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Identification of Three HLA-A*0201-Restricted Cytotoxic T Cell Epitopes in the Cytomegalovirus Protein pp65 That Are Conserved Between Eight Strains of the Virus

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The Ag specificity of the CTL response against CMV is directed almost entirely to a single CMV tegument protein, the phosphoprotein pp65. We report the identification of three peptides derived from the protein pp65 that displayed a high or intermediate binding to HLA-A*0201 molecules, which were also able to induce an in vitro CTL response in peripheral blood lymphocytes from CMV seropositive individuals. The peptide-specific CTLs generated were capable of recognizing the naturally processed pp65 either presented by CMV-infected cells or by cells infected with an adenovirus construct expressing pp65 in an HLA-A*0201-restricted manner. Thus, we were able to demonstrate responses to dominant CTL epitopes in CMV-pp65 that were not detected in polyclonal cultures obtained by conventional stimulations. We also found that the amino acid sequences of the three peptides identified as HLA-A*0201-restricted CTL epitopes were conserved among different wild-type strains of CMV obtained from renal transplant patients, an AIDS patient, and a congenitally infected infant, as well as three laboratory strains of the virus (AD169, Towne and Davis). These observations suggest that these pp65 CTL peptide epitopes could potentially be used as synthetic peptide vaccines or for other therapeutic strategies aimed at HLA-A*0201-positive individuals, who represent ~40% of the European Caucasian population. However, strain variation must be taken in consideration when the search for CTL epitopes is extended to other HLA class I alleles, because these mutations may span potential CTL epitopes for other HLA molecules, as it is described in this study. *The Journal of Immunology, 1999, 163: 5512–5518.

Cytomegalovirus infection in immunocompetent individuals is normally asymptomatic, and CTLs specific for CMV are present with a high frequency in the peripheral blood of seropositive individuals (1). In contrast, in immunocompromised patients such as allogeneic bone marrow transplant recipients, CMV infection can be a significant complication. In these patients the recovery of CMV-specific CD8+ CTL response has been correlated with an improved outcome from CMV disease (2, 3). These observations suggest an important role for the cellular immune response in the control of CMV infection, and have encouraged studies to identify the target Ags recognized by CMV-specific CTLs in the context of different HLA class I molecules.

One of the predominant viral Ags recognized by CMV-specific CTLs is the lower matrix 65-kDa phosphoprotein (pp65) (4, 5), one of the most abundant structural proteins of the virion (6). Currently, several strategies have been attempted to identify the particular peptide epitopes derived from this protein responsible for CTL recognition (5, 7, 8). Some of these studies have employed autologous fibroblasts infected with CMV, or vaccinia constructs expressing pp65, for the in vitro induction and expansion of pp65-specific CTLs (5, 8). Such stimulation protocols can lead to the activation of polyclonal CTL responses, where effector cells are likely to recognize only the more dominant epitopes derived from the pp65 protein (9, 10). Because immunodominant viral epitopes are often selected for mutation in vivo, this could hamper their effectiveness as therapeutic targets (11–14). In an attempt to avoid biasing the CTL response to immunodominant epitopes, alternative protocols of CTL generation have made use of APC pulsed with potential CTL peptide epitopes. This might allow the triggering of CTL responses to epitopes, which are not detected using conventional stimulation protocols, and could potentially make useful peptide based vaccines. We have therefore used the latter approach to identify CTL epitopes in the CMV protein pp65.

The prediction of epitopes that have the potential of eliciting a CTL response has been greatly facilitated by the identification of binding motifs for different MHC class I alleles. Based on the reported binding motifs for the HLA-A*0201 molecule (15–17), we have searched the amino acid sequence of the pp65 protein for peptides that could bind to this HLA molecule. We chose to use HLA-A*0201 due to its high frequency in the Caucasian population, with an average allele frequency of 25%, reaching 39% in selected populations (18).

The peptides thus identified were then assessed for their ability to bind to the HLA-A*0201 molecule, and a subset of binding peptides was then analyzed for their ability to induce an HLA-A*0201-restricted CTL response in vitro. We showed that the pp65

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protein contains multiple HLA-A*0201-restricted CTL epitopes, which were conserved among eight different strains of CMV. Only one of these peptide epitopes had previously been identified using other epitope mapping approaches (5, 8).

Materials and Methods

Cell lines

The human fetal lung fibroblast cell line MRC-5 (HLA-A*0201, A29, B13, B44 Cw7) and the human foreskin fibroblast line, Hs68 (HLA A1, 29; B8, 44; Cw7, 16) were obtained from the European Collection of Cell Cultures (ECACC, Witshire, U.K.) and used from passages 20 to 35. The primary human embryonal kidney cell line 293 was obtained from the American Type Culture collection (ATCC, Manassas, VA). These cell lines were grown in MEM (Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine, 1% nonessential amino acids, and 10% FCS. Cells were cultured in a 5% CO2 humidified atmosphere at 37°C. The EBV transformed B cell line JY (HLA type: A*0201, B7, Cw7, DR4, DR6x, DPw2) was grown in RPMI 1640 medium supplemented with 10% FCS (RPMI/10 FCS). The cell line 174xCEMT2 (T2) (19) was maintained in either RPMI/10% FCS or the serum-free medium X-vivo 10 (BioWhittaker, Walkersville, MD). PHA-activated T cell blasts were obtained by stimulation of PBMC with 2 μg/ml PHA (Murex Biotech, Dartford, U.K.) in RPMI 1640 with 10% AB human serum for 3 days, and expanded in medium supplemented with 25 IU/ml IL-2 (Boehringer Mannheim, Mannheim, Germany). Cell lines and PBMC were HLA-typed by serology using standard microtiter typing techniques, and HLA-A*0201 typing was accomplished by using Sequence Specific Oligonucleotide Probing (SSOP) as described elsewhere (20).

Virus

The following strains of CMV were used: laboratory strains AD169 (passage 94), Davis (passage 76), Towne (passage 132), and the low passage clinical isolates Toledo (passage 12), C1F (passage 8), C1FE (passage 8) and CRV (passage 5). The AD169, Davis, and Towne strains were obtained from the American Type Culture Collection (Manassas, VA), and the Toledo strain was a gift from Dr. Stuart Starr (Philadelphia, PA). C1F, R7, and CRV are clinical isolates which have been passed through fibroblasts, whereas C1FE was derived from C1F by subsequent passage through endothelial cells and has enhanced pathogenicity for the latter cell type. With the exception of strain C1F, virus was propagated by serial passage through confluent monolayers of human embryonic lung fibroblasts, in MEM supplemented with 4% FCS and stored at −70°C. Virus stocks, which had a titer of 6 × 10⁷ PFU/ml were used to infect fibroblasts at a multiplicity of infection of 2–4. This virus dose was confirmed to produce >95% infection in fibroblasts as determined by flow cytometric analysis of the expression of the CMV immediate early Ag (21). Virus stocks of CMV strain AD169 were screened for Mycoplasma and confirmed to be negative using the Mycoplasma TCI test kit (Gen-Probe, San Diego, CA).

The recombinant adeno virus encoding the CMV protein pp65 (RAd-pp65) was generated by homologous recombination between the shuttle vector pMV60-pp65 and the plasmid pJMI7, which contains the entire Ad type 5 d309 genome, and the sequence for the plasmid pBR inserted into the E1a gene region. These two plasmids were cotransfected into the 293 cell line, which constitutively expresses the Ad E1 protein as a helper function. As a result, the pp65 gene under the control of the CMV major immediate-early promoter was inserted into the adenovirus genome in place of the E1 sequences. An adenovirus construct expressing β-galactosidase (RAd-35), obtained from Dr. Gavin Wilkinson (Cardiff University, Cardiff, U.K.), was used as a control (22).

Synthetic peptides

CMV-pp65 and control peptides were synthesized at the Department of Biochemistry, Nottingham University using Fmoc chemistry on solid phase, and were purified by HPLC. Peptides were dissolved in DMSO at a concentration of 10 mg/ml and stored at −70°C before use. The HLA-A*0201-specific peptide FLPSDYFPSV (11, 23) was synthesized and flu­orescein (FL)-labeled at the Department of Immunohematology and Blood Bank (University Hospital Leiden, Leiden, The Netherlands). This peptide was synthesized as a Cys derivative in which a tyrosine was substituted with a cysteine to tag a FL group to the peptide FLPSDC(FL)FPSV, as described previously (24, 25)

T2 stabilization assay

Peptide-induced stabilization assay of the HLA-A*0201 class I molecule expressed by the T2 cell line was performed using a modification of the method described elsewhere (26). Briefly 5 × 10³ T2 cells were incubated in the presence of 100 μM of peptide in X-vivo 10 medium for 18 h at 37°C. Fully conformed cell surface HLA class I molecules were detected by indirect immunofluorescence using the Ab W6/32 (27, 28). Labeled cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, Oxford, U.K.) as described previously (29).

Peptide competition assay

To determine the relative affinity of putative peptides for stable HLA-A*0201 molecules at the cell surface, peptides were tested using the protocol described by Burg et al. (24, 25). Briefly, JY cells were treated with acid for 5 min using ice-cold citric acid-Na2HPO4 buffer (pH 3.2) for 90 s (24, 30). Cells were immediately buffered with IMDM medium, resuspended in the presence of 1.5 mg/ml β2-microglobulin, and mixed with 150 nM of a FL-labeled reference peptide plus competitor peptide at a range of 0.5–32 μM. After 12 h at 4°C, cells were washed and analyzed by flow cytometry. The mean fluorescence (MF) values obtained with the FITC-labeled peptide in the absence of competitor peptide was taken as the maximal binding and equated to 0% inhibition. The MF value obtained without FITC-labeled peptide was taken as being 100% inhibition (triplicates were assayed for all test wells).

The percentage inhibition of binding was calculated using the following formula: [(MF-labeled peptide)/(MF-labeled peptide − MF control without labeled peptide)] × 100. The relative binding affinity of the peptide was expressed as the peptide concentration needed to inhibit 50% of the binding of the reference labeled peptide (IC₅₀). The binding affinity was categorized as follows: high < 5 μM, intermediate ≥ 5 < 15 μM, or low ≥ 15 μM.

Generation of peptide-specific CTL

PBMC from two CMV seropositive and HLA-A*0201-positive individuals (HLA A2, 3; B7, 60 and HLA-A2, 19, B7, 15) were stimulated with 5000 irradiated (6000 rads) autologous PHA blasts pulsed with 50 μg/ml peptide per well. Cultures were incubated at various responder to stimulator ratios (10:1, 5:1, 2.5:1, 0.4:1) in RPMI 1640 with 10% AB serum. On days 3 and 6, the medium was supplemented with 10 IU/ml IL-2. On day 10, each well received irradiated (9000 rads) T2 cells pulsed with peptide, irradiated (3000 rads) autologous PBMC as feeder cells containing 10 IU/ml IL-2. Responding T cells were expanded by weekly stimulations and the phenotype of the responder cells was determined by flow cytometric analysis using the following mAbs: CD3/FITC; CD4/PE; CD8/PE; CD16/PE; CD14/PE; CD19/FITC; CD56/PE (Becton Dickinson). After 24 days, the CD4+ T cell fraction in the responder cell population was depleted by incubation with anti-CD4 magnetic beads (Dynabeads M-450 CD4; Dynal, Wirral, U.K.). The resulting CD8+ enriched population was replated at 1 × 10⁶ responder cells/well and restimulated as described above.

Cytotoxicity assays

Cytotoxic activity was measured by a standard 4-h ⁵¹Cr-release assay (31). Target cells, comprising of either peptide-pulsed T2 cells (50 μg/ml) or fibroblasts infected either with CMV strain AD169 or the recombinant adeno viruses, were labeled with 100 μCi of ⁵¹NaCrO₄. Labeled target cells were incubated with effector cells. E/T cell ratios varied between 1:5 and 1:50. The spontaneous release controls were normally <25% of the total release. The data from the ⁵¹Cr release assays shown are representative of three similar experiments.

ELISPOT assays

The production of IFN-γ was determined by ELISPOT assay, essentially as described by McCutcheon et al. (32) with minor modifications. Briefly, cultures were incubated with 50 μg/ml of the relevant peptides overnight, and the following day stimulator cells were removed and the ELISPOT plates processed as previously described (32).

DNA sequencing by PCR

Viral DNA was extracted from cell-free stock of various CMV strains, using Quiagen (Qiagen, Crawley, U.K.) following the manufacturer’s protocol. DNA (0.1–250 ng) was amplified in a 25 μl reaction mix using cloned Pfu DNA polymerase (Stratagene, La Jolla, CA) and the following

4 Abbreviations used in this paper: FL, fluorescein; ELISPOT, enzyme-linked immunospot.

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Identification of HLA-A*0201 binding peptides

The amino acid sequence of the pp65 protein from the CMV strain AD169 (33) was searched for sequences containing the consensus anchor motifs for HLA-A*0201 binding (P2(L or I), P9(V or L)) (15–17). In total, 17 peptides were found that contained this binding motif. These CMV-pp65 peptides were then analyzed for their capacity to stabilize HLA-A*0201 molecules on the surface of the Ag processing deficient cell line T2 (34). T2 cells were incubated with the relevant peptide and the resultant increase in the cell surface expression of HLA-A*0201 was measured. In this assay, a nonameric peptide from the influenza matrix protein (Flu-M158–66), which has been described as having a high binding affinity for the HLA-A*0201 molecule (35–37), was used as a positive control. This peptide resulted in a 6-fold increase in cell surface HLA-A*0201 stabilization (Table I). In contrast, an HLA-B27-binding peptide used as a negative control, residues 383–392 from influenza nucleoprotein (Flu-NP383–392), showed no increase above the background of T2 cells with no peptide added (Table I). The CMV-pp65 peptides with the HLA-A*0201 binding motif displayed varying levels of stabilization of HLA-A*0201 molecules on T2 cells. We found that peptides bearing the anchor residues leucine and valine at positions 2 and 9, respectively, generally showed good HLA class I stabilization (Table I). The highest levels of stabilization were observed for peptides AE42, AE44, and AE45, which also possessed in their sequence several hydrophobic residues. For example, in peptide AE42, where valine and alanine were present in positions 6 and 7. In contrast, peptides AF88, AE47, AE48, and AE49 showed minimal or no stabilization (Table I), although they also possessed the same anchor residues. However, the latter peptides contained at least one charged residue in secondary positions, which might explain their poor binding.

The relative affinity of all the peptides bearing the leucine binding (L2) and valine (V9) motif for the HLA-A*0201 molecule was then determined using a competition-based HLA class I binding assay (24). In this assay, the lymphoblastoid cell line JY, was used after acid treatment, aimed to remove the peptides from the HLA molecules present on its surface. These stripped cells were then incubated with a mixture of a fixed amount of a fluoresceinated reference peptide, and different concentrations of the pp65 peptides under test (Fig. 1). Peptides with affinity for the HLA-A*0201 molecule will compete for the binding of the fluoresceinated reference peptide. The pp65-derived peptides with the L2-V9 motif showed either a high IC50 (2–15 μM) (AE45, AE48, and AE47) or intermediate IC50 (5–15 μM) (AE42, AE44, and AE49) binding affinity for HLA-A*0201 (Fig. 1, Table I). Interestingly, peptides AE47 and AE48, which were unable to stabilize HLA-A*0201 molecules on T2 cells, appeared to have intermediate and high affinities, respectively, for HLA-A*0201 in the peptide competition assay. This lack of correlation is likely to reflect the different parameters of peptide binding that the two-assays measure. Although the T2 stabilization assay measures peptide off rates and thus the stability of binding, the peptide binding competition assay is likely to be more influenced by on rates, which, given that the off-rates of bound peptides are usually slow, means that those peptides which bind faster compete better. A similar discrepancy was previously found when these peptides were used in an optical biosensor system, which measures the relative binding affinity of the peptides for purified HLA-A*0201 molecules fixed to a solid matrix (29). As expected, the HLA-A*0201-specific Flu-M1 58–66 peptide bound with a high relative affinity (≤5 μM; data not shown), whereas the HLA-B27-specific peptide Flu-NP383–392 showed negligible affinity for HLA-A*0201 molecules in the competition binding assay (Fig. 1, Table I).

Stimulation of pp65 peptide-specific T cells in vitro

Previous observations have shown that known CTL peptide epitopes display binding to HLA class I molecules with a high to intermediate affinity (IC50, 2–15 μM) (24, 38). Therefore, we evaluated the ability of four pp65-derived peptides, which showed reproducibility of binding to HLA-A*0201 molecules between the T2 and the peptide-binding competition assays, for their ability to elicit a CMV-specific CTL response.

To achieve this, PBMC from two normal CMV seropositive HLA-A*0201 individuals were isolated and stimulated in vitro with peptides AE42, AE44, AE45, and AE47. The cytotoxic activity of the responding cells was measured after three consecutive stimulations against T2 cells pulsed either with the peptide used in the stimulations or with an irrelevant peptide, Flu-M1 58–66, as target cells. When the peptide-pulsed T2 cells were utilized in the
To obtain this level of inhibition represents the binding capacity of that particular peptide (IC50). Samples were assayed in triplicate, and the results shown are representative of two independent experiments. The figure shows the mean ± SD of the three triplicates.

Competition of binding of CMV-pp65-derived peptides against a FL-labeled reference peptide to empty HLA-A2 molecules. Acid-treated JY cells were incubated with 150 nM of the FL-labeled reference peptide derived from the hepatitis B virus core protein (positions 18–27) FLPSDC(F1)FPSV, and increasing amounts (in μg/ml) of pp65 peptides bearing the HLA-A2 binding motif L2-V9/10. Peptide Flu-NP 383–392 was also used as a negative control. Inhibition of binding was calculated as described in Material and Methods and is shown in relation to the amount of labeled reference peptide used. The dotted line represents 50% inhibition of binding of the control peptide. The extrapolation of the concentration of each peptide necessary to obtain this level of inhibition represents the binding capacity of that particular peptide (IC50). Samples were assayed in triplicate, and the results shown are representative of two independent experiments. The figure shows the mean ± SD of the three triplicates.

initial rounds of stimulation, we found very strong non-peptide-specific responses, which masked the underlying peptide-specific response, as demonstrated by the fact that the responder cells recognized non-peptide-pulsed T2 cells (data not shown). To circumvent this nonspecific response, we used autologous peptide-pulsed PHA blasts in the first two rounds of stimulation and found that this was sufficient to focus the response, and to obtain specific recognition of the peptide used as the stimulator. This peptide-specific recognition was maintained when peptide-pulsed T2 cells were used for subsequent stimulations. Following this strategy, three of the peptides that displayed relatively high binding affinity for HLA-A*0201 molecules (Table I) were able to induce peptide-specific CTL responses. These peptides were AE42, (495–503, NLVPMVATV), from the carboxyl terminus, and AE44 (14–22, VLCPGISGHY) and AE45 (120–128, MLNIPSINV) from the amino terminus of the pp65 protein. In contrast, peptide AE47 failed to do so (data not shown). The failure of AE47 to induce CTLs correlated with poor ability to stabilize HLA-A*0201 molecules on T2.

The CTL lines generated lysed T2 cells pulsed with peptide AE44, but also lysed cells pulsed with an irrelevant peptide, Flu-M1 58–66. Similar nonspecificity against the irrelevant peptide was seen when peptides AE42 and AE45 pulsed cells were used as stimulators (data not shown), and this was attributed to the polyclonal nature of the cultures. No significant differences were found when different stimulators to responder cell ratios were used for the generation of the CTL lines. In an attempt to reduce the observed nonspecificity and to obtain a predominant CD8+ population, some of these bulk cultures generated using peptides AE42, AE44, and AE45 were depleted of CD4+ cells, and the resultant CD8+ cell population was then replated (1000 cells/well), and long-term lines were generated.

Following two rounds of stimulation and cell expansion, these CD4+ depleted CTL lines were again tested for peptide specificity in a cytotoxicity assay. Approximately 20% of the sublines then displayed more clearly defined specificity for the relevant peptides than before the CD4+ depletion. These lines no longer cross-reacted with T2 cells alone, and displayed minimal recognition of irrelevant peptides derived from the same pp65 protein (Fig. 2), or the less related peptide from Flu-M1 58–66 (data not shown).

It is therefore clear from these results that the CD4+ depletion strategy followed by the generation of sublines was successful in selecting only those lines that were specific for the pp65 peptides AE42, AE44, and AE45, excluding cells responsible for the non-specific lysis previously observed in cytotoxicity assays.

Peptide-specific CTL recognize endogenously processed pp65

A frequent feature of stimulation with APC pulsed with synthetic peptides is the induction of low affinity CTL that lyse target cells in the presence of relatively high concentrations of exogenous peptide and which do not recognize epitopes from endogenously processed Ags (39, 40). To investigate if the CTL lines generated were able to recognize endogenously processed pp65, representative peptide-specific CTL lines were tested in a cytotoxicity assay against HLA-A*0201 positive fibroblasts (MRC5) infected with the CMV strain AD169. The lines tested were H4, C11, and D11 generated against the peptides AE42, AE44, and AE45, respectively.

The results from the cytotoxicity assay showed that all three CTL lines were able to recognize HLA-A*0201 fibroblasts infected with CMV (Fig. 3), with minimal recognition of the uninfected HLA-A*0201-positive fibroblasts. Lines generated against the peptide AE45 showed a comparable percentage of lysis to lines generated against peptides AE42 or AE44, despite the fact that a low level of lysis of peptide-pulsed target cells had been observed in previous experiments shown in Fig. 2. One possible explanation for this might be that the number of effector cells in the earlier assays was suboptimal, and that the extent of the response had been underestimated.

To further examine this inconsistency and to determine whether the results could be attributed to an assay-based problem, PBMC from a number of HLA-A*0201 and CMV-positive individuals were challenged with the peptides and their response assessed using the ELISPOT system. As can be seen from the results in Fig. 4, when peptides AE42 and AE45 are presented by PBMC, the predominant response is to peptide AE42, with a much reduced
response to peptide AE45, thus confirming the data from Fig. 2. Thus in vivo, the response to peptide AE45 is subdominant in comparison to AE42. This serves to further illustrate that the binding values of a given peptide to a particular HLA allele are not indicative of its functional abilities, and highlights the need to incorporate some form of functional tests in initial peptide screens rather than relying solely on selection with binding assays. To confirm that the reactivity of pp65 peptide-specific CTLs was restricted by the HLA-A*0201 allele, they were tested against HLA-A*0201-negative fibroblasts following natural infection with CMV. The target cell type used in this assay was the fibroblast cell line Hs68, infected with CMV strain AD169. As shown in Fig. 3, CTL lines H4, C11, and D11 were unable to recognize endogenously processed pp65 in CMV-infected target cells which did not express HLA-A*0201 molecules. These results confirmed that CTLs generated against peptides AE42, AE44, and AE45 clearly recognized the CMV-infected target cells in an HLA-A*0201-restricted manner. To demonstrate that the pp65 peptide-specific CTL lines reacting against CMV-infected fibroblasts were recognizing a peptide derived from the endogenously processed pp65, and not from any other CMV proteins, they were tested further for their ability to lyse fibroblasts infected with an adenovirus construct expressing pp65 (RAd-pp65). Fibroblasts infected with a control adenovirus construct, RAd-35, expressing the enzyme β-galactosidase were

FIGURE 2. Peptide-specific cytotoxicity of CD4\(^+\) depleted CTL lines stimulated with pp65-derived peptides. The figure shows representative CTL lines stimulated with pp65 peptides AE42, AE44, or AE45, tested for cytotoxicity 3 wk after CD4\(^+\) depletion and generation of sublines. Target cells in these experiments were \(^{51}\)Cr-labeled T2 cells pulsed with 50 μg/ml of relevant peptides AE42, AE44, and AE45, respectively. T2 cells pulsed with an irrelevant peptide or T2 cells alone were used as negative controls. The E:T cell ratio used in these experiments was 40:1.

FIGURE 3. The recognition of endogenously processed pp65 protein by peptide-specific CTLs. CD4\(^+\) depleted CTL lines H4 (a), C11 (b), and D11 (c), generated against peptides AE42, AE44, or AE45, respectively, were tested in a \(^{51}\)Cr-release assay. Fibroblasts were infected with the CMV strain AD169 or with the adenovirus construct RAd-pp65, and at 24 h postinfection these cells were labeled with \(^{51}\)Cr and used as target cells. CTLs were analyzed against either HLA-A2 positive fibroblasts: uninfected (UI) or infected with CMV or infected with the adenovirus constructs RAd-pp65 or RAd-35 or HLA-A2-negative fibroblasts infected with CMV or uninfected (UI). These experiments were conducted at an E:T cell ratio of 40:1, and the results are representative of two separate experiments.
used as a control in these experiments. Fig. 3 shows that these CTL lines were able to lyse fibroblasts infected with the RA-d-pp65 construct with minimal nonspecific lysis against the control RA-d-35-infected fibroblasts. These results confirm that the pp65-derived peptide epitopes identified here are present in association with HLA-A*0201 on the surface of infected cells as a result of endogenous pp65 processing.

Two independent groups following different strategies to ours have previously identified one of the peptides found to be immunogenic in this study, peptide AE42 (positions 495–503, NLVP-MVATV). By designing overlapping peptide sequences from pp65 and testing them for their recognition by CMV-specific CTLs, Wills et al. (5) reported that such CTLs recognized a 15-mer (positions 493–507). When this peptide was shortened they found that the actual CTL epitope presented in the context of HLA-A*0201 was the 10-mer, positions 495–504. Diamond et al. (8), using recombinant vaccinia constructs expressing truncated sequences of pp65, mapped the recognition of a pp65-specific T cell clone restricted by HLA-A*0201 to an 84-aa region in the carboxy-terminal end of pp65. From this region they designed synthetic peptides based on MHC binding motifs and found the peptide pp65 (positions 495–503, NLVP-MVATV) as the only epitope recognized by their pp65-specific T cell clone. This group also reported that this peptide was able to induce both in vivo and in vitro CTLs that lysed HLA-A*0201 positive CMV infected fibroblasts (8). With the strategy employed in the present study, we have been able to identify not only peptide pp65 (positions 495–503), but also two more peptides which are presumably subdominant and therefore were not identified by other strategies.

### Analysis of the HLA-A*0201-restricted pp65 CTL epitopes in a range of CMV isolates

Previous results from Riddell et al. (41) found that CTL clones from different donors were able to lyse autologous fibroblasts infected with the two laboratory strains AD169 and Towne and with four wild-type CMV isolates. These results suggested that the immunodominant CTL response is specific for epitopes that are conserved among several genetically distinct CMV strains. To confirm this and to demonstrate that the peptides identified within this study have the potential for use in the prevention of CMV infection in the clinical situation, we performed direct sequencing on eight different CMV strains, three laboratory adapted strains (AD169, Towne, and Davis), and five clinical isolates (Toledo, CRV, R7, CIF, and CIFE). When the DNA sequences were compared with the published sequence of the CMV strain AD169 (Table II) several nucleotide differences were found; however, the differences were mainly synonymous and would not change the amino acid sequence of the pp65 protein. Only four of the differences found at the nucleotide level would give rise to amino acid differences when compared to the strain AD169 (Table II). The first substitution present only in Toledo encoded a Ser-Asn mutation at position 371. The second was found in both Towne and Toledo and was a Ser-Ala mutation at position 447, whereas the third change that encoded an Asp-Glu mutation at position 545 was found in three strains, Davis, Toledo, and CRV. The fourth substitution observed was an Ile-Ala change found in the Towne strain which had also been described by Pande et al. (42). Importantly, as shown in Table II, we found that the three peptide epitopes described in the present study lie in conserved regions of the pp65 amino acid sequence in all the strains analyzed. As these three peptides were found to be conserved in several examples of clinical CMV isolates, a synthetic peptide vaccine or alternatively the adoptive transfer of CTLs generated against these peptides could potentially provide protection against the multiple CMV strains to which bone marrow transplant recipients are exposed.

However, it was considered that the amino acid substitutions observed in the pp65 protein in some of the CMV strains might affect CTL epitopes to other HLA class I Ags. With this in mind, 25 HLA class I Ags commonly found in the Caucasian population were analyzed by a peptide binding computer algorithm. This algorithm predicts the number of potential binding peptides for a given MHC molecule, by ranking the potential octamer, nonamer, or decamer peptides, based on a predicted half-time of dissociation to HLA class I molecules (16). The most interesting results were found with HLA-A*4403 in which a CMV-pp65 peptide at positions 364–372 was found to bear the appropriate binding motif and displayed high scores for the binding prediction with putative peptides. This peptide spans the mutation found in Toledo at position 371. Two additional peptides found to be potential binders for HLA-A*2705 (amino acids 539–547) and HLA-B*3701 (amino acids 544–552) were found to span the substitution in position 545 found in the Davis, CRV, and Toledo strains of virus as indicated in Table II.

Our approach in the present study of searching the whole pp65 amino acid sequence for binding motifs for HLA-A2 not only identified the peptide previously reported to be a pp65-derived CTL epitope, but also two more peptides that are recognized in vivo in the context of HLA-A*0201.

This result serves to emphasize the potential value of the combined use of motif analysis and MHC binding assays and functional testing in the selection and identification of CTL immunogenic peptides. Furthermore, this approach does not bias the outcome to the identification of immunodominant epitopes. We have been able to demonstrate that by the judicious application of different techniques we have established a simple and reproducible system for the generation of peptide-specific CTL that importantly retain the ability to respond with native Ag as well as with peptide as seen in Fig. 4.

It has been suggested that the pp65 protein entering the cell as part of the tegument of the virion is processed and presented by MHC class I molecules before viral DNA synthesis and replication (4). This is particularly relevant in the CTL recognition of CMV, as there are several CMV proteins expressed at immediate early, early, and late times after infection which have shown to interfere with the processing and presentation of class I Ags. This family of proteins, known as US2–11, down regulates HLA class I molecules using several mechanisms (43–45). The fact that pp65 may be presented before the expression of US2–11 suggest that the down-regulation of MHC class I molecules may not have any effect on the recognition of pp65. Also, this could explain the high dominance of pp65 as a target Ag for CMV-specific CTL responses (5, 7, 46). Taken together, this evidence suggests that pp65 is a prime

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**Table II. Amino acid sequences with coding substitutions in different CMV strains**

<table>
<thead>
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<th>CMV Strain</th>
<th>Residue Number (amino acid)</th>
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<th>448</th>
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<td>S</td>
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* The DNA obtained from the different CMV strains was amplified by PCR using pp65-specific primers, and the DNA sequences obtained were translated into their coding amino acids. Predicted amino acid changes with respect to the AD169 CMV sequence in four different regions of the pp65 protein are shown in bold and underlined.
candidate to target in the development of immunotherapies against CMV, namely peptide or subunit vaccines or adoptive T cell therapy. The work described here permitted us to identify three peptides which are CTL epitopes recognized in the context of HLA-A*0201, the most common Caucasian HLA class I molecule. In the design of a potential peptide vaccine the ideal situation would be to produce a product with the ability to target as high a number of the affected population as possible, and the strategy proposed could be used in the identification of additional peptide epitopes in the context of other HLA class I molecules.

References


