by analysis on a FACsort flow cytometer (Becton Dickinson). The MAbs used were anti-CD3 (PERCP), -CD4 (phycoerythrin conjugated), -CD16 (fluorescein isothiocyanate conjugated) and anti-CD3 (PERCP), -CD8 (phycoerythrin conjugated), -CD56 (fluorescein isothiocyanate conjugated). The TcR Vβ chain usage by unstimulated $CD8^+$ T cells in PBMC and in $CD8^+$ T cells generated in response to HCMV-infected fibroblasts was determined by using a

TABLE 1. HCMV-specific CTLp frequencies in a group of HCMV-seropositive and -seronegative donors

Donor	HCMV IgG	HLA type	HCMV-specific CTLp/10^6 $CD3^+$ $CD8^+$ T cells
001	+	A2 B12(44) B27	648
002	+	A9(24) A19(33) B7 B12(44)	1,398
003	+	A1 B8 B12(45)	303
004	+	A2 B16(38)	350
005	+	A33 A31 B35 B12(44)	1,459
006	+	A28 A32 B39 B41	219
007	−	A2 A9(24) B15(62) B35	<8
008	−	A1 A2 B8 B40(60)	<8

panel of TcR-specific fluorescein isothiocyanate-conjugated MAbs, including Vβ 2, 3, 6.1, 12, 13.6, 14, 17, 20, 21.3, and 22 (Immunotech); Vβ 5.1, 5.2, 5.3, 6.7, 8, and 12.1 (T-Cell Diagnostics); and Vβ 13.1/13.2 (Serotech).

RESULTS

Production of HCMV-specific CTL. PBMC from HCMV-seropositive donors were cocultured with HCMV-infected autologous fibroblasts in the presence of IL-2 in order to stimulate resting HCMV-specific CTLp to proliferate. However, this method of stimulation also activated CD16-positive NK cells: the presence of NK cells in the effector population produced high background levels of MHC-nonrestricted killing, making the quantitative determination of antigen-specific MHC-restricted CTL killing difficult. Depletion of the CD16-positive cell fraction by an IgM anti-CD16 MAb and complement was therefore used prior to antigen stimulation to reduce $CD16^+$ NK cells to <1%. The effector population produced from antigen-stimulated CD16-depleted PBMC was predominantly T cells as determined by flow cytometry and allowed the accurate determination of antigen-specific MHC-restricted CTL activity (data not shown).

Determination of HCMV-specific CTLp frequency in normal healthy donors. HCMV-specific CTLp frequencies were determined by LDA for six normal, healthy HCMV-seropositive donors. Two HCMV-seronegative donors were also included in order to control for the generation of primary CTL responses with the in vitro stimulation system. The results showed that all HCMV-seropositive donors had high frequencies of HCMV-specific CTLp; HCMV-seronegative donors had no detectable HCMV-specific CTL (Table 1).

Determination of the relative frequencies of CTLp specific for HCMV proteins IE1, pp65, and gB. By using split-well analysis, it was possible to determine the relative frequencies of CTLp specific for the HCMV IE1, pp65, and gB proteins. Limiting-dilution cultures were set up as before for each of the HCMV-seropositive donors and stimulated with HCMV-infected autologous fibroblasts; each microculture was split into five aliquots and assayed against autologous BCL targets infected with recombinant vaccinia virus IE1, pp65, or gB or the negative control T7. An MHC-mismatched vaccinia virus pp65-infected BCL target was also included so that MHC-restricted killing could be calculated.

All HCMV-seropositive donors tested had high frequencies of CTLp specific for the pp65 protein. CTL specific for the IE1 protein were also present in some (three of five) donors tested but at approximately 10-fold-lower frequencies than pp65-specific CTL. CTL specific for gB were not detected over the range of input cells used in these assays (Fig. 1).

Determination of the proportion of total HCMV-specific CTLp which were specific for the pp65 protein. The HCMV CTLp frequency was determined by using HCMV-infected autologous primary fibroblasts (Table 1), whereas the pp65-specific CTLp frequency was determined by using autologous EBV-transformed BCL infected with recombinant vaccinia virus expressing pp65 (Fig. 1). We considered that the target cells and vectors were sufficiently different that a direct comparison between them may not be valid. For this reason, in

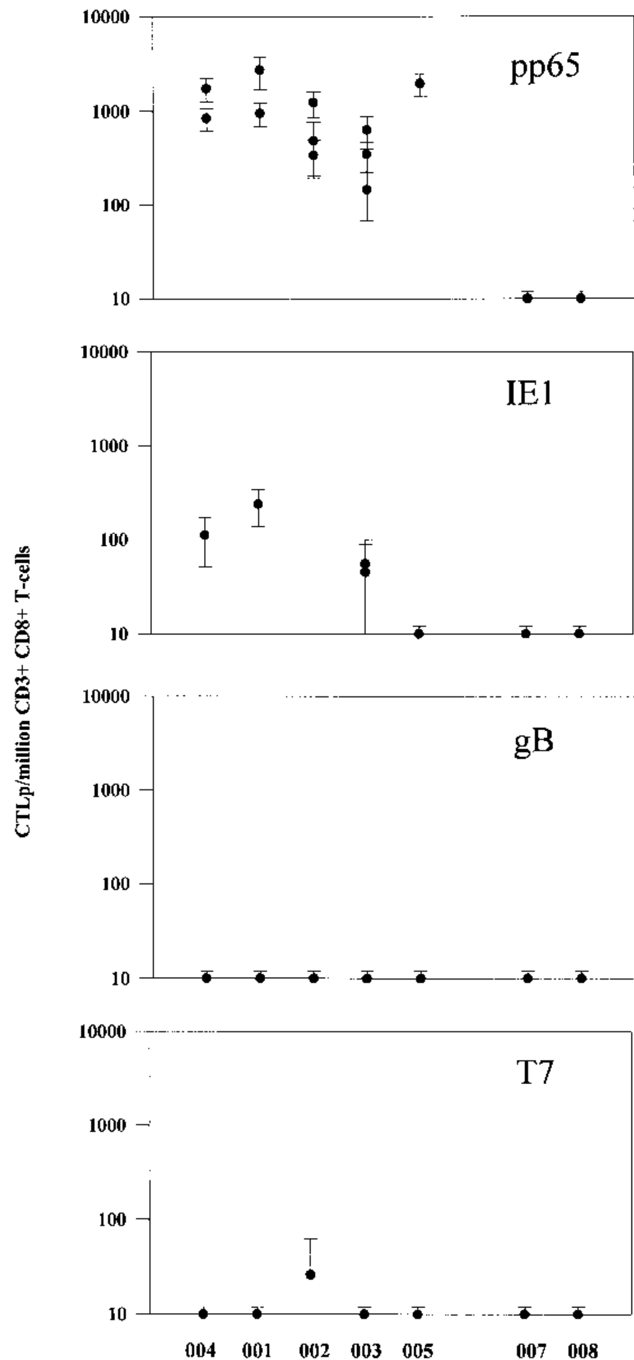


FIG. 1. Split-well HCMV LDA with five seropositive donors (001 to 005), some of whom were tested on multiple occasions, and two seronegative donors (007 and 008). Target cells were recombinant vaccinia virus-infected BCL expressing HCMV proteins pp65, IE1, and gB. BCL expressing T7 acted as negative controls for antigen specificity. CTLp frequencies are expressed as number per 10^6 $CD3^+$ $CD8^+$ T cells; error bars represent 95% confidence limits.

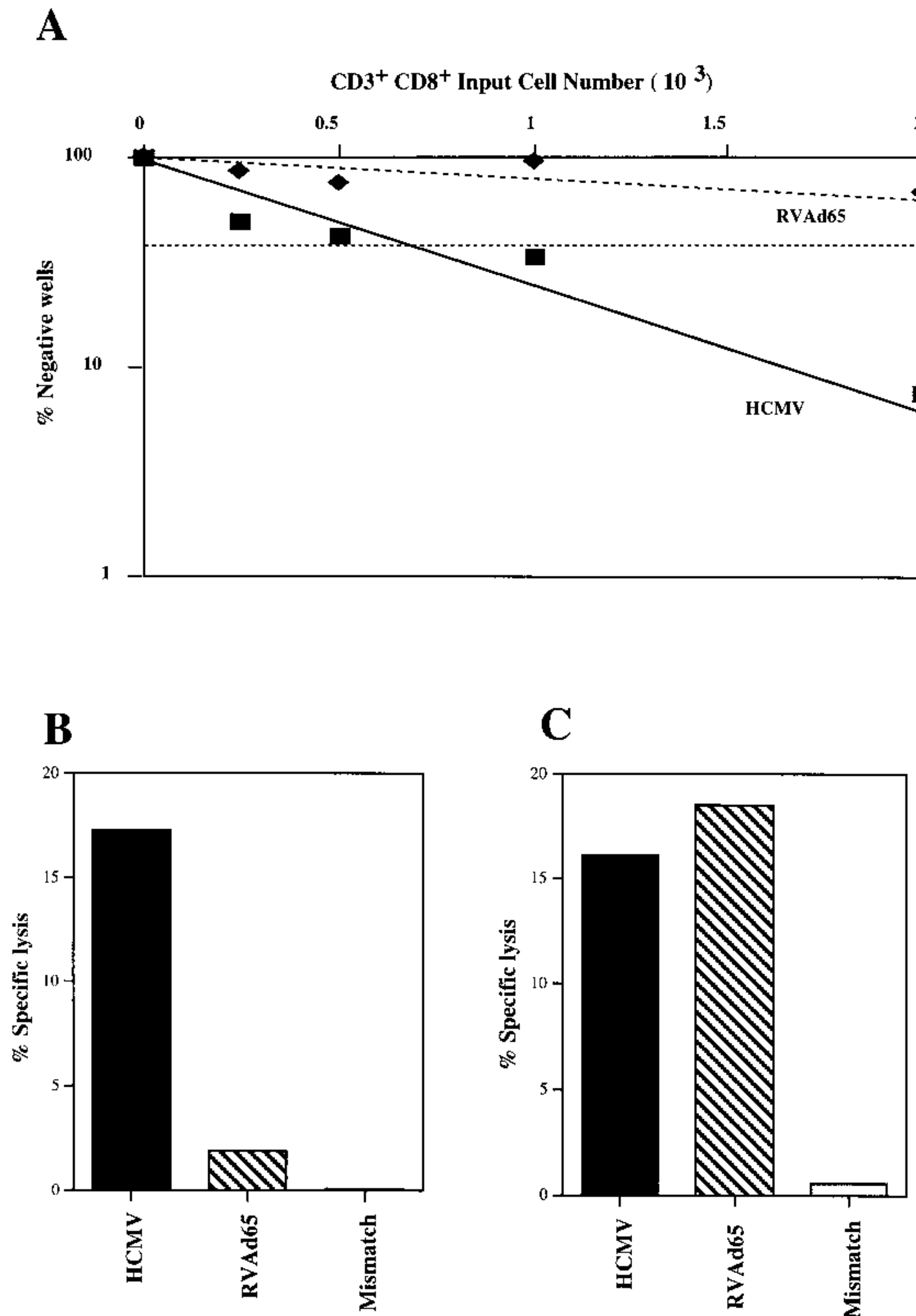


FIG. 2. (A) LDA plot of two-way split-well analysis. Target cells were HCMV (■)- and RVAd65 (◆)-infected fibroblasts. (B) Individual well from LDA showing killing against HCMV but not the pp65 deletion mutant RVAd65 or an HCMV-infected mismatched control. (C) Individual well from LDA showing killing against HCMV and also the pp65 deletion mutant RVAd65 but not an HCMV-infected mismatched control.

order to use the same target cell types, we determined the percentage of HCMV-specific CTL which were pp65 specific by comparing the frequencies of CTLp specific for autologous primary fibroblasts infected with wild-type virus and with virus with pp65 deleted. Limiting-dilution cultures were established as before, using wild-type HCMV-infected cells to stimulate CTL responses. Split-well analysis was performed against target cells infected with either HCMV AD169 or RVAd65; a wild-type-HCMV-infected MHC-mismatched control target was also included. The results showed that the majority (70 to 90%) of all HCMV-specific CTLp detectable in LDA were specific for the pp65 protein, i.e., recognized the wild-type-infected cells but not the RVAd65-infected cells (Fig. 2A and Table 2). This analysis is valid only if RVAd165 is able to present antigens other than pp65 as effectively as wild-type AD169. The CTL experiments clearly show that RVAd165 can present other viral antigens to HCMV-specific CTL, as demonstrated by the fact that a proportion of the polyclonal HCMV-specific CTL were able to kill fibroblasts infected with the deletion mutant. Figure 2B shows the percent specific lysis

TABLE 2. Comparison of frequencies of CTLp directed towards HCMV- and RVAd65-infected fibroblasts

Donor	CTLp/10^6 CD3$^+$ CD8$^+$ T cells		% pp65-specific CTL[a]
	HCMV	RVAd65	
001	664	60	91
003	389	113	71
005	1,459	250	83

[a] Calculated as 100 − [(RVAd65 frequency/HCMV frequency) × 100].

from an LDA well which could kill only the wild-type virus and not RVAd165, while Fig. 2C shows a well killing both wild-type and RVAd165, suggesting CTL specific for HCMV proteins other than pp65; the magnitudes of the killing are also similar, suggesting that presentation of the antigen being recognized by either wild-type- or RVAd165-infected fibroblasts is similar.

Maintenance of pp65-specific CTLp frequency over time. The pp65-specific CTLp frequencies in two of the HCMV-seropositive donors were further studied over a period of 18 to 22 months on three or four occasions. The level of CTLp specific for the pp65 protein remained high, and the absolute values were similar over this period (Fig. 3).

Phenotypic analysis of responder cells. Responder cells from HCMV LDA cultures were pooled and stained with MAbs to CD4 and CD8 cell surface markers; there was a mixture of CD4$^+$ and CD8$^+$ T cells (Fig. 4A). The CD4$^+$ and CD8$^+$ T-cell populations from donor 002 were separated by flow cytometric cell sorting, giving sorted populations which were >98% pure, and used as effector cells in a cytotoxicity assay against BCL targets expressing pp65. Only the CD8$^+$ T-cell fraction demonstrated cytotoxic activity at the E/T ratios used (Fig. 4B). The analysis was repeated for donor 003 at E/T ratios of 10:1 and 20:1, and again the results showed that only the CD8$^+$ T-cell fraction was cytotoxic (data not shown).

Screening of HCMV-specific CTL against overlapping pools of pp65 peptides. HCMV-specific CTL generated from five of

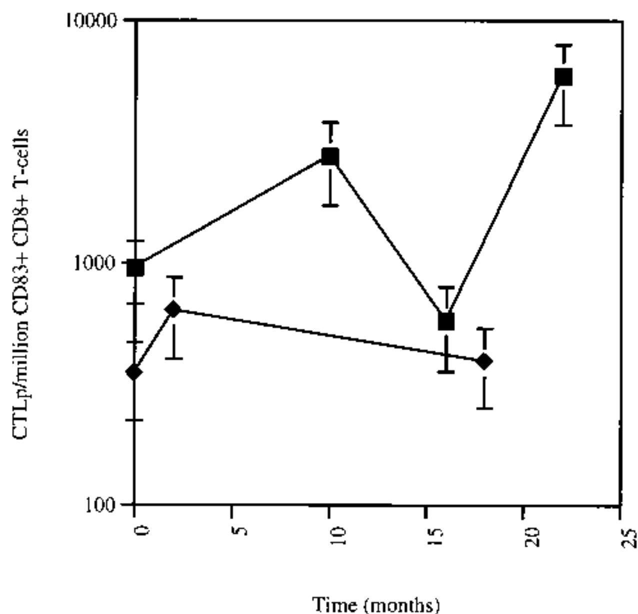
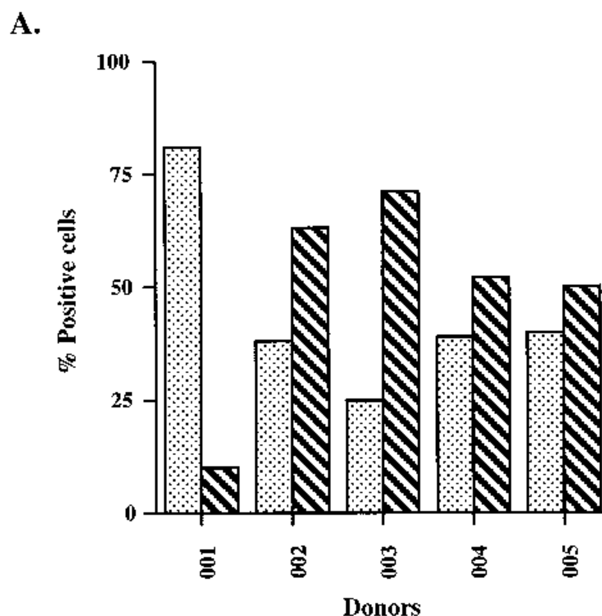


FIG. 3. pp65-specific CTLp frequencies expressed as number per 10^6 CD3$^+$ CD8$^+$ T cells (error bars represent 95% confidence limits) for HCMV-seropositive donors 001 (■) and 003 (◆) over a period of 18 to 22 months.



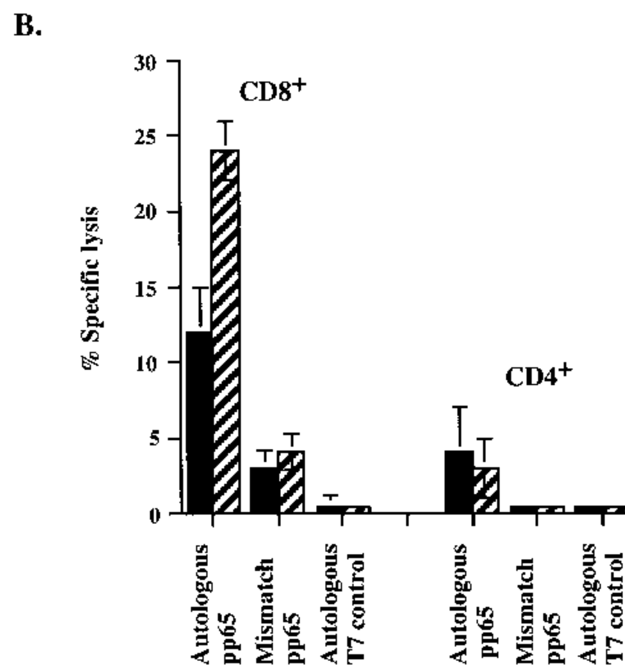


FIG. 4. (A) Comparison of the percentages of CD4$^+$ (▧) and CD8$^+$ (▨) responder T cells derived from HCMV LDA cultures in five donors. (B) Comparison of the cytotoxicities of sorted CD4$^+$ and CD8$^+$ T cells from donor 002 against autologous and mismatched BCL expressing HCMV pp65; autologous T7 targets were included for antigen specificity controls. E/T ratios of 5:1 (■) and 10:1 (▨) were used. Error bars represent ±1 standard deviation.

the HCMV seropositive donors were screened for reactivity against the pools of pp65 synthetic 15-mer peptides. The results in Fig. 4b indicated that the CTL responses were mediated by $CD8^+$ CTL and thus were class I restricted; in addition, comparative LDA of CD16- and CD4/CD16-depleted PBMC showed that there was no difference in the pp65-specific CTLp frequency (data not shown). We therefore depleted $CD4^+$ lymphocytes from PBMC prior to polyclonal HCMV stimulation for these experiments. The CTL were generated by stimulation of PBMC, which had been stained with CD4 and CD16 MAbs and separated by flow cytometric cell sorting, with HCMV (AD169)-infected autologous fibroblasts; PBMC were plated out at 10,000 cells per well, and 27 replicates were used for each peptide pool. This allowed the screening of cytotoxic activity with a given pool to be assessed by using a semiquantitative one-point LDA technique. The number of wells which showed killing among the 27 replicate wells gives an indication of the abundance of CTL specific to a peptide contained within that pool (Fig. 5). Peptide-specific CTL were detected in all five of the donors tested; however, the pattern of reactivity against the pooled peptides differed between donors, as expected from their different MHC haplotypes.

Determination of CTL peptide specificity and HLA class I presenting allele. CTL that recognized peptide pools were retested against target cells incubated with individual peptides making up that pool; an example is shown in Fig. 6A, and the results for all subjects are summarized in Table 3. In some cases, a single peptide in a single pool was recognized strongly, such as for donor 001, who recognized peptide 69 (aa 493 to 507); in others, two overlapping peptides were recognized equally, such as for donor 005, who recognized peptides 17 and 18 (aa 187 to 201 and 193 to 207, respectively). Individual peptides were also retested with a panel of partially class I HLA-matched EBV-transformed B-cell lines; this allowed the identification of the individual class I HLA allele presenting that peptide to the specific CTL (Fig. 6B and Table 3).

MAb analysis of TcR Vβ usage by peptide-specific CTL. Unstimulated PBMC and HCMV-stimulated $CD8^+$ cells generated from five of the HCMV-seropositive donors were stained by immunofluorescence with a panel of 17 MAbs specific for various Vβ gene families. The stained cells were analyzed by flow cytometry, and the results were expressed as a percentage of $CD8^+$ T cells (Fig. 7). Two donors showed a highly restricted TcR Vβ gene usage: 67% of the HCMV-stimulated responder $CD8^+$ T cells from donor 001 were Vβ 13.1^+, and 76% of the responder $CD8^+$ T cells from donor 002 were Vβ 14^+. Other donors demonstrated lower usages of a particular TcR Vβ chain (donor 005, 29% Vβ 20^+; donor 003, 17% Vβ 17^+). We performed further experiments with the cells from donor 005, in which we purified the Vβ 20 CTL from the polyclonal HCMV-specific CTL by using a specific MAb and flow cytometric sorting and then used these cells as effectors against autologous targets expressing the five identified peptides; this demonstrated that the purified Vβ 20^+ CTL could kill only target cells expressing peptides 17 and 18 (specific lysis, 39.1% at an E/T ratio of 20:1) and none of the other peptide-pulsed target cells (specific lysis, $<2\%$ at E/T ratio of 20:1). The TcR Vβ chain usage by donor 004 was not detected by this panel of MAbs. It was noteworthy that in those donors whose responder $CD8^+$ T cells showed restricted TcR Vβ usage, the same Vβ gene segment was also present at the highest frequency of all Vβ segments tested on $CD8^+$ cells from their unstimulated PBMC.

Recognition of peptide 69 by CTL from HCMV-seropositive HLA A2 donors. Two of the five donors in this study were HLA A2, and both recognized peptide 69 of pp65 (aa 493 to 507).

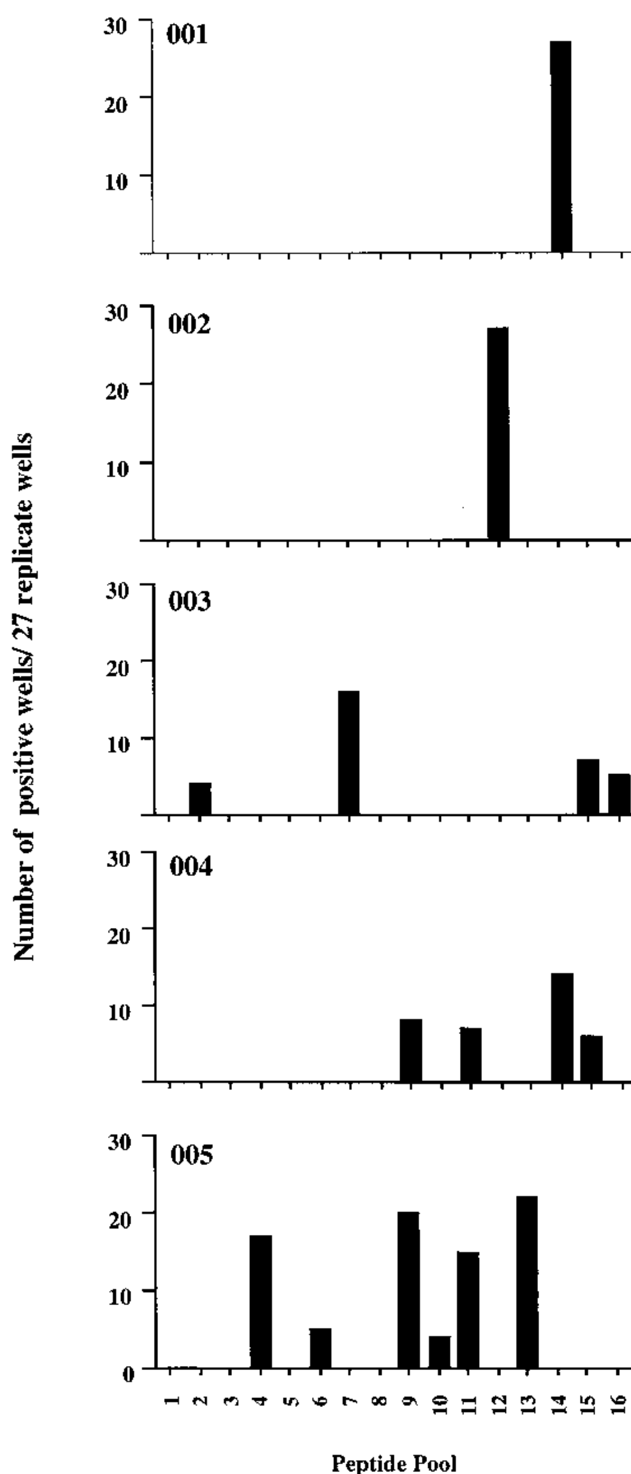


FIG. 5. Screening of polyclonal HCMV-specific CTL from five donors against pools of overlapping peptides (15-mer overlapping by 9 amino acids). Replicate microcultures ($n = 27$) of CTL were assessed for cytotoxicity against each pool of peptides; the results are expressed as number of positive wells for each pool, giving a semiquantitative estimate of the abundance of peptide-specific CTL contained within the input cell population.

We therefore wished to determine whether this peptide was selected for CTL recognition in other HLA A2 donors. The stimulation of HCMV-specific CTL responses has hitherto depended for presentation upon the use of HCMV-infected

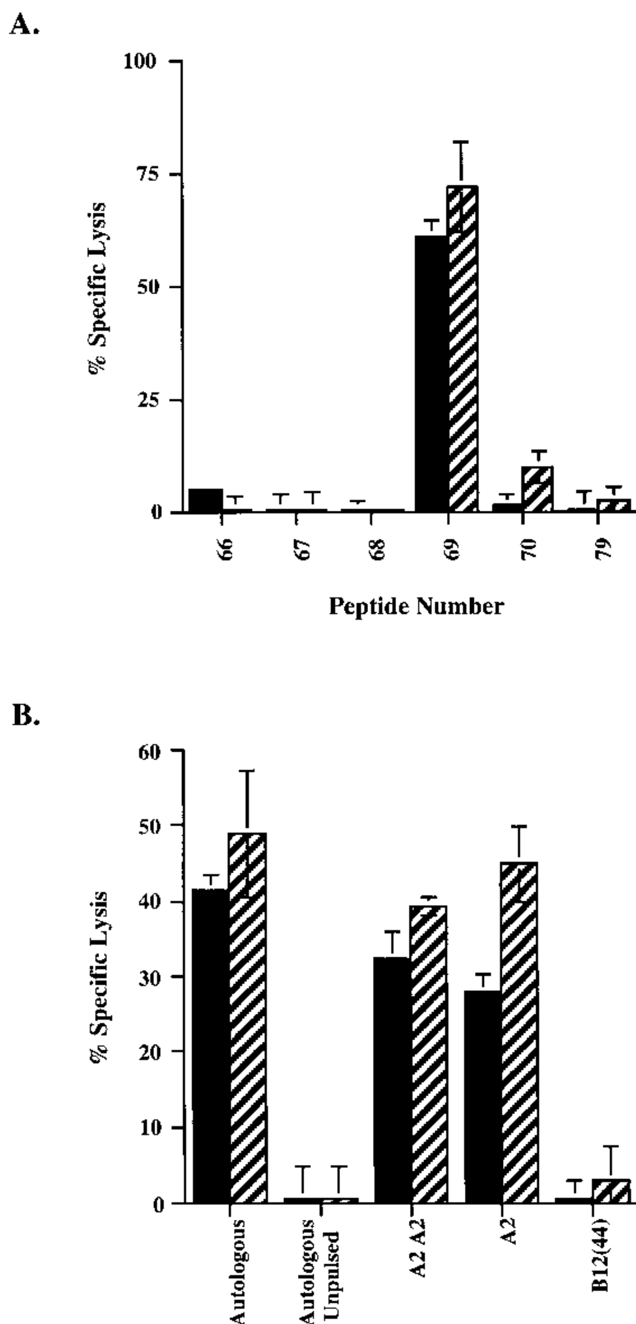


FIG. 6. (A) Determination of peptide specificity within a pool of peptides. (B) Determination of HLA class I presenting allele for peptide 69 by using a panel of partial matched BCL for donor 001, autologous class I haplotype A2 A2 B12(44) B27. E/T ratios of 20:1 (■) and 40:1 (▨) were used. Error bars represent ±1 standard deviation.

autologous primary fibroblast lines established from skin biopsies. However, this procedure is time-consuming and demanding on the donors. Direct stimulation of CTL responses by using peptide-pulsed irradiated autologous PBMC is an efficient alternative when a candidate allele-specific peptide of interest has been identified. Two additional HCMV-seropositive HLA A2 donors were identified, and their PBMC were stimulated with peptide 69; the CTL generated were assayed for cytotoxicity against autologous BCL pulsed with peptide 69, with unpulsed autologous BCL as target cells serving as a specificity control. The results showed that both of these additional HLA A2 HCMV-seropositive donors had peptide 69-specific CTLp responses (Fig. 8). Peptide 69 (aa 493 to 507, ARNLVPMVATVQGQN) contains the consensus HLA A2-binding motif leucine at position 2 and valine at position 9 (23). The peptide was resynthesized and purified (>99%) by high-pressure liquid chromatography as a 10-mer (NLVPMVATVQ); peptide 69-specific CTL were retested to confirm that they could lyse target cells expressing this shorter, high-purity peptide which contained the minimal HLA A2 consensus binding sequence. CTL which recognized the 15-mer peptide were also able to lyse cells expressing the shorter 10-mer peptide (Fig. 8B). We examined the TcR Vβ gene usage of the peptide 69-specific CTL from all of the donors by using a panel of Vβ specific MAbs. We did not identify a common Vβ gene used by CTL from all HLA A2 donors; however, two donors did utilize Vβ 13.1/3 for peptide recognition, in addition to other unidentified Vβ genes. One donor utilized Vβ 22 in addition to other unidentified Vβ genes.

DISCUSSION

In this study all HCMV-seropositive donors had a high frequency of HCMV-specific CTLp, which were not detected in HCMV-seronegative donors. Split-well LDA showed that all of the seropositive donors tested had CTL specific for the pp65 protein, and a very high proportion of all HCMV-specific CTL in any given donor was specific for this single virus protein. CTL directed to the IE1 protein were also detectable in some donors but at a 10-fold-lower frequency. gB-specific CTL were not detected in these assays; gB-specific CTL have previously been generated by using bulk culture techniques (5), and the failure to demonstrate them in this study suggests that they may be present at a very low frequency, as the minimum resolution of this LDA is $7/10^6$ $CD3^+$ $CD8^+$ PBMC. Split-well analysis with target cells expressing wild-type HCMV or the HCMV deletion mutant RVAd65 showed that pp65-specific CTLp constituted between 70 and 90% of the total circulating HCMV-specific CTLp that could be restimulated in vitro. The pp65-specific CTLp frequency was maintained over a prolonged period of time. Mapping of the fine specificity of pp65-specific CTL generated from a number of donors showed that two donors (001 and 002) recognized only a single peptide, and in both cases a very large proportion (>60%) of the CTL were utilizing a single Vβ gene product, which differed for the two individuals (Vβ 13.1 and Vβ 14, respectively). pp65-specific CTL from other donors recognized multiple peptides located throughout the pp65 protein. However, in some cases they also demonstrated restricted TcR Vβ gene segment usage in the recognition of particular peptides. In all, nine new pp65-specific CTL peptides have been identified, and the HLA restriction element has been identified for five of these peptides.

Our results show that the pp65-specific CTLp frequency is maintained over a period of time (18 to 22 months). This continued high level of memory CTL specific for a structural protein may reflect its continued expression and would support the idea that either periodic reactivation or chronic low-level replication of the virus allows for the restimulation of HCMV pp65-specific CTLp and maintenance of the CTL memory pool. This can be contrasted with an acute virus infection, such as influenza, in which a CTL response is mounted during infection, but following clearance of the viral infection from the host the magnitude of influenza virus-specific CTLp memory then progressively declines over time (19).

These studies confirm and extend the previous work of Riddell, Greenberg, and colleagues showing that the matrix pro-

TABLE 3. Identification of individual pp65 peptide epitopes recognized by five HCMV-seropositive donors and determination of HLA presenting allele

Donor	Pool	Peptide(s)	% Specific lysis (mean ± SD)[a]										Presenting HLA allele
			Autologous		Partial matches								
			With peptide	Without peptide	A1	A2	A9(24)	A33	B7	B8	B12(44)	B35	
001	14	69 (aa 493–507, ARNLVPMVATVQGQN)	49 ± 6	1 ± 5		39 ± 2					3 ± 5		A2
002	12	56 (aa 415–429, RKTPRVTGGGAMAGA)	45 ± 10	1 ± 1			1 ± 1		35 ± 3		5 ± 2		B7
003	15	72 (aa 511–525, QEFFWDANDIYRIFA)	29 ± 2	8 ± 3	2 ± 4					20 ± 3			B8
004	14	69 (aa 493–507, ARNLVPMVATVQGQN)	48 ± 2	3 ± 3		50 ± 3							A2
005	4	17 and 18 (aa 181–195, YYTSAFVFPTKDVAL, and aa 187–201, VFPTKDVALRHVVCA)	99 ± 5	20 ± 20				19 ± 12			10 ± 7	95 ± 8	B35
	11	53 (aa 397–411, DDVWTSGSDSDEELV)	68 ± 3	2 ± 1							7 ± 3	73 ± 10	B35

[a] Lysis was determined with E/T ratios of 20:1 and 40:1; results for E/T ratios of 40:1 are shown.

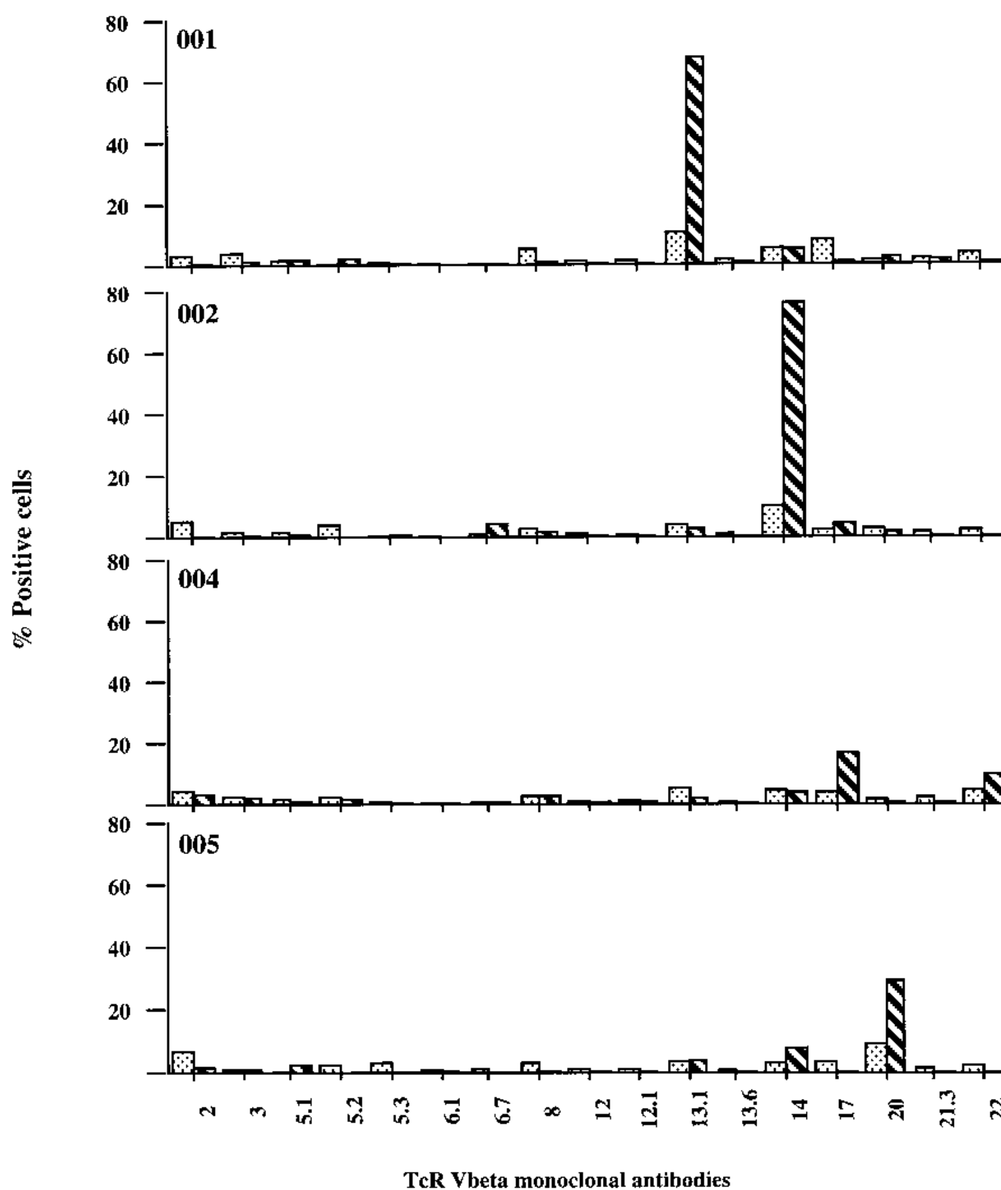


FIG. 7. Analysis of Vβ gene usage by $CD8^+$ T cells in fresh PBMC (▨) and polyclonal HCMV-specific CTL derived from LDA (▧) from four HCMV-seropositive donors, using a panel of Vβ-specific MAbs. Results are expressed as percent Vβ-positive cells within the $CD8^+$ T-cell fraction.

tein pp65 is a target antigen for HCMV-specific CTL in most HCMV-seropositive donors tested (18). CTL specific for pp65 were detectable in all of the donors we tested. We have extended this observation by quantitating the pp65-specific CTLp frequency and showing this to be very high. In addition, by the use of a pp65 gene deletion mutant of HCMV, it was possible to calculate the contribution of pp65-specific CTL to the total HCMV-specific CTL response: in the donors tested, they were found to represent 70 to 90% of the total HCMV-specific CTL. This calculation assumes that all HCMV-specific CTLp present in the PBMC of a donor have been restimulated by in vitro culture with autologous infected fibroblasts in the presence of IL-2 and that wild-type virus and the deletion mutant express all viral genes other than pp65 equally well. Recently, clinical isolates have been shown to contain additional gene segments that are not present in AD169 (9), and it is possible that these gene products may be CTL targets. However, the absolute frequency of pp65-specific CTL is strikingly high in all donors that we have tested; if CTL specific to other HCMV proteins were present and we had failed to stimulate them, this would of course affect our estimation of the number of pp65-specific CTL expressed as a percentage of total HCMV-specific CTL. However, despite this caveat it is clear that pp65-specific CTLp are present in all donors tested at a very high frequency, strongly suggesting that this protein is an important target for the CTL response to HCMV.

CTL recognition of pp65 peptides presented on the surface of HCMV-infected cells may have a number of advantages. pp65 is a major constituent of the virus tegument (27) and is consequently available to the class I processing pathway immediately after HCMV infects a cell, even before immediate-early gene expression. This property has been demonstrated in vitro, as UV-inactivated HCMV can sensitize cells to killing by pp65-specific CTL (25). In addition, we have shown that fibroblasts exposed to UV-inactivated HCMV, which is incapable of de novo virus gene expression, can act as antigen-presenting cells for stimulation of pp65-specific CTLp (data not shown).

It has previously been reported that pp65-specific clones were able to lyse HCMV-infected fibroblasts, whereas in contrast, IE1-specific clones failed to lyse HCMV-infected cells while retaining their ability to lyse cells expressing IE1 in isolation (13); it was suggested this might be due to inhibition of IE1 presentation consequent to class I MHC downregulation. The capacity of HCMV to interfere with normal class I MHC processing and presentation at early times after infection (3, 4, 7, 31, 32) might thus explain why the specificity of the CTL response should be focused on structural proteins which are relatively abundant immediately after infection derived from input virus. It is difficult to find a parallel for the clear immunodominance of pp65 in other human virus CTL systems so far described. EBV is probably the best-studied analogous persistent virus infection; however, no single viral protein is clearly immunodominant, as CTL specific for numerous latent cycle gene products (EBNA 2, 3a, 3b, and 3c and LMP 2) have been observed in EBV-seropositive donors (29). In human immunodeficiency virus-seropositive subjects, CTL with specificity to Env, Gag, Nef, Vif, and Rev proteins have been observed (28). The CTL responses observed in acute virus infections such as influenza (nucleoprotein, matrix and hemagglutinin [14]) or measles (hemagglutinin, fusion protein, and nucleoprotein [11]) are also specific for multiple virus-encoded proteins.

Different patterns of peptide recognition were observed in the donors we studied: two of five recognized a single peptide fragment, whereas the other three donors recognized a number of peptides scattered throughout the pp65 protein. These

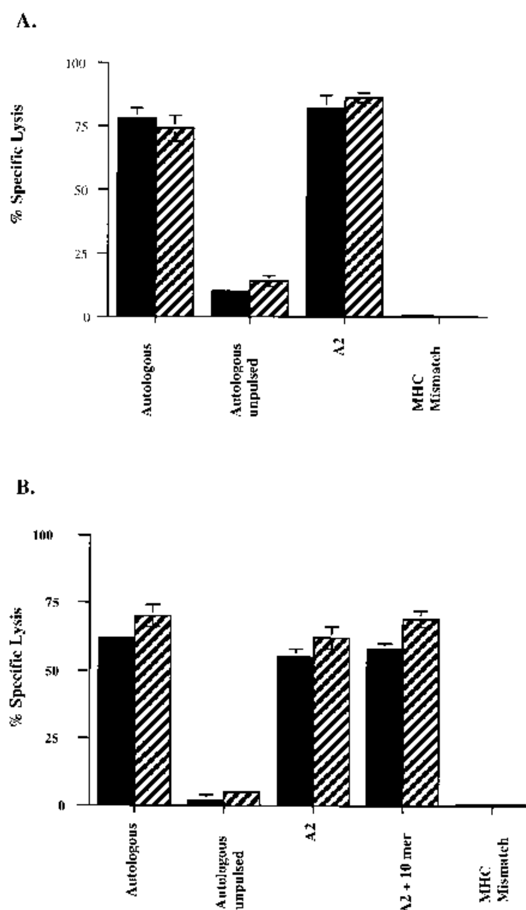


FIG. 8. Determination of CTL specific to peptide 69 (ARNLVPMVATVQG QN) in two additional HLA A2-bearing HCMV-seropositive donors, donor 009 (HLA A2 A3 B7 B37) (A) and donor 010 [A2 A9(24) B15(62) B12(44), A2 + 10-mer (NLVPMVATVQ)] (B). E/T ratios of 20:1 (■) and 40:1 (▨) were used. Error bars represent ±1 standard deviation.

determinations of peptide fine specificity have mostly been performed at a single time point. It is not known for how long a particular donor has been infected with HCMV; it is conceivable that the peptide specificities seen in HCMV-seropositive donors may change over time. Over a period of time, there might be progressive selection in favor of particular peptide-HLA class I associations. Equally, the particular combination of HLA alleles and TcR α/β gene repertoires will influence the range of CTL epitopes which evoke a response in any given donor, and once selected, clones with these specificities may be detectable over a long period of time. CTL clones with defined antigen specificities for human immunodeficiency virus type 1 gp41 (aa 584 to 592) and molecularly characterized TcR Vα/β gene rearrangements have been isolated from the same donor over a 30-month period (15), which would support the idea that individual CTL clones can be long lived.

It was interesting that both donors who recognized only a single peptide in pp65 had a highly restricted TcR Vβ usage in HCMV-responsive $CD8^+$ T cells. In another donor (005), for whom at least five pp65 peptide epitopes were identified, 29% of the $CD8^+$ CTL were TcR Vβ 20 and specific for peptides 17 and 18. Studies of virus-specific CTL in murine model systems have examined the relationship between TcR usage and recognition of particular peptide epitopes and have often shown preferential use of Vα and Vβ gene segments. However, a

more detailed characterization of the hypervariable V-J and V-D-J regions has shown considerable heterogeneity (1, 33, 34). Studies of TcR usage by human CTL in both acute and persistent viral infections have also identified restricted TcR Vβ usage, both within a donor and between donors bearing the same HLA class I alleles. Again, various degrees of heterogeneity have been identified by using molecular techniques to characterize the hypervariable regions of the TcR (6, 15, 17, 20). We have observed restricted Vβ usage at the level of the framework of the TcR, using family-specific MAbs;it is not clear whether these CTL result from monoclonal or oligoclonal expansions, and further characterization of the V-D-J regions is needed to answer this question. Such analysis in conjunction with a longitudinal study of a number of donors whose pp65 peptide-specific CTL have been characterized would also be valuable in understanding the long-term immune response to this persistent virus infection and to address the question of whether the specificity of the pp65 CTL response changes or becomes more focused over time.

One aim in characterizing the fine specificity of pp65-specific CTL from a number of donors was to determine whether donors who shared HLA class I alleles also had CTL specific to the same pp65 peptide. Four donors shared the HLA A2 allele, and all of these recognized the same peptide (peptide 69, aa 493 to 507). HLA A0201 is one of the most frequent class I alleles, being present in over 40% of the Caucasoid population. A similar phenomenon has been observed after influenza A virus infection: most HLA A0201 subjects have CTL specific for the matrix protein M58-66 which preferentially use a TcR Vβ 17 gene rearrangement (17). We did not observe the use of a particular Vβ gene segment in the four HLA-A2 donors for pp65 peptides. Most of the donors used at least two different Vβ genes to recognize the HLA A2-peptide 69 combination; the majority (67%) of HCMV-responsive $CD8^+$ T cells from donor 001 bore TcR Vβ 13.1/3, which was also used by donor 009 but by only a minority (11%) of the latter's responder cells. Our current panel of TcR Vβ-specific MAbs can identify only about 60% of the known TcR Vβ gene segments, and we note that the Vβ gene usages of a proportion of HCMV-specific responder cells in all four HLA A2 donors remain unidentified: until the TcR Vβ genes utilized by these CTL are characterized, it will be unclear whether a common TcR rearrangement exists for peptide 69-HLA A2 recognition. Detailed knowledge of common pp65 peptide-HLA class I associations could inform attempts to enhance HCMV-specific CTL immunity in at risk groups such as bone marrow transplant recipients.

In summary, this work shows a striking concentration of the CTL response to a common persistent virus infection, on a single protein among the 200 proteins which HCMV encodes. This structural protein of the virus tegument is, by inference, capable of accessing the class I MHC pathway after entering the cell on input infecting virus. This situation appears to be different from common CTL recognition elements in other virus infections. Further work is needed to determine the detailed mechanisms of T-cell recognition of pp65 and the extent to which these can be exploited for preventive strategies against HCMV.

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