Characterization of neutralizing monoclonal antibodies to murine cytomegalovirus

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Monoclonal antibodies (MAbs) and polyclonal antibodies raised in mice to murine cytomegalovirus (MCMV) were characterized in vitro by their virus-neutralizing activity and their reactivity with MCMV polypeptides and MCMV-infected mouse embryo fibroblasts (MEF). MCMV was neutralized by the MAbs prior to virus adsorption to MEF by a complement-dependent mechanism. Although the neutralization of MCMV prior to virus adsorption to MEF by polyclonal antibodies was enhanced in the presence of complement, MCMV was also neutralized by a complement-independent mechanism after virus adsorption. No correlation was observed between the level of neutralization of MCMV and the ability of MAbs or polyclonal antibodies to interfere with the binding of the virus to MEF. As the neutralization of MCMV with polyclonal antibodies by both complement-dependent and -independent mechanisms may reflect the interaction of antibodies with different specificities, the ability of each MAb to interact with a second MAb was investigated. One MAb, 1E8, inhibited the neutralizing activity of several other MAbs. Several MAbs reacted with multiple polypeptides by immunoprecipitation and Western blotting analysis; MAb AC1 cross-reacted with a neutralizing 92K/98K MCMV domain and a 70K ribonucleoprotein, the latter with which sera from patients with connective tissue diseases also reacted. This suggests that an homology exists between these proteins, which may lead to the development of autoimmune manifestations in vivo.

Introduction

The humoral and cell-mediated responses of mice to murine cytomegalovirus (MCMV) infection have provided a useful experimental model of the immune responses elicited by human cytomegalovirus (HCMV) in man (reviewed by Griffiths & Grundy, 1987). In both the human and murine host, CMV infection elicits antibodies that possess neutralizing activity in vitro (Stalder & Ehrensberger, 1980; Lawson et al., 1988) and reduce the severity of CMV-induced disease when passively administered in vivo, particularly in the immunocompromised host (Araulho-Cruz et al., 1978; Shanley et al., 1981; Winston et al., 1987). As considerable heterogeneity in the recognition of HCMV proteins by different human sera has been observed Landini & Michelson (1988), the murine model has proved useful for the analysis of viral proteins recognized during seroconversion and for studying the parameters that may contribute to the heterogeneous response in man. Immunoblotting studies have shown that antibodies to both immediate-early and structural MCMV proteins are elicited in response to MCMV infection; the temporal appearance and level of serum antibodies reactive to individual proteins were influenced by the genetic constitution and immune status of the host, as well as by the dose of the virus inoculum (Farrell & Shellam, 1989).

In addition to the protection afforded by the humoral response, antibodies induced during HCMV and MCMV infection also react with normal cell components and may contribute to the autoimmune manifestations that accompany CMV infections in mice and man (Wager et al., 1968; Andersen & Andersen, 1975; Bartholomaeus et al., 1988). This phenomenon may be triggered by immunological cross-reactivity between viral and self proteins (Fujinami et al., 1988), or the incorporation of host cell components into the viral envelope (Michelson et al., 1989).

Further analysis of the CMV proteins which possess epitopes for either virus-neutralizing or self-reactive antibodies necessitates the use of monoclonal antibodies (MAbs). Indeed, such antibodies have been raised in mice to HCMV proteins for characterizing the viral proteins that elicit an antibody response in man (Nowak et al., 1984), including those which react with self
components (Michelson et al., 1989). However, as HCMV does not cause a productive infection in mice, the viral determinants recognized by mice may differ from those that elicit antibody responses during natural HCMV infections in man.

Accordingly, we have produced and characterized MAbs to structural proteins of MCMV in mice and investigated their ability to neutralize MCMV in vitro and to react with host cell proteins. Evidence is presented for the presence of neutralizing determinants on a number of different MCMV proteins and for the mechanisms of neutralization for monoclonal and polyclonal antibodies. A MAb which reacted with both a neutralizing MCMV epitope and with a ribonuclear protein in uninfected cells is also described.

**Methods**

**Virus.** The Smith strain of MCMV was maintained by salivary gland passage in weaning female BALB/c mice (Chalmers et al., 1977). The virus was attenuated by a single passage in mouse embryo fibroblasts (MEF) derived from outbred CD-1 mice and was used as the source of infectious virus for all subsequent in vitro assays. For the preparation of MCMV antigen, the virus was clarified, pelleted at 100000 g and passed through a Bio-Gel A-15m column (Bio-Rad), as previously described (Farrell & Shellam, 1989). Viral protein estimations were performed by the method of Bradford (1976).

**Reference sera.** For the preparation of hyperimmune anti-MCMV sera, both resistant (B10.BR and BALB/K) and susceptible (C57BL/10 and BALB/c) mice were inoculated intraperitoneally (i.p.) with 10⁸ p.f.u. MCMV and challenged with 10³ p.f.u. 4 weeks later. Sera were collected 14 days after the final challenge and pooled. Normal mouse serum (NMS) was obtained from uninfected mice. All sera were heat inactivated (56 °C, 30 min) and stored at −70 °C until use. Human anti-ribonuclear protein sera [designated anti-(RNP + Sm) sera] were obtained from patients with mixed connective tissue disease and were a kind gift from Dr Michael Garlepp, Department of Medicine, University of Western Australia.

**MAb production.** Mice were inoculated subcutaneously with 1 µg of MCMV antigen emulsified in Freund's complete adjuvant. After 30 days mice were inoculated with 0.1 µg of MCMV antigen in phosphate-buffered saline (PBS) having the osmolality of mouse serum (330 mOsm) by both the i.p. and intravenous routes. Spleens were removed 4 days later and were used as the source of extracellular MCMV for immunoprecipitation studies (described below). The binding of radiolabelled MCMV to MEF was inhibited by purified, unlabelled MCMV in competition experiments (not shown).

**Preparation of radiolabelled MCMV.** Confluent MEF were infected with MCMV at an m.o.i. of 10 in MEM-2. Following an 8 h incubation at 37 °C the medium was replaced with MEM-2 containing 20% of the normal concentration of unlabelled methionine and [³⁵S]methionine (1329 Ci/mmol; Amersham) was added to a final concentration of 30 µCi/ml. Uninfected MEF were also labelled with [³⁵S]methionine under the same conditions. Following a labelling period of 22 h the extracellular fluid was harvested and the virus pelleted from the MCMV-infected cells at 100000 g. This preparation was used as the source of extracellular [³⁵S]methionine-labelled MCMV for binding studies (described below). The binding of radiolabelled MCMV to MEF was inhibited by purified, unlabelled MCMV in competition experiments (not shown).

**Preparation of radiolabelled MCMV.** Confluent MEF were infected with MCMV at an m.o.i. of 10 in MEM-2. Following an 8 h incubation at 37 °C the medium was replaced with MEM-2 and infected and uninfected cells were lysed with buffer (0.05 M-Tris HCl pH 7.2, 0.15 M-sodium chloride, 1% Triton-X 100, 1% sodium deoxycholate, 1 mM-phenylmethylsulphonyl fluoride and 100 units of aprotinin per ml) sonicated for 1 min at 4 °C and clarified at 100000 g for 1 h. All radiolabelled preparations were stored at −70 °C until use.

**Immunoprecipitation.** Radiolabelled proteins from infected and uninfected MEF (0.04 ml) were mixed with 0.04 ml MAb (100 µg) and incubated for 1 h at 4 °C. Immune precipitates were collected with Protein A-Sepharose (Pharmacia) and unreacted antigen was removed by precipitation procedures the cell lysates were pretreated by incubation at 4 °C with NMS and Protein A-Sepharose. Samples were prepared for SDS–PAGE by boiling for 3 min in sample buffer (0.05 M-Tris–HCl pH 6.8, 20% SDS, 5% 2-mercaptoethanol and 0.005% bromophenol blue).

**SDS–PAGE and fluorography.** Immune complexes were separated by SDS–PAGE and the separated proteins were transferred to nitrocellulose (Towbin et al., 1979). The reactivity of MAbs (diluted to 100 µg/ml) with polypeptides was detected with an alkaline phosphatase-conjugated goat anti-mouse IgG + IgM F(ab')₂.
Neutralizing monoclonal antibodies to MCMV

Preparation of ribonuclear protein extracts. Ribonuclear protein was prepared according to the method of Tan & Peebles (1976).

Densitometry. The level of reactivity of monoclonal and polyclonal antibodies with MCMV structural proteins and infected-cell extracts was determined using a video densitometer (Bio-Rad). The Mr of viral proteins were determined by comparison with standards of known Mr (Bio-Rad).

Neutralization of MCMV prior to adsorption to MEF. Detection of virus-neutralizing antibody in vitro by plaque reduction on MEF has previously been described (Lawson et al., 1988). Briefly, serial dilutions of MAbs were diluted in MEM-2 in the presence or absence of 5% fresh guinea-pig complement (GPC) and incubated with MCMV for 90 min at 37 °C in 5% CO₂. Following the incubation the virus-antibody mixture was added to washed MEF monolayers for a further 60 min at 37 °C, after which time it was replaced with methyl cellulose (Fisher Scientific) containing 2% FCS. The MEF monolayers were stained with methylene blue after 5 days at 37 °C in 5% CO₂ and the plaques counted. Control cultures contained virus incubated with heat-inactivated (56 °C, 30 min) NMS.

Neutralization after MCMV adsorption to MEF. MCMV was diluted in cold MEM-2 and incubated with MEF monolayers for 60 min at 4 °C. Following the incubation the monolayers were washed five times with cold MEM to remove unbound MCMV and incubated for a further 60 min at 4 °C with serial dilutions of MAbs containing 5% FCS. The virus-antibody mixture was replaced with methyl cellulose and incubated for 5 days at 37 °C, as described above.

Virus binding studies. To determine the effect of monoclonal and polyclonal antibodies on the binding of MCMV to MEF, extracellular [³⁵S]methionine-labelled MCMV ([³⁵S]metMCMV) was incubated with serial dilutions of antibodies and GPC for 90 min at 37 °C. Following the incubation the virus-antibody mixture was chilled to 4 °C and added in quadruplicate to MEF coverslip cultures for a further 60 min at 4 °C. Cells were then washed five times with cold MEM and the coverslip cultures were added to scintillation vials containing 1 ml NCS tissue solubilizer (Amersham). Radioactivity of the samples was determined by liquid scintillation spectrometry. The level of bound MCMV in control cultures (measured in d.p.m.), which contained NMS, was determined from the mean of six wells.

Double antibody neutralization assay. The ability of MAbs to interact and either augment or inhibit the neutralization of MCMV was assessed using a two antibody plaque reduction assay (Lussenhop et al., 1988). Equal volumes of two MAbs (each at 100 µg/ml) were combined and serially diluted in MEM-2 containing 5% GPC. MAbs were diluted individually with equal concentrations of NMS in MEM-2 as controls. MCMV was added to all cultures for 90 min at 37 °C, prior to the addition to washed MEF monolayers for a further 60 min incubation at 37 °C, as described above.

Statistical analysis. Statistical analysis of the data was performed using Student's t-test.

Results

Frequency of hybrid cell lines secreting anti-MCMV MAbs

Culture supernatants from 4% of the total wells showed reactivity for MCMV by ELISA. Eight hybridomas were produced in ascites fluid and further characterized by immunoblotting, immunoprecipitation, virus neutralization and immunofluorescence staining of MCMV-infected MEF. The isotype of these antibodies is indicated in Table 1 and all MAbs possessed the κ light chain.

Table 1. MAbs to MCMV: isotype, protein specificity and neutralization in vitro

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>MCMV protein specificity (Mr × 10⁻²)</th>
<th>Neutralizing titre* before MCMV adsorption to MEF†</th>
<th>Neutralizing titre after MCMV adsorption to MEF‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ GPC</td>
<td>- GPC</td>
</tr>
<tr>
<td>1E8</td>
<td>IgG2b</td>
<td>56, 60$</td>
<td>60</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>2F7</td>
<td>IgM</td>
<td>98</td>
<td>3</td>
<td>125</td>
</tr>
<tr>
<td>3B2</td>
<td>IgG2b</td>
<td>50</td>
<td>0-5</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>4F9</td>
<td>IgG2b</td>
<td>28, 34, 36, 48-50, 60, 68, 68, 76, 92</td>
<td>2</td>
<td>375</td>
</tr>
<tr>
<td>EA4</td>
<td>IgM</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>AC1</td>
<td>IgM</td>
<td>92, 98</td>
<td>3</td>
<td>250</td>
</tr>
<tr>
<td>1B4</td>
<td>IgM</td>
<td>87, 92, 98, 102, 110</td>
<td>200</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>1G4</td>
<td>IgM</td>
<td>68, 123</td>
<td>500</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>Hyperimmune anti-MCMV Ig</td>
<td>Multiple</td>
<td></td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

* Titres are expressed as the µg/ml of antibody yielding a 50% reduction in the number of plaques compared with control cultures, which were incubated with NMS.
† MCMV was incubated with antibody for 90 min at 37 °C prior to the addition to MEF.
‡ MCMV was adsorbed to MEF for 60 min at 4 °C prior to the addition of antibody.
§ Values in bold indicate the MCMV protein(s) to which MAbs exhibited the highest reactivity.
¶ ND, Not determined.
Fig. 1. Detection of MAbs reactive with MCMV-infected and uninfected MEF by immunofluorescence. Typical immunofluorescence patterns of MAbs 1E8, 2F7, 3B2, 4F9 and EA4 (a), 1B4 (b), 1G4 (c), AC1 (d), hyperimmune antibodies (e) and NMS (f) with MCMV-infected MEF. Reactivity of MAb AC1 with uninfected MEF is shown in (g). No reactivity was observed between MAbs 1E8, 2F7, 3B2, 4F9, 1B4, 1G4 and EA4 or polyclonal antibodies with uninfected MEF (not shown). Bar marker represents 30 µm.
Identification of MCMV proteins recognized by MAbs

The reactivity of MAbs with purified MCMV virions and MCMV-infected cell lysates was determined by Western blotting and immunoprecipitation, respectively, and is summarized in Table 1. No reaction was observed between MAbs and proteins from mock-infected MEF purified by the same procedure (not shown). Polyclonal antibodies reacted with multiple structural MCMV polypeptides (Fig. 2, lane 1) confirming previous studies (Farrell & Shellam, 1989). MAb 2F7 reacted with a 98K protein (lane 3), whereas a 50K protein was recognized by 3B2 (lane 4). Proteins of M, 92K and 98K were detected by MAbs AC1 (lane 6) and 1B4 (lane 7), although 1B4 recognized 87K, 102K and 110K proteins as well. 1G4 was weakly reactive with 68K and 123K proteins (lane 8).

MAbs 1E8, 4F9, EA4 and NMS did not react with any viral proteins using the Western blotting method (Fig. 2, lanes 2, 5, 9 and 10, respectively), which was possibly due to the conformational disruption of viral epitopes by reduction and SDS treatment. The MCMV proteins detected by these MAbs was achieved by immunoprecipitation with [35S]methionylMCMV (Fig. 3, lanes 1 to 4).

MAb 4F9 reacted strongly with a 60K protein and exhibited moderate reactivity with 28K, 34K, 48K to 50K and 92K M, proteins and weak reactivity with 36K, 68K, 76K and 83K M, proteins (Fig. 3, lane 1). EA4 reacted with multiple MCMV proteins (lane 2), a phenomenon which has been observed with immunoprecipitates of MAbs with HCMV (Pereira et al., 1982). Thus, the precise specificity of EA4 remains to be elucidated. 1E8 exhibited low reactivity with 56K and 60K proteins (lane 3), whereas polyclonal sera recognized in excess of 35 proteins in MCMV-infected cells in the M, range of 25K to 200K (lane 4). No reactivity was observed between MAbs or polyclonal sera with uninfected MEF (not shown).

Reactivity of MAbs with intracellular antigens specified by MCMV

The reactivity of MAbs with MCMV-infected and uninfected MEF was detected by immunofluorescence and is shown in Fig. 1 (a to g). MAbs 1E8, 2F7, 3B2, 4F9 and EA4 reacted with antigens that were perinuclear at 20 h p.i. (Fig. 1a), whereas grainy and diffuse cytoplasmic reactivity was observed with MAbs 1B4 and 1G4, respectively (Fig. 1b and e). Finally, MAb AC1 reacted strongly with the nucleus of MCMV-infected MEF, resulting in a speckled immunofluorescence pattern (Fig. 1d). Viral antigens in both the cytoplasm and nucleus of infected cells were detected by hyperimmune serum (Fig. 1f), whereas normal mouse serum gave no reaction (Fig. 1f). Interestingly, MAb AC1 also exhibited a speckled nuclear fluorescence pattern on uninfected MEF (Fig. 1g), although the reactivity was less than in infected cells.

Characterization of the self-reactive MAb AC1

To define further the nuclear reactivity of MAb AC1, illustrated in Fig. 1(d), proteins from ribonuclear extracts were separated by SDS-PAGE, blotted onto nitrocellulose and incubated with MAb AC1. The reactivity of the other MAbs, hyperimmune anti-MCMV sera, human anti-(RNP + Sm) sera and NMS with the ribonuclear protein extract was also investigated. Both MAB AC1 and anti-(RNP + Sm) sera recognized a 70K polypeptide, which was not detected by NMS (Fig. 4) or by the remaining unreactive MAbs (not shown). Hyperimmune anti-MCMV sera also weakly recognized the 70K ribonuclear polypeptide, although this reaction is not visible in the photograph.
Neutralization of MCMV with MAb prior to the adsorption of the virus to MEF

To compare the neutralizing activities of individual MAb, serial dilutions of antibodies were incubated with MCMV for 90 min at 37 °C and added to MEF monolayers for a further 1 h at 37 °C. Control cultures were incubated at 37 °C in the absence of antibodies. The concentration of MAb required to reduce the total number of virus plaques in MEF by 50% is shown in Table 1. In the presence of GPC MAb 3B2 exhibited the highest neutralization titre, exceeding that of hyperimmune serum, and although MAb AC1 2F7, 4F9 and EA4 exhibited neutralizing titres comparable with hyperimmune serum, 20- to 100-fold higher concentra-

tions of MAb 1E8 and 1B4 were required to achieve 50% neutralization. In contrast, MAb 1G4 did not neutralize MCMV at any concentrations below 500 µg/ml. Interestingly, the maximum reduction of virus plaques by MAb AC1 was 67% and an increase in the concentration of antibody failed to reduce further the quantity of infectious virus. Neutralization of MCMV by MAb was dependent on the presence of complement, although MAb 2F7, 4F9 and AC1 did neutralize the virus at much higher antibody concentrations in the absence of complement. A five- to six-fold enhancement in neutralization by polyclonal antibodies was observed in the presence of complement, but low concentrations of these antibodies were required to neutralize the virus by 50% in the absence of complement.

Neutralization of MCMV with MAb after the adsorption of the virus to MEF

Binding of MCMV to MEF was performed at 4 °C for 1 h to quantify virus adsorption, as these conditions are known to minimize virus uptake and macromolecular synthesis (Dimmock, 1984). Following incubation the monolayer was washed five times to remove unbound virus. The ability of MAb and polyclonal sera to reduce plaque titres in MEF was determined by incubating the bound MCMV with serial dilutions of antibody for a further 1 h prior to the addition of methyl cellulose.
Neutralizing monoclonal antibodies to MCMV

Table 2. Effect of temperature on the dissociation of \[^{35}\text{S}\]metMCMV from MEF following binding at 4 °C

<table>
<thead>
<tr>
<th>Treatment following virus binding at 4°C*</th>
<th>Number of virus plaques (±S.D.)†</th>
<th>Reduction in virus plaques following 1 h incubation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Methyl cellulose added immediately after removal of unbound MCMV (maximum number of virus plaques)</td>
<td>192 ± 9</td>
<td>-</td>
</tr>
<tr>
<td>(2) Incubation for 1 h at 4 °C prior to the addition of methyl cellulose</td>
<td>185 ± 10</td>
<td>4‡</td>
</tr>
<tr>
<td>(3) Incubation for 1 h at 37 °C prior to the addition of methyl cellulose</td>
<td>140 ± 11</td>
<td>27§</td>
</tr>
</tbody>
</table>

* [35S]metMCMV was incubated with MEF for 60 min at 4 °C and washed with cold MEM-2 prior to treatments (1), (2) and (3).
† Plaque numbers are derived for the mean of 24 individual cultures; all treatments were performed in the same experiment.
‡ Not significant compared with (1).
§ Significant reduction in the number of virus plaques following incubation compared with (1) (P < 0.05).

Table 3. MCMV neutralization titres* using combinations of MAbs†

<table>
<thead>
<tr>
<th>MAb</th>
<th>1E8</th>
<th>2F7</th>
<th>3B2</th>
<th>4F9</th>
<th>AC1</th>
<th>1B4</th>
<th>1G4</th>
<th>EA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1E8</td>
<td>60‡</td>
<td>9-3</td>
<td>1-5</td>
<td>2-8</td>
<td>12-6</td>
<td>125</td>
<td>150</td>
<td>1-5</td>
</tr>
<tr>
<td>2F7</td>
<td>3-0</td>
<td>1-0</td>
<td>2-0</td>
<td>0-3</td>
<td>0-6</td>
<td>0-7</td>
<td>0-7</td>
<td>0-7</td>
</tr>
<tr>
<td>3B2</td>
<td>0-6</td>
<td>1-0</td>
<td>1-0</td>
<td>0-5</td>
<td>0-8</td>
<td>0-8</td>
<td>0-8</td>
<td>0-8</td>
</tr>
<tr>
<td>4F9</td>
<td>1-0</td>
<td>3-0</td>
<td>3-0</td>
<td>3-0</td>
<td>3-0</td>
<td>3-0</td>
<td>3-0</td>
<td>3-0</td>
</tr>
<tr>
<td>AC1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>1-5</td>
</tr>
<tr>
<td>1B4</td>
<td></td>
<td>100</td>
<td>150</td>
<td>1-5</td>
<td></td>
<td>150</td>
<td>1-5</td>
<td></td>
</tr>
<tr>
<td>1G4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>1-5</td>
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<tr>
<td>EA4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-5</td>
</tr>
</tbody>
</table>

Ⅰ Titres are expressed as the concentration of MAbs (µg/ml) that gave 50% reduction in plaque numbers.
† Equal volumes of MAbs (at 100 µg/ml) were combined and serially diluted in MEM-2 containing 5% GPC. Following a 90 min incubation with MCMV at 37 °C the virus–antibody mixtures were added to MEF monolayers, as described in Methods. All titrations were performed in the same experiment.
‡ Values in bold are the neutralization titres of MAbs when titrated with heat-inactivated NMS.

Previous studies have shown that the early stages of binding of MCMV to MEF is reversible and that the virus readily dissociates (Hodgkin et al., 1988), a phenomenon which is enhanced with increasing temperature. Accordingly, an initial experiment was performed to determine whether MCMV was eluted from MEF during the 1 h incubation with antibodies, following virus adsorption. MEF cultures containing cell-bound MCMV were further incubated at either 4 °C or at 37 °C in MEM-2 for 1 h before the addition of methyl cellulose. To determine the maximum number of plaques produced by the bound virus following adsorption at 4 °C, methyl cellulose was immediately added to MEF after removal of unbound virus. Further incubation of bound virus at 4 °C for 1 h did not significantly reduce the number of plaques compared with control cultures, whereas a significant reduction in plaques was observed in cultures incubated at 37 °C (Table 2). Therefore, to prevent the reduction of infectivity attributable to the dissociation of MCMV from MEF, all incubations of bound virus with MAbs were performed at 4 °C.

The incubation of MAbs with MEF following adsorption of MCMV at 4 °C failed to reduce plaque titres below that of MEF treated with NMS (Table 1). In contrast, MCMV was effectively neutralized by hyper-immune immunoglobulin, although higher concentrations of antibody were required to achieve the 50% neutralization, compared with the amount required when virus and antibody were incubated prior to virus adsorption to MEF.

Effect of monoclonal and polyclonal antibodies on the binding of virus to susceptible cells

To determine whether neutralizing and non-neutralizing MAbs differentially affected the binding of MCMV to
cells, serial dilutions of MAbs or polyclonal hyper-
immune immunoglobulin were preincubated with
\(^{35\text{S}}\text{MCMV} to MEF (a and b) and the level of virus neutralization
(c and d). IgG2b MAbs and hyperimmune antibodies are shown in (a)
and (c); IgM MAbs are shown in (b) and (d). Serial twofold dilutions of
hyperimmune antibodies (\(\triangle\)), MAbs 1E8 (■), 3B2 (△), 4F9 (□), 2F7
(○), AC1 (●), 1B4 (○) and 1G4 (○) were incubated with
\(^{35\text{S}}\text{MCMV} and 5\% GPC for 90 min at 37 °C. The virus–antibody
mixtures were divided in half and added to MEF for the binding and
plaque reduction assays, as described in Methods. All dilutions
performed in quadruplicate; S.D. values were <10\% of the mean and
are not shown.

Inhibition of MCMV binding was observed with high
concentrations (≥62 \(\mu\text{g/ml}\)) of polyclonal antibodies and
MAb AC1, but no correlation was observed between the
level of inhibition of virus binding and neutralization.
Firstly, virus binding was not always blocked at the
concentrations of polyclonal and MAbs that achieved
100\% neutralization. Secondly, the inhibition of MCMV
binding by MAb AC1 at high antibody concentrations
(Fig. 5b) was not accompanied by a loss of virus
infectivity (Fig. 5d). Interestingly, high concentrations of
the IgG2b MAbs 1E8, 3B2 and 4F9, which were all
neutralizing, showed an enhancement of bound virus
compared with controls, which was possibly due to the
binding of MCMV–antibody complexes via the Fc
receptors.

Two antibody plaque reduction assay
To determine whether each MAb affected the efficacy
of neutralization of other MAbs, the neutralizing titres of
two MAbs, when combined at equal concentrations,
were compared with the titres of individual MAbs (Table
3). No interaction between combinations of highly
neutralizing MAbs 2F7, 3B2, 4F9, AC1 and EA4 was
observed, with the neutralizing titres of combined MAbs
being equal, or within a two-fold dilution, to the more
highly neutralizing MAb. Similarly, no interaction was
observed between each of the low neutralizing antibodies
1E8, 1B4 and 1G4. However, with combinations of
highly and weakly neutralizing MAbs, 1E8 inhibited the
neutralization of AC1 and 2F7 by 4-2-fold and 3-1-fold,
respectively, and modest inhibition by this MAb was
also observed with MAbs 3B2 and 4F9. In contrast, no
interaction was observed between other combinations of
weakly and highly neutralizing MAbs.

Discussion
In this study, the reactivity of eight MAbs to MCMV
structural polypeptides and the ability of these MAbs to
neutralize virus infectivity \textit{in vitro} was investigated. The
MAbs recognized MCMV proteins with different im-
munological and electrophoretic properties; indeed, all
MAbs except 2F7 and 3B2 were reactive with more than
one protein by either the Western blotting or immuno-
precipitation assays. Proteins with shared antigenic
determinants and different electrophoretic properties
have also been precipitated by MAbs to HCMV (Pereira
et al., 1982) and HSV types 1 and 2 (Pereira et al., 1980,
1981; Balachandran et al., 1981). The faster migrating
polypeptides recognized by MAbs may be cleavage
products of the more slowly migrating species. Alterna-
tively, polypeptides with similar electrophoretic mobili-
ties may represent different levels of glycosylation of a
single protein. In addition, immunoprecipitated MCMV
proteins from infected cell lysates may represent
antigenically related precursors of mature forms of the
proteins.

The localization of MCMV antigens recognized by the
MAbs in infected MEF identified four patterns of
reactivity by immunofluorescence: nuclear, perinuclear,
granular cytoplasmic and diffuse cytoplasmic. All
MAbs, except AC1, were specific for MCMV-infected
MEF; the speckled nuclear reactivity exhibited by MAb
AC1 on uninfected MEF is reminiscent of the reactivity
of sera from autoimmune patients with ribonuclear
The development of autoimmunity as a result of HCMV which cross-reacts with a normal cell membrane protein. Recently, Michelson et al. (1989) identified a MAb to HCMV which cross-reacts with a normal cell membrane protein. Cross-reactivity of MAbs with viral and host cell proteins has been described for other viruses and has been implicated as a mechanism for virus-induced autoimmunity (reviewed by Oldstone & Notkins, 1986). The development of autoimmunity as a result of HCMV infection has not been established, although patients with Sjogren’s syndrome, an autoimmune disease characterized by antibodies directed against cytoplasmic and ribonuclear proteins, have significantly higher antibody titres to HCMV than normal controls (Shillitoe et al., 1982). The development of anti-nuclear (Wager et al., 1968) and anti-smooth muscle (Andersen & Andersen, 1975) antibodies has also been associated with episodes of acute HCMV infection. Recent studies by Keil et al. (1987) have identified homology between the major immediate early 89K MCMV protein and the cellular histone H2B protein. In addition, Fujinami et al. (1988) identified sequence homology and immunological cross-reactivity of the immediate early HCMV protein with the HLA-DR β chain. These authors suggest that the production of these cross-reactive antibodies following HCMV infection may contribute to graft rejection after transplantation. As multiple autoantibodies have been observed in sera from mice after MCMV infection (Bartholomaeus et al., 1988) further analysis of the specificity of MAb AC1 and its possible immunopathological significance in vivo is being currently investigated.

Our data show that all of the MAbs, regardless of isotype, neutralized MCMV prior to virus adsorption and that complement was required to promote effective neutralization. In contrast, hyperimmune immunoglobulin was able to neutralize infectivity after virus adsorption and, whereas neutralization was enhanced in the presence of complement, the virus was neutralized at low antibody concentrations even in the absence of complement. These results are consistent with previous reports of the requirement of complement for neutralization of MCMV and HCMV by some sera (Kim & Carp, 1973; Rasmussen et al., 1985; Masuho et al., 1987; Lawson et al., 1988), but not by others (Rasmussen et al., 1984). Although the role of complement is generally attributed to an increase in protein bulk on the virion surface, which hinders adsorption, studies of the neutralization of other enveloped viruses have indicated that complement may act by direct lysis of the virion in virus–antibody complexes (Oldstone, 1975).

With the exception of high concentrations of AC1 all MAbs, regardless of neutralizing capacity, were unable to prevent the adsorption of MCMV, a phenomenon that has been observed in other virus systems, including influenza virus (Dimmock, 1984; Possee & Dimmock, 1981), rabies virus (Dietzschold et al., 1987) and herpes simplex virus type 1 (Fuller & Spear, 1987). Our results suggest that MCMV that has been neutralized by antibody can still adsorb to the cell membrane, although infection does not ensue. The binding of neutralized virus to the cell surface has also been observed for poliovirus (Mandel, 1967) and influenza virus (Possee & Dimmock, 1981).

Results from the present study suggest that the neutralization of MCMV by polyclonal antibodies occurs by several mechanisms, which may reflect the cooperative interaction of antibodies of different specificities. However, this study demonstrated the ability of MAb 1E8 to inhibit the neutralization of several other MAbs, although the mechanism of this interference is unknown. As these MAbs recognized proteins with different electrophoretic properties, the possibility that the inhibition was caused by steric hindrance is unlikely. Alternatively, the binding of MAb 1E8 may cause conformational changes to distal epitopes. The phenomenon of a poorly neutralizing antibody inhibiting the neutralizing activity of other MAbs in vitro has been observed for HCMV (Lussenhop et al., 1988). Therefore, it is possible that MAb 1E8 recognizes an epitope that induces non-neutralizing or non-protective antibodies. The ability of MAb 1E8 to inhibit protection against MCMV infection in vivo remains to be determined.

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References


