Humoral Immune Response to Proteins of Human Cytomegalovirus Latency-Associated Transcripts

Maria Paola Landini,[1] *Tiziana Lazzarotto,*[1] *Jiake Xu*[3] *Adam P. Geballe*[2], *Edward S. Mocarski*[3]*

[1]Department of Clinical and Experimental Medicine, Division of Microbiology, University of Bologna, Bologna, Italy; [2]Divisions of Molecular Medicine and Clinical Research, Fred Hutchinson Cancer Research Center and Departments of Medicine and Microbiology, University of Washington, Seattle, Washington; [3]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California

*Correspondence and reprint requests: Edward S. Mocarski, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5124; 650-723-6435; fax: 650-723-1606; e-mail: mocarski@stanford.edu

(Received June 16, 1999; accepted December 30, 1999)

ABSTRACT

Latent human cytomegalovirus (CMV) infection of hematopoietic progenitor cells is associated with the presence of latency-associated transcripts that may express 6 proteins larger than 44 amino acids in size (open reading frame [ORF] 55, ORF45, ORF94, ORF59, ORF154, ORF152/UL124). The serologic response to these proteins was evaluated in healthy seropositive individuals as well as in individuals undergoing active CMV infection. Individual recombinant GST-fusion proteins, prepared from bacteria, were found by enzyme-linked immunosorbent assay to be recognized by between 8% and 44% long-term healthy seropositive individuals, with ORF94 and ORF55 being the most broadly and significantly recognized. Although nearly all of serum samples (85%) recognized at least 1 of these proteins, none reacted with all 6. Patterns of antibody prevalence to these proteins in long-term seropositive individuals were similar to many antigens expressed during productive replication (IE1, ppUL57, ppUL83/pp65), but none were broadly detected by a majority of individuals, a characteristic of only a few productive-phase antigens, including ppUL44/ICP36 and ppUL32/pp150. Consistent with prevalence in long-term seropositive individuals, commercial preparations of pooled human gamma globulin were also found to recognize latency-associated proteins. Serologic reactivity to latency-associated proteins was slow to develop following primary infection, in a pattern distinct from any of the characterized replication-phase proteins tested here, and was boosted late after secondary infection or reactivation in solid-organ transplant recipients without showing a correlation with viremia or disease. These results provide evidence that proteins expressed from the latent region during natural infection exhibit immunogenicity comparable with most other characterized viral antigens, although the narrow response to individual latency-associated proteins likely precludes their use in serologic assays to investigate clinical correlates or outcome in transplant recipients.

INTRODUCTION

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus and a common cause of opportunistic infections, often as a result of reactivation of latent virus that follows from decreased immunosurveillance [1]. The widespread distribution of latent virus in the general population has made the occurrence of CMV disease following blood and marrow allograft transplantation particularly significant, even with the available antiviral therapy [2]. Although latency remains poorly understood, and sites of latent virus residence have not been fully characterized, reactivation of latent virus remains the single most important determinant of CMV disease following transplantation or immunosuppression. Bone marrow–derived granulocyte-macrophage progenitors that give rise to peripheral blood monocytes and tissue macrophages have been implicated as important reservoirs of latent virus [3-9]. Although the CMV genome is highly complex and contains over 200 putative genes [9,10,11], viral gene expression is highly restricted during latency [9,10,12]. Two types of transcripts—denoted sense and antisense CMV latency-associated transcripts—specifically detected in latently infected granulocyte-macrophage progenitors have been mapped to the *ie*1/*ie*2 region of the CMV genome [8] (Figure 1). Sense transcripts have been more frequently detected than antisense transcripts in bone marrow–derived mononuclear cells from naturally infected individuals [8]. Sense transcripts are detected in a small percentage of $CD33^+$ progenitors of monocytes,

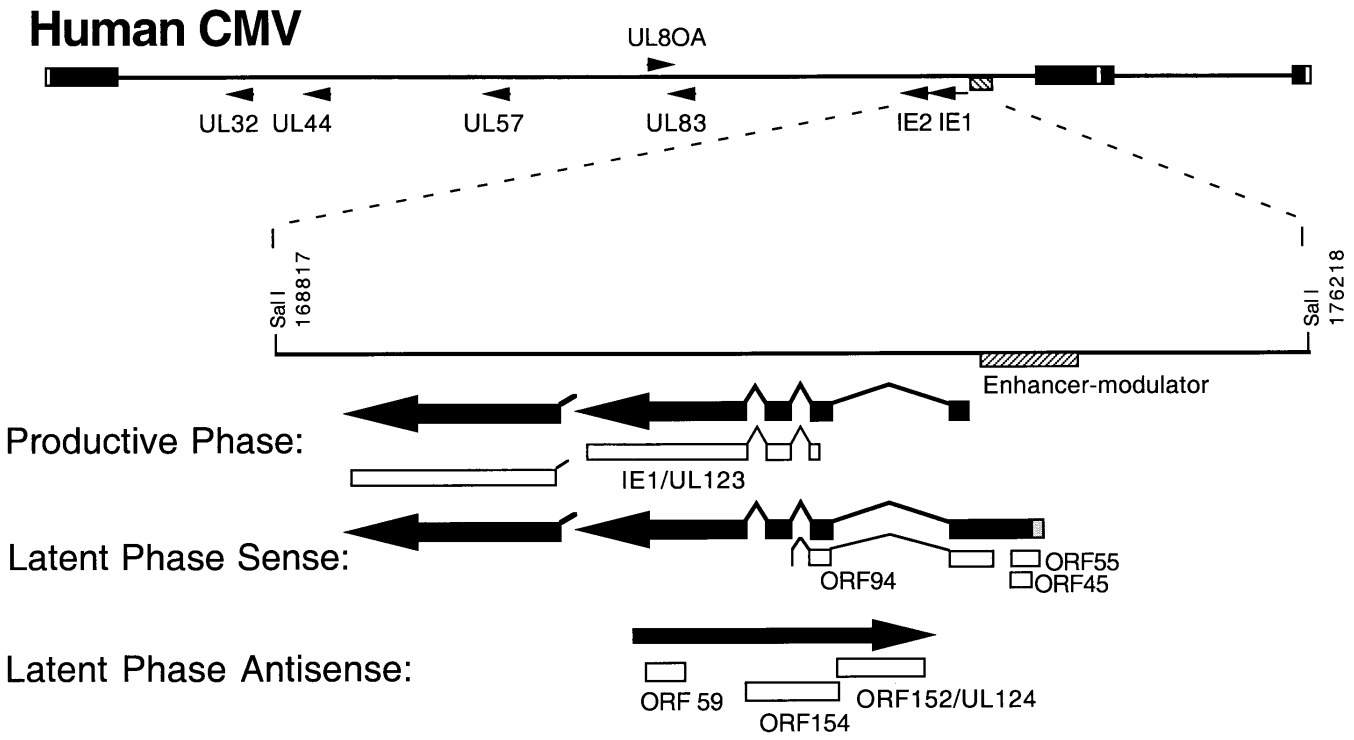

Figure 1. *Genomic map position of the ORFs for LP and PP proteins used in this study. The top line shows the CMV genome with PP ORFs UL32, UL44, UL57, UL83, UL80a, IE2/UL122 and IE1/UL123 indicated [10]. The expanded segment shows a schematic of ie1/ie2 region transcripts detected in this region during the productive phase, with IE1/UL123 indicated, and latent phase [9], with ORF94, ORF45, ORF55 indicated on sense transcripts and ORF 59, ORF154, and ORF152/UL124 indicated on antisense transcripts.*

granulocytes, and monocyte-derived dendritic cells, but are not detectable in T-cells, B-cells, mature monocytes, or mature granulocytes from naturally infected bone marrow donors [10,12].

Both types of latency-associated transcripts contain open reading frames (ORFs) that are associated with the latent phase (LP) of infection [7,8]. These ORFs are not contained on any known productive phase (PP) transcript. Sense latent transcripts carry 3 ORFs (ORF55, ORF45, and ORF94) and antisense transcripts carry 3 ORFs (ORF59, ORF154, and ORF152, which is also designated UL124) larger than 44 codons that are conserved among CMV strains (Figure 1). Although LP transcripts have been detected in hematopoietic progenitors from healthy seropositive carriers, the only evidence suggesting that they encode proteins has been derived from serologic analyses. Consistent with the expression of transcripts, antibodies specific for ORF94 and ORF152 have been detected in sera by immunoblot from 7 and 3, respectively, of 15 from healthy CMV-seropositive individuals [8].

Productive CMV infection elicits a strong humoral immune response to both structural and nonstructural viral proteins. A detailed analysis of this response in different clinical settings has been the subject of considerable work over the past 15 years [13]. Certain PP proteins are highly immunogenic, a characteristic that has led to the use of recombinant protein-based assays to monitor seroconversion during secondary infection and longevity of serologic responses in healthy carriers [14-18]. When assayed individually, the immunogenicity of PP proteins can be divided into 4 categories: (1) proteins that represent most viral antigens such as IE1 (ppUL123) and ppUL54 (DNA polymerase) induce a weak response in a small percentage of seropositive individuals or a response of short duration [17,18]; (2) proteins such as ppUL83 (pp65) induce a strong but short-lived antibody response (Landini, unpublished data); (3) proteins such as pUL80a (virion-assembly protein) or ppUL57 (single-stranded DNA binding protein) induce an IgM-specific response [15,19,20], and ppUL90 (pp28) [21] induces an IgG-specific response; (4) proteins such as ppUL32 (pp150) elicit a strong and lasting response detectable years after resolution of acute infection [14,22], or proteins such as ppUL44 (p52, ICP36) that elicit a strong early and lasting response in a majority of individuals following active infection. Because of a broad pattern of recognition, ppUL32 and ppUL44 have been used as surrogate markers to follow the serologic response to CMV [15,23,24]. These proteins contain linear epitopes that are highly immunogenic in many animal species.

Although both the existence and character of the humoral immune response to PP proteins is well established, nothing is known about the characteristics of the immune response against predicted LP proteins. The purpose of this study was to determine whether antibodies to predicted LP gene products appeared in sera and whether the patterns of response to these antigens would provide

information about the virus–host interaction. The results suggest that 85% of long-term seropositive individuals recognize 1 or more of the LP proteins, but that the percentage of individuals responding to any 1 of these proteins is relatively low, an overall pattern similar to category 1 PP proteins. Consistent with the relative weakness of the response, the serologic response to LP proteins following active CMV infection is delayed relative to PP proteins.

MATERIALS AND METHODS

Construction of Plasmids and Fusion Proteins

Plasmids pON2501, pON2303 (GST-ORF94$_{1-94}$), pON2304 (GST-ORF154$_{6-120}$), pON2305 (GST-ORF152$_{1-152}$), and pON2307 (GST-IE1$_{232-400}$) have been described previously [9]. Plasmid pEQ593 (GST-ORF55$_{1-55}$) was prepared by amplifying the 5′ end of the sense transcript by polymerase chain reaction (PCR) using 5′-GTTTGGTAC CGAGTCACTCTTGGCACGGGGAA-3′ and 5′-GTT TAAGCTTGTGTATCATATGCCAAGT-3′ as primers and CMV (Towne) DNA as the template. This PCR product was digested with *Hind*III and *Hinc*II, overhanging ends were filled in using Klenow polymerase, and the resulting fragment was cloned into the *Sma*I site of pGEX1 to generate pEQ593. To construct GST-IE1$_{132-274}$ (pON2990), a 429 bp *Eco*RV fragment from pON308G was cloned into the *Sma*I site of pGEX-3X. To construct GST-ORF45$_{2-45}$ (pON2991), a 380-bp *Hae*III fragment from pON2501 was cloned into *Sma*I site of pGEX-1N. To construct GST-ORF59$_{1-59}$ (pON2993), plasmid pON308G was amplified with PCR with primer ORF59A (5′-CGGGATCCGCG ATGGCCCGTAGGT-3′) and primer ORF59B (5′-GTGAATTCTGTCGGGTGCTG-3′) and the PCR product was cloned into pGEM-T (Promega) to make pON2992. A 216 bp *Bam*HI/*Eco*RI fragment from pON2992 was then cloned between the *Bam*HI and *Eco*RI sites of PGEX-2TK [25] to make pON2993. All clones were subjected to nucleotide sequencing using pGEX-specific primer 5′-ATAGCATGGCCTTTGCAGGG-3′. Growth of bacteria for preparation of fusion proteins was performed according to the methods described by Kondo et al. [7]. The PP CKS-fusion proteins used in this work were obtained as described previously [26,27].

Purification of Fusion Proteins

GST-ORF55 and GST-ORF59 were prepared as described previously [9]. GST-ORF45, GST-ORF94, GST-ORF152, and GST-ORF154 were purified by electroelution using standard protocols [26,27]. Briefly, after separation on 10% polyacrylamide preparative gels, proteins were stained with $CuCl_2$, the bands corresponding to fusion proteins were cut out, placed into dialysis tubing (Spectra/Pore membrane MW 6000-8000; Spectrum, Laguna Hills, CA), and electroeluted for 5 hours at 120 V. Electroeluted proteins were concentrated with a Centricon 30 (Amicon, Beverly, MA) and protein concentration was determined by Bio-Rad protein assay (Bio Rad, Richmond, CA).

Insoluble CKS-CMV fusion proteins (containing portions of pUL32, ppUL44, ppUL83, ppUL57) were initially purified after lysis by a combination of detergent washes followed by being made soluble in 8 mol/L urea as previously described [23]. After being made soluble in 8 mol/L urea, the fusion proteins were purified by Sepharose Q chromatography (Pharmacia Biotech, Piscataway, NJ). Soluble CKS-fusion protein ppUL80a was purified after cell lysis on preparative SDS-PAGE using a Bio-Rad Prep Cell (Bio-Rad). Soluble control CKS protein was purified by DEAE Sephadex chromatography after cell lysis and ammonium sulfate precipitation.

Human Serum Samples

The first group of sera were used to determine cutoff values and were obtained from 30 subjects who were randomly chosen as blood donors and 16 healthy adults without detectable CMV IgG by either conventional enzyme immunoassay or immunoblot assay. The second group of sera was obtained from 36 subjects who were randomly chosen as blood donors judged seropositive (IgG) for CMV by both conventional enzyme immunoassay and immunoblot assay. The third group of sera was obtained from 39 immunocompetent subjects (mainly pregnant women) who had undergone recent active CMV infection and were judged seropositive (IgM) for CMV by conventional enzyme immunoassay and immunoblot assay. A fourth group of 185 serum samples was obtained from 29 immunocompetent (21 pregnant women and 8 young adults) and 13 immunocompromised individuals (10 heart and 3 kidney transplant recipients) undergoing culture-confirmed [28] or antigenemia-confirmed [29,30] primary CMV infection. A fifth group of sera consisted of 149 samples from 7 immunocompetent adults (pregnant women) and 12 immunocompromised individuals (9 heart and 3 kidney transplant recipients) undergoing culture-confirmed or antigenemia-confirmed secondary CMV infection.

Pregnant women were examined at the clinic in Bologna to confirm an active CMV infection. Two or 3 serum samples were sequentially obtained from each of these women at 2- to 4-week intervals. Young adults with CMV infectious mononucleosis were hospitalized, and serum was obtained 2 to 3 times at 2- to 4-week intervals. Transplant recipients were virologically (urine cultures) and serologically monitored for CMV infection weekly during the first 2 months after transplantation, at 2-week intervals during the third month, at monthly intervals until the twelfth month, and then periodically.

CMV Serology Assays

Conventional enzyme immunoassay and recombinant protein enzyme immunoassay for anti-CMV IgG was performed with a commercial kit (Enzygnost anti-HCMV/IgG EIA alpha method, Behring AG, Marburg, Germany). Plates were read on a microEIA automatic reader (Behring AG). Conventional and recombinant protein enzyme immunoassay for anti-CMV IgM was performed using the Enzygnost Anti-HCMV/IgM kit (Behring AG). Both kits were used and the results were interpreted as suggested by the manufacturers. For recombianant protein enzyme immunoassay, 0.1 µg per well of purified recombinant protein in bicarbonate buffer (pH 9.6) was used to coat enzyme immunoassay 96 well plates (A/S NUNC, Roskilde, Denmark). After an overnight incubation at 4^{o}C, plates were rinsed 3 times with phosphate-buffered sodium (PBS)-Tween

20 (sigma chemicals, St. Louis Missouri) (0.05%) and incubated with bovine serum albumin, (BSA) (1%) in bicarbonate buffer (pH 9.6). After 1 hour of incubation at room temperature, wells were rinsed 3 times with PBS-0.05% Tween 20 and then incubated at 37°C for 2 hours with human serum samples used at a dilution of 1:100 in PBS (50 µL final volume per well). After 3 washes with PBS-Tween 20, peroxidase-conjugated antihuman γ or µ chain antibodies were added to wells, and plates were incubated at 37°C for 1.5 hours. After 3 washes with PBS-Tween 20, the presence of antibody was detected by the addition of chromogen tetramethylene benzidine dihydrochloride (TMB). The reaction was stopped after 1 hour by the addition of sulfuric acid (0.5 N), and the results were read on the MicroEIA reader. For each sample, the immunoreaction level was determined as the difference (recombinant antigen minus the control fusion protein antigen) in readings at the absorbance (OD_{450}). The cutoff value for each fusion protein was set at a value higher than any value from 46 sera that were CMV IgG-seronegative by either conventional enzyme immunoassay or immunoblot analysis.

Six standard sera whose reactivity was previously assayed in 4 independent experiments were included to perform linear regression analyses and to standardize each run. Runs were considered acceptable when the values of the internal control sera were within an interval defined by 2 SD around the mean. Immunoblot assays were perfomed by a recently developed modification [31] that allowed the convenient addition of 3 recombinant CMV proteins (ppUL32, ppUL44, and ppUL57), as well as a positive (human γ or µ chains) and a negative (GST or CKS) control to blots of CMV-infected cell extracts. Statistical evaluation of data employed Statview 5.0.1 (SAS Institute, Cary, NC) run on a Macintosh computer.

RESULTS

In order to investigate the presence of IgG antibody directed against LP proteins in a large number of serum samples, 6 predicted latent gene products as well as 2 groups of immunogenic PP proteins were evaluated in parallel by recombinant protein enzyme immunoassay. The serologic response to each of the 6 LP proteins (ORF55, ORF45, and ORF94 from sense transcripts; ORF59, ORF154, and ORF152 from antisense transcripts) were compared with 6 PP fusion proteins (nonstructural proteins IE1, ppUL44, and ppUL57; structural proteins ppUL83, pUL80a, and ppUL32) that had been the subject of previous detailed evaluation [13,14,17,20,23,24,32]. The immune response to these proteins was investigated in 409 total serum samples derived from both healthy individuals and patients in different clinical settings. For comparison and to provide a range of examples, the immunogenicity of the CMV PP proteins chosen for comparison was known to vary and to represent a range of possible response patterns. The nonstructural proteins chosen for inclusion included IE1, which elicits a poor humoral response [17,32]; ppUL44, which elicits a strong and lasting response [23,24]; and ppUL57, which elicits mainly an IgM response [20]. The structural proteins and protein fragments chosen for comparison (ppUL83, pUL80a, and ppUL32) include the most immunogenic antigens in the viral particle [13]. The fragment ppUL32, in particular, is known to elicit the strongest and longest lasting antibody response in a large number of infected individuals [14,22].

Initially, 46 human sera that were CMV IgG-negative by both conventional enzyme immunoassay and immunoblot assay were used to determine background reactivity of each fusion protein in an enzyme-linked immunosorbent assay (ELISA). The background values for different proteins ranged from 80 to just over 200 OD_{450} units. Overall, predicted LP proteins exhibited a significantly lower overall background reactivity than did PP proteins when compared by the Mann-Whitney test (P = <.0001). Initially, a cutoff value for each recombinant fusion protein was established as described in "Materials and Methods", using the highest background value observed for each protein in the large group of CMV-seronegative human sera. Figure 2 shows that cutoffs, which in most cases represented a cluster of several sera, were lower for LP than PP proteins because of a lower overall background reactivity with LP proteins. The proportion of ELISA readings above the cutoff ranged from 8% to 44% for different LP proteins (ORF55, 33%; ORF45, 25%; ORF94, 44%; ORF59, 8%; ORF154, 22%; and ORF152, 11%), with ORF55 and ORF94 exhibiting the highest percentage as well as the most significant difference from controls as determined by the Mann-Whitney test (P = <.0001 and P = .02, respectively) (Figure 2). Although this statistical evaluation is difficult to apply in settings where only a small percentage of individuals respond, such responses to antigens can certainly be reproducible and informative [18].

The authors believe that the small differences in the distribution observed with other LP proteins was attributable to the very small numbers of CMV seropositive sera that recognize these antigens, and supports the use of the cutoff as an operational measure of specificity. The proportion of ELISA readings above the cutoff ranged from 11% to 78% for different PP proteins (IE1, 25%; UL44, 47%; UL57, 11%; UL83, 19%; UL80A, 36%; and UL32, 78%), which was as expected from previous work [13]. Four of the PP proteins, but only 2 of the LP proteins, exhibited significant differences when reactivity with CMV-seropositive sera was compared with control sera by use of the Mann-Whitney test. As further evidence that reactivity above the cutoff was specific, sentinel samples of sera that exhibited reactivity with a particular LP protein were subjected to adsorption with either the GST or GST-LP fusion protein for 1 hour at 4°C. In every sample tested, ELISA reactivity toward an LP protein was removed by the homologous antigen but not by GST alone (data not shown). This supports the conclusion that responses to some latent antigens were stronger than responses to others, but that a subpopulation of infected individuals mount a humoral response to each of the LP proteins.

Comparison of Reactivity of Sera From Long-Term Seropositive Individuals (Igg+/Igm–) With Sera From Individuals Undergoing Active or Recent Infection (IgG+/IgM+)

Individual LP proteins were recognized by at least 8%, but no more than 44%, of serum samples from long-term seropositive individuals (IgG+/IgM– by conventional enzyme immunoassay) (Figures 2 and 3). Products of sense

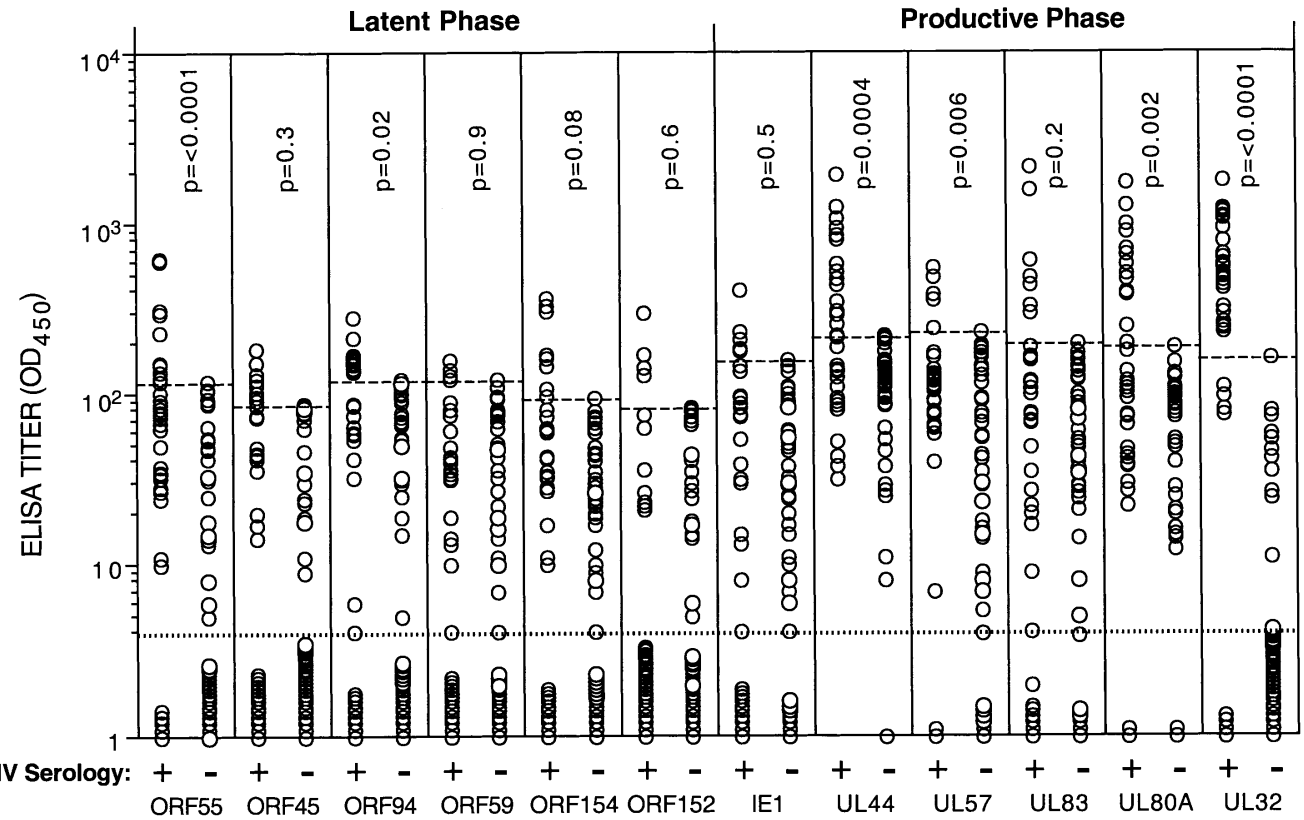

Figure 2. *Antibody responsive to LP and PP fusion proteins. Each antigen was assayed for IgG responses against sera from 46 seronegative (the first group in the subheading Human Sera Samples in "Materials and Methods") and 36 seropositive (second group) individuals as determined by conventional enzyme immunoassay and immunoblot assay and described in "Materials and Methods." The cutoff values (dashed lines) were set above the highest OD_{450} values obtained with CMV-negative serum samples. The nonparametric Mann-Whitney test was used to compare the ranks of the seronegative and seropositive data, with the resulting P value indicated above the scatter plot. The dotted line represents the limit of detection of the assay.*

transcripts (ORF94, ORF55, and ORF45) were recognized by the highest percentage of sera (44%, 31%, and 25%, respectively), whereas products of antisense transcripts (ORF154, ORF152, and ORF59) were recognized by fewer CMV-seropositive individuals (25%, 11%, and 8%, respectively). The level and breadth of reactivity to these antigens was reminiscent of responses to nonstructural proteins IE1, ppUL57, and ppUL54 [17,18] (Figure 2), but was not as strong as occurred with highly immunogenic antigens ppUL44, ppUL83, and ppUL80A or the broadly recognized antigen ppUL32 (Figure 2). A total of 85% of the sera from CMV-seropositive individuals recognized at least 1 LP protein, but none recognized the entire set of 6 (data not shown). In contrast to LP proteins, but as expected from previous analyses [13,14,17,20,23,24,32], a larger percentage of long-term seropositive individuals had antibody responses to PP proteins ppUL32, ppUL44, and ppUL80a, with a total of 97% of serum samples detecting at least 1 PP protein; 1 sample recognized all 6 proteins (data not shown).

Using the cutoff approach for each of the antigens, the serologic responses to predicted LP proteins was found to be less prevalent in individuals experiencing a recent active CMV infection (IgG+/IgM+) than in long-term seropositive individuals (Figure 3). Within the group of CMV IgG+/IgM+ samples, IgG responses to ORF94 and ORF45 were the most dramatically reduced (8% and 5%, respectively) when compared with the group of IgG+/IgM- samples (44% and 25%, respectively). As was the case for IgG+/IgM– sera, ORF55 exhibited a high prevalence as well as the most significant difference in IgG+/IgM– samples when compared with controls when analyzed by the Mann-Whitney test (P = <.0001), and 2 other proteins exhibited a clear significant difference by this rank test (ORF45, 0.01; ORF154, 0.002). Only about 50% of the IgG+/IgM+ serum samples detected any 1 LP protein, and none recognized all 6 proteins (data not shown), suggesting that antibody to LP proteins was slow to develop following active viral infection. The sera that was reactive with LP proteins did not contain any detectable LP protein-specific IgM, whereas PP proteins were recognized by specific IgM as well as IgG in many sera (data not shown).

Overall, the serologic response to LP proteins in individuals with acute CMV infection appears to be low. Consistent with previously published data [13], the serologic response to PP proteins was variable (Figure 3). As expected [23,24], reactivity to ppUL44 was widespread (80%), and the percentage of sera reacting with ppUL57 and ppUL83 was also more prevalent among IgG+/IgM+ samples (33%

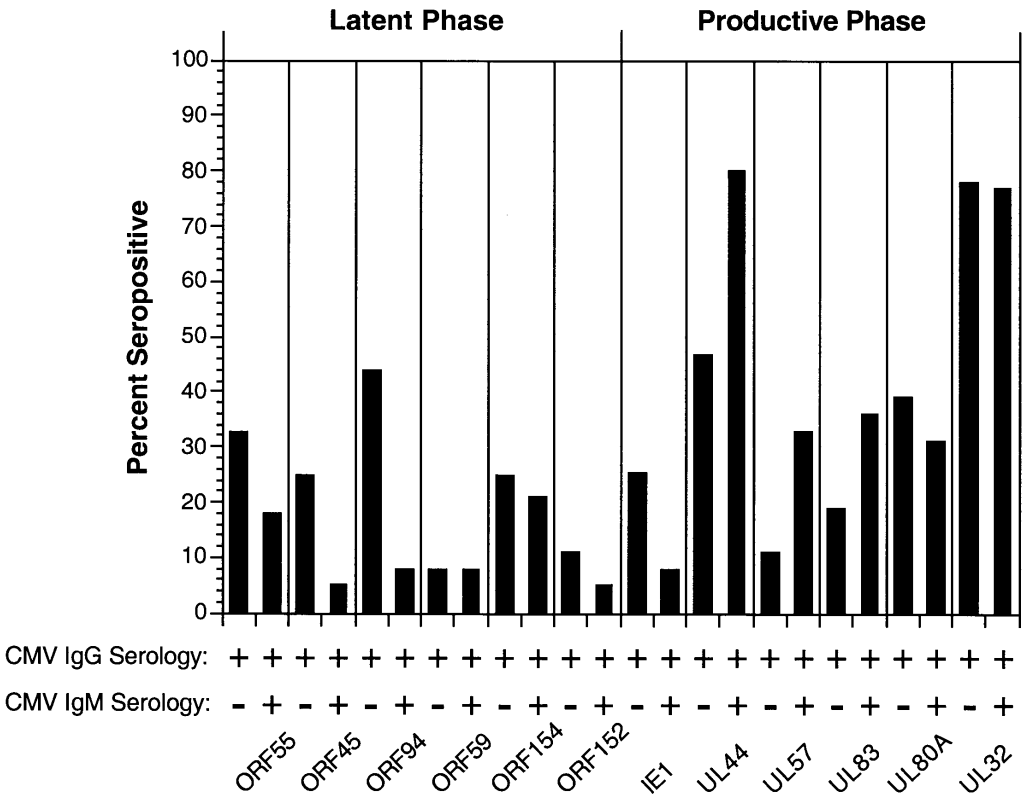

Figure 3. *Percentage of serum recognizing LP and PP fusion proteins in long-term seropositive individuals and in individuals with acute or recent infection. There were 36 serum samples from long-term seropositive individuals (IgG+/IgM–, second group under Human Sera Samples in "Materials and Methods") and 39 serum samples from individuals with acute or recent infection (IgG+/IgM+, third group) that were assayed by ELISA for reactivity with LP and PP fusion proteins.*

and 36%, respectively) as opposed to IgG+/IgM– samples (11% and 19%, respectively). A total of 97% of serum samples detected at least 1 PP protein and 2 sera recognized all 6 PP proteins (data not shown). These results suggest that the immune response to LP antigens develops very slowly following active infection, a possible result of low or delayed expression relative to PP antigens.

Reactivity With Sera From Vir ologicall y Confirmed Primar y and Secondary Inf ection

To further investigate the level of serologic response to individual LP proteins, reactivity to LP and PP proteins was followed in sera of individuals undergoing virologically confirmed primary and secondary CMV infections. One group consisted of 185 sera from 29 immunocompetent and 13 immunocompromised individuals undergoing a confirmed primary infection, and the other group consisted of 149 samples from 7 immunocompetent and 12 immunocompromised individuals undergoing secondary infection. Less than 10% of sera from immunocompetent individuals with ongoing primary CMV infection recognized any of the LP proteins, even when these individuals were followed for 1 to 3 months after infection (data not shown). Consistent with their seroconversion by conventional enzyme immunoassay, 80% eventually developed antibody to 1 or more PP protein (Figure 4). When CMV-seronegative individuals who had received a heart or kidney from a CMV-seropositive donor were followed for 2 years, all patients experienced CMV infection and all seroconverted to 1 or more PP proteins within the first 9 months (mean, 3.5 months; range, 1-9 months after transplant). In contrast, seroconversion to LP proteins was delayed and appeared in only 7 patients (58%) after a very long interval (mean, 12 months; range, 2-26 months after transplant). In 5 of these cases, antibody to LP proteins was first detected more than 10 months after active virus infection (assessed by antigenemia assay or virus culture) and seroconversion to PP proteins occurred months earlier, suggesting that the seroconversion to LP proteins might not have been linked to the levels of viral replication, as was seroconversion to PP proteins. Following primary infection, the antibody levels to LP proteins among those who seroconverted reached 2 to 6.5 times the cutoff values in a large majority of individuals, with the highest antibody titers observed to ORF59 and ORF154 (data not shown). The LP products reacted with a smaller percentage of sera (from a low of 1% for ORF55 to a high of 14% for ORF45; Figure 4) than PP products (from a low of 7% for IE1 to a high of 83% for ppUL44; Figure 4), and none of these serum samples reacted with all 6 LP or all 6 PP proteins (data not shown).

Following a secondary infection, ORF59 was the most widely recognized LP protein (48% of sera), followed by ORF55 and ORF154 (37% and 25% of sera, respectively);

however, none of the serum samples reacted with all LP proteins (data not shown). The antibody levels among seroconverters to LP proteins varied from 2 to 13 times the cutoff, with the highest being observed against ORF59 and ORF154 (data not shown). As expected, the broadest reactivity to PP proteins was to ppUL32 (96% of sera), followed by ppUL80a (70% of sera) and ppUL44 (63% of sera); however, none of the serum samples reacted with all 6 PP proteins (data not shown).

Presence of Antibody to Predicted LP Proteins in Commercial Gamma Globulin Preparations

In 2 cases of primary infection in transplant recipients, the presence of IgG to LP and PP proteins was detected immediately following transplantation and before any sign of CMV infection. Clinical records indicated that both patients had been treated with anti-CMV hyperimmune gamma globulins that have measurable levels of antibody to PP proteins. To determine whether commercially available IgG preparations contained antibodies to LP proteins, 2 different preparations (Cytotek from Biotest, Marburg, Germany, and Endobulin, from Immuno AG, Vienna, Austria) were evaluated. Both preparations showed reactivity to at least 1 LP protein. One in particular (Biotest), which was described as hyperimmune for CMV by the manufacturer, showed the presence of antibodies to all 3 predicted LP proteins (ORF45, ORF94, and ORF55) encoded by sense transcripts and 1 protein (ORF154) from antisense transcripts, as well as antibodies to 4 (ppUL44, UL57, ppUL83, and ppUL32) of 6 PP proteins (data not shown). The nature of the sera used to produce this batch of gamma globulin is not known, but it is expected that long-term seropositive individuals predominated in the proportion of donors who were seropositive. Thus, commercial preparations of gamma globulins used for immunoprophylaxis of CMV contain antibodies to both LP and PP proteins.

DISCUSSION

This study analyzed the level of the humoral immune response to 6 proteins, made in bacteria as GST-fusion proteins, predicted to be encoded by latency-associated ORFs, 3 from sense (ORF55, ORF45, and ORF94) and 3 from antisense (ORF59, ORF154, and ORF152) transcripts. The results indicate that specific antibody to all 6 LP proteins is induced in a subset of individuals infected with CMV, indicating that all of these proteins are expressed during natural infection. Seroconversion to LP proteins was delayed following the resolution of active CMV infection in immunocompromised individuals. Direct detection of PP antigens by antigenemia or viral DNA by PCR are effective means to identify active CMV infection [33], and serologic assays for PP antigens provide a useful way to identify long-term, latently infected individuals in the population. Serologic responses to latency-associated proteins do not appear to rise within a time frame that would be useful in any clinical setting. The response to these proteins appeared more variable in both strength and breadth than the response to PP

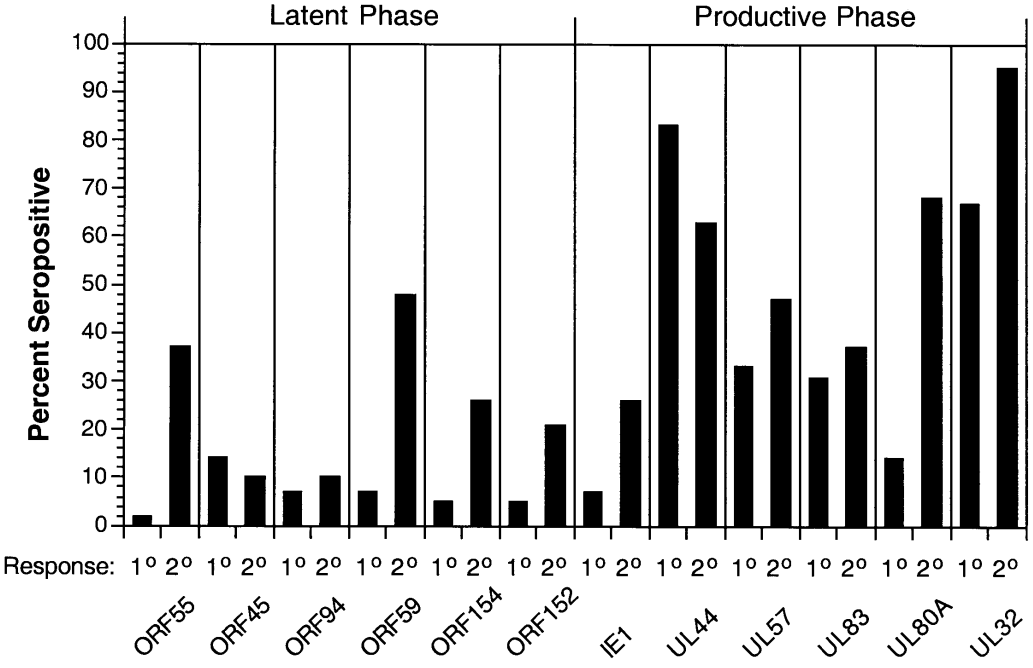

Figure 4. *Percentage of individuals undergoing primary and secondary CMV infection whose serum show reactivity to LP and PP fusion proteins. There were 85 sera from 29 immunocompetent and 13 immunocompromised subjects undergoing primary CMV infection (fourth group under Human Sera Samples in "Materials and Methods"), and 149 samples from 7 immunocompetent adults and 12 immunocompromised individuals undergoing a secondary CMV infection (fifth group) that were assayed by ELISA for reactivity with LP and PP fusion proteins. A patient was considered seropositive to a particular LP or PP if any of multiple serum samples obtained from that individual recognized the antigen above the cutoff. These results represent the percentage of patients with antigen-specific antibody.*

proteins chosen for comparison in healthy seropositive individuals as well as in individuals who had undergone active CMV infections. ORF55 and ORF154 induced the strongest antibody response of LP proteins, and ORF55 and ORF94 induced a response in the greatest number of individuals. In terms of breadth and level of response, LP proteins induced a response that was most similar to PP proteins IE1, ppUL57, and ppUL83 rather than more highly immunogenic PP proteins. As was the case for weakly immunogenic PP proteins, the breadth of responses to individual LP proteins appears to be too limited to consider these antigens as markers to identify latently infected individuals, although the fact that 85% of sera reacted to any 1 protein suggests that they should be evaluated as a group. The presence of a serologic response to LP proteins does not appear to be predictive of viral reactivation or disease, and screening for this response in donors would not likely provide any benefit.

The percentage of individuals with antibodies to any 1 LP protein varied from a minimum of 5% to a maximum of 48%, and only an IgG response was detected. As observed here for LP proteins, previous evaluation of the PP protein ppUL54 (viral DNA polymerase) [18] had revealed an IgG response in the absence of an IgM response. When the total number (409) of CMV-seropositive sera tested are considered, most of the LP protein-positive samples also recognized at least 1 PP protein (146 of 409), although a significant number of PP protein-positive sera did not recognize any of the LP proteins (199 of 409), and many failed to recognize any of the 12 recombinant CMV proteins at all (58 of 409). Very few sera failed to recognize PP proteins and yet recognized 1 of the LP proteins (6 of 409). Thus, sera that recognize LP proteins also typically contain antibody to PP proteins.

On follow-up examination, transplant recipients were found to develop antibodies to LP protein with a significantly delayed time course relative to PP proteins. There was not sufficient data to undertake a similar evaluation in immunocompetent subjects because they were not followed for a sufficient period of time. However, the delayed antibody response may not be unique to LP proteins. Schoppel et al. [32] described a 2- to 4-month delay in the development of antibody to CMV envelope glycoproteins relative to some of the same PP proteins used in the current study following primary infection in immunocompromised and immunocompetent individuals. The delay that was observed in responses to LP proteins was more pronounced than that reported to occur with envelope glycoproteins. Direct comparisons are complicated by the fact that the response to any PP glycoproteins was not investigated in the current study. The significant delay in detecting an LP protein-specific immune response is consistent with their expression during prolonged latency rather than during productive infection; however, this may also have resulted from lower levels of expression or a reduced immunogenicity of this class of proteins relative to PP proteins. It is also possible that individuals who develop antibodies to LP proteins over a prolonged 2-year period may also have experienced additional undetected CMV infections that boosted their response. The detection of a greater percentage of sera with reactivity for LP proteins in individuals who had experienced a secondary infection supports the suggestion that a booster effect can occur.

In conclusion, this work establishes the presence of antibody to LP proteins in both healthy long-term seropositive individuals as well as in some individuals undergoing active or recent infection. These results support the detection of transcripts from this region of the CMV genome in a large fraction of the population [8], and are consistent with the detection of these protein expressions during natural infection.

ACKNOWLEDGMENTS

The authors would like to thank Sohail Jarrahian and Melanie Miller (Fred Hutchinson Cancer Research Center and University of Washington, Seattle, WA) for preparing PEQ593 (ORF55), and G. T. Maine (Abbott Laboratories, Abbott Park, IL) for supplying construct 32 (UL57). This work was supported by grants from the Italian Ministry of Public Health, Istituto Superiore di Sanita', Progetto Sangue and Progetto AIDS (MPL), and by US Public Health Service grants RO1 AI26672 (APG) and Project IX of PO1 CA49605 (ESM).

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