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Antibody Response to Recombinant Lambda gt11 Fusion Proteins in Cytomegalovirus Infection

M. P. LANDINI,* T. LAZZAROTTO, A. RIPALTI, M. X. GUAN, AND M. LA PLACA

Medical Faculty, Institute of Microbiology, St. Orsola General Hospital, 40138 Bologna, Italy

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Significant epitopes of two of the major cytomegalovirus antigens, a nonstructural DNA-binding protein of 52 kilodaltons (kDa) and a structural phosphoprotein of 150 kDa, expressed as fusion proteins with the β-galactosidase, were induced in *Escherichia coli* after infection with recombinant lambda gt11 clones. The epitopes were then used in immunoblotting to assay specific immunoglobulin G (IgG) and IgM in several groups of sera from long-term seropositive subjects and from patients undergoing primary or secondary virus infection. The data obtained showed that IgM reacting with the 52-kDa nonstructural antigen are linked to primary virus infection and can therefore be considered a serological marker of this infection.

Symptomatic cytomegalovirus (CMV) infection is usually manifest in congenitally infected infants, organ transplant recipients, and other patients, such as those who have neoplastic disease or a compromised immune system. A close correlation has been documented between primary CMV infection and severe clinical symptoms. Less severe clinical symptoms are the result of viral reactivation or exogenous reinfection. Detection of immunoglobulin M (IgM) antibody to CMV has been proposed as a rapid method of diagnosing primary infection with this virus. Studies have shown that IgM antibody to CMV in pregnant women generally signifies primary infection (2, 5, 17, 20, 21), while in organ transplant populations CMV-specific IgM antibody has been observed in several patients undergoing nonprimary CMV infections (2, 8, 13, 14). Procedures other than IgM detection have been proposed to identify CMV primary infection (1, 15, 18), but none of them is completely straightforward. Therefore, the differentiation between primary and recurrent CMV infection in transplant patients still needs a lot of attention.

In this study, we used immunoblotting to detect the presence of antibody (IgG and IgM) against two of the major CMV antigens: a nonstructural DNA-binding protein of 52 kilodaltons (kDa) (3, 4, 6, 11, 15) and a structural phosphoprotein of 150 kDa (6, 7, 16, 19) in renal transplant recipients with virological, serological, or both kinds of evidence of viral primary or secondary infection as well as in long-term seropositive subjects. Since no attempt has been made here to distinguish between viral reactivation and exogenous reinfection, for infections other than primary infection the term secondary infection is used.

Significant epitopes of the two CMV antigens, expressed as fusion proteins with β-galactosidase (12, 16), were induced in *Escherichia coli* after infection with recombinant lambda gt11 clones (16) and used as antigenic material in this work.

MATERIALS AND METHODS

Cells and virus. Human embryo fibroblasts were grown in minimal essential medium with 10% fetal calf serum. The Towne strain of CMV was used in all the experiments. The virus was propagated and purified as previously described in detail (10).

Fusion proteins. Fusion proteins of lambda gt11 clones were produced in Lac$^-$ *E. coli* Y1089. A 200-μl portion of an overnight culture of Y1089 in LB medium was diluted 1:2 and infected with recombinant bacteriophages G2 and D1/F3 (16), at a multiplicity of infection of 5, for 20 min at room temperature. The cell suspension was then diluted into 3 ml of LB medium and incubated with shaking at 37°C until an optical density at 600 nm of 0.2 was reached (approximately for 1 h). The synthesis of the fusion protein was then induced by the addition of 10 mM isopropyl-β-D-thiogalactopyranoside, and the temperature was shifted to 44°C for 15 min. After an additional 2 h at 37°C, the cells were collected, solubilized in sodium dodecyl sulfate-gel sample buffer and used for polyacrylamide gel electrophoresis and immunoblotting (8).

Serum samples. A total of 197 serum specimens from pregnant women and renal transplant recipients were used in this work. Most of the sera were sent to our diagnostic laboratory for serological monitoring of CMV infection during pregnancy and after renal transplants. The samples were stored at −20°C until tested.

The first group of sera consisted of 130 randomly selected serum specimens which were divided into four subgroups on the basis of their anti-CMV antibody titers as determined by enzyme-linked immunosorbent assay (ELISA). Most of these sera were from pregnant women. The second group consisted of 42 specimens from 12 renal transplant recipients undergoing a primary CMV infection. These patients were CMV seronegative at the time of transplantation and became CMV seropositive after some time (11). The third group consisted of 25 specimens from 10 transplant patients with a CMV secondary infection. These patients were already CMV seropositive at the time of transplantation and their ELISA value had increased at least 1.5 times (8).

Conventional serological procedures. ELISA for both IgM and IgG was performed with the following commercial ELISA kits: (i) an indirect CMV ELISA for IgG detection (from M. A. Bioproducts, Walkersville, Md.) and (ii) an antibody capture ELISA for IgM detections (from Tecnogenetics, Hamburg, Federal Republic of Germany). The results

* Corresponding author.

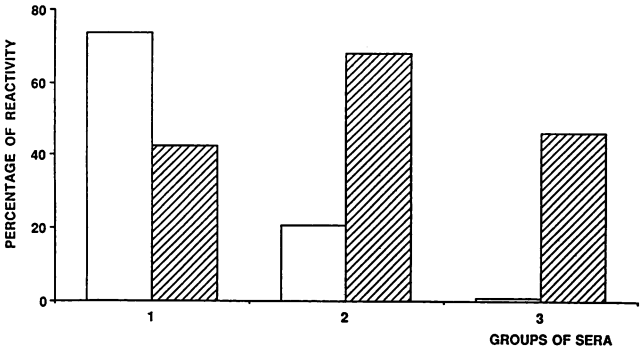

FIG. 1. Reactivity of antibody present in human sera with CMV-recombinant proteins. A total of 130 human serum specimens were divided into four groups on the basis of their anti-CMV ELISA titers (described in Materials and Methods). Sera were then tested by immunoblotting for total antibody reactivity to the two CMV-recombinant proteins, rp52 (□) and rp150 (▨). Group 1, 34 IgM-positive specimens; group 2, 53 IgM-negative specimens with a medium-to-high IgG level: group 3, 26 IgM-negative specimens with a low IgG titer; group 4, 17 IgM-negative specimens (not shown in the graphic as no reactivity was detected).

for both kits were interpreted as suggested by the manufacturers.

Immunoblotting. The procedure for immunoblotting has been previously described in detail (9). Briefly, CMV (Towne strain) was purified from the extracellular supernatant through a sorbitol cushion and linear sorbitol gradient. Purified CMV was denatured in the presence of sodium dodecyl sulfate and β-mercaptoethanol, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 9% acrylamide gels. Separated polypeptides were electrotransferred to nitrocellulose, and the immune reaction was performed in a miniblotter chamber (Biometra; Göttingen, Federal Republic of Germany). A peroxidase-coupled rabbit anti-human IgG or IgM was purchased from Dako (Glostrup, Denmark). Sera were tested under code in a single-blind manner at a 1:100 dilution. All the sera that tested positive for IgM were tested for the presence of rheumatoid factor by latex agglutination (Rheuma Wellcotest; Wellcome Research Laboratories, Beckenham, England). All the sera studied gave negative results. Furthermore, to exclude possible false-positive reactions due to anti-*E. coli* β-galactosidase antibodies, all the sera were also tested against a bacterial lysate from *E. coli* cells infected with nonrecombinant lambda gt11 and induced by isopropyl-β-D-thiogalactopyranoside.

Virus isolation. Urine specimens were inoculated into standard human fibroblast culture at a volume of 0.1 ml into two wells of a 24-well plastic plate. Cultures were maintained for 2 weeks and were examined twice weekly for CMV cytopathic effects.

RESULTS

Distribution of antibody to rp52 and rp150. A total of 130 serum specimens, mostly from pregnant women, were grouped on the basis of their anti-CMV antibody titers and class, as defined by ELISA (as described in Materials and Methods), into the following four groups: (i) IgM-positive sera (34 specimens), (ii) IgM-negative sera with a medium-to-high IgG level (53 specimens), (iii) IgM-negative sera with a low IgG level (26 specimens), and (iv) IgM- and IgG-negative sera (17 specimens). All these sera were tested by immunoblotting for total antibody reactivity against the two recombinant proteins, rp52 (β-galactosidase fused with a portion of p52, the CMV nonstructural protein of 52 kDa) and rp150 (β-galactosidase fused with a portion of p150, a major structural antigen and a phosphoprotein of 150 kDa). The results obtained are summarized in Fig. 1 and show that, while the reactivity to rp52 is very high (73.5%), particularly in the first group of sera (IgM-positive sera), the reactivity to rp150 is much more widespread (42.2, 68, and 46% in the first, second, and third groups of sera, respectively). The difference between the percentage of reactivity to rp52 in the first group and that in the second group of sera is statistically significant ($\chi^2 = 21.7$, $P < 0.001$ with 5 degrees of freedom). No nonspecific reactions were found in CMV-negative sera (data not shown), and only two samples from the whole study group showed the presence of a low-degree reactivity against *E. coli* β-galactosidase.

TABLE 1. Comparison between antibody reactivity to recombinant p150 (rp150) and native viral p150 (vp150) in 50 human serum specimens

Result for reactivity to vp150	No. of specimens with indicated reactivity to rp150			
	IgG		IgM	
	Positive	Negative	Positive	Negative
Positive	18	7	20	6
Negative	5	20	5	19

Comparison between antibody reactivity to rp150 and virion p150. The percentage of sera reacting with rp150 in the three groups of sera studied was lower than expected on the basis of results obtained in previous works where the percentage of sera reacting with p150 was evaluated by using polypeptides separated from whole virions (8, 9, 11). We therefore compared the reactivity to rp150 with the reactivity to virion p150 (vp150) in 50 serum specimens randomly selected among our study group. The results are summarized in Table 1 and show that the results for detection of IgG and IgM in sera reactive to vp150 and to rp150 are in agreement in 76 and 78% of cases, respectively. In particular, 72% of the sera that had IgG reactive to the whole vp150 were also found positive for reactivity to rp150, and 77% of the sera that had IgM reactive to vp150 also reacted with rp150.

Antibody reactivity to rp52 and rp150 in sera from patients with primary CMV infection. Of 12 serum specimens obtained in the acute phase of primary CMV infection, 9 showed the presence of IgM reacting with rp52 (Table 2). All rp52-positive sera but one were also positive by IgM ELISA. Viruria was detected in most patients with CMV-specific IgM. The two specimens that did not contain IgM reactive to rp52 were also negative by IgM ELISA. IgM reactive to rp150 was present in five patients; all of them were positive by IgM ELISA. The presence of IgG reactive to rp52 and rp150 was limited to eight and four serum specimens, respectively. An example of the reactivity against rp52 and rp150 observed in sera of this group is shown in Fig. 2A.

Antibody reactivity to rp52 and rp150 in sera from patients with CMV secondary infection. Only one patient from a group undergoing secondary CMV acute infection had serum with IgM reactive to rp52 (Table 3). This is in contrast to the result obtained with the ELISA for CMV-specific IgM; in fact, with this serological procedure, eight of 10 serum specimens gave positive results. Four specimens also had IgG reacting with rp52, and all were positive for rp150. An example of the reactivity to rp52 and rp150 obtained with the sera of this group is shown in Fig. 2B.

TABLE 2. Behavior of sera obtained from patients undergoing primary CMV infection

Serum code no.[a]	Presence (+), strong presence (++), or absence (−) of:								Time[b]
	IgM reactive to:			IgG reactive to:			IgM[c]	Viruria	
	rp52	rp150	vp150	rp52	rp150	vp150			
3515	++	+	+	−	−	+	+	+	18
4373	++	−	−	++	−	−	+	+	7
1532	−	−	−	+	+	−	−	+	37
2811	++	++	−	++	++	−	+	+	13
4811	++	−	−	−	−	−	+	+	11
5235	++	++	+	−	−	−	+	+	60
880	−	−	−	++	−	−	−	+	15
3926	+	−	−	−	−	−	−	+	60
1903	++	−	+	++	−	−	+	+	7
738	++	−	−	+	−	+	+	ND[d]	14
534	++	++	+	++	+	+	+	ND	24
665	+	+	+	+	+	+	+	ND	62

[a] The sera shown in this table correspond to the first sera that gave a positive result by ELISA for CMV-specific IgM, IgG, or both. All these sera were negative for anti-*E. coli* β-galactosidase antibody.
[b] Days after the previous CMV-negative serum sample.
[c] As determined by ELISA.
[d] ND, Not determined.

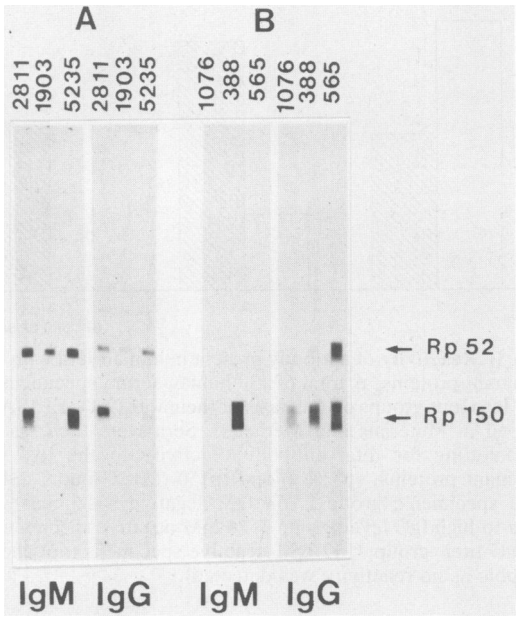

FIG. 2. Differential detection of recombinant CMV proteins by sera from patients with primary infection (A) and sera from subjects with secondary viral infection (B). Lysates of infected *E. coli* cells were run in a 7% acrylamide gel and electrotransferred to nitrocellulose. Sera were used diluted 1:100, and the immune reaction for both IgG and IgM detection was carried out as previously described in detail (9). Numbers above the lanes represent serum code numbers. The positions of rp52 and rp150 are shown on the right of the figure.

DISCUSSION

Recombinant proteins induced in *E. coli* after infection with recombinant lambda gt11 clones (16) were used in an immunoblotting assay to study the specific IgG and IgM reactivity of human sera.

While rp52 contains a large portion (or approximately 30 kDa) of the viral nonstructural protein of 52 kDa and can be considered representative of the whole protein, rp150 contains a small portion (approximately 15 kDa) of the viral structural phosphoprotein of 150 kDa. Nevertheless, immunodominant epitopes should be present in the latter recombinant protein, since the great majority of the sera that were shown to have antibodies to the whole viral protein (vp150) also gave a positive result with rp150. However, some sera reacted with vp150 and not with rp150, probably because antibodies were mainly specific for epitopes of the protein not present in rp150. Furthermore, some other sera did not react with vp150 and reacted with rp150, suggesting that the small amount of p150 present in a virion preparation is not enough to reveal their presence.

By using rp150 and rp52 as antigenic material to detect specific antibodies in human sera, we were able to show that immunoglobulins reactive to rp52 are present in a significantly greater percentage of IgM-positive sera than in the other two groups, while immunoglobulins reactive to rp150 are much more widespread. This result, which suggests that immunoglobulins to rp52 could be linked to active CMV infection, led us to investigate the presence of IgG and IgM separately in patients with primary CMV infection as well as in patients with secondary viral infection.

A significantly greater percentage of IgM reactive to rp52 was observed among sera from patients with primary CMV infection than in patients undergoing secondary infection. While IgM detected by ELISA as well as IgG reactive to rp52 and IgM reactive to rp150 were evenly distributed in the three groups, IgG reactive to rp150 was preferentially present in secondary-infected subjects. This finding confirms previous data suggesting that antibodies to p150 rise more slowly during primary infection (3, 11). IgG reactive to rp150 cannot be considered a marker of secondary viral infection as it is known that antibodies to this protein persist for a long period of time after infection (8, 9, 19, and data of this work where antibodies to rp150 are still present in 46% of sera from long-term seropositive subjects). On the other hand, IgM reactive to rp52 can be considered a marker to identify a primary CMV infection in renal transplant recipients.

Extension of the data presented in this report to groups of CMV-infected individuals other than renal transplant recipients and pregnant women and the development of a serological procedure simpler than immunoblotting are two important steps to be taken in the near future.

TABLE 3. Behavior of sera obtained from patients undergoing a secondary viral infection

Serum code no.[a]	Presence (+), strong presence (++), or absence (−) of:							Time[b]
	IgM reactive to:			IgG reactive to:			IgM[c]	
	rp52	rp150	vp150	rp52	rp150	vp150		
1076	−	−	−	−	++	+	−	22
263	−	−	−	−	++	+	−	15
493	−	−	+	+	+	+	+	62
134	−	−	+	++	+	+	−	30
817	−	+	+	++	++	+	+	70
568	+	++	+	−	++	+	+	63
388	−	++	+	−	++	+	+	45
833	−	++	+	−	++	+	+	37
565	−	−	−	++	++	+	+	45
1062	−	−	−	−	++	+	+	40

[a] The sera shown in this table are the first sera in which a significant increase in CMV-specific antibody was detected. All these sera were negative for the presence of anti-*E. coli* β-galactosidase antibody.
[b] Days after the previous serum sample.
[c] As determined by ELISA.

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