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Recombinant Mono- and Polyantigens To Detect Cytomegalovirus-Specific Immunoglobulin M in Human Sera by Enzyme Immunoassay

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Serological detection of human cytomegalovirus (HCMV)-specific antibody varies greatly because of antigen composition and the lack of antigen standardization. Antigenic materials composed of single well-characterized viral proteins or portions of them, produced via molecular biology, have proven to be promising tools in improving serodiagnosis. We constructed a recombinant protein containing two regions of ppUL32 (p150) and half of ppUL44 (p52) and compared the immunoglobulin M (IgM) reactivity of this triple-antigen fusion protein with that of a double-antigen fusion protein containing the two ppUL32 fragments and that of a monoantigen fusion protein containing half of ppUL44. We also constructed and tested two other monoantigen fusion proteins containing a large fraction of ppUL80a and a fraction of ppUL83. More than 700 serum samples from different groups of immunocompetent and immunosuppressed subjects were tested for the presence of HCMV IgM by recombinant enzyme immunoassay (rec-EIA) and by a commercially available EIA. Western blotting (immunoblotting) and (in the case of immunosuppressed individuals) antigenemia tests by immunofluorescence and PCR of polymorphonuclear leukocytes were also carried out. The results obtained demonstrate that (i) the triple-antigen fusion protein can replace the individual proteins; (ii) the triple-antigen fusion protein cannot be used alone to replace the virus or infected cells in the serological detection of anti-CMV IgM; (iii) the addition of the fusion proteins containing portions of ppUL83 and ppUL80a is essential for the formation of an antigenic mixture that can replace the virus for the search of HCMV-specific IgM; (iv) rec-EIA is very specific and is more sensitive than the commercially available EIA, and the results obtained are consistent with those obtained by Western blotting; and (v) rec-EIA can reliably be used to detect HCMV-specific IgM in different groups of patients with active HCMV infection.

Human cytomegalovirus (HCMV) is an ubiquitous herpesvirus in humans. It is rarely pathogenic in healthy adults, but it is associated with several diseases in immunocompromised individuals (such as human immunodeficiency virus-infected people and transplant recipients). Furthermore, HCMV is the most common cause of congenital infection in humans. Primary intrauterine HCMV infections are second only to Down's syndrome as a known cause of mental retardation (for a review, see reference 8). Because infections either are asymptomatic or are accompanied by symptoms that are not specific for HCMV (such as fever and leukopenia), laboratory techniques are the sole means of diagnosing acute HCMV infection. Diagnosis of HCMV infection can be obtained by direct demonstration of the virus or virus components in pathological materials or indirectly through serology (13). Diagnosis of primary HCMV infection is exclusively accomplished by serological methods. HCMV-specific immunoglobulin M (IgM) is a sensitive and specific indicator of primary HCMV infection in immunocompetent subjects, while it is very often produced during viral reactivation in transplant recipients (2, 21). However, detection of HCMV-specific IgM varies widely, and poor agreement has been found among the results obtained with different commercially available kits (18). Antigenic materials composed of single well-characterized viral proteins or portions of them, produced via molecular biology or peptide chemistry, have proven to be promising tools for improving HCMV serology (for a review, see reference 13). The aim of the present work was the large-scale testing of the most promising recombinant antigens for the detection of HCMV-specific IgM in sera from different groups of subjects.

The analysis of the humoral immune response elicited during natural infection has repeatedly shown that the basic phosphoprotein of 150 kDa encoded by UL32 (ppUL32) (9) and localized in the viral tegument is highly immunogenic and is recognized by sera from nearly 100% of the HCMV-seropositive subjects tested (10, 16). In this molecule at least two epitopes have been identified and shown to react efficiently with human immunoglobulins. In particular, the analysis of several ppUL32 fusion proteins showed that a region localized in the last 43 amino acids at the carboxy terminus of the molecule (amino acids 1006 to 1048) reacts with more than 80% of IgM-positive serum samples (17, 27). When chemically synthesized, another region localized between amino acids 595 and 614 gave a positive reaction with almost 100% of IgG-positive serum samples (22). When it is expressed as recombinant protein, this region also reacted efficiently with HCMV-specific IgM (27). The two coding regions were recently fused together and were shown to produce a double-epitope fusion protein which can replace the entire p150 molecule in its IgM-binding ability (28). Another HCMV protein which reacts very well with IgM is ppUL44 (14, 26, 33), the nonstructural DNA-binding protein of 52 kDa. The carboxy-terminal part of the molecule was chosen because it does not contain relevant amino acid sequences cross-reacting with the homologous protein of other members of the *Herpesviridae* family (12). The carboxy-terminal half of this protein was expressed and tested

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with human sera alone and in combination with the double-ppUL32 epitope fusion protein. Besides ppUL32 and ppUL44, two other HCMV proteins were chosen: the major matrix protein ppUL83 and the assembly protein (ppUL80a). The first is known to induce a strong IgM response, and antibodies reacting exclusively to this protein were described during primary infection (15). The second protein was included in the study because in HCMV-seropositive transplant recipients undergoing viral reactivation, IgM to this protein is very often the first marker to be detected (12a).

Fusion proteins were purified and used in an enzyme immunoassay (EIA) to detect IgM antibodies in more than 700 serum samples from healthy individuals and from subjects with different clinical conditions.

MATERIALS AND METHODS

Virus propagation and preparation of viral cDNA. HCMV AD169 was propagated in human embryo fibroblasts by standard methods. Five days after infection at a multiplicity of infection of approximately 1 PFU per cell, the infected cells were harvested, washed with phosphate-buffered saline, and homogenized with a glass-Teflon homogenizer. Total RNA was isolated from the homogenized cells by using an RNA isolation kit (Stratagene Cloning Systems, La Jolla, Calif.), and poly(A)$^+$ RNA was isolated by using an mRNA isolation kit (Pharmacia Biotech, Piscataway, N.J.). HCMV cDNA was synthesized from purified viral mRNA by using a ZAP-cDNA Synthesis Kit (Stratagene Cloning Systems).

Cloning and expression of HCMV genes. All HCMV gene fragments encoding antigens were obtained by PCR amplification with PCR primers designed to amplify specific nucleotide sequences. These gene fragments were cloned into an *Escherichia coli* CMP–2-keto-3-deoxyoctulosonic acid synthetase (CKS) expression vector as described previously (4), resulting in the expression of the HCMV polypeptides in fusion with CKS. Standard hot-start PCR (Perkin-Elmer, Norwalk, Conn.) was used to generate the HCMV DNA fragments encoding fusion proteins 1A, 3B, 4, and 26, and DNA plasmids pMB33 (28), pMB33 (26), pMB33 and pMB13 (26, 28), and pMB38 (13a), respectively, were used as templates. Nested hot-start PCR (Perkin-Elmer) was used to amplify the UL83 fragment encoding fusion protein 9, and viral cDNA was used as template DNA. The DNA sequences of all cloned HCMV genes were determined and confirmed. Bacterial clones expressing HCMV fusion proteins and the control bacterial strain expressing unfused CKS were grown to the log phase in rich medium containing ampicillin, and the synthesis of the CKS-HCMV fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside as described previously (29). After 4 h postinduction, the cells were harvested and the cell pellets were stored at −80°C until protein purification.

Purification of recombinant CKS-HCMV fusion proteins. Insoluble CKS-HCMV fusion proteins (1A, 3B, 4, and 9) were initially purified after lysis by a combination of detergent washes and then solubilization in 8 M urea (29). After solubilization in 8 M urea, the fusion proteins were purified by Q-Sepharose chromatography (Pharmacia Biotech). Fusion proteins 4 and 9 were subjected to additional purification by Sephacryl S-200 chromatography (Pharmacia Biotech). Soluble CKS-HCMV fusion protein 26 was purified after cell lysis by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a Bio-Rad Prep Cell (Bio-Rad, Richmond, Calif.). Soluble CKS protein was purified after cell lysis by ammonium sulfate precipitation and then DEAE chromatography.

Human serum samples. Many groups of human serum samples were used in the present work. The first group of samples, which was used for the determination of the cutoff values, consisted of 210 serum samples from blood donors (n = 150) and healthy adults (n = 60). While 68 serum samples were IgG and IgM negative, 142 serum samples were IgG positive and IgM negative, as judged by both a commercially available EIA (conv-EIA) and Western blotting (immunoblotting). The second group of serum samples consisted of 150 HCMV-positive serum samples from immunocompetent subjects (50 with a high IgM titer to HCMV, as detected by conv-EIA, 50 with a medium level of HCMV-specific IgM, and 50 with a low level of HCMV-specific IgM). The presence of HCMV-specific IgM in this group of sera was determined by conv-EIA and was confirmed by Western blotting. A third group of serum samples consisted of 51 serum samples from pregnant women (18 were from HCMV-uninfected women, 26 were from HCMV-infected women who did not transmit the infection to their offspring, and 7 were from HCMV-infected pregnant women who transmitted the infection to their offspring). All of these serum samples were obtained between 22 and 24 weeks of gestation. We also tested a group of 35 serum samples from 35 newborns. Six serum samples were obtained during the first week of life from 6 congenitally infected newborns; 19 serum samples were from newborns (1 to 8 months of life) from whom the virus was repeatedly isolated from their urine. Twelve newborns had different symptoms (maculopapular rash, encephalopathy, growth delay); the others were asymptomatic shedders. Among the 35 serum samples obtained from newborns, only 2 were from the newborns of the pregnant women studied. Finally, 10 serum samples were from HCMV-uninfected newborns during the first 8 months of life. Another group of samples consisted of 104 serum samples from 23 transplant recipients (20 heart and 3 kidney transplant recipients) who were found to have HCMV infection during the first 3 months after transplantation. Eight patients had a primary infection, while infection in 15 patients was a result of viral reactivation. The last group of serum samples consisted of 200 serum samples randomly selected among sera from renal transplant recipients (n = 150) and pregnant women (n = 50) which came to our diagnostic laboratory for HCMV monitoring after transplantation and during pregnancy.

Determination of HCMV infection in pregnant women and newborns. HCMV infection in pregnant women was determined by one or more of the following parameters: virus isolation from urine, saliva, or blood or seroconversion for anti-HCMV antibodies. A congenital HCMV infection in a newborn was determined by HCMV isolation from urine during the first week of life. An HCMV infection in newborns was determined by virus isolation from urine.

Determination of HCMV infection in transplant recipients. HCMV infection in transplant recipients was determined by the presence of pp65-positive polymorphonuclear leukocytes (PMNLs) (antigenemia) in peripheral blood, as determined by indirect immunofluorescence, and the presence of the HCMV genome in the same cells, as determined by PCR.

HCMV serology. (i) Conv-EIA. The evaluation of anti-HCMV IgG was carried out with a commercially available kit (Enzygnost anti-HCMV/IgG EIA alpha method; Behring AG, Marburg, Germany). Plates were read on a microEIA automatic reader (Behring AG). The evaluation of anti-HCMV IgM was performed by using the Enzygnost anti-HCMV/IgM kit (Behring AG). Both kits were used, and the results were interpreted as suggested by the manufacturers. IgM-positive sera were divided into three groups on the basis of the EIA titer. In particular, sera with optical densities (ODs) of between 210 and 400 were considered to have a low IgM titer, those with ODs from 401 to 800 were considered to have a medium titer, and those with ODs greater than 800 were considered to have a high IgM titer.

(ii) Western blotting. Protein extracts from purified viral particles (Towne strain) were run in a 9% acrylamide gel, and the electrophoretically separated polypeptides were then transferred to nitrocellulose paper. Infection of cells, virus purification, protein extraction, blotting, and immune reaction with sera were done as previously described in detail (16).

(iii) Rec-EIA. Recombinant EIA (rec-EIA) was performed as described previously (25) with the reagents from the commercially available Enzygnost anti-HCMV/IgG and anti-HCMV/IgM kits (Behring AG). For each sample the immunoreaction level was considered to be the difference between the measured absorbances (absorbance of the recombinant antigen − absorbance of the control antigen) that were obtained. The cutoff value for each individual fusion protein was determined by testing 210 serum samples that did not contain IgM to HCMV (determined by both conv-EIA and Western blotting). For the determination of IgM antibodies, the rheumatoid factor absorbent (Behring AG) was used to remove rheumatoid factor from the serum samples. To perform linear regression analysis and to standardize the test run, three serum calibrators were included on each plate. Reproducibility was controlled by including in each EIA plate six standard serum samples whose reactivities were previously assayed in four independent experiments. Test runs were considered acceptable when the value for the internal control serum sample was within an interval of 2 standard deviations from the mean value established previously.

Virological detection of HCMV. (i) HCMV isolation. The shell vial procedure (6) was used to isolate HCMV from urine and saliva. The inoculated cells were fixed 24 to 48 h after inoculation and were stained in an indirect immunofluorescence assay by using a monoclonal antibody reacting with the gene products of HCMV immediate-early genes 1 and 2 (E13; Bioline, Paris, France).

(ii) Antigenemia. The presence of HCMV pp65 (ppUL83) in PMNLs was determined as originally described by van der Biji et al. (32) and modified by Revello et al. (24) by using an HCMV pp65-specific pool of two monoclonal antibodies (Biotest, Frankfurt, Germany) in indirect immunofluorescence tests.

(iii) Determination of the presence of HCMV genome by PCR. Aliquots of 5 × 10^5 PMNLs were used for PCR assay. Sequences were from the fourth exon of the HCMV immediate-early I gene (*Eco*RI-J fragment of the AD169 strain) and correspond to nucleotides 1767 to 1786 and nucleotides 1894 to 1913. With these primers, a fragment of 147 bp was amplified. A third oligonucleotide consisting of nucleotides 1807 to 1847 was complementary to the antisense DNA strand in the region between the binding sites of the other oligonucleotides and was used for hybridization (11). PCR was carried out, and its specificity and sensitivity were determined as described previously (19).

RESULTS

Five different fusion proteins were obtained and used in the study. The first fusion protein (fusion protein 1A) carries the two ppUL32 major antigenic regions (amino acids 595 to 614 and 1006 to 1048) fused together. The second fusion protein (fusion protein 3B) carries the carboxy-terminal half of ppUL44 (amino acids 202 to 434). The third fusion protein (clone 4) carries three regions (two regions of ppUL32 and one

TABLE 1. Rec-EIA results obtained by testing 150 IgM-positive serum samples

No. of serum samples	HCMV serology[a]		No. (%) of serum samples positive for:						
	IgG	IgM	1A	3B	4	9	26	4 + 9 + 26	All fusion proteins
50	+	High	42	49	49	44	39	50	50
50	+	Medium	31	47	44	42	37	50	50
50	+	Low	29	40	42	31	32	47	48
Total (150)			102	136	135	117	108	147 (98)	148 (98.7)

[a] Sera were divided into three groups on the basis of IgM titers determined by EIA. All serum samples were confirmed to be positive for IgM by Western blotting. They were all IgG positive, as shown by EIA.

of ppUL44) fused together. Fusion proteins 9 and 26 carry large fragments of ppUL83 (amino acids 297 to 510) and ppUL80a (amino acids 117 to 373), respectively. These regions were chosen because of their high degree of reactivity to IgM, as determined in previous studies (14). As described in the Materials and Methods section, 210 human serum samples defined as IgM negative to HCMV by both conv-EIA and Western blotting were used to determine the rec-EIA cutoff values for each individual fusion protein. The cutoff values correspond to OD values higher than the maximum OD obtained among the 210 serum samples (they approximately correspond to the mean of the negative values plus 4 to 5 standard deviations).

To evaluate the sensitivity of the rec-EIA, 150 serum samples were selected among several hundred serum samples because they contained IgM to HCMV, as determined by the fact that they gave an IgM-positive result by both conv-EIA and Western blotting. As shown in Table 1, all serum samples with medium and high IgM titers to HCMV reacted with one or more fusion proteins. Among the serum samples with a low IgM titer, only two were not detected by any fusion protein. The reactivity is therefore greater than 98%. The highest degree of reactivity obtained was against fusion proteins 3B and 4, and the combination of proteins 4, 9, and 26 gave a reactivity of 98%. Only one serum sample did not react with this combination and was found to be reactive with fusion protein 3B alone.

After determining the sensitivity of rec-EIA compared with that of conv-EIA, we tested several serum samples obtained from different groups of subjects. Table 2 compares the results obtained by rec-EIA with a group of 51 serum samples from pregnant women with the results obtained by conv-EIA. Among 18 HCMV-uninfected pregnant women, none was found to be IgM positive by either conv-EIA or rec-EIA. On the contrary, among 26 HCMV-infected pregnant women who did not transmit the infection to their offspring, rec-EIA detected IgM in 20 women, while conv-EIA detected IgM in only 12 women. Among a group of eight HCMV-infected pregnant women who transmitted the infection to their offspring, all eight women were found to be IgM positive by rec-EIA and six were found to be IgM positive by conv-EIA. The highest degree of reactivity was always obtained with the triple-antigen fusion protein (fusion protein 4). The combination of proteins 4, 9, and 26 gave the maximum reactivity. As shown in Table 2, a significant difference in IgM titer to fusion protein 4 was observed between HCMV-infected pregnant women who did not transmit the infection (lower titers) and those who transmitted the infection (higher titers). While the antibody titer against clone 26 was also significantly different in the two groups, the differences in titers to fusion proteins 3B and 9 were not significant.

We also tested a group of 35 serum samples from newborns (Table 3). Among 10 HCMV-uninfected newborns, none was found to be IgM positive by either conv-EIA or rec-EIA. On the contrary, among six serum samples obtained from six congenitally infected newborns during the first week of life, two gave a positive reaction by rec-EIA and none gave a positive reaction by conv-EIA. The maximum IgM reactivity that was observed was specific for fusion proteins 9 and 26. In another group of 19 serum samples from newborns persistently excreting HCMV in urine during the first year of life (these newborns were not checked for HCMV at birth), rec-EIA detected IgM in 10 newborns, while conv-EIA detected IgM in only 2 newborns. The maximum IgM reactivity obtained was against fusion proteins 4 and 26, and the combination of fusion proteins 4, 9, and 26 gave the maximum reactivity.

Results of IgM detection by rec-EIA compared with that by conv-EIA in a follow-up study of a group of 23 transplant recipients with HCMV infection are provided in Table 4. The patients were also followed by the pp65 antigenemia test and

TABLE 2. Comparison between conv-EIA and rec-EIA with sera from pregnant women by determination of IgM titers to individual fusion proteins

Group of subjects[a]	No. of women	No. of positive samples							Conv-EIA	IgM titers (OD [10^3]) detected by rec-EIA with fusion protein:			
		Rec-EIA with fusion protein(s):											
		1A	3B	4	9	26	4 + 9 + 26	All		3B	4	9	26
HCMV-infected pregnant women who did not transmit the infection	25	1	15	19	9	10	20	20	12	461 (±211)	838 (±324)[b]	164 (±59)	487 (±220)[c]
HCMV-infected pregnant women who transmitted the infection	8	1	6	8	3	6	8	8	6	724 (±344)	1,450 (±379)	191 (±52)	1,064 (±490)
HCMV-uninfected pregnant women	18	0	0	0	0	0	0	0	0	ND[d]	ND	ND	ND

[a] Samples were obtained at 22 to 24 weeks of gestation.
[b] $P = 0.012$.
[c] $P = 0.023$.
[d] ND, not done.

TABLE 3. Comparison between conv-EIA and rec-EIA with sera from newborns

Group of subjects	No. of newborns	No. of positive samples							
		Rec-EIA with fusion protein(s):							Conv-EIA
		1A	3B	4	9	26	4 + 9 + 26	All	
Congenitally infected newborns[a]	6	0	1	1	2	2	2	2	0
Newborns excreting HCMV during the first year of life[b]	19	0	4	7	1	7	10	10	2
HCMV-uninfected newborns[b]	10	0	0	0	0	0	0	0	0

[a] Sera were obtained 1 to 7 days after birth.
[b] Sera were obtained between 1 and 12 months after birth.

by the detection of the HCMV genome by PCR in PMNLs. PCR detected HCMV infection in all 23 patients, the antigenemia test and rec-EIA detected HCMV infection in 22 patients, and conv-EIA detected HCMV infection in 17 patients (data not shown). No false-positive reactions occurred by the rec-EIA testing blood samples obtained from 21 patients before infection (these samples were all PCR and antigenemia test negative and were obtained 0 to 12 days after transplantation), while conv-EIA gave a false-positive reaction for one serum sample. At the very beginning of infection, when only PCR could detect the HCMV genome in PMNLs (eight serum samples from 14 to 40 days after transplantation), rec-EIA detected two patients who were IgM positive, while conv-EIA judged one patient to be IgM positive; the latter patient corresponds to the same patient in the previous group whose serum was false positive for IgM. When PCR positivity was accompanied by the antigenemia assay positivity (in 21 patients, from 10 to 78 days after transplantation), conv-EIA and rec-EIA detected IgM in 9 and 15 patients, respectively. Later during infection, when PCR positivity was still accompanied by positivity by the antigenemia assay (in 19 patients), conv-EIA and rec-EIA detected IgM in 9 and 17 patients, respectively. We obtained a third sample from 12 patients at 36 to 82 days after transplantation, and these samples were still positive by the antigenemia test and PCR. Among these samples, 11 and 8 samples were IgM positive by rec-EIA and conv-EIA, respectively. At the end of the acute phase of infection, when the antigenemia test gave a negative result and PCR was still positive (in 17 patients, 42 to 158 days after transplantation), conv-EIA and rec-EIA detected IgM in 14 and 15 patients, respectively. Finally, at the end of infection (69 to 185 days after transplantation) when PCR and the antigenemia test were both negative (we only had six patients), conv-EIA detected IgM in one patient, while rec-EIA detected IgM in 5 patients. The highest degree of reactivity obtained was against fusion protein 4, and the combination of fusion proteins 4, 9, and 26 gave the maximum number of positive reactions. Among the 23 transplant recipients with HCMV infection (8 with primary and 15 with secondary infections), 17 experienced symptomatic infection (8 with primary and 7 with secondary infections) and were treated with ganciclovir. The results of IgM detection by conv-EIA and rec-EIA at the time of symptoms are given in Table 5. In the first serum sample taken when symptoms appeared (17 patients), conv-EIA detected IgM in 9 patients, while rec-EIA detected IgM in 13 patients. The antigenemia test was positive for 13 patients, and PCR was positive for all 17 patients. At 3 to 7 days after the onset of symptoms, we obtained samples from 10 patients. PCR and the antigenemia test gave positive results for 10 and 8 patients, respectively, while conv-EIA and rec-EIA detected IgM in 7 and 9 patients, respectively. Also, in this case the highest degree of reactivity was obtained with fusion protein 4, and the combination of proteins 4, 9, and 26 detected the maximum number of positive samples.

In the same group of patients, we compared the ability of the antigenemia test and rec-EIA to be the first indicators of an active HCMV infection in relationship to the serological status of the donor and the recipient and the development of a symptomatic or asymptomatic infection. The antigenemia test detected three symptomatic infections earlier than rec-EIA, and rec-EIA detected three symptomatic infections and one asymptomatic infection earlier than the antigenemia test. Furthermore, among eight patients with primary infection, the antigenemia test detected four symptomatic infections earlier than rec-EIA, and rec-EIA detected one symptomatic infection earlier than the antigenemia test (data not shown).

Representative examples of the virological and serological

TABLE 4. Comparison between IgM reactivity detected by conv-EIA and rec-EIA, PCR, and antigenemia test for sera from 23 transplant recipients with HCMV infection

Serum samples[a]	Days after transplantation (mean)	No. of positive samples							
		Rec-EIA with fusion protein(s):							Conv-EIA
		1A	3B	4	9	26	4 + 9 + 26	All	
AG−/PCR−, obtained before infection (n = 21)	0–12 (5.3)	0	0	0	0	0	0	0	1
AG−/PCR+ at beginning of infection (n = 8)	14–40 (25.6)	1	1	1	2	1	2	2	1
First AG+/PCR+ (n = 21)	10–78 (42.2)	5	7	12	7	12	15	15	9
Second AG+/PCR+ (n = 19)	25–77 (51.9)	7	11	17	9	15	17	17	9
Third AG+/PCR+ (n = 12)	36–82 (58.9)	4	9	11	7	7	11	11	8
AG−/PCR+, at end of infection (n = 17)	42–158 (74.5)	7	11	14	7	10	15	15	14
AG−/PCR−, at end of infection (n = 6)	69–340 (185)	0	2	3	2	3	5	5	1

[a] The antigenemia test and PCR were carried out on PMNLs.; AG−, antigenemia test negative; AG+, antigenemia test positive; PCR−, PCR negative; PCR+, PCR positive.

TABLE 5. Presence of HCMV IgM at the time of symptoms in 17 transplant recipients with symptomatic HCMV infection

Samples[a]	No. of positive samples									
	Rec-EIA with fusion protein(s):							Conv-EIA	AG[b]	PCR[b]
	4	1A	3B	9	26	4 + 9 + 26	All			
First (n = 17)	11	4	10	7	9	13	13	9	13	17
Second (n = 10)	8	2	4	5	4	9	9	7	8	10

[a] First, the first serum sample obtained in coincidence with the appearance of symptoms; second, serum sample obtained 3 to 7 days later, during treatment.
[b] The antigenemia test (AG) and PCR were carried out on PMNLs.

monitoring of HCMV infection in transplant recipients is shown in Fig. 1.

From the data presented here, fusion protein 4 gave the highest degree of reactivity with IgM antibodies present in the sera of healthy adults, pregnant women, infected newborns, and transplant recipients. However, fusion protein 4 alone could not represent the entire complex of viral antigens reacting with anti-HCMV IgM. To determine the relative importance of the other fusion proteins in the overall IgM reactivity to the virus, we studied the different combinations of IgM-positive reactions to the five recombinant proteins with 253 serum samples that were judged to be IgM positive by rec-EIA. A total of 35.6% of the serum samples reacted with all five fusion proteins. It was interesting that 5.5% of the serum samples reacted exclusively with fusion protein 4, 3.2% reacted exclusively with fusion protein 26, 1.6% reacted exclusively with fusion protein 9, and only 0.4% reacted exclusively with fusion protein 3B (none reacted exclusively with fusion protein 1A) (data not shown). We also determined the cumulative IgM reactivity to the different fusion proteins. A total of 90.1% of the serum samples that were judged to be IgM positive by rec-EIA had IgM that reacted with fusion protein 4 and 99.6% had IgM that reacted with the combination of fusion proteins 4, 9, and 26. Only 1 of 253 IgM-positive serum samples did not react with this combination and gave a weak reaction exclusively to fusion protein 3B (data not shown).

Rec-EIA seems more sensitive than conv-EIA except for the samples with very low IgM titers (Table 1). Therefore, we

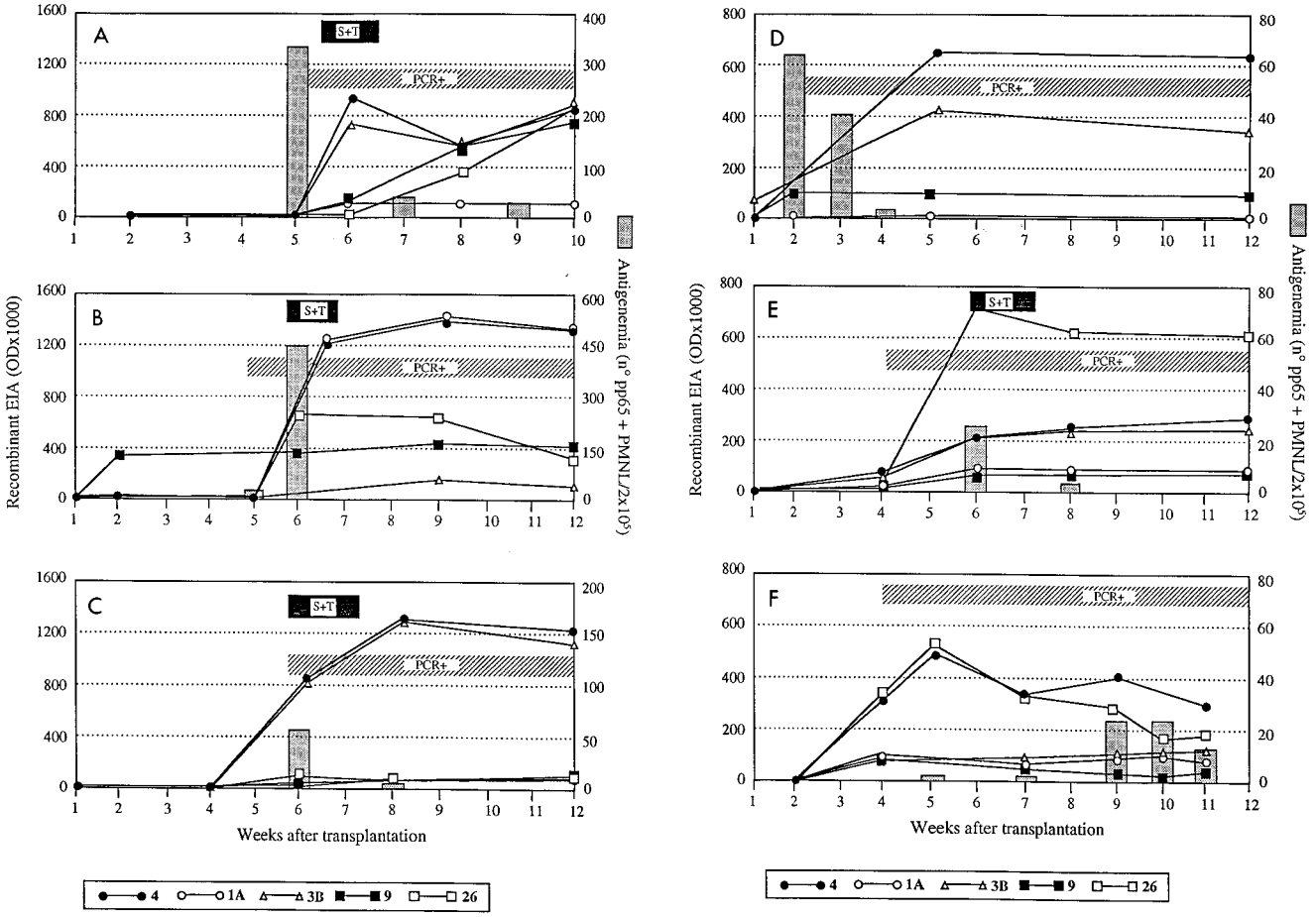

FIG. 1. Six representative examples of follow-up of transplant recipients with primary (A to C) and secondary (D to F) HCMV infection during the first 3 months after transplantation. (A and B) follow-up of kidney transplant recipients; (C to F), follow-up of heart transplant recipients. S+T, symptoms and ganciclovir treatment; PCR+, positive detection of HCMV genome in polymorphonuclear cells. ●, fusion protein 4; ○, fusion protein 1A; △, fusion protein 3B; ■, fusion protein 9; □, fusion protein 26.

TABLE 6. Comparison between conv-EIA and rec-EIA (fusion proteins 4, 9, and 26) and Western blotting with 200 serum samples randomly selected from renal transplant recipients and pregnant women

Western blotting result	No. of samples			
	Conv-EIA[a]		Rec-EIA[b]	
	Positive	Negative	Positive	Negative
Positive (n = 137)	86	51	136	1
Negative (n = 63)	2	61	0	63
Total (n = 200)	88	112	136	64

[a] Sensitivity, 86 of 137 = 63%; specificity, 61/63 = 97%.
[b] Sensitivity, 136/137 = 99.3%; specificity, 63/63 = 100%.

compared the reactivities of rec-EIA and conv-EIA with that obtained by Western blotting, which is a very sensitive and specific method of detecting HCMV-specific IgM (17, 18). A total of 200 serum samples randomly selected from among serum samples from renal transplant recipients (n = 150) and pregnant women (n = 50) were tested by rec-EIA by using fusion proteins 4, 9, and 26, by conv-EIA, and by Western blotting using polypeptides separated from purified viral particles. The results are given in Table 6. Among 200 serum samples, conv-EIA detected IgM in 88, rec-EIA detected IgM in 136, and Western blotting detected IgM in 137. Among the 88 serum samples found to be IgM positive by conv-EIA, 2 were not confirmed by either rec-EIA or Western blotting, suggesting that they were false positives. Among 112 serum samples that were judged to be IgM negative by conv-EIA, 50 were found to be positive by both rec-EIA and Western blotting. This indicates that those sera were false negative by conv-EIA. Finally, one serum sample judged to be IgM negative by both conv-EIA and rec-EIA gave a positive reaction by Western blotting. The protein that was recognized had a molecular mass of 150 kDa (data not shown).

DISCUSSION

Many recombinant antigens have been produced and characterized for their ability to bind to HCMV-specific antibodies. However, the exact composition of the antigenic mixture representative of the entire complex of HCMV antigens to be used for detecting HCMV antibodies is still a matter of study. Analysis of the humoral immune response elicited during natural HCMV infection has repeatedly shown that ppUL32 is highly immunogenic (10, 16, 23, 30). We recently produced a single-fusion protein containing the two ppUL32 antibody-binding regions. This double-recombinant antigen reacted with IgM in a way that is representative of the reactivity obtained with the entire ppUL32 (25, 28).

However, IgM reactivity to ppUL32 is not representative of the whole IgM reactivity to the entire complex of HCMV proteins. Therefore, to the double ppUL32 epitope fusion protein we have now added a large portion of ppUL44, which is another HCMV protein which reacts very well with IgM. The triple-epitope fusion protein (fusion protein 4) as well as the double ppUL32 (fusion protein 1A) and the mono ppUL44 fusion protein (fusion protein 3B) were purified and tested by EIA with more than 700 serum samples from different groups of subjects. The results obtained strongly indicate that the triple-epitope fusion protein can replace the three individual components in IgM binding. Moreover, 5.5% of the IgM-positive serum samples reacted with fusion protein 4 but not with fusion proteins 1A and 3B, which express portions of ppUL32 and ppUL44 contained in fusion protein 4. This result confirms previous findings (28) and may be explained as a simple cumulative effect, but the formation of a bridge epitope cannot be ruled out. The results obtained also indicate that fusion protein 4 alone cannot entirely replace the virus or virus-infected cells in searching for anti-HCMV IgM. In fact, protein 4 reacts with 90% of IgM-positive serum samples. Therefore, proteins other than ppUL32 and ppUL44 seem to be necessary for optimal IgM detection. For this reason we produced, purified, and tested by rec-EIA significant portions of ppUL83 (fusion protein 9) and ppUL80a (fusion protein 26), which are two proteins that have repeatedly been reported to react efficiently with HCMV-specific IgM (10, 14, 18). The results obtained indicate that 3.2% of the IgM-positive serum samples react only with clone 26 and that 1.6% react exclusively with clone 9. Our results also indicate that the combination of fusion proteins 4, 9, and 26 can efficiently replace the virus in IgM detection. In fact, 99.6% of IgM-positive serum samples gave a positive IgM reaction with one or more of these three fusion proteins, indicating that this cocktail of epitopes is very sensitive for IgM capture and can address potential strain differences among CMV isolates. When the rec-EIA with the three clones was compared with a conv-EIA and Western blotting, we found that rec-EIA and Western blotting gave the same results, and both procedures are much more sensitive than conv-EIA. This means that the sensitivity and specificity of rec-EIA are very similar to those of Western blotting, which is a very sensitive and specific procedure for HCMV IgM detection (7, 16).

Therefore, a mixture of recombinant antigens that can replace the virus in IgM binding is now available, and a new generation of serological procedures for anti-HCMV IgM detection can be set up. Serological procedures are cheaper than the other diagnostic tests, require a short execution time, and can be completely automated. Therefore, we explored possible applications in which the use of rec-EIA for HCMV-specific IgM detection is justified. For this purpose, we tested for the presence of HCMV-specific IgM in the sera of different groups of subjects with active HCMV infection by rec-EIA and compared the results with those obtained by other diagnostic procedures.

In a group of HCMV-infected pregnant women, rec-EIA detected most of those who did not transmit the infection to their fetuses and 100% of those who transmitted the infection to their fetuses (they were all primary maternal infections). No aspecific reactivities were detected in HCMV-uninfected pregnant women. Conv-EIA showed a much lower sensitivity. Rec-EIA would therefore be useful for screening for HCMV infection during pregnancy, allowing subsequent virological tests and prenatal diagnosis in the case of positivity. Interestingly, we found that the IgM titers to fusion protein 4 were much higher in HCMV-infected women who transmitted the infection than in those who did not. This result is in agreement with data in the literature (1), suggesting that HCMV-infected pregnant women who transmit the infection in utero have higher levels and more sustained levels of production of virus and viral antigens than HCMV-infected pregnant women who do not transmit the infection, and this induces a higher degree of stimulation of the immune system. Further studies are necessary in order to establish a possible cutoff value indicative of the risk of virus transmission.

Rec-EIA was also used to assay the sera of HCMV-infected newborns. It was shown to be very specific and was able to detect 50% of infected newborns. Conv-EIA gave a very poor result because it detected only 8% of the infected newborns.

Therefore, in this group of subjects, although rec-EIA is definitively much better than conv-EIA, HCMV isolation from urine or saliva, or both, continues to be the diagnostic procedure of choice.

We have also followed by rec-EIA a group of 23 transplant recipients with acute HCMV infection within the first 3 months of transplantation. In this group of patients, the antigenemia test and serology are equally sensitive, and this confirms previously reported data (31). For 70% of the patients, the rec-EIA for IgM gave a positive signal coinciding with the positivity detected by antigenemia (but later than by PCR). In two patients, the positivity by rec-EIA preceded that obtained by the antigenemia test. This result is in contrast to published data (3, 5, 20), indicating that a positive antigenemia test result appeared earlier than positive IgM serology and can be explained by the much higher sensitivity of the rec-EIA compared with that of conv-EIA. Since rec-EIA consistently gives a positive result coinciding with the appearance of symptoms, it could be useful as an early diagnostic tool to ascertain an HCMV infection after transplantation as a diagnostic parameter complementary to antigenemia. As expected, the rec-EIA for IgM cannot distinguish between symptomatic and asymptomatic HCMV infections. Furthermore, the IgM detected by rec-EIA lasts much longer than the symptoms and the positivity detected by the antigenemia test, and therefore, it cannot be considered useful for monitoring therapy. Even though the follow-up of other transplant recipients is still under way, we believe that the features of HCMV infection observed in the group of transplant recipients that we studied are consistent with those common to all organ allograft recipients, and our findings may be of general significance. Although serological diagnosis in general is less attractive than virological diagnosis because of the immunological disorders occurring in transplant recipients, when performed by a sensitive and specific rec-EIA for IgM, it could represent an additional test for obtaining an early diagnosis of ongoing HCMV infection.

REFERENCES

1. **Alford, C. A., K. Hayes, and W. Britt.** 1988. Primary cytomegalovirus infection in pregnancy: comparison of antibody responses to virus-encoded proteins between women with and without intrauterine infection. J. Infect. Dis. **5:**917–924.
2. **Basson, J., J. C. Tardy, and M. Aymard.** 1989. Pattern of anti-cytomegalovirus IgM antibodies determined by immunoblotting. A study of kidney graft recipients developing a primary or recurrent CMV infection. Arch. Virol. **108:**259–270.
3. **Boland, G. J., G. C. deGast, R. J. Hené, G. Jambroes, R. Donckerwolcke, T. H. The, and G. C. Mudde.** 1990. Early detection of active cytomegalovirus (CMV) infection after heart and kidney transplantation by testing for immediate early antigenemia and influence of cellular immunity on the occurrence of CMV infection. J. Clin. Microbiol. **28:**2069–2075.
4. **Bolling, T. J., and M. Mandecki.** 1990. An Escherichia coli expression vector for high-level production of heterologous proteins in fusion with CMP-KDO synthetase. BioTechniques **8:**488–490.
5. **Dolan, J., J. D. Briggs, and G. B. Clements.** 1989. Antibodies to cytomegalovirus in renal allograft recipients: correlation with isolation of virus. J. Clin. Pathol. **42:**1070–1077.
6. **Gleaves, C. A., T. F. Smith, E. A. Shuster, and G. R. Pearson.** 1984. Rapid detection of cytomegalovirus in MRC5 cells inoculated with urine specimens by use of low speed contrifugation and monoclonal antibody to an early antigen. J. Clin. Microbiol. **19:**917–919.
7. **Gold, D., R. Ashley, H. H. Handsfield, M. Verdon, L. Leach, J. Mills, L. Drew, and L. Corey.** 1988. Immunoblot analysis of the humoral immune response in primary cytomegalovirus infection. J. Infect. Dis. **157:**319–325.
8. **Ho, M.** 1991. Cytomegalovirus. Biology and infection, II ed. Plenum Medical Press, New York.
9. **Jahn, G., T. Kouzarides, M. Mach, B. C. Scholl, B. Plachter, B. Traupe, E. Preddie, S. C. Satchwell, B. Fleckenstein, and B. G. Barrell.** 1987. Map position and nucleotide sequence of the gene for the large structural phosphoprotein of human cytomegalovirus. J. Virol. **61:**1358–1367.
10. **Jahn, G., B. C. Scholl, B. Traupe, and B. Fleckenstein.** 1987. The two major phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties. J. Gen. Virol. **68:**1327–1337.
11. **Jiwa, N. M., G. W. Gemert, A. K. Raap, F. M. Van der Rijjke, A. Mulder, P. F. Lens, M. M. M. Salimans, F. E. Zwaan, W. Van Dorp, and M. Van der Ploeg.** 1989. Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation **48:**72–76.
12. **Karlin, S., E. S. Mocarski, and G. A. Schachtel.** 1994. Molecular evolution of herpesviruses: genomic and protein sequence comparisons. J. Virol. **68:** 1886–1902.

12a. **Kraat, et al.** Submitted for publication.

13. **Landini, M. P.** 1993. New approaches and perspectives in cytomegalovirus diagnosis. Prog. Med. Virol. **4:**157–177.

13a. **Landini, M. P.** Unpublished data.

14. **Landini, M. P., M. X. Guan, G. Jahn, W. Lindenmeier, M. Mach, A. Ripalti, A. Necker, T. Lazzarotto, and B. Plachter.** 1990. Large scale screening of human sera with cytomegalovirus recombinant antigens. J. Clin. Microbiol. **28:**1375–1379.
15. **Landini, M. P., T. Lazzarotto, A. Ripalti, M. X. Guan, and M. La Placa.** 1989. Antibody response to recombinant lambda gt11 fusion proteins in cytomegalovirus infection. J. Clin. Microbiol. **27:**2324–2327.
16. **Landini, M. P., G. Mirolo, B. Baldassarri, and M. La Placa.** 1985. Human immune response to cytomegalovirus structural polypeptides studied by immunoblotting. J. Med. Virol. **17:**303–311.
17. **Landini, M. P., A. Ripalti, K. Sra, and P. Pouletty.** 1991. Human cytomegalovirus structural proteins: immune reaction against pp150 synthetic peptides. J. Clin. Microbiol. **29:**1868–1872.
18. **Lazzarotto, T., B. Dalla Casa, B. Campisi, and M. P. Landini.** 1992. Enzyme-linked immunoadsorbent assay for the detection of cytomegalovirus-IgM: comparison between eight commercial kits, immunofluorescence and immunoblotting. J. Clin. Lab. Anal. **6:**216–218.
19. **Lazzarotto, T., G. Furlini, M. C. Re, E. Ramazzotti, B. Campisi, and M. P. Landini.** 1994. Human cytomegalovirus replication correlates with differentiation in a hematopoietic progenitor cell line and can be modulated by HIV. Arch. Virol. **135:**13–28.
20. **Marsano, L., R. P. Perrillo, M. W. Flye, D. W. Hanto, E. D. Spitzer, J. R. Thomas, P. R. Murray, D. W. Windus, E. M. Brunt, and G. A. Storch.** 1990. Comparison of culture and serology for the diagnosis of cytomegalovirus infection in kidney and liver transplant recipients. J. Infect. Dis. **161:**454–461.
21. **Nielsen, S. L., I. Sorensen, and H. K. Andersen.** 1988. Kinetics of specific immunoglobulins M, E, A, and G in congenital, primary, and secondary cytomegalovirus infection studied by antibody-capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. **26:**654–661.
22. **Novak, J., P. Sova, V. Krchnak, E. Hamsikova, and H. E. Zavadova.** 1991. Mapping of serologically relevant regions of human cytomegalovirus phosphoprotein pp150 using synthetic peptides. J. Gen. Virol. **72:**1409–1413.
23. **Plachter, B., L. Wieczorek, B.-C. Scholl, R. Ziegelmaierand, and G. Jahn.** 1992. Detection of cytomegalovirus antibodies by an enzyme linked immunosorbent assay using recombinant polypeptides of the large phosphorylated tegument protein pp150. J. Clin. Microbiol. **30:**201–206.
24. **Revello, M. G., M. Zavattoni, E. Percivalle, P. Grossi, and G. Gerna.** 1989. Correlation between immunofluorescent detection of human cytomegalovirus immediate early antigens in polymorphonuclear leukocytes and viremia. J. Infect. Dis. **160:**159–160.
25. **Ripalti, A., M. C. Boccuni, F. Campanini, G. Bergamini, T. Lazzarotto, M. C. Battista, B. Dalla Casa, and M. P. Landini.** 1994. Construction of a polyepitope fusion antigen of human cytomegalovirus ppUL32 and detection of specific antibodies by ELISA. Microbiologica **18:**1–12.
26. **Ripalti, A., P. Dal Monte, M. C. Boccuni, F. Campanini, T. Lazzarotto, B. Campisi, Q. Ruan, and M. P. Landini.** 1994. Prokaryotic expression of a large fragment of the most antigenic cytomegalovirus DNA-binding protein (ppUL44) and its reactivity with human antibodies. J. Virol. Methods **46:** 39–50.
27. **Ripalti, A., M. P. Landini, E. S. Mocarski, and M. La Placa.** 1989. Identification and preliminary use of recombinant lambda gt11 fusion proteins in cytomegalovirus diagnosis. J. Gen. Virol. **70:**1247–1251.
28. **Ripalti, A., Q. Ruan, M. C. Boccuni, F. Campanini, G. Bergamini, and M. P. Landini.** 1994. Construction of a polyepitope fusion antigens of human cytomegalovirus ppUL32: reactivity with human antibodies. J. Clin. Microbiol. **32:**358–363.
29. **Robinson, J. M., T. J. Pilot-Matias, S. D. Pratt, C. B. Patel, T. S. Bevirt, and J. C. Hunt.** 1993. Analysis of the humoral response to the flagellin protein of *Borrelia burgdorferi*: cloning of regions capable of differentiating Lyme disease from syphilis. J. Clin. Microbiol. **31:**629–635.
30. **Scholl, B. C., B. Von Hintzestein, B. Borisch, B. Traupe, M. Broker, and G. Jahn.** 1988. Procaryotic expression of immunogenic polypeptides of the large phosphoprotein (pp150) of human cytomegalovirus. J. Gen. Virol. **69:**1195–1204.
31. **Thè, T. H., A. P. van der Berg, W. J. van Son, A. M. Tegzess, M. J. H. Sloof, I. J. Klompmaker, E. B. Haagsma, W. van der Bij, and M. van der Giessen.**

1991. Recent advances in the early and reliable immunodiagnosis of cytomegalovirus infection in immunocompromised hosts, p. 209–220. *In* M. P. Landini MP (ed.), Progress in cytomegalovirus research. Elsevier, Amsterdam.

32. **van der Biji, W. J., R. Torensma, W. J. Son, J. Schirm, A. M. Tegzess, and T. H. The.** 1988. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leukocytes. J. Med. Virol. **25:**179–188.
33. **Vornhagen, R., B. Plachter, W. Hinderer, T. H. Thè, J. Van Zanten, L. Matter, C. A. Schmidt, H. H. Sonneborn, and G. Jahn.** 1994. Early serodiagnosis of acute cytomegalovirus infection by enzyme-linked immunosorbent assay using recombinant antigens. J. Clin. Microbiol. **32:**981–986.