Monoclonal Antibodies to Guinea-pig Cytomegalovirus: An Immunoelectron Microscopic Study

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SUMMARY

Three groups of monoclonal antibodies which reacted with cells infected by guinea-pig cytomegalovirus (GPCMV) were prepared. The first group of antibodies immunoprecipitated a 50 000 mol. wt. (50K) polypeptide of GPCMV-infected cells. This polypeptide was identified as part of the nuclear inclusion by immunofluorescence. This nuclear fluorescence was markedly diminished when the infected cells were incubated in the presence of phosphonoacetic acid. By immunoelectron microscopy the antibodies reacted mainly with filamentous structures (26 to 28 nm in diameter) in nuclear inclusions and occasionally stained nucleocapsids. Neither intracytoplasmic nor extracellular virions reacted with the antibodies. Therefore, the 50K protein with which the monoclonal antibodies reacted was nuclear inclusion-specific and a non-structural protein. The second group of antibodies reacted with a 76K polypeptide of the infected cells which was a matrix protein found in both cytoplasmic inclusions and extracellular dense virions. The third group of antibodies mainly reacted with a virion core protein by immunoelectron microscopy.

INTRODUCTION

Guinea-pig cytomegalovirus (GPCMV) infection resembles human cytomegalovirus (HCMV) infection with respect to the growth pattern of the virus in vitro, development of cytopathology and transplacental infection of the foetus. Therefore, GPCMV provides an experimental model for HCMV infection, useful especially in the analysis of intrauterine infection (Choi & Hsiung, 1978; Kumar & Nankervis, 1978).

The cytopathology induced in man, in animal hosts and in various cell cultures has been recognized as characteristic of cytomegalovirus infection, notably cell rounding and formation of nuclear and cytoplasmic inclusions (Smith, 1956; Hartley et al., 1957; Albrecht et al., 1980).

The ultrastructural development of GPCMV-infected cells has been reported by Middelkamp et al. (1967), Patrizi et al. (1967), Fong et al. (1979, 1980) and Fong (1982). The nuclear inclusions in GPCMV-infected cells consist of a variable number of associated subunits: nucleocapsids, tubular structures, electron-dense fibrils and amorphous matrices (Fong et al., 1979, 1980). Antigenic relationships between these subunits and cytoplasmic and extracellular virions or dense bodies have been investigated using polyvalent hyperimmune sera (Fong et al., 1979; Fong & Brigati, 1982).

In this study, we produced monoclonal antibodies reacting specifically with intranuclear inclusions, matrix protein and viral core proteins of GPCMV, and we investigated their reactivity with infected cells by immunoelectron microscopy.

METHODS

Virus and cell cultures. GPCMV, prototype strain 22122, was obtained from the American Type Culture Collection and was grown in guinea-pig embryo (GPE) cell culture. Primary GPE cell cultures were prepared from a 35-day-old embryo of a Hartley strain guinea-pig. GPE cells were grown in Eagle's MEM containing penicillin...
(100 units/ml) and streptomycin (50 μg/ml) and 10% foetal calf serum (FCS). The titre of the virus stock was 5 × 10^6 p.f.u./ml.

Production of monoclonal antibody-producing cell lines. Six-week-old BALB/c mice were immunized with nuclei of GPCMV-infected GPE cells. Nuclei were prepared 72 h after infection as described previously (Tsutsui et al., 1983), sonicated for 15 s with a sonicator (Kontes Co. Ltd) at maximum setting, and emulsified in an equal volume of Freund’s complete adjuvant. Four weeks later, animals were injected with the same antigen in incomplete Freund’s adjuvant. Three days after the final booster injection, spleen cells were fused to myeloma (P3-NS/1-Ag4-1) cells as described by Köhler & Milstein (1975). Hybrid cells were obtained by growing the cells in a selective medium containing hypoxanthine, aminopterin and thymidine (HAT) in 96-well tissue culture plates. For screening and isolation of hybridoma cells, RPMI 1640 medium (Gibco) was used throughout the experiments. Hybridoma culture fluids were screened for their reactivity with sonicated nuclei of the GPCMV-infected GPE cells, and with control sonicated nuclei of uninfected GPE cells, by an ELISA that was essentially the same as that of Kimura-Kuroda & Yasui (1983). The selected hybridoma cells were cloned twice by limiting dilution. The cloned hybridoma cells were injected into the peritoneal cavity of BALB/c mice and ascites antibody-containing fluid was collected.

Labelling of infected cultures with radioisotope, cell extraction and immunoprecipitation. The GPE cells plated in 25 cm² flasks 72 h after infection with GPCMV (or uninfected cells) were labelled with [35S]methionine (50 μCi/ml, Amersham) for 3 h in methionine-free MEM supplemented with 10% FCS. Radiolabelled cultures were washed with Hank’s solution three times, and 1.5 ml of extraction buffer (EB) was added as described by Blanton & Tevethia (1981). The cell extracts were incubated with 20 μl normal rabbit serum and 100 μl of a 50% suspension of Protein A-agarose (E Y Laboratories) for 15 min at room temperature. The suspension was centrifuged in an Eppendorf microfuge at 10000 r.p.m. for 2 min, and 120 μl of the supernatant was added to 10 μl of ascites of the hybridoma cells. The mixtures were agitated overnight at 4°C, added to 40 μl of a 50% suspension of Protein A–agarose, incubated for 15 min at room temperature and centrifuged in an Eppendorf microfuge at 10000 r.p.m. for 2 min. The pellets were washed three times with 1 ml of washing buffer as described by Blanton & Tevethia (1981) and suspended in 40 μl SDS sample buffer as described by Laemmli (1970). The suspensions were heated at 100°C for 2 min and centrifuged in an Eppendorf microfuge for 2 min. The supernatants were subjected to SDS-PAGE (10% polyacrylamide) according to the method of Laemmli (1970). After electrophoresis, gels were fixed in 7% TCA, immersed in 99% ethanol and then in 1M-sodium salicylate, and dried. The gels were exposed on Fuji RXO-H X-ray film at −70°C for 3 to 10 days. [14C]Methylated polypeptide molecular weight standards (Amersham) were co-electrophoresed.

Preparation of labelled virions. GPE cells plated in 175 cm² flasks 72 h after infection with GPCMV were labelled with [35S]methionine (20 μCi/ml) for 6 h as described above. The labelled culture fluid was centrifuged at 5800 g for 20 min. The supernatant was centrifuged in a Hitachi RSP-27 rotor at 75000 g for 90 min. The pellets were suspended in TBS buffer (Tris–HCl pH 7.4, 0.15 M-NaCl) and centrifuged on a 30/55% discontinuous sucrose gradient in a Hitachi RSP-40T rotor at 152000 g for 90 min at 4°C. The band at the interface was collected and centrifuged at 152000 g for 90 min in the same rotor. The pellets were used as a virion preparation.

Immunofluorescence. GPE cells plated on coverslips were infected with GPCMV or left uninfected. At appropriate times after infection the monolayers were fixed with 4% paraformaldehyde in 0.1 M-phosphate buffer pH 7.4, and post-fixed with cold acetone. The fixed cells were incubated for 30 min with hybridoma culture fluid at 37°C, then washed with three changes of phosphate-buffered saline (PBS) and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Miles Laboratories) diluted 30-fold. After a final wash the preparations were dehydrated with ethanol, mounted with Entellan (Merck), and viewed under a fluorescence microscope.

Immunoelectron microscopy. GPE cells plated on glass slides were infected with GPCMV at a multiplicity of 5 p.f.u./cell. At 27 h and 72 h after infection, the cell monolayers were washed with PBS and fixed in periodate–lysine–paraformaldehyde (PLP) as described by McLean & Nakane (1974) for 3 h on ice. The cell monolayers were incubated in 10% 15%, and 20% sucrose in PBS for 30 min each, then frozen in solid CO 2–ethanol for 3°C and then in 20% sucrose in PBS. The cells were incubated with monoclonal antibodies (ascites diluted 1:100) overnight at 4°C, then washed for 3 h with several changes of PBS. The cells were incubated with peroxidase-conjugated anti-mouse IgG (Cappel Laboratories) at 1:100 dilution for 3 h at 4°C and washed in the same manner as described above. Before the benzidine reaction, the cells were fixed in 0.5% glutaraldehyde in PBS for 5 min and washed with PBS. The cells were immersed in diaminobenzidine solution (0.02% diaminobenzidine, 50mM Tris–HCl pH 7.6) for 15 min, then incubated in diaminobenzidine solution with 0.05% H 2 O 2 for 5 min at room temperature. After washing with distilled water the cells were fixed in 1% osmium tetroxide for 1 h on ice, then subjected to the routine process of dehydration and embedding. The cell monolayers were covered with gelatin capsules filled with Epon mixture. After polymerization the capsules were removed from the glass by heating on a hot plate at 80°C for 5 min. Ultrathin sections were cut parallel to the monolayer surface in a LKB 8800 Ultrotome III using a diamond knife. Unstained thin sections were examined in a JEOL (JEM-100B) electron microscope at 60 kV.
Monoclonal antibodies to GPCMV

Fig. 1. Photographs of immunofluorescence reactions of GPCMV-infected GPE cells using monoclonal antibodies E-16 (a), A-29 (b) and A-13 (c). After adsorption with GPCMV, GPE cells incubated for 24 h were used for the reaction with monoclonal antibody E-16 and cells incubated for 72 h were used for the reaction with monoclonal antibodies A-29 and A-13. Fixation and immunofluorescence procedures are described in Methods. Magnification ×266.

Table 1. Monoclonal antibodies to GPCMV-infected cells*

<table>
<thead>
<tr>
<th>Group</th>
<th>Monoclonal antibody</th>
<th>Polypeptide apparent mol. wt.†</th>
<th>Immunofluorescence‡</th>
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<tr>
<td>A</td>
<td>B-16</td>
<td>50K</td>
<td>Nuclear</td>
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<tr>
<td></td>
<td>B-29</td>
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<td></td>
<td>D-13</td>
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<td></td>
<td>E-16</td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>A-29</td>
<td>76K</td>
<td>Cytoplasmic granular</td>
</tr>
<tr>
<td></td>
<td>B-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>A-9</td>
<td>78K</td>
<td>Faint granular cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>A-13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>D-22</td>
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* Guinea-pig whole embryonal fibroblasts were infected with GPCMV. Three days after infection the nuclei were isolated from the infected cells and sonicated before immunization.
† Molecular weights were determined after immunoprecipitation and SDS-PAGE.
‡ Immunofluorescence tests of the infected GPE cells were performed using culture fluids from antibody-producing hybridoma cell lines.

RESULTS

Isolation of hybridoma cell lines producing monoclonal antibodies to GPCMV-infected cells

Ten hybridoma cell lines which produced antibodies reacting with GPCMV-infected nuclei but not with uninfected nuclei in ELISA were cloned (Table 1). In immunofluorescence tests of GPE cells infected with GPCMV, three patterns were observed with these monoclonal antibodies: the first group showed nuclear fluorescence (Fig. 1a), the second group showed granular fluorescence mainly in the cytoplasm (Fig. 1b), and the third group showed faint granular fluorescence mainly in the cytoplasm (Fig. 1c). Fluorescence was not detected with any of the monoclonal antibodies in cells which had been treated with cycloheximide (50μg/ml) for 16 h after infection and released from the block by washing and adding new medium for 2 h. This means that these antibodies do not detect the immediate-early viral antigens. Nuclear fluorescence in tests using the first group of antibodies and cells incubated for 24 h after
infection was markedly reduced if the cells had been incubated in the presence of phosphonoacetic acid (PAA; 100 μg/ml). Using the second and third groups of antibodies, only trace amounts of fluorescence were observed in the cells 24 h after infection and no fluorescence was observed in cells treated with 100 μg/ml PAA. These results suggested that the monoclonal antibodies used react with late viral antigens. The nuclear antigen detected by the first group of antibodies, however, apparently began to appear prior to the antigens detected by the second and third groups of antibodies.

Identification of polypeptides recognized by the monoclonal antibodies

At 72 h after infection, GPE cells were labelled with [35S]methionine for 3 h and the polypeptides in the cell lysate were separated by 10% SDS–PAGE. Infected cell-specific polypeptides (ICSPs) were defined by comparison with the polypeptides from uninfected cells.

Fig. 2. Fluorogram of immunoprecipitated GPCMV-infected cell-specific polypeptides using monoclonal antibodies to GPCMV-infected nuclei. At 72 h after infection, GPE cells were labelled with 50 μCi [35S]methionine/ml for 3 h. Cell extracts were immunoprecipitated with monoclonal antibodies A-29 (b), B-9 (c), E-16 (d), A-13 (f) and normal mouse serum (g). (a) Infected cell extract; (e) mol. wt. markers.
Monoclonal antibodies to GPCMV

Major ICSPs with molecular weights of 125000 (125K), 105K, 76K, 65K, 56K and 50K were recognized, as shown in Fig. 2(a). By immunoprecipitation, monoclonal antibodies that showed nuclear fluorescence precipitated a polypeptide with a mol. wt. of about 50K (Fig. 2d); antibodies in the second group, which showed granular fluorescence in the cytoplasm, precipitated a polypeptide of mol. wt. about 76K (Fig. 2b, c); antibodies in the third group, which showed faintly granular fluorescence, precipitated a polypeptide of mol. wt. about 78K (Fig. 2f) (Table 1).

An extract from mock-infected cells labelled with [35S]methionine in the same way as the infected cells was also immunoprecipitated with each of the three groups of antibodies but no definite band was obtained (data not shown).

Immunoprecipitation of extracellular virions and dense bodies was performed using the three groups of antibodies. The molecular weights of the major polypeptides of the virions and the dense bodies were 105K, 84K, 76K to 78K, 70K and 64K (Fig. 3d). The first group of antibodies, which precipitated the 50K polypeptide, did not react with the virions or the dense bodies (Fig. 3a), while the second and third groups of antibodies did react with the virions and the dense bodies (Fig. 3b, c). In the case of antibody A-13, a faint band was also detected at 105K in addition to that at 78K. Although not established in the present study, it is possible that A-13 might cross-react with a common epitope of these two molecules.
Nuclear inclusion-specific antigen detected by monoclonal antibodies

By immunoelectron microscopy, peroxidase labels were detected in nuclear inclusions of GPE cells 27 h after infection, by the first group of monoclonal antibodies which recognized the 50K polypeptide (Fig. 4). The peroxidase labels corresponded to nuclear inclusions which were forming fibrillar networks (Fig. 4). At 72 h after infection, the peroxidase label had spread all over the nucleus, although the nuclear inclusions were more intensely labelled (Fig. 5). The nucleocapsids in the inclusions showed various types of staining pattern with the antibodies to 50K antigen: nucleocapsids with double-ringed staining, occasionally with spikes between the rings, nucleocapsids with strongly stained cores and nucleocapsids with hollow unstained centres were found (Fig. 6a). In the nuclear inclusions that had less intense labelling with the antibodies to 50K antigen in their central regions, unstained nucleocapsids were prominent in the peripheral part of the inclusions (Fig. 6b). The peroxidase-labelled structures in the nuclear inclusions were filamentous (26 to 28 nm diam.) (Fig. 4; Fig. 6a, b).

Although nucleocapsids and cores were sometimes stained in the nuclear inclusions, neither the virions in the dense matrices in the cytoplasm nor those in the extracellular space were stained in cells by antibodies to the 50K antigen (Fig. 6c).

Detection of viral matrix protein and viral core protein by monoclonal antibodies

Immunoelectron microscopy with the monoclonal antibodies in the second group was performed using GPE cells 72 h after infection. The dense matrices in the cytoplasm (Fong et al., 1979) and the matrix protein of the extracellular dense bodies and dense virions (Fong et al., 1979) were stained in cells with this group of antibodies. The staining pattern of the dense matrices of the cytoplasm was loosely stranded, while that of the extracellular dense bodies and
Monoclonal antibodies to GPCMV

Fig. 5. Electron micrograph of GPE cell 72 h after infection with GPCMV subjected to an immunoperoxidase reaction using monoclonal antibody E-16. Peroxidase label was observed all over the nucleus. The nuclear inclusion (NI) was intensely labelled where nucleocapsids were labelled in various patterns (arrows). Bar marker represents 1 μm.
Fig. 6. Nuclear inclusions, extracellular dense virions and dense bodies in GPCMV-infected GPE cells (72 h after infection) subjected to an immunoperoxidase reaction using monoclonal antibody E-16. (a) Nucleocapsids showed various types of staining patterns; some were stained inside the capsids (long arrows) and the others were ring-shaped with unstained centres (short arrows). Arrowheads show labelled filamentous structures. NE, Nuclear envelope. (b) Hollow unstained capsids were prominent in a nuclear inclusion of a different cell. (c) Extracellular dense virions (DV) and dense bodies (DB) were not stained. Bar markers represent 0.5 μm.
Fig. 7. Electron micrographs of GPE cells 72 h after infection, immunoperoxidase-stained using monoclonal antibody A-29. DM, Dense matrix; NI, nuclear inclusion; DV, dense virion; DB, dense body. Bar markers represent 1 μm (a, b) and 0.5 μm (c).
dense virions was compact (Fig. 7a, b). Some dense bodies were stained only in the outer membrane. The interiors of the virions (Fig. 7c) and the nuclear inclusions were not stained by this group of antibodies (Fig. 7a).

Using the third group of antibodies, virion cores in both the cytoplasm and the extracellular spaces were stained, but the matrix of the dense virions and dense bodies was hardly stained (Fig. 8a, b). The nuclear inclusions were not stained with this group of antibodies.

**DISCUSSION**

We generated hybridomas producing monoclonal antibodies to GPCMV-infected cells. According to the staining patterns by immunofluorescence and the polypeptides detected by immunoprecipitation, three groups of monoclonal antibodies were defined: antibodies to a 50K polypeptide which is located in nuclear inclusions, antibodies to a 76K polypeptide which is in the cytoplasmic inclusions and virions and dense bodies in the extracellular space, and antibodies to a 78K polypeptide which is present in the core of the virions.

Monoclonal antibodies to HCMV have been prepared using infected cells (Pereira et al., 1982; Goldstein et al., 1982) and virions (Nowak et al., 1984; Rasmussen et al., 1984) as immunogens and from a human × human hybridoma using Epstein–Barr virus-transformed lymphoblastoid cells (Emanuel et al., 1984). There has been no report so far about monoclonal antibodies to GPCMV-infected cells or to the virions.

By immunoelectron microscopy using monoclonal antibodies to the 50K polypeptide, peroxidase labelling was associated first with nuclear inclusions, then extended to the rest of the nuclear regions. Nucleocapsids in the nuclear inclusions had different staining patterns with these antibodies. Some of the capsids were apparently filled with the label and others did not contain the antigen.

It is possible that 50K antigens are enclosed in nucleocapsids in the nuclear inclusion and are then transported through the cytoplasm to the extracellular space as a core protein in the virions. An important question is whether the nuclear antigen detected by the monoclonal antibodies is a viral structural protein or a non-virion protein. We concluded that the nuclear antigen is a non-virion protein for the following reasons: staining of intracytoplasmic and extracellular virions was not detected by immunoelectron microscopy, and the majority of nucleocapsids in the peripheral region of the nuclear inclusions were not stained. It is possible that once the antigen becomes embedded inside the capsids or the virions, antibodies are not able to reach it. Using antibodies to the 78K polypeptide, however, peroxidase labelling was detected inside the virions. Furthermore, no detectable precipitate was obtained when immunoprecipitation of the extracellular virions and dense bodies was performed using antibodies to the 50K polypeptide. Similarly, Gibson et al. (1981) reported that cells infected with the Colburn strain of
Monoclonal antibodies to GPCMV also contained a large amount of non-virion nuclear protein which had a DNA-binding ability.

Ultrastructurally, nuclear inclusions in GPCMV-infected cells consist of several components such as electron-dense amorphous matrices, tubular structures, bundles of electron-dense fibrils and electron-dense granules (Fong et al., 1979; Fong, 1982). It might be interesting, from the standpoint of viral morphogenesis, to establish which components of the nuclear inclusion react with antibodies to the 50K polypeptide. By immunoelectron microscopy the nuclear inclusions were stained as filamentous structures (26 to 28 nm diam.). These structures did not correspond to any of the components of the nuclear inclusions observed in routinely stained thin sections. Considering the apparent increase in size of the structures as detected by indirect immunological reactions, the filamentous structures may correspond to the 10 nm fibrils of the inclusions. Fong (1982) demonstrated by electron microscopic autoradiography that virus DNA is present in both the electron-dense amorphous matrices and the fibrils.

The fact that the nucleocapsids in the inclusions stain in different patterns and finally appear unstained suggests that the 50K nuclear antigen may play a special role in viral morphogenesis, such as providing the sites for assembly of the virus proteins in addition to its possible role in viral DNA synthesis.

A unique characteristic of GPCMV-infected GPE cells is the formation of enveloped dense virions containing one or more capsids, and dense bodies in the cytoplasm of infected cells, although the mechanism by which the enveloped dense virions form is not clear. By immunoelectron microscopy using monoclonal antibodies which immunoprecipitated the 76K polypeptide, peroxidase labelling was observed in the cytoplasmic dense matrix, the matrices of the dense bodies and dense virions in extracellular spaces. These results suggest that an antigenic determinant of the matrix protein in the intracytoplasmic dense matrix cross-reacts with that of the matrix protein of the virions and the dense bodies in the extracellular spaces. In contrast, almost no peroxidase label was observed in the nuclear inclusion. Formation of dense bodies in HCMV-infected human cells has been reported (Fiala et al., 1976) and antigenic analysis of dense bodies and dense virions in HCMV-infected cells showed a similar cross-reactivity. In fact, Fong et al. (1979) observed that the dense bodies and the dense virions in GPCMV-infected cells share a common surface antigen(s). Thus, monoclonal antibodies against 76K polypeptide provide a tool for studying the mechanism of formation of these unique structures.

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