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Early Serodiagnosis of Acute Human Cytomegalovirus Infection by Enzyme-Linked Immunosorbent Assay Using Recombinant Antigens

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DNA fragments from eight different reading frames of human cytomegalovirus (HCMV) were generated by PCR and subsequently cloned and expressed in *Escherichia coli* in fusion with glutathione *S*-transferase. The recombinant viral antigens were evaluated in immunoblot analyses. The most reactive antigens were purified and further evaluated in ELISAs. For this, sera from healthy blood donors and immunocompetent individuals with acute HCMV infection, and follow-up sera from transplant recipients with acute primary HCMV infection were used. The results of our experiments indicate that only three particular recombinant polypeptides from two viral proteins are necessary for serodiagnosis. While a fragment covering amino acids (aa) 495 to 691 of pp150 (150/1) was the most suitable antigen for the identification of infected individuals in general, immunoglobulin M antibodies against the C-terminal parts of pp150 (aa 862 to 1048; 150/7) and p52 (aa 297 to 433; 52/3) proved to be excellent serological markers to monitor acute HCMV infection. The selected recombinant antigens enable the improvement of serodiagnosis of HCMV-related diseases, especially during the early stages of infection.

Human cytomegalovirus (HCMV) is a widely spread human pathogen. Primary infection in immunocompetent hosts generally is asymptomatic and has lifelong persistence. Congenital infection as well as infection of immunocompromised individuals such as transplant recipients or AIDS patients, however, may lead to severe and life-threatening illness (12). To ensure efficient and timely treatment, sensitive and reliable diagnostic methods must be available. In addition, to prevent HCMV transmission by infected blood donations or organs, methods must be suitable for screening large numbers of samples. For the identification of HCMV-seropositive individuals and for the diagnosis of acute infection, serological methods such as enzyme-linked immunosorbent assay (ELISA) are widely used (7). However, recent evaluations have clearly shown that despite their advantages in handling, serological tests need to be improved. While most immunoglobulin G (IgG)-specific assays reveal acceptable specificity and sensitivity (13), IgM-specific ELISAs are of limited use because of the high number of discordant results (18). All currently available serological assays use poorly defined natural viral antigens derived from HCMV-infected fibroblast cultures. It has been shown, however, that only a few viral antigens present in cell culture antigen preparations are essential for serodiagnosis of HCMV (16). Furthermore, numerous HCMV antigens show substantial homology with their counterparts in other herpesviruses (6). Therefore, careful selection of antigens is the most promising way to improve serodiagnosis. Recombinant technology offers the possibility of producing selected viral antigens or antigen fragments in large amounts. The diagnostic potential of different recombinant HCMV antigens has been evaluated in several comparative studies (14, 15, 19, 21). Those studies used viral antigens expressed in fusion with different bacterial proteins, and seroreactivity was evaluated solely by immunoblot technology. In addition, because of the limitations of conventional cloning techniques, portions of essential antigens were omitted. As a result of this, contradictory conclusions about the usefulness of recombinant antigens for the improvement of serodiagnosis, especially during the acute phase of infection, were reached.

In the present study, portions of different viral proteins were expressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST). As a first step, all antigens were evaluated in Western blot (immunoblot) experiments. According to the results of these analyses, selected recombinant proteins were purified and IgM- and IgG-specific reactivities were further tested by ELISA. The results of these experiments indicate that only three particular viral polypeptides from two viral proteins, pp150 and p52, may be sufficient to improve the serodiagnosis of acute and past HCMV infections.

MATERIALS AND METHODS

Cloning and expression of antigens. DNA fragments coding for the different HCMV antigens were generated by PCR amplification. The general cloning strategy and the detailed methods have been described previously (28). Briefly, amplification was performed with pairs of PCR primers containing recognition sequences of the restriction endonucleases *Bam*HI and *Eco*RI (*Ava*I), respectively 5′ and 3′ of the original priming sequence, to facilitate the following cloning steps.

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Cosmids and plasmids harboring defined genomic fragments or cDNAs of strain AD169 served as templates for PCR amplification (1, 8). After initial cloning and identification of recombinant clones with the standard vector pUC8, DNA fragments were subcloned without modification into pGEX-3X (25), a vector which enables the expression of polypeptides in fusion with GST.

Western blots. Western blot experiments were performed with unpurified bacterial lysates according to the method of Baur et al. (4). To ensure comparable amounts of viral antigens in each sample, the bacterial lysates were prediluted until the different recombinant proteins were visible as faint bands of comparable intensities in a Coomassie-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel. A lysate of a clone expressing GST alone was included as a control in each Western blot. After separation by SDS–15% polyacrylamide gel electrophoresis (PAGE), the proteins were transferred to polyvinylidine difluoride membranes (Millipore) under semidry conditions. Membranes were blocked for 1 h with 1% bovine serum albumin (Sigma) and incubated overnight with 1:100-diluted human sera. After membranes were washed, bound immunoglobulins were visualized with horseradish peroxidase-conjugated polyclonal rabbit anti-human IgG or IgM (Dako) and were developed with diaminobenzidine.

ELISA with purified recombinant fusion proteins. Selected HCMV fusion proteins were purified and further evaluated in ELISAs. Soluble antigens (150/1, 150/7, 52/3, 28, and GST) were purified after lysis of bacteria by glutathione-Sepharose affinity chromatography (25). Insoluble antigens (65/3 and IE1) were purified by differential washing and centrifugation steps using variable concentrations of detergents and chaotropic reagents at different pH values (11). The final purity of the recombinant proteins was higher than 95%. Polystyrene microdilution plates (96 wells each; Nunc) were coated with 100 ng of purified HCMV-GST fusion proteins or purified GST as a control solubilized in 100 μl of 0.01 M carbonate buffer, pH 9.5. ELISA plates were incubated for 2 h with 1:50-diluted human sera in a water bath at 40°C. Bound immunoglobulins were detected by incubation with IgG- and IgM-specific conjugates (Dako) for 30 min at 40°C. Color was developed with 1,2-phenylenediamine for 15 min at room temperature. All other reagents were standard components used in commercially available ELISA kits (anti-HIV 1/2 recombinant and anti-EBV recombinant; Biotest, Dreieich, Germany). All optical densities (OD) reported here were measured at 495 nm (reference, 620 nm) and are uncorrected values. Samples with OD values above 0.5 and a GST-specific reactivity below 0.25 were considered positive.

In order to identify false-positive results caused by rheumatoid factors in IgM ELISA, sera were retested after treatment with rheumatoid factor absorbent (Behring, Marburg, Germany) according to the manufacturer's instructions.

Serum samples. In this study recombinant HCMV antigens were evaluated with human sera from different sources. Sera from healthy blood donors were obtained from a local transfusion center (DRK, Frankfurt, Germany), and HCMV-specific seroprevalence was determined with the Biotest anti-CMV IgG ELISA (Biotest), which uses cell-culture-derived antigens. Patients receiving a renal allograft at the University Hospital Groningen, Groningen, The Netherlands, were monitored with the pp65-specific antigenemia assay (27) and IgG- and IgM-specific solid-phase ELISAs utilizing unpurified cell-culture-derived antigens developed at the University Hospital Groningen (27). Sera obtained from immunocompetent individuals with primary infection proven by seroconversion (n = 3) and from immunocompetent patients with CMV excretion and acute disease with fever and/or lymphadenopathy (n = 8) were examined for HCMV-specific immunoglobulins at the Institute of Medical Microbiology, Bern, Switzerland, by latex agglutination (Becton Dickinson AG, Basel, Switzerland), IgG-specific indirect ELISA with a cell-culture-derived HCMV antigen (Biotest AG), and two μ-capture ELISAs. In these assays, bound IgM molecules were detected with cell-culture-derived enzyme-labelled antigens (Medac, Hamburg, Germany) or with immunocomplexes consisting of a cell-culture-derived antigen and an enzyme-linked HCMV-specific mouse monoclonal antibody (Sorin Biomedica, Saluggia, Italy). Virus was isolated from leukocytes (after separation by Histopaque [Sigma Chemie, Buchs, Switzerland]), throat swabs, or urine from immunocompetent individuals by using shell vial and standard cell culture techniques with MRC-5 cells (10). Shell vials were processed after 1 day, or, for leukocytes, after 1 and 2 days, and cell cultures were maintained for 3 weeks. Acute primary HCMV infection of liver transplant recipients was monitored by PCR with primer pairs from the major immediate-early gene (IE1) and the late antigen pp65 with alkaline lysates of peripheral buffy coat leukocytes (24).

RESULTS

Cloning and expression of HCMV antigens. DNA fragments coding for different HCMV antigens were generated by PCR technology and subsequently cloned and expressed in *E. coli*. In most cases, the entire coding sequences of the selected viral proteins were cloned as 500- to 700-bp nonoverlapping DNA fragments. The C-terminal part of p52 (ICP36) was expressed by two different clones. While 52/2 codes for the complete C-terminal half of the protein, 52/3 has an N-terminal deletion. The deleted part shows a high degree of homology with a phosphoprotein of human herpesvirus 6 (5). Despite numerous attempts with modified primer pairs, no stable clone representing the middle part of pp65 was obtained, although PCR amplification always resulted in a distinct DNA band. In a previous study it could be shown that the central part of pp65 is only weakly reactive with human sera (22). Therefore, this antigen fragment was not included in our study. All other cloned DNA fragments were highly expressed in fusion with GST.

Western blot evaluation of recombinant HCMV antigens. A total of 16 different expression clones were included in our major Western blot evaluation (Table 1). pp150 and p52 were represented by 150/1, 150/7, 52/2, and 52/3. In a previous analysis, in which we evaluated antigen fragments representing the entire coding sequences of these two reading frames, these polypeptides were shown to be the most reactive fragments of pp150 and p52 (28). In all Western blot experiments reported here, 52/2 and 52/3 showed similar results. Therefore, only the results concerning 52/3 are included.

In the first set of experiments, the 16 antigens were tested with 30 serum samples obtained from healthy blood donors. Each sample had given a positive result in the Biotest IgG ELISA, which utilizes cell-culture-derived antigens. Table 2 summarizes the prevalence of IgG antibodies against the major reactive antigens in ELISA-positive sera. With one exception, all positive sera reacted with 150/1 and 150/7. The single nonreactive serum sample, which had given a weakly positive result in the Biotest ELISA, was also negative with all other recombinant antigens. Strong reactivity was also observed with the clone representing the entire sequence of pp28. All other antigens were less reactive. With regard to pp65, pp71, and IE2, the C-terminal portions were by far the most reactive parts of the antigens. With ten serum samples giving a negative

TABLE 1. Recombinant HCMV fusion proteins evaluated in this study

Antigen (reading frame)[a]	Clone	aa
pp150 (UL32)	150/1	495–691
	150/7	862–1048
p52 (UL44)	52/2	217–433
	52/3	297–433
pp65 (UL83)	65/1	2–161
	65/3	372–546
pp71 (UL82)	71/1	1–187
	71/2	188–389
	71/3	390–559
pp28 (UL99)	28	3–190
gp116/58 (UL55)	58/1	460–679
	58/2	687–905
IE1 (UL123)	IE/1	1–234
	IE/8	301–491
IE2 (UL122)	IE/3	1–259
	IE/4	261–504

[a] According to Chee et al. (6).

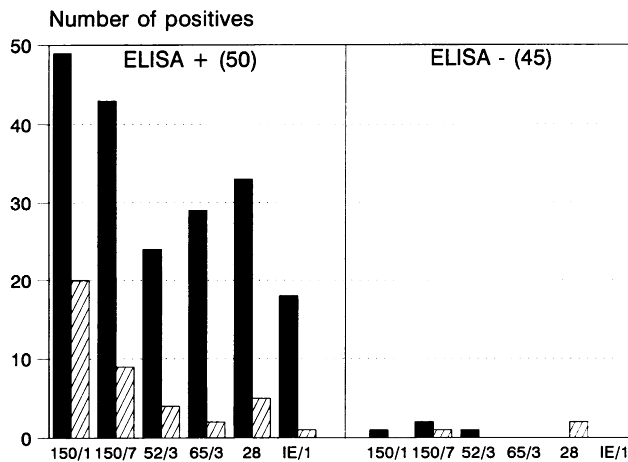

FIG. 1. Reactivities of purified recombinant HCMV antigens in experimental ELISAs with sera obtained from healthy blood donors positive or negative in the Biotest IgG ELISA. ■, IgG; ▨, IgM.

result in the Biotest IgG ELISA, only the N-terminal fragment of pp65 (65/1) gave a potentially false-positive result in Western blot, while all other antigens were negative (data not shown).

In the second set of experiments, the recombinant antigens were evaluated with sera obtained from kidney transplant recipients with acute HCMV infection. Eight serum samples were obtained from patients with acute primary infection, and 13 serum samples originated from patients with acute reactivation (Table 2). All serum samples from these individuals were drawn 3 to 4 weeks after the onset of antigenemia, at a time when a clearly positive result appeared in the reference ELISAs which use cell-culture-derived antigens (27). All sera from patients with acute primary infection contained IgM antibodies against 150/1, 150/7, and 52/3 and IgG antibodies against 150/7, 52/3, and 28. Only two antigens, 150/7 and 52/3, revealed IgM- as well as IgG-specific reactivity with all sera from primary infections. With sera from individuals with acute reactivation, high IgM- as well as IgG-specific reactivity was observed only with 150/1, 150/7, 52/3, and 28. All other antigens were less consistently reactive.

ELISA evaluation of selected recombinant HCMV antigens. (i) Healthy individuals. On the basis of the results of the Western blot experiments, we selected the six most reactive recombinant HCMV antigens from clones 150/1, 150/7, 52/3, 28, 65/3, and IE/1 for further evaluation. Together with unfused GST, which served as a control, these antigens were purified and examined in ELISAs. Figure 1 summarizes the IgG- and IgM-specific reactivities of the recombinant antigens with randomly selected sera obtained from healthy blood donors. The highest IgG-specific prevalence with sera positive in the Biotest CMV IgG ELISA was observed with the recombinant antigen from clone 150/1. Only one serum sample was just below cutoff in our experimental ELISA. This sample, which was weakly positive in three conventional ELISAs, was also negative with all other antigens even in our recombinant Western blot analyses. High levels of reactivity were also found with the C-terminal end of pp150 (150/7), while all other antigens revealed intermediate seroprevalence. OD values with 150/1 in the experimental ELISA were high. Ninety percent of the serum samples gave values above 1.0, and 50% of the samples gave values above 2.5. High levels of IgM-specific reactivity were observed with 150/1 in 20 serum samples. By preabsorption of rheumatoid factors, only one positive result for 150/1 became negative, while all results obtained with the other recombinant antigens remained unal-

TABLE 2. Western blot results of the major reactive recombinant HCMV antigens with different serum panels

Serum sample[a]	No. of samples	Antibody	No. of samples with positive results									
			150/1	150/7	52/3	65/3	71/3	28	58/1	58/2	IE/1	IE/4
Samples from healthy blood donors												
ELISA + (OD, <1.0)	10	IgG	9	9	4	2	4	8	0	3	1	1
ELISA ++ (OD, ≥1.0 to <1.5)	10	IgG	10	10	6	5	3	9	0	4	7	5
ELISA +++ (OD, >1.5)	10	IgG	10	10	8	7	4	10	3	7	8	7
Samples from patients with acute primary infection[b,c]	8	IgM	8	8	8	7	7	4	6	0	4	5
	8	IgG	7	8	8	4	3	8	1	1	5	6
Samples from patients with acute secondary infection[c,d]	13	IgM	12	12	11	5	3	11	2	1	3	1
	13	IgG	13	13	9	9	3	13	1	9	8	8

[a] HCMV-seropositive healthy blood donors grouped according to their results in the Biotest CMV IgG ELISA.
[b] Renal transplant recipients with acute primary infection proven by seroconversion.
[c] All sera from transplant recipients were drawn 3 to 4 weeks after the outset of antigenemia.
[d] Renal transplant recipients with acute secondary infection. All patients were HCMV seropositive prior to transplantation.

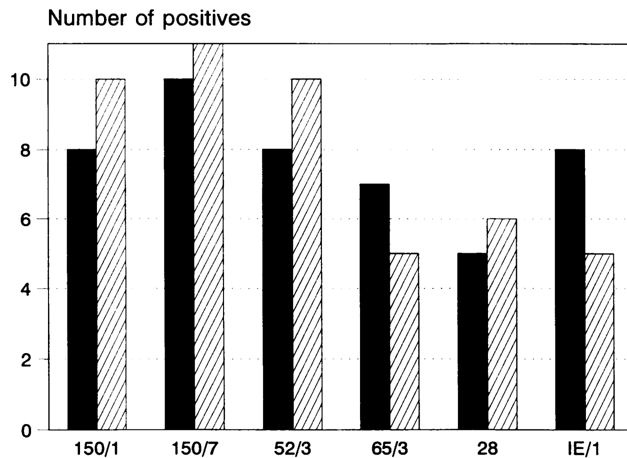

FIG. 2. Reactivities of purified recombinant HCMV antigens in experimental ELISAs with 11 sera obtained from immunocompetent individuals with acute HCMV infection. Sera were obtained from three individuals with acute primary infection proven by seroconversion and eight patients with HCMV excretion revealing acute disease symptoms such as fever and/or lymphadenopathy. All sera were positive for HCMV-specific IgG and IgM in reference ELISAs. ■, IgG; ▨, IgM.

tered. Very low levels of IgG and IgM reactivity were found with sera which were negative in conventional ELISAs.

(ii) Individuals with acute HCMV infection. Figure 2 summarizes the IgG- and IgM-specific ELISA reactivities of the recombinant HCMV antigens with 11 serum samples from immunocompetent individuals with active HCMV infection. IgM-specific reactivity with all samples was observed only with 150/7. High seroprevalence was also found with 150/1 and 52/3, while all other antigens were less reliably reactive. The most important information about the course of the humoral immune response against the recombinant antigens during acute HCMV infection was obtained with follow-ups from transplant recipients. Figure 3 shows the IgM-specific reactivities in our experimental ELISA of the three most reactive recombinant antigens with four typical follow-ups from renal transplant recipients with acute HCMV primary infection. Infection had been monitored with the pp65-specific antigenemia assay and an IgM ELISA which uses cell-culture-derived antigens. In two patients (A and C), positive results with our experimental ELISA were first observed with samples no. 3. These samples were also the first to be positive in the antigenemia assay. While 150/1, 150/7, and 52/3 gave comparable results with sera obtained from patient A, 150/7 and 52/3 were the first recombinant antigens to react with sera obtained from patient C. In the two other patients (B and D), antibodies against 150/1, 150/7, and 52/3 could be detected 1 week after the onset of antigenemia. IgM-specific reactivity with recombinant antigens occurred with sera from two patients (B and C) earlier than in the standard IgM ELISA, while with sera from patients A and D and three additional patients (data not shown), experimental ELISA and standard IgM ELISA revealed similar sensitivities. IgG antibodies against 150/1, 150/7, and 52/3 were found 1 or 2 weeks later than IgM antibodies. All other recombinant antigens (65/3, 28, and IE/1) were much less reactive and generally showed positive results 2 or more weeks after the onset of antigenemia.

Comparable results were obtained with three follow-ups from liver transplant recipients with acute primary infection. The course of HCMV infection in these patients was monitored by PCR. In contrast to the renal transplant recipients, this group of patients was treated with hyperimmunoglobulins. IgM-specific antibodies against recombinant HCMV antigens could be observed 7 to 21 days after the first PCR-positive sample was obtained. Again antibodies against 150/1, 150/7, and 52/3 were the most reliable serological markers for acute infection, while all other proteins appeared to be less reactive. In all three patients, IgM-specific ELISA reactivities against 150/7 and 52/3 reverted in the last sample obtained from each (85 to 152 days after transplantation). IgM-specific serology was not affected by prophylactic immunoglobulin treatment, while all experimental ELISAs with recombinant antigens revealed IgG-specific reactivities with serum samples obtained before active HCMV infection.

DISCUSSION

In the present study, antigen fragments from eight different HCMV proteins were analyzed for their usefulness in serodiagnosis. Our data demonstrate that only three particular polypeptides from two different viral proteins, pp150 and p52, may be necessary and sufficient to monitor HCMV infection. pp150 was by far the most useful antigen for the identification of infected individuals. A very high level of IgG-specific reactivity with sera from HCMV-seropositive healthy blood donors was observed, especially with polypeptide 150/1. These results fully agree with a previous report showing that a recombinant protein covering amino acids (aa) 555 to 701 of pp150 is the most useful antigen for the identification of HCMV-seropositive individuals (23). In addition, a study utilizing synthetic peptides of pp150 identified major IgG-specific epitopes between aa 594 and 643 (26).

During our experiments we did not find a single serum sample obtained from a healthy, HCMV-seropositive individual which did not react with 150/1 while being reactive with any other recombinant antigen. These data suggest that a combination of recombinant pp150 with one or more of the less reactive recombinant antigens evaluated in this study will not improve detection of HCMV-specific IgG in infected individuals. However, further investigations with large numbers of serum samples are required to elucidate whether in rare cases antigens other than pp150 may be helpful for the identification of HCMV-seropositive individuals.

The question of whether recombinant antigens are able to improve serodiagnosis of acute HCMV infection has been addressed in several studies (14, 15, 19, 21). All these studies were carried out by immunoblot analysis with recombinant HCMV polypeptides fused to different heterologous proteins. In all cases of acute infection evaluated in this study, IgM antibodies against 150/7, 52/3, and 150/1 were the first serological markers. IgM as well as IgG antibodies against pp150 were detectable very early during acute infection. The usefulness of 150/1 for the identification of acutely infected individuals is limited by its high level of IgM-specific reactivity. Forty percent of sera from healthy blood donors without any evidence of acute HCMV infection reacted with this antigen. In comparison, IgM-specific prevalence as determined with different IgM ELISAs in comparable populations normally ranges from 1 to 13% (2, 9, 20). Therefore, the high level of reactivity of 150/1 in the recombinant ELISA must be considered, at least in part, false positive. Our data, however, indicate that the sole utilization of antigen 150/7 in combination with 52/3 will enable reliable serodiagnosis of acute primary infections. With these antigens, IgM antibodies may become detect-

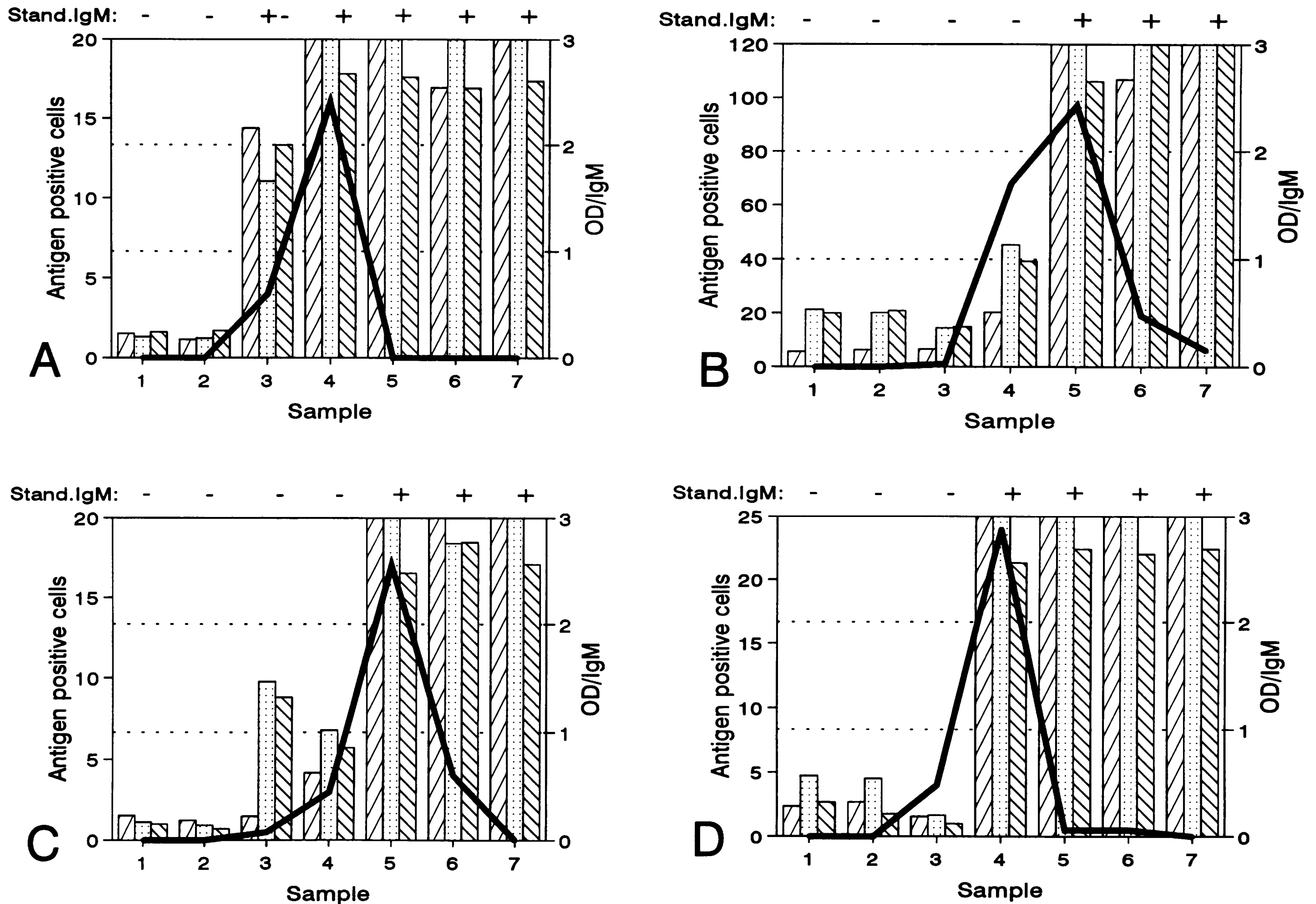

FIG. 3. IgM-specific reactivities of purified recombinant HCMV antigens in experimental ELISAs with four follow-ups from renal transplant recipients (A to D) with acute HCMV primary infection monitored by the pp65 antigenemia assay and an IgM ELISA (stand. IgM), which utilizes cell-culture-derived antigens. Samples were drawn at different times as follows: sample 1, pretransplantation; sample 2, 14 days posttransplantation; sample 3, at the onset of antigenemia; samples 4 to 7, 1 to 4 weeks after the onset of antigenemia. All patients showed symptoms of CMV disease associated with positive results in the antigenemia assay. Patients B and D received antiviral chemotherapy. Dashed lines indicate the number of antigen-positive cells per 50,000 cells. ▨, OD with 150/1 in IgM ELISA; ◻, OD with 150/7 in IgM ELISA; ▧, OD with 52/3 in IgM ELISA.

able significantly earlier than with assays which use antigens derived from cell culture.

The finding that especially the carboxy-terminal part of pp150 is an important antigen for the detection of IgM-specific antibodies is in contrast to the results of previous immunoblot studies (14, 15), in which antibodies against pp150 were detected only in late stages of infection. On the other hand, experiments with synthetic peptides have identified a major IgM-specific epitope within the last 38 aa of pp150 (17), a portion covered by 150/7. Recombinant viral antigens of the delayed-early DNA-binding protein p52 (ICP36) have also already been shown to be useful for the serological identification of acute HCMV infections (15, 19). The results of those studies, however, did not indicate that the use of recombinant p52 might improve assay sensitivity compared with that of established serological assays. On the other hand, immunoblot experiments with antigens derived from cell culture identified a protein with a molecular mass of 45 to 47 kDa—presumably p52—which revealed IgM-specific reactivity 1 to 2.5 weeks earlier than established IgM ELISAs (3). Our results indicate that both 150/7 and p52 are required to achieve an early and reliable detection of IgM antibodies during acute HCMV infection.

In conclusion, we have shown that only three particular recombinant antigen fragments from two viral proteins seem to be necessary for the serodiagnosis of HCMV infection, whereas all other recombinant antigens evaluated in this study are of minor importance. While 150/1 proved to be the most suitable antigen for the identification of HCMV-positive donors, the use of 150/7 and 52/3 will enable the improvement of IgM-specific serology during the acute phase of HCMV infection.

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