Cytomegalovirus Early and Late Membrane Antigens Detected by Antibodies in Human Convalescent Sera

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Using indirect immunofluorescence and a panel of human convalescent-phase sera, we identified cytomegalovirus (CMV) early and late membrane antigens (CMV-EMA and CMV-LMA, respectively) as separate entities on the surfaces of viable CMV-infected fibroblasts starting at 6 to 12 and 36 to 48 h postinoculation, respectively. For expression of CMV-EMA and CMV-LMA, infectious virus and active protein synthesis were required, whereas the expression of CMV-LMA, in addition, required viral DNA synthesis. Our data suggest that CMV-EMA and CMV-LMA form an individual set of CMV antigens that are different from intracellular CMV antigens and possibly (partly) different from the viral envelope.

The cell-associated nature of human cytomegalovirus (CMV) points to the importance of virus-induced neoantigens on the surfaces of CMV-infected cells (CMV-MA) as primary targets for the immunological defense of the host. Both cellular (11) and humoral (2, 8) mechanisms may be involved, although their relative contributions to the development of effective antiviral immunity during the acute stages of CMV infections remain to be established. Recent reports have detailed the effectiveness of passive immunization in the prevention (5) and treatment of CMV infections (13). However, the nature of the protective antibody is not known.

Antibodies against CMV-MA have been detected in sera from patients recovering from acute CMV infections (2, 4, 8, 9, 20). In contrast to neutralizing antibodies (10, 14), CMV-MA antibodies are detectable only temporarily early after the onset of disease symptoms (2, 8, 20) and may help to limit viral replication by means of their capacity to mediate the killing of CMV-infected cells (2, 8; J. M. Middeldorp, J. Jongsma, and T. H. The, unpublished data). Recently, evidence was provided for the dissection of the CMV-MA complex into early and late membrane antigens (CMV-EMA and CMV-LMA, respectively) appearing before and after the onset of viral DNA synthesis, respectively (3, 16). To allow further study of the role of humoral immunity against CMV-MA, we investigated the time course of induction of CMV-EMA and CMV-LMA with a panel of human sera.

Sera from 24 CMV-infected patients were obtained within 4 to 30 weeks (mean, 11.8) after the onset of clinical symptoms (Table 1). Control sera were obtained from 35 healthy donors who were either seropositive ($n = 25$) or seronegative ($n = 10$) for CMV. Routine CMV serological testing was performed with an enzyme-linked immunosorbent assay (ELISA) (7). Antibodies against CMV-MA were detectable in 100% of the patients but only in 20% of the CMV-seropositive controls (Table 1). Antibody titers against CMV-MA were significantly lower in the latter controls ($P < 0.001$, Wilcoxon rank sum test). For further studies of CMV-MA induction, we used the 24 patient sera as outlined below.

Cell culture conditions for human fibroblasts and propagation of cell-free stocks of human CMV strain AD169 have been described elsewhere (7). For the specific visualization

of CMV-MA staining on viable cells, we used an improved indirect immunofluorescence procedure as described in detail elsewhere (J. M. Middeldorp, J. Jongsma, and T. H. The, submitted for publication). Briefly, viable CMV-infected cells were washed with ice-cold Hanks balanced salt solution containing 2.6 mM Ca^{2+} , 2.2 mM Mg^{2+} , 180 mM glucose, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) (HH), incubated with appropriate dilutions of human sera (made in HH) for 1 h on ice, washed twice with ice-cold HH and incubated with purified fluorescein isothiocyanate-labeled rabbit immunoglobulin G (IgG) F(ab)₂ fragments specific for human IgM or IgG heavy chain (each at 1 mg/ml; FITC/protein molar ratio [18], 2.4 and 2.1, respectively) at a 1:20 dilution in HH. After three washes with ice-cold HH, the cells were fixed with methanol at -20°C .

The induction of CMV-MA was analyzed by inoculating fibroblasts in five eight-well slides (no. 4838; Miles Scientific, Div. of Miles Laboratories, Inc.; 5×10^4 cells per well) with CMV in parallel for each time point studied, i.e., 0, 3, 6, 12, 18, 24, 36, 48, 72, 96, and 120 h postinoculation (p.i.) (Fig. 1 and 2). Infection at ≥ 2 PFU per cell was essential to achieve simultaneous antigen induction in $>90\%$ of the cells. After inoculation, the cells were washed with HH and maintained at 37°C Dulbecco modified Eagle medium with 3% fetal calf serum until used. At each time point, one slide was fixed for visualization of CMV nuclear antigens (6, 19), and one slide was used for membrane fluorescence with HH and seven control sera negative for CMV antibody but containing antibodies against other herpesviruses, as assessed by complement binding or indirect immunofluorescence or both (17) (all used at a 1:20 dilution in HH). In the remaining three slides (24 wells), each well received a different sample (1:20 dilution) from a set of patient sera (Table 1), and membrane fluorescence was assessed as described above with γ -chain-specific conjugate. None of the sera used in this study showed reactivity against viable noninfected human fibroblasts.

The results are shown in Fig. 1 and 2. At 0 to 3 h p.i., 46 to 66% of the sera (Fig. 1) showed small dots of fluorescence (inoculum staining) which were occasionally concentrated over a small number of cells (Fig. 2a) and which gradually disappeared between 3 and 6 h p.i. (3). Starting at 6 to 12 h p.i. (Fig. 2b), a smooth rim of fluorescence appeared on a small number of cells, and became more prominent on an

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TABLE 1. CMV serology of the sera used in this study

Antigen	Antibody	No. (%) of patients with positive sera (<i>n</i> = 24) ^a	Median titer of patient sera (range) ^b	No. (%) of controls with positive sera (<i>n</i> = 25) ^c	Median titer of control sera (range) ^b
CMV-EMA ^d	IgM	7 (29)	20 (<20–40)	0 (0)	
	IgG	14 (58)	20–40 (<20–80)	0 (0)	
CMV-LMA ^e	IgM	19 (79)	40 (<20–80)	0 (0)	
	IgG	24 (100)	80 (20–320)	5 (20)	<20 (<20–40)
CMV late nuclear antigens ^f	IgG	24 (100)	10,240 (2,560–>40,960)	25 (100)	2,560 (160–40,960)

^a Sera were taken from 24 CMV-infected patients during the acute or convalescent phase of infection. The infection was manifested as CMV mononucleosis-hepatitis (*n* = 6) in previously healthy adults (except for pulmonary complications in 2 patients) or as primary (*n* = 7) or secondary (*n* = 11) symptomatic infections in renal allograft recipients. CMV infection was proven by serology (positive CMV IgM or at least a fourfold rise in CMV IgG [determined by ELISA] or both in all of the patients) and by viral culturing or histopathology or both in 16 of 24 patients.

^b Antibody titers (expressed as the reciprocal dilution) were determined with heavy-chain-specific second antibodies by a microplate modification of the membrane fluorescence procedure described elsewhere (Middeldorp et al., submitted for publication).

^c CMV-seropositive controls were negative for CMV IgM (7) and did not have a known history of symptomatic CMV infections.

^d Antibodies were detected on CMV-infected cells cultured for 48 h p.i. in the presence of 0.5 mM PFA.

^e Antibodies were detected on CMV-infected cells at 96 h p.i.

^f Antibodies were determined by ELISA (7).

FIG. 1. Schematic compilation of membrane fluorescence data at different times after CMV infection for sera from 24 CMV-infected patients. The induction of nuclear antigen staining and the development of cytopathology are shown at the top of the figure. Further details are described in the text and in the legend to Fig. 2. Nuclear fluorescence was studied on CMV-infected cells fixed in situ with acetone or methanol (6, 19). IEA, Immediate early antigen staining; EA, homogeneous early nuclear antigen staining; EA/LA, small condensed nuclear inclusions appearing in a homogeneous background staining; LA, large nuclear inclusions with reduced or absent EA staining. CPE, Cytopathic effect. SP, Speckled inoculum staining (Fig. 2a); +, weak fluorescence not on all cells (Fig. 2b); ++, weak fluorescence on >90% of all cells (Fig. 2c and d); +++, strong fluorescence not on all cells (Fig. 2e and f); ++++, strong fluorescence on >90% of all cells (Fig. 2g and h). —, Nontreated cultures; - - - -, cultures treated with 0.5 mM PFA after infection with CMV.

FIG. 2. Appearance of CMV-EMA and CMV-LMA on the surfaces of CMV-infected fibroblasts as detected with human sera. Infection of fibroblasts and membrane fluorescence staining were done as described in the text. Fluorescence was visualized with a Leitz Diavert fluorescence microscope. (a) Inoculum staining at 3 h p.i.; (b) CMV-EMA staining at 12 h p.i.; (c) CMV-EMA staining at 24 h p.i.; (d) CMV-EMA staining at 72 h p.i. in the presence of 0.5 mM PFA. At 36 to 48 h p.i. in nontreated cultures, the fluorescence intensity per cell increased (CMV-LMA), starting as a low percentage of the cells (e, at 48 h p.i.) and increasing thereafter (f, g, and h, at 72, 96, and 120 h p.i., respectively). Multinucleated fused cells positive or negative for CMV-MA are indicated by arrows. Fluorescein isothiocyanate-labeled rabbit IgG F(ab)₂ fragments specific for human IgG (heavy chain) were used for the visualization of CMV-MA detected by human antibodies.

increasing number of cells (>90%) during the next 12 to 24 h (Fig. 2c). This fine fluorescent lining along the cell boundary (referred to as CMV-EMA staining, see below) was detectable with only 14 of 24 sera (Fig. 1 and Table 1) during the early stages of CMV infection, i.e., 6 to 36 h p.i. Upon further culturing of CMV-infected cells (48 to 120 h p.i.), membrane fluorescence became detectable with all sera (Fig. 1), and the fluorescence intensity per cell increased considerably and became more granular with time p.i. (CMV-

LMA staining, Fig. 2e through h). At very late times p.i. (≥ 120 h p.i.), a small number of cells (<5%) lost CMV-MA expression, whereas nuclear inclusions remained visible by light microscopy (Fig. 2h). No membrane fluorescence was observed at any time p.i. when CMV-antibody-negative sera were used. This demonstrates the virus specificity of the CMV-MA reaction, as these sera all contained antibodies against other human herpesviruses. Heat inactivation of CMV (30 min at 60°C) or treatment of the cells with 0.178

mM cycloheximide 2 h before, during, and after CMV inoculation abolished CMV-MA induction. No antibody bound to the CMV-induced Fc receptor at 0°C, as was shown elsewhere (Middeldorp et al., submitted for publication). CMV-induced cytopathology (Fig. 1, top) was similar to that described by Albrecht et al. (1). When infected at 2 PFU per cell, a small number of multinucleated fused cells that were present from 24 h p.i. onward (Fig. 2c) that also contained CMV-MA. Their number increased with the virus input and with time p.i. Nuclear antigens (Fig. 1, top) were detected with human CMV-seropositive control sera and classified according to their morphology, as described by others (6, 19).

When the 10 sera that were negative for CMV-MA at early times p.i. (Fig. 1) were used in an even more concentrated form (1:4), no membrane fluorescence was observed before 24 h p.i., but some sera reacted strongly (a titer of up to 160) at 72 h p.i. This suggests that different antigens were detected at early and late times p.i. When CMV-infected cells were treated with 0.5 mM phosphonoformate (PFA) to block viral DNA synthesis (7, 21), no differences in staining patterns were observed initially as compared with nontreated cultures (Fig. 2a through c); however, the intensity of membrane staining (CMV-LMA staining) did not increase but rather decreased from 72 to 120 h p.i. (Fig. 2d). Positive staining remained restricted to the 14 sera previously shown to be positive for CMV-EMA (Fig. 1). Adsorption of three sera positive for CMV-EMA (and CMV-LMA) with "early" CMV-infected cells (48 h p.i.) in the presence of 0.5 mM PFA reduced CMV-LMA reactivity and completely removed CMV-EMA reactivity from these sera. Adsorption with "late" CMV-infected cells (96 h p.i., in the presence of 0.5 mM phosphonoformate) completely removed both CMV-EMA and CMV-LMA reactivities, whereas adsorption with noninfected human fibroblasts did not (data not shown). All adsorptions were done with 2×10^7 viable cells per ml of serum for 2 h at 37°C.

From the experiments described above, we conclude that some patient sera ($n = 14$) (Table 1) detected an additional set of CMV-MA whose induction does not require viral DNA synthesis. These antigens, CMV-EMA, were detectable as early as 6 to 12 h p.i., which is earlier than previously described (3, 15, 16, 20). CMV-LMA staining was detectable from ca. 36 to 48 h p.i. as a bright and increasingly granular fluorescence paralleled by the appearance of condensed nuclear inclusions indicative of the assembly of viral progeny (12). As fluorescence staining was performed at 0°C, it is assumed that the observed differences in membrane staining reflect the original antigen distribution on the infected cell membrane (Middeldorp et al., submitted for publication) at different times p.i. This may reflect differences in antigen distribution or in the number of different CMV antigens or both before and after the onset of viral DNA replication. The latter suggestion is supported by adsorption experiments with early infected cells, as shown in this paper and by Boldogh et al. (3).

Sera from healthy CMV-seropositive individuals, who may have high antibody titers to CMV as measured by conventional techniques, such as ELISA (Table 1) and immunofluorescence on fixed CMV-infected cells, are frequently negative for CMV-MA antibodies (2, 8, 9, 20; Middeldorp et al., submitted for publication). In this study, no correlation was found (by linear regression analysis) between the antibody titer CMV late nuclear antigens as measured by ELISA (Table 1) and the CMV-LMA antibody titer, as measured by membrane immunofluorescence in

individual sera from healthy individuals ($r = 0.096$) or CMV-infected patients ($r = 0.27$). The lack of correlation suggests that both techniques measure antibodies against different sets of CMV antigens. This implies that the CMV-MA complex may form an individual set of CMV antigens not related to intracellular (nuclear) CMV antigens, which form the bulk of CMV antigens in glycine extracts (7).

It is interesting that sera from normal healthy seropositive individuals contain neutralizing antibodies (11, 15) and, in addition, that these neutralizing antibodies develop rather late after the onset of CMV infection and remain present long after convalescence (10, 14), whereas CMV-LMA and cytolytic antibody responses are limited to the early stages of CMV infection (2, 8). Therefore, different antigens may be detected by neutralizing and CMV-MA antibodies. This indicates that the antigenic composition of the viral envelope which is acquired at nuclear and (occasionally) cytoplasmic membranes (12) may (partly) differ from the composition of the plasma membrane of the infected cell. Further studies are in progress to substantiate this and to determine the molecular composition of the CMV-MA complex (P. Bruins, J. M. Middeldorp, and T. H. The, Abstr. Eighth Int. Herpesvirus Workshop, abstr. 87, 1983; J. M. Middeldorp, P. Bruins, and T. H. The, submitted for publication). The presence of antibodies against CMV-MA, especially in human convalescent-phase sera, suggests a possible important role of humoral immunity during the acute stage of human CMV infection.

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