17D Yellow Fever Virus Infection of P388D1 Cells Mediated by Monoclonal Antibodies: Properties of the Macrophage Fc Receptor

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SUMMARY

Thirteen IgG monoclonal antibodies to the envelope protein of 17D yellow fever virus (17D YF) were produced. All of the antibodies, whether type-specific to 17D YF or flavivirus cross-reactive, mediated antibody-dependent enhancement (ADE) of virus growth in P388D1 cells. There was no consistent relationship between ADE titres and the degree or pattern of neutralizing and/or haemagglutination inhibition activity. Monoclonal antibodies of different isotypes were used to investigate further the properties of P388D1 Fc receptors. The effects of trypsin treatment of P388D1 on ADE were similar to those previously described in experiments measuring direct binding of IgG proteins or rosetting of sheep red blood cells (SRBC) by macrophages, demonstrating sensitivity to digestion by trypsin of the Fc receptor for monomeric IgG2a but not for IgG2b. Aggregated myeloma proteins of IgG2a and IgG2b isotypes competed equally well with either IgG2a or IgG2b monoclonal antibodies to 17D YF in inhibition of ADE. However, selective inhibition by the homologous isotype was observed when rosetting by P388D1 of SRBC coated with IgG2a or IgG2b monoclonal antibodies was examined. These results may help to explain apparent discrepancies previously reported between experiments utilizing direct binding of IgG proteins and those using rosetting of antibody-coated SRBC to examine Fc receptor properties and indicate that immune complexes of virus and antibody resemble aggregated immunoglobulins with respect to macrophage Fc receptor function and differ from antibody-coated SRBCs.

INTRODUCTION

Antibody-dependent enhancement (ADE) of flavivirus replication in macrophages has been observed with dengue (Halstead & O'Rourke, 1977), West Nile (Peiris & Porterfield, 1979) and yellow fever viruses (Schlesinger & Brandriss, 1981) but the mechanism of ADE is incompletely understood. Evidence for the role of the Fc receptor has been presented: monoclonal antibody to an Fc receptor of P388D1, a continuous mouse macrophage-like cell line, blocked ADE of West Nile virus growth (Peiris et al., 1981); ADE of 17D yellow fever virus (17D YF) replication in U937, a continuous human macrophage-like cell line, was prevented by pretreating the cells with human myeloma proteins of high affinities for the U937 Fc receptor (Schlesinger & Brandriss, 1981).

Although most observations concerning ADE have been made with hyperimmune sera, ascitic fluids or their IgG fractions, ADE of replication of dengue (Brandt et al., 1982) and of West Nile virus (Peiris et al., 1982) by monoclonal antibodies has been described. We have recently produced 19 antibody-secreting hybridomas to 17D YF (Schlesinger et al., 1983); most were type-specific for yellow fever virus but several cross-reacted with other flaviviruses. The ability of these antibodies to enhance growth of 17D YF in P388D1 cells was examined and some of the antibodies were also used to investigate further properties of the P388D1 Fc receptor.
METHODS

Virus and cells. Avian leukemia virus-free strain 17D yellow fever virus (lot no. 2091, Connaught Laboratories) was passed once in Vero cells (from Dr Karl Johnson), maintained in minimum essential medium supplemented with 5% foetal calf serum (Hyclone) and stored in multiple small aliquots at −75 °C. A single virus pool containing $10^7$ p.f.u. was used throughout. P388D1 cells (from Dr Clark Anderson) were maintained in continuous spinner culture in RPMI 1640 medium supplemented with 2 mM-glutamine (both Gibco) and 10% foetal calf serum (RPMI-FCS).

Monoclonal antibodies. The production and characterization of mouse monoclonal antibodies to the 17D vaccine strain of yellow fever virus have been described by Schlesinger et al. (1983). Briefly, using the method of Fazekas de St Groth & Scheidegger (1980), 19 hybridomas were produced by fusing non-secretor P3X63Ag8.653 mouse myeloma cells (Kearney et al., 1979) with spleen cells from BALB/c mice immunized with 17D YF vaccine (lot. no. 2091, Connaught). Five hybridomas produced monoclonal antibodies (IgG) against 17D YF virus-specified non-structural proteins and 14 hybridomas produced antibody (13 IgG and one IgM) against the envelope protein (E) of 17D YF virus as determined by radiocommunoprecipitation and gel electrophoresis. The serological properties of the IgG monoclonal antibodies to E are summarized in Table 1. All were of the IgG2a isotype except for SE6 (IgG2b) and 3A3 (IgG1). Control or monoclonal antibody-rich ascitic fluids were prepared by intraperitoneal injection of pristane-primed syngeneic BALB/c mice with $10^7$ P3X63Ag8.653 or cloned hybridoma cells respectively, and a single pool of each ascitic fluid was used for all assays and experiments.

An IgG2a mouse monoclonal antibody, UN-2, against sheep red blood cells (SRBC) was a gift from Dr Betty Diamond and SP/2, an IgG2b anti-SRBC monoclonal antibody, was from Accurate Chemical and Scientific Co. Both antibodies were obtained as immune ascitic fluids.

Myeloma proteins. Mouse myeloma proteins were produced by intraperitoneal injection of $10^7$ PC-5 (IgG2a) or MOPC-141 (IgG2b) cells into pristane-primed BALB/c mice. Myeloma proteins were purified by Protein A-Sepharose CL-4B (Pharmacia) affinity chromatography (Ey et al., 1978). Fractions were examined by Ouchterlony double gel diffusion using goat antisera (Meloy & Litton Bionetics) with heavy chain specificities. With this method the fractions used contained no detectable immunoglobulin of another subclass.

Infection of P388D1 cells. Plastic 12-well (16 mm) cluster plates (Costar) were inoculated with $10^5$ P388D1 cells in 1-5 ml of RPMI-FCS and incubated 18 h at 37 °C in a humidified 7% CO2 atmosphere. Monolayers were washed with phosphate-buffered saline (PBS) pH 7.2, and 100 μl of diluted control or monoclonal antibody-rich ascitic fluid was added. This was followed immediately by addition of 20 μl of diluted stock 17D YF virus for a multiplicity of infection of 0.1. The monolayers were incubated for 60 min at 37 °C after which residual virus and antibody were removed by copious washing with PBS, antibody-free RPMI-FCS (1-5 ml) was added, and the infected monolayers were incubated for 36 h at 37 °C after which the cluster plates were frozen at −75 °C. Titres of infectious virus present in the supernatants of freeze-thawed P388D1 monolayers were determined by plaque assay in Vero cells (Schlesinger & Brandriss, 1981). Enhancement of 17D YF replication by antibody was considered significant if there was a threefold or greater increase in mean virus p.f.u. compared to controls.

Trypsin treatment. PBS-washed P388D1, monolayers were incubated with trypsin (Worthington), 1-0 mg per ml PBS, for 30 min at 37 °C. The trypsin was inactivated by addition of soybean trypsin inhibitor (Worthington), 0-1 to 1-0 mg per ml PBS. Control cells were treated with soybean trypsin inhibitor alone. Trypsin-treated and control monolayers were washed three times with cold PBS and kept at 4 °C until used.

Competition experiments. Solutions of bovine serum albumin (BSA, Sigma) and purified mouse myeloma proteins were dialysed against PBS pH 7.2, and filter-sterilized through a 0.45 μm Millipore TF filter before use. Monomeric myeloma proteins were prepared by centrifugation of the protein solutions at 150000 g for 3 h. Aggregated IgG2a or IgG2b were prepared by heating at 63 °C PBS solutions (2 to 5 mg/ml) of the myeloma proteins until slight opalescence. The ability of aggregated myeloma proteins to interfere with rosette formation between P388D1 cells and monoclonal antibody-coated SRBC was measured as described previously (Diamond et al., 1978).

RESULTS

Antibody-mediated enhancement of viral growth

ADE of viral growth was not observed with antibodies to non-structural proteins, nor with the single IgM antibody to the envelope protein. In Table 1, ADE, plaque-reduction neutralization (PRNT50) and haemagglutination inhibition (HAI) activities are compared for 13 monoclonal IgG antibodies to the envelope protein of 17D YF. Nine of the antibodies were type-specific for yellow fever virus by HAI and PRNT50 and four cross-reacted with one or more of the other flaviviruses tested: Zika, Banzi and dengue 2. All the antibodies enhanced replication of 17D YF in P388D1 cells with no consistent relationship between ADE titres and degree or pattern of
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Table 1. Enhancing (ADE), neutralizing (PRNT₉₀) and haemagglutination inhibition (HAI) titres of 17D yellow fever virus type-specific and cross-reactive monoclonal antibodies

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>ADE</th>
<th>PRNT₉₀</th>
<th>HAI</th>
</tr>
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<tbody>
<tr>
<td>Type-specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4E8</td>
<td>8.6*</td>
<td>4.3</td>
<td>7.6</td>
</tr>
<tr>
<td>2C9</td>
<td>6.6</td>
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<tr>
<td>4E1</td>
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<td>1.6</td>
</tr>
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<td>3E9</td>
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<td>&lt;1.3</td>
<td>1.6</td>
</tr>
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<td>4.3</td>
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<td>4.3</td>
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<tr>
<td>3A3</td>
<td>3.6</td>
<td>&lt;1.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Cross-reactive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5E5</td>
<td>7.5</td>
<td>&lt;1.3</td>
<td>&lt;1.0-3.1†</td>
</tr>
<tr>
<td>5E6</td>
<td>5.6</td>
<td>&lt;1.3</td>
<td>&lt;1.0-5.2</td>
</tr>
<tr>
<td>4E11</td>
<td>5.6</td>
<td>&lt;1.3-2.8</td>
<td>3.4-5.2</td>
</tr>
<tr>
<td>5H3</td>
<td>3.6</td>
<td>&lt;1.3-2.5</td>
<td>4.6-7.3</td>
</tr>
</tbody>
</table>

* All titres are shown as –log₁₀.
† Two values indicate range of titres to 17D, Zika, Banzi and/or dengue 2 viruses.

PRNT₉₀ and HAI reactivity. With three antibodies (5E5, 4E1 and 3E9) ADE titres were at least 10⁴- to 10⁵-fold greater than PRNT₉₀ and HAI titres. Antibodies without neutralizing activity against 17D yellow fever virus exhibited ADE activity at the lowest dilution tested (10⁻²) but it was usually necessary to dilute the neutralizing antibodies to sub-neutralizing concentrations before ADE was seen.

Effects of trypsin on antibody-mediated infection

Evidence has been presented that IgG₁ and IgG₂b antibodies are bound to mouse macrophages by a trypsin-resistant Fc receptor (Diamond & Scharff, 1980) whereas IgG₂a antibodies are bound by a separate, trypsin-sensitive Fc receptor (Unkeless & Eisen, 1975). To examine further the properties of P388D₁ Fc receptors with respect to ADE, trypsin-treated and untreated cells were infected with virus in the presence of an IgG₂a (4E8) or an IgG₂b (5E6) monoclonal antibody. Trypsin treatment resulted in a 55% to 90% decrease in virus titre when infection was established in the absence of antibody; however, trypsin treatment of P388D₁ had no effect on ADE mediated by either the IgG₂a or the IgG₂b monoclonal antibody (Fig. 1a). In these experiments, virus and antibody were added simultaneously to P388D₁, offering an opportunity for virus–antibody complexes to form before attachment to the cells. In another series of experiments, trypsin-treated and untreated P388D₁ cells were first incubated with either control ascitic fluid or the same monoclonal IgG₂a or IgG₂b antibodies for 45 min at 4 °C. Excess antibody was removed by extensive washing, 17D YF virus (m.o.i. 0.1) was then added and the temperature was brought to 37 °C. The results are illustrated in Fig. 1(b). Under these conditions, we reasoned that any ADE observed could be ascribed primarily to attachment of virus to antibody that was already cell-bound via the Fc receptor with minimal opportunity for formation of virus–antibody complexes prior to cell attachment. Antibody-mediated enhancement of viral growth was observed in non-trypsinized cells and in trypsinized cells preincubated with IgG₂b monoclonal antibody. However, trypsin treatment eliminated ADE by the IgG₂a monoclonal antibody.

Competition experiments

ADE

P388D₁ monolayers were incubated for 60 min at 4 °C with 200 μg of either BSA, monomeric or aggregated IgG₂a or IgG₂b myeloma protein. 17D YF virus (m.o.i. 0.1) and either normal ascitic fluid or IgG₂a (4E8) or IgG₂b (5E6) monoclonal antiviral antibody were then added.
Fig. 1. (a) P388D1 cells treated with trypsin (T) or untreated (C) and infected by simultaneous addition of 17D YF and $10^{-6}$ final dilutions of IgG$_{2a}$ monoclonal antibody 4E8, $10^{-4}$ final dilution of IgG$_{2b}$ monoclonal antibody 5E6, or control ascitic fluid. (b) Trypsin-treated and untreated P388D1 cells incubated with $10^{-2}$ final dilutions of antibodies 4E8, 5E6 or control ascitic fluid for 45 min at 4 °C. After excess antibody was removed by washing with cold RPMI, the cells were infected with 17D YF. Each value represents the mean and standard deviation of an experiment performed in triplicate. VI, Control ascites; [], IgG$_{2a}$ anti-17D YF ascites; I, IgG$_{2b}$ anti-17D YF ascites.

After a 1 h adsorption period at 37 °C, the monolayers were washed and were processed as usual for infectious virus. The results are presented in Fig. 2. In cells preincubated with BSA, a marked increase in yield of virus (27- to 47-fold) was observed when infection was established in the presence of either monoclonal antibody. Preincubation of P388D1 with either aggregated IgG$_{2a}$ or IgG$_{2b}$ myeloma proteins resulted in a 90% or greater reduction of viral yield mediated by either the IgG$_{2a}$ or the IgG$_{2b}$ antibody and viral titres were not significantly greater in the presence of antibody than in its absence. Preincubation of P388D$_1$ cells with monomeric myeloma proteins had no effect on antibody-mediated virus growth (data not shown).

In similar experiments we examined the effects of concentration of aggregated myeloma protein on inhibition of ADE by the same monoclonal antibodies. The results using the IgG$_{2a}$ myeloma protein are illustrated in Fig. 3. Equal inhibition of ADE by the IgG$_{2a}$ and by the IgG$_{2b}$ monoclonal antibodies was observed over a broad range of concentration of the IgG$_{2a}$ myeloma protein.

Rosetting of antibody-coated sheep red blood cells (E-rosetting)

P388D$_1$ monolayers were incubated for 45 min at 37 °C with 500 μg of either BSA or the same
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Fig. 2. P388D1 cells incubated with 200 μg of aggregated (agg) IgG2a (PC-5) or IgG2b (MOPC-141) myeloma proteins or bovine serum albumin (BSA) before infection with 17D YF in the presence of IgG2a monoclonal antibody 4E8 or IgG2b monoclonal antibody 5E6 or control ascitic fluid. Each value represents the mean and standard deviation of an experiment performed in triplicate. □, Control ascites; ■, IgG2a anti-17D YF ascites; ■, IgG2b anti-17D YF ascites.

Table 2. Effect of aggregated myeloma proteins on rosetting of sheep red blood cells coated with monoclonal antibody

<table>
<thead>
<tr>
<th>Pretreatment with aggregated IgG myeloma</th>
<th>% Rosettes with anti-SRBC</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IgG2a</td>
</tr>
<tr>
<td>None</td>
<td>95</td>
</tr>
<tr>
<td>IgG2a</td>
<td>8</td>
</tr>
<tr>
<td>IgG2b</td>
<td>88</td>
</tr>
</tbody>
</table>

aggregated IgG2a or IgG2b myeloma proteins that were employed in the previous experiments on ADE. The P388D1 cells were then incubated with SRBC coated with either an IgG2a or an IgG2b monoclonal antibody to SRBC. The results are given in Table 2. In contrast to ADE, the inhibitory effects of myeloma proteins on E-rosetting were selective. Rosetting of IgG2a-coated SRBC was almost completely inhibited by preincubation of P388D1 cells with the aggregated IgG2a myeloma protein, but the IgG2b protein had no significant inhibitory effect. Some inhibition of rosetting of IgG2b-coated SRBC was observed with the aggregated IgG2a myeloma protein but the IgG2b protein was clearly a more effective inhibitor.

DISCUSSION

Halstead et al. (1980) reported that the capacity of a rabbit antiserum to enhance replication of dengue 2 virus in human peripheral blood macrophages is proportional to the degree of crossreactivity of the antiserum against related flaviviruses as measured by HA1. Their results were supported by similar experiments measuring enhancement of dengue 2 replication in U937 cells.
by anti-dengue 2 monoclonal antibodies (Brandt et al., 1982). In contrast, Peiris et al. (1982) found that both a strain-specific and a group-specific monoclonal antibody enhanced the growth of West Nile virus in P388D1 cells. Our results, using a larger number of monoclonal antibodies, are similar to those of Peiris et al. (1982) and indicate that, unlike ADE of dengue 2 by rabbit anti-flavivirus sera or dengue 2 monoclonal antibodies, type-specific and cross-reactive 17D YF monoclonal antibodies are equally effective mediators of ADE. The reasons for this disparity are not immediately apparent but could reflect as yet undescribed differences between dengue 2 and 17D YF viruses in the distribution of the antigenic sites on the envelope protein that express flavivirus type- and group-specificities.

It is widely assumed, but not proven, that attachment of antibody to a critical site(s) on the flavivirus envelope protein results in neutralization (Della-Porta & Westaway, 1978). That neutralizing monoclonal antibodies to 17D YF are capable of enhancing infection when present in sub-neutralizing concentrations supports the 'multihit' mechanism of neutralization, requiring attachment of antibody to multiple sites on the virion at least for this virus. In this regard, Peiris et al. (1982) have also reported enhancement of West Nile virus growth by sub-neutralizing concentrations of a high titred neutralizing monoclonal antibody to the virus.

Most experiments concerning the affinities of Fc receptors for immunoglobulin isotypes have been performed by measuring the binding or the inhibition of binding of radiolabelled antibodies or myeloma proteins to the cell. The evidence suggests that IgG1 and IgG2b immunoglobulins are bound to mouse macrophages by a trypsin-resistant Fc receptor (Diamond & Scharff, 1980), whereas monomeric IgG2a is bound by a trypsin-sensitive Fc receptor (Unkeless & Eisen, 1975). There are some discrepancies in experimental results as to whether or not aggregated IgG2a binds to the same receptor as IgG1 or IgG2b (Heusser et al., 1977). Previous observations (Peiris et al., 1981; Schlesinger & Brandriss, 1981) indicate that attachment of virus to macrophages via the cell membrane Fc receptor is a necessary step in antibody-mediated enhancement of viral growth; ADE, therefore, can be regarded as another measure of
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Fc receptor function. Our results are consistent with the selective trypsin sensitivity of the Fc receptor for monomeric IgG2a previously described, in that antibody-mediated enhancement of viral growth by an IgG2a but not an IgG2b monoclonal antibody was abolished by trypsinization of P388D1 cells when infection was established in a manner that did not allow for formation of virus–antibody complexes prior to cell attachment. When infection was established in a manner that did allow formation of virus–antibody complexes prior to attachment to P388D1 cells, trypsinization had no effect on enhancement mediated by either the IgG2a or the IgG2b monoclonal antibodies. This is also consistent with earlier observations indicating that aggregated IgG2a and IgG2b (and IgG1) are bound by the same trypsin-resistant Fc receptor (Heusser et al., 1977). Similar to earlier observations on infection of U937 cells (Schlesinger & Brandriss, 1981) trypsinization of P388D1 cells resulted in a reduced yield of virus when infection was established in the absence of antibody. This probably represents sensitivity of the cell virus receptor to digestion by trypsin.

In our experiments prior incubation of P388D1 cells with either aggregated IgG2a, or IgG2b myeloma proteins resulted in equal inhibition of antibody-mediated enhancement by both IgG2a, and IgG2b monoclonal antibodies. This observation is consistent with those of Heusser et al. (1977) who reported cross-inhibition of binding between aggregates of IgG1 and IgG2a. It is also consistent with the observations of Segal & Titus (1978) who interpreted their results to indicate that IgG1, IgG2a, and IgG2b, aggregated or monomeric, share the same Fc receptor although an additional trypsin-sensitive receptor that bound monomeric IgG2a only was postulated. In contrast, Diamond et al. (1978), utilizing rosetting of SRBC to examine Fc receptor function, found that pretreatment of macrophages with aggregated myeloma proteins of the homologous but not the heterologous isotype inhibited rosetting of SRBC complexed with IgG2a, or IgG2b, anti-SRBC monoclonal antibodies. Using the same myeloma proteins that gave cross-inhibition of ADE between IgG isotypes, we found evidence of isotype specificity when inhibition of E-rosetting was examined, in confirmation of the results of Diamond et al. (1978). It is likely that this variation in results is the consequence of different assay systems and that immune complexes of antibody and virus more closely resemble aggregated immunoglobulins with respect to Fc receptor function than they do antibody-coated red cells. The recent work of Peiris et al. (1981) suggests that this may indeed be the case. These authors found that a monoclonal antibody against the trypsin-resistant Fc receptor of P388D1 cells inhibited E-rosetting of IgG2a-coated, but not of IgG2b-coated, SRBCs whereas it inhibited ADE of West Nile virus by both an IgG2a and an IgG2b monoclonal antibody to the virus.

REFERENCES


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