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Immune Responses to Isolated Human Cytomegalovirus Envelope Proteins

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A group of envelope proteins of human cytomegalovirus, gA protein (L. Pereira, M. Hoffman, M. Tatsuno, and D. Dondero, Virology 139:73–86, 1984; L. Pereira, p. 383–404, *in* B. Roizman, ed., *The herpesviruses, vol. 3*, 1985), and two protein mixtures (58,000-molecular-weight [58K]-66K and 130K-66K), separated by serial columns prepared with anti-gA immunoglobulin G from sera of immunized guinea pigs, induced neutralizing antibodies and a cellular immune response in the animals. The gA is a disulfide-linked protein complex consisting of high-molecular-weight (>200K), 130K-150K, and 55K-58K proteins.

Human cytomegalovirus (HCMV) infection is involved in a wide range of diseases (see reference 17 for a review), and vaccination might prevent or modify these infections (13). A first step toward the development of a subunit HCMV vaccine is the identification, isolation, and structural analysis of proteins that confer protection.

We have shown previously that HCMV envelope proteins are able to induce humoral and cellular immune responses in guinea pigs (5). Based on this observation, we studied a mixture of antigenically related proteins (gA protein) isolated by Pereira et al. (10, 12) from HCMV-infected cell lysates by immunoaffinity column chromatography with a monoclonal antibody designated CH28-2. The gA preparation was tested again in our laboratory by immunoblot assay under reducing conditions with CH28-2 monoclonal antibody and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The results (not shown) were largely consistent with those of Pereira et al. (10, 12). Thus, two strong bands at the 130,000-molecular-weight (130K)-140K (11, 12; gA3; 138K) and 58K (11, 12; gA6; 58.5K) positions, a weak smear at the 140K-150K position (11, 12; gA1, gA2; 160K-148K, 142K), and two weak bands at the 115K (11, 12; gA4; 123K-107K) and 55K (11, 12; gA7; 56.5K) positions were detected.

To test the immunogenicity of the gA protein preparation, we immunized guinea pigs with gA preparation or, for comparison, with complete envelope preparation (Table 1, experiment 1) and analyzed the sera. The HCMV Towne strain, used for the neutralization assay and for all of the experiments, was propagated on MRC-5 human embryo lung fibroblasts as previously described (5). The guinea pigs developed a high level of neutralizing antibodies quantitated in the presence of complement (16) and also a cellular immune response to HCMV antigens as determined by lymphocyte proliferation assay (5). The humoral immune response directed against the individual glycoproteins was analyzed by immunoblot and immunoprecipitation assays. Infected and uninfected cell lysates were prepared by extraction from infected cells in RIPA buffer (0.01 M Tris hydrochloride [pH 7.4], 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid sodium salt, 50,000 Klett units of aprotinin per 500 ml) on the third day after infection, centrifuged at 100,000 × *g* for 1 h at 4°C, aliquoted, and stored at −80°C. For immunoblot assay, cell lysates were mixed with reducing buffer (50 mM Tris hydrochloride [pH 7.0], 2% SDS, 5% 2-mercaptoethanol [2-ME], 10% glycerol, bromophenol blue), electrophoresed (7), and transferred to a nitrocellulose sheet as previously described (2). Reactivity of immune sera with immobilized polypeptides was assessed as previously described (3). For immunoprecipitation, antigen was prepared as for immunoblotting except that, 48 to 52 h after infection, cells were radiolabeled with 10 μCi of [^{35}S]methionine (Amersham Corp.; specific activity, 1,330 Ci/mmol) per ml in methionine-free minimal essential medium (GIBCO Laboratories) containing 10% dialyzed fetal calf serum (GIBCO). Aliquots of radioactively labeled cell lysates containing equal counts per minute were immunoprecipitated as described by Britt (1) with anti-gA antibodies; immune complexes were eluted from bacteria with a buffer consisting of Tris buffer-saline plus 2% SDS

TABLE 1. Immune response of guinea pigs to HCMV-gA protein preparation and two subsets of this preparation

Expt and no. of guinea pigs	Immunizing antigen[a]	Neutralization[b]	Lymphocyte stimulation index[c]
Expt no. 1			
1	gA	512	10.2
1	gA	512	8.6
12	Viral envelope	137	10.5
Expt no. 2			
1	130K-66K	512	16.5
1	130K-66K	512	ND[d]
1	58K-66K	256	8.2
1	58K-66K	512	ND

[a] Guinea pigs in experiment no. 1 received 40 μg of gA protein or 50 μg of viral envelope preparation per injection per animal, and in experiment no. 2 they received 10 μg of protein per injection per animal. These preparations were mixed with an equal volume of complete Freund adjuvant and injected into animals three times at 2-week intervals. The animals were bled 2 weeks after the last injection.

[b] Data are given as the geometric means of reciprocal dilutions of postimmune sera; preimmune sera did not neutralize the virus in a dilution of 1:8.

[c] Lymphocyte stimulation index is given as mean counts per minute in HCMV antigen-stimulated lymphocytes/mean counts per minute in MRC-5 control antigen-stimulated culture.

[d] ND, Not determined.

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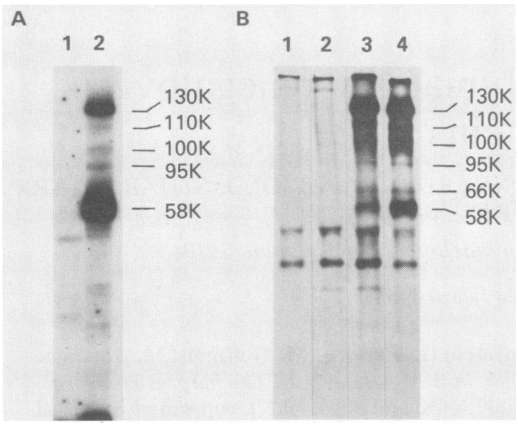

FIG. 1. Autoradiogram of an immunoblot assay for detection of infected cell-specific proteins and fluorograph of electrophoretically separated viral infected cell-specific proteins after immunoprecipitation with polyclonal guinea pig serum specific to gA protein preparation. (A) Immunoblot assay of infected cell lysate (20 μg of protein per lane) with a 10% SDS-polyacrylamide gel. Lanes: 1, mixture of two preimmune guinea pig sera (dilution 1:200); 2, mixture of two anti-gA guinea pig sera (dilution 1:200). (B) Immunoprecipitation assays with a 10% SDS-polyacrylamide gel. Lanes: 1, uninfected cell lysate, mixture of two anti-gA guinea pig sera; 2, infected cell lysate, mixture of two preimmune sera; 3, infected cell lysate, anti-gA serum no. 1; 4, infected cell lysate, anti-gA serum no. 2. Numbers on the right are molecular weights in thousands.

and 5% 2-ME and analyzed on an SDS-polyacrylamide gel. By immunoblot assay of infected cell lysates and anti-gA guinea pig serum, 130K, 110K, 100K, 95K, and 58K protein species were detected (Fig. 1A). SDS-PAGE analysis of viral proteins in infected cell lysates immunoprecipitated with anti-gA antibodies revealed the same species and an additional 66K protein (Fig. 1B). Immunoblot assay was done to determine which of the denatured viral proteins were recognized by the anti-gA serum after electrophoretic separation of the purified virion proteins (Fig. 2) and of the gA protein complex (Fig. 3) in the presence or absence of 2-ME. Anti-HCMV human serum was also used to detect virion proteins in the gA preparation. Virions were purified by centrifugation on a sucrose gradient as previously described (6) and lysed in either reducing buffer or the same buffer without 2-ME (nonreducing buffer). In some experiments, 0.05 M iodoacetamide (Sigma Chemical Co.) was included in the buffers used in the preparation of purified virions and in the nonreducing lysing buffer to prevent artifactual formation of disulfide-linked complexes. Immunoblot assays were carried out as described in the legend to Fig. 1. In the presence of 2-ME, the anti-gA serum recognized 130K and 55K-58K species in the purified virion preparation, whereas in the absence of 2-ME, the same antibodies revealed a broad band ranging from 130K to 150K and also a high-molecular-weight protein (>200K) (Fig. 2). Figure 3A shows that, in the presence of 2-ME, a broad band of gA complex proteins at the 55K-58K position and a light smear at the 130K position were detected by anti-gA serum. In the absence of 2-ME, the 130K-150K protein and a high-molecular-weight protein (>200K) were resolved when anti-gA guinea pig serum was used. The respective results were identical with human HCMV-positive serum (Fig. 3B). Detection of the 55K-58K species in both the purified virion and gA-protein complex preparations, but only in the presence of 2-ME, suggests that the 55K-58K protein is a cleavage product of the 130K protein or of a high-molecular-weight protein bound together or to other gA forms by disulfide bridges or both.

To determine whether the anti-gA antibodies coupled to a cyanogen bromide-activated Sepharose 4B column are able

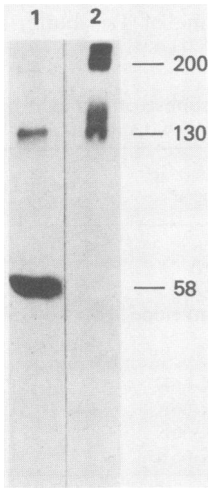

FIG. 2. Autoradiogram of an immunoblot assay for detection of proteins in purified virions lysed in the presence or absence of 2-ME. Purified virions (10 μg of protein per lane) were lysed in the presence or absence of 2-ME as described in the text, electrophoresed on a 7.5% SDS-polyacrylamide gel, and transferred to a nitrocellulose sheet. The strips were then incubated with anti-gA guinea pig serum as described in the legend to Fig. 1A. Lanes: 1, 2-ME, anti-gA serum; 2, no 2-ME, anti-gA serum. Preimmune guinea pig serum produced no reaction with the virion proteins. Numbers on the right are molecular weights in thousands.

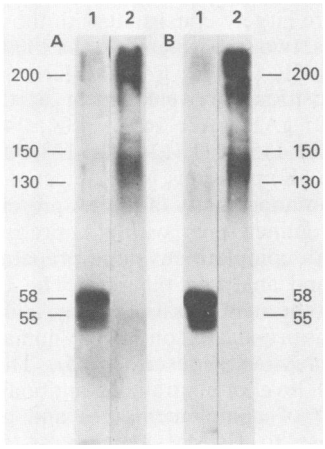

FIG. 3. Autoradiogram of an immunoblot assay for detection of viral proteins in gA protein complex. The gA preparation (5 μg per lane) was lysed in the presence (lanes 1) or absence (lanes 2) of 2-ME, electrophoretically separated on a 7.5% SDS-polyacrylamide gel, and transferred to a nitrocellulose sheet. Incubation with sera was as described in the legend to Fig. 1A. (A) Lanes: 1, 2-ME, anti-gA serum; 2, no 2-ME, anti-gA serum. (B) Lanes: 1, 2-ME, HCMV-positive human serum (tested previously by immunofluorescence and neutralization assay); 2, no 2-ME, HCMV-positive human serum. Preimmune guinea pig serum and HCMV-negative human serum (Cetus) produced no reaction with the gA preparation. Numbers to the left and right are molecular weights in thousands.

to select viral proteins that induce neutralizing antibodies and a cellular immune response, we carried out immunoaffinity column chromatography and immunization of guinea pigs with the isolated proteins. Figure 4 shows two bands at the 58K and 66K positions (subset I) and at the 130K and 66K positions (subset II), respectively (a 10% polyacrylamide gel was used). Using 5% polyacrylamide in the separation gel and no 2-ME in the lysing buffer, we observed a single 300K band in both subsets (data not shown). For immunization of guinea pigs, the pooled dialyzed protein fractions were first concentrated by lyophilization and dissolved in appropriate volumes of phosphate-buffered saline. Two guinea pigs were immunized three times at 2-week intervals with 10 μg of each of the isolated protein preparations. The animals developed high-titer neutralizing antibodies and a cellular immune response (Table 1, experiment 2). In immunoblot assays of infected cell lysates under reducing conditions, the anti-130K-66K and anti-58K-66K sera reacted strongly with the 55K-58K and 130K-150K proteins, whereas the same assay with purified virions as the antigen revealed strong reactivity of these sera with the 55K-58K species but only weak reactivity with the 130K-150K protein. A protein at the 90K-92K position was also detected, which might be an intermediate or breakdown product of the 130K-58K complex. Under nonreducing conditions, the 130K-150K protein and a high-molecular-weight protein (>200K) were detected (data not shown).

Monoclonal antibodies have been described that reportedly coprecipitate 130K and 58K glycoproteins (9) and 142K-138K and 58.5K protein species (10–12). Britt (1) described a disulfide-linked glycoprotein complex within the envelope of HCMV composed of 300K, 160K, 116K, and 55K species. Pereira et al. (12) also recognized the instability of the 138K protein and the abundance of the 58.5K species under reducing conditions but did not find evidence for disulfide linkage. Rasmussen et al. (15) published the presence of 130K and 55K polypeptides existing in the HCMV-infected cell lysate as disulfide-linked multimers and suggested that the 130K and 55K proteins arise from a common high-molecular-weight protein by disruption of disulfide bonds. We found a disulfide-linked protein complex containing 300K, 130K-150K, and 55K-58K protein species in the virion. Since iodoacetamide, which binds to free sulfhydryl groups, was included in the nonreducing buffer, the possibility of artifactual formation of the high-molecular-weight complexes was ruled out.

The presence of the 66K protein in the original gA preparation as detected by SDS-PAGE and silver staining and the isolation by the anti-gA–immunoglobulin G immunoadsorbent column of a 66K protein together with the 58K species (subset I) and together with the 130K species (subset II) were unexpected. Of several possible explanations, we favor the possibility that the 66K protein is an immunoglobulin Fc receptor and is bound nonspecifically to the columns.

By loading the unbound material on the immunoaffinity column after the first cycling, we were able to fractionate the gA proteins into two subsets (I and II). Strong detection by both anti-130K-66K and anti-58K-66K sera of the 130K and 58K proteins in purified virions and infected cell lysates also indicated that common antigenic sites are present in the 130K and 58K proteins and that some of these are involved in the neutralization of virions.

An 86K protein and a mixture of two 130K and 55K proteins isolated from infected cell lysate induced neutralizing antibody in vivo (14). We found that not only humoral but also cell-mediated immunity was stimulated by the isolated gA protein complex and by two subsets of it. Our results also demonstrated the presence of the 130K-58K complex in the virion envelope, as our starting material for the isolation was envelope. We detected the 130K protein and a higher-molecular-weight protein (~300K) of the gA complex in purified virion preparations by immunoblot assays, in contrast to Rasmussen et al. (15) and Nowak et al. (9), who were unable to detect the 130K polypeptides in extracellular virions. Further studies are needed to determine whether (i) the primary structures of the 130K and 58K proteins have the same or different antigenic determinants or (ii) the presence of conformational determinants present in the isolated protein mixtures is responsible for the immune response.

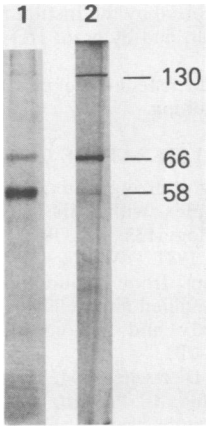

FIG. 4. SDS-PAGE analysis of the bound fractions of anti-gA–immunoglobulin G–Sepharose 4B column. Immunoglobulin G from sera from guinea pigs immunized with gA protein was purified as previously described (4) and coupled to CNBr-activated Sepharose 4B (Pharmacia, Inc.) at a concentration of 3.5 mg of protein per cm^3 of packed beads. A column (1 by 20 cm) containing approximately 41 mg of purified anti-gA immunoglobulin G coupled to 11.4 cm^3 of Sepharose 4B beads was prepared per manufacturer instructions. The precolumn containing immunoglobulin G from sera of guinea pigs immunized with uninfected MRC-5 cells was prepared as described above. The HCMV envelope material (8 mg of protein), prepared as previously described (5), was mixed with ^{35}S-labeled, purified virions (200 μg of protein; infected cells were incubated with 20 μCi of [^{35}S]methionine per ml starting on day 3 after infection and purified) treated with 0.5% Nonidet P-40 for 1 h at 4°C, loaded on the anti-MRC-5–immunoglobulin G–Sepharose 4B precolumn, and cycled over three times. The flowthrough fraction of the last cycle was loaded onto the anti-gA–immunoglobulin G–Sepharose 4B column and cycled five times, and the unbound material was stored. The column was then washed extensively, and the bound material was eluted with 0.1 M diethylamine buffer (pH 11.5) containing 0.5% (wt/vol) Nonidet P-40. Fractions were collected in tubes containing neutralizing buffer (1 M Tris hydrochloride [pH 7.2]), and radioactivity was determined by scintillation counting. Peak fractions were pooled and dialyzed against distilled water overnight (subset I). The stored, unbound material was loaded back onto the column and cycled five additional times. The washing and elution procedures were the same as before. The peak fractions were pooled and dialyzed (subset II). Total protein content of subsets I and II was 600 μg, determined as described by Lowry et al. (8), and the protein composition of these fractions was analyzed by SDS-PAGE. The electrophoretically separated proteins were visualized by silver staining (18). Lanes: 1, 1.2 μg of protein of the first bound material (subset I); 2, 1.0 μg of protein of the second bound material (subset II). Numbers on the right are molecular weights in thousands.

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