Role of Complement and the Fc Portion of Immunoglobulin G in Immunity to Venezuelan Equine Encephalomyelitis Virus Infection with Glycoprotein-Specific Monoclonal Antibodies

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We have previously characterized with monoclonal antibodies (MAbs) seven unique epitopes on the two envelope glycoproteins of Venezuelan equine encephalomyelitis (VEE) virus vaccine strain TC-83. The epitopes important in protection from VEE virus infection were determined in passive antibody transfer studies, with virulent VEE (Trinidad donkey) virus as the challenge virus. Selected high-avidity MAbs to the three major protective epitopes (E2γ, E1β, and E1γ) were assayed for in vitro complement activity. All three fixed murine complement to high titer. Limited pepsi digestion of the anti-E2γ in the presence of cysteine resulted in a rapid decrease and complete loss of complement-fixing ability by 2 h, but the majority of mice, except at the lowest dilution of MAbs, were protected until the Fc termini were cleaved at 3 h. Anti-E2γ Fab′2 would neutralize VEE (Trinidad donkey) virus more efficiently than either Fab or Fab'; none of the fragments would fix complement or was effective in passive protection. C5-deficient mice and mice depleted of C3 with cobra venom factor were still protected from VEE (Trinidad donkey) virus challenge after passive transfer of either anti-E2γ or anti-E1β MAb. The results show that the anti-E2β MAB mediates neutralization through bivalent binding at a critical site on the virion and that Fc effector functions, other than complement, are necessary for protection. Although the ability of the anti-E2γ MAb to fix complement was associated with its ability to protect in vivo, no direct cause-and-effect relationship was found. Since the epitope defined by the anti-E1β antibody is found on the cell membrane, but is not expressed on the infectious virion, protection in mice was most likely mediated at the cellular level, possibly by inhibition of the final stages of virion maturation.

The role of the complement system in virus immunity has been extensively investigated and recently reviewed in detail (5, 14, 33). Although most effects of activating complement are beneficial to the host in terms of more rapid abrogation of virus infections, complement cleavage products can magnify an infection through immunopathological manifestations (31). The Fc portion of the immunoglobulin molecule is also important in immunity, primarily via effector functions. The Fc can aggregate and may bring about allosteric changes in virions, and it can exert adjuvant effects (10, 28). Also, receptors for the Fc region of immunoglobulin molecules are found on phagocytic cells (4).

The involvement of both complement pathways, both independent of and with antibody, has been demonstrated for viruses (6, 16, 32, 48). Although virus alone can activate the alternate complement pathway, it appears that bivalent antibody must be present for actual lysis of virions or infected cells (43). F(ab′)2 can be efficient for alternate complement pathway lysis or in vitro neutralization, but Fab, Fab′, and Fc are not (7, 10, 36, 43, 44). However, F(ab′)2 is not effective in lysis when the classical complement pathway is activated by virus, since the C42 region of the Fc, the site of Clq binding, is cleaved by proteolytic digestion (9, 30, 49). Lysis of retroviruses with complement appears to depend primarily on the spatial orientation of the antibody-antigen complex with respect to the membrane bilayer (34). Neutralization can also proceed through deposition of complement proteins on the virion surface, either causing agglutination or sterically blocking adsorption to cell surfaces (1, 33). The presence of C3b on the virus enhances the uptake and destruction of virus by phagocytic cells (33). Conversely, increased flavivirus replication has been noted via a complement receptor on macrophages (2).

The relevance of these complement functions, as defined primarily in vitro to the role of complement in virus immunity in vivo, is difficult to ascertain. In general, whole antibody has been more effective than antibody fragments in protecting animals from virulent virus challenge (4, 7, 30). Therefore, effector functions via an intact Fc appear to be important in vivo immunity.

Our laboratory has been involved in long-term molecular, biochemical, and immunological studies with Venezuelan equine encephalomyelitis (VEE) virus, an alphagovirus. Most of these studies have revolved around the virulent 1A subtype Trinidad donkey (TrD) parent virus and its vaccine derivative TC-83 (25, 46). Using VEE (TC-83) virus, we originally characterized with monoclonal antibodies (MAbs) seven antigenic epitopes on the two envelope glycoproteins (39). Three epitopes were mapped on the larger glycoprotein (E2, 56,000 daltons), and four were mapped on the smaller glycoprotein (E1, 50,000 daltons). We have more recently characterized five more epitopes on E2, three of which map into the critical neutralization site as determined by competitive binding assays (40). The biological functions of hemagglutination and neutralization reside primarily on E2, and four epitopes (E2γ, E2β, E2γ, and E2β), which mapped into the same critical site, expressed one or both of these biological functions. An MAb to another epitope (E1α) with little or no in vitro biological activity had the same cross-reactivity pattern as the anti-E2γ. Competitive binding assays indicated that these epitopes have a close structural relationship on the virion spike. Recently, passive transfer of
these MAb into mice subsequently challenged with virulent VEE (TrD) virus showed not only that high-avidity MAb to the critical neutralization site (E2e) was effective in protecting in vivo but that both non-neutralizing MAb to the E1b was also protective (23). Nonneutralizing MAb to vesicular stomatitis and Sindbis viruses have been found to be important in vivo immunity (20, 42).

In this report, MAb to the E2c, E1b, and E1d epitopes of VEE (TC-83) virus as well as proteolytic digests of anti-E2c were selectively investigated in passive transfer studies with complement-sufficient and decomplemented mice and mice genetically deficient in C5 to determine whether any correlation existed between in vitro complement fixation and protection. We found that in vivo protection in mice by these MAb is Fc dependent, but not complement dependent.

MATERIALS AND METHODS

Mice and cells. BALB/cAnNCrlBr mice were obtained from the Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). The Jackson Laboratory (Bar Harbor, Maine) was the source of C5-deficient B10.D2/oSn and C5-sufficient B10.D2/nSm mice. National Institutes of Health outbred general-purpose white Swiss mice were provided by the Animal Production unit of our laboratory. The SP2/0-Ag14 nonsecting myeloma cell line was used for all fusions.

Viruses and antisera. The two VEE subtype 1A viruses used in this study were the virulent parent (TrD) and its vaccine derivative (TC-83). Cloned virus seed stocks were prepared in Vero or BHK-21 cell cultures. Viruses were purified in 30% glycerol–45% potassium tartrate gradients as previously described (39). VEE (TrD) and normal polyclonal hyperimmune ascitic fluids were provided by the Arbovirus Reference Branch, Centers for Disease Control. Goat anti-mouse immunoglobulin G (IgG) antisera specific for F(ab)2 and Fc fragments, goat anti-mouse C3 peroxidase conjugate, and rabbit anti-mouse (RAM) IgG were obtained from Cappel Laboratories (West Chester, Pa.). Rabbit polyclonal anti-E2 and anti-E1 antisera were provided by one of us (D.W.T).

MAb. The immunization, cell fusion, cloning, and screening procedures were described by Roehrig et al. (39). Ascitic fluids containing VEE (TC-83) MAbs were purified by a two-step ammonium sulfate precipitation, followed by chromatography over protein A-Sepharose. The MAbs used in this study were anti-E2c (3B4C-4), anti-E1b (3B2A-9), and anti-E1d (3A5B-1). So dilutions would be uniform, MAbs were all adjusted to 1 mg/ml and considered to be undiluted at that concentration. The antibody characterizations, including epitope specificities, binding affinities, monoclonality, and isotype determinations, have been described previously (39).

Antibody fragmentation. Pepsin 1:60,000 (3,630 U/mg), papain (22 U/mg), and pl-cysteine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). A time-course pepsin digestion of anti-E2c MAb was done to correlate Fc degradation with decreased complement activity (8). Antibody (1 to 2 mg/ml) in phosphate-buffered saline was added to an equal volume of 0.2 M ammonium acetate (pH 4.5). Pepsin was added at a ratio of 1:100 enzyme to substrate, and pl-cysteine hydrochloride was added to a final concentration of 0.1 M. This mixture was incubated at 37°C and sampled periodically for 3 h. The digestion was stopped by neutralizing with 1 N NaOH. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the digests in both reduced and nonreduced 7.5 or 10% gels was performed by the method of Laemmli (19). Gels were stained with either Coomassie blue or silver stain (50). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of these digests in unreduced 7.5% gels yielded a 50-kilodalton Fab'-like fragment.

The generation of F(ab')2 from mouse IgG1 MAb (150 to 160 kilodaltons) has been described previously (35). After pepsin digestion, the mixture was first run over protein A-Sepharose 4B to remove any residual Fc. The final eluted product gave a uniform band of 110 to 120 kilodaltons in 7.5% unreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fab fragments of anti-E2c were prepared by the procedure of Mishell and Shiigi (26). The concentration of papain was 1:50 (wt/wt) enzyme to substrate.

Immunological analysis of fragmentation products. An enzyme-linked immunosorbent assay (ELISA), similar to one routinely employed in our laboratory, was used to evaluate Fab, Fab', F(ab')2, and Fc reactivity in all of the anti-E2c peptide and papain digests (39). Purified VEE (TrD) virus (1 to 2 µg per well) was used as the source of antigen, and goat anti-mouse F(ab)2 and Fc were conjugated to alkaline phosphatase by the method of Voller et al. (47). Anti-E2c Fab fragments were also evaluated in a micro-double immunodiffusion procedure against the same unconjugated goat anti-mouse antisera.

Neutralization assays. A microtiter modification of the standard constant virus varying serum dilution plaque reduction neutralization macrotest was performed. Into each well of 96-well, round-bottomed tissue culture plates (Linbro/Titertek, Hamden, Conn.) was placed 0.05 ml of minimal essential medium containing 10% fetal bovine serum and 0.15% sodium bicarbonate. After 0.05-ml samples of appropriate serum dilutions were placed in the first wells, serial twofold dilutions were made. VEE (TrD) virus suspension (0.05 ml) containing 90 to 120 PFU was added to all wells. The plates were incubated in a humidified 5% CO2 atmosphere at 37°C for 1 h. The plates were then placed on ice, and 0.08 ml from each well and 0.04 ml from the virus back titrations were inoculated in duplicate onto Vero cells grown in six-well tissue culture panels (Linbro, Hamden, Conn.). Procedures for adsorption, agar overlay, and staining have been described previously (27). Plaque reduction neutralization titers were determined by plaques remaining after incubation of serum-virus mixtures (0.05 and 0.025 ml, respectively) for 30 min at 37°C and the addition of 0.025 ml of either anti-mouse F(ab')2 or anti-mouse Fc (final dilution 1:50), followed by another 30-min incubation period before plating.

Complement fixation. The fixation of murine complement by VEE antibodies was determined by ELISA. Dilutions of antibody in Veronal-buffered saline (pH 7.1) containing 0.25% polyoxyethylene sorbitan monolaurate (Tween 20) and 1% heat inactivated (56°C, 30 min) normal mouse hyperimmune ascitic fluid were added to wells coated with 1 µg of VEE (TrD) virus. An equal volume (0.05 ml) of murine complement in Veronal-buffered saline was added to give a final complement concentration of 1:40. The source of murine complement was freshly drawn sera from normal outbred Swiss mice. The sera were pooled, aliquoted, and stored at −70°C. The conjugate used was peroxidase coupled to anti-mouse C3; the substrate of choice was [2,2'-azino-di(3-ethyl-benzthiazoline sulfonate)]. Optical densities were read at 405 nm with a Titertek Multiskan ELISA reader. When it was necessary to titrate complement activity in murine serum samples instead of determining the complement fixation titers of the VEE antibodies, a constant...
TABLE 1. Immunological characterization of protective anti-VEE (TC-83) MAb

<table>
<thead>
<tr>
<th>Antibodiesa</th>
<th>Epitope</th>
<th>Isotype</th>
<th>ELISAa</th>
<th>Plaque reduction titer</th>
<th>Complement fixation</th>
<th>Mouse protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>3B4C-4</td>
<td>E2c</td>
<td>IgG1</td>
<td>25,600</td>
<td>40,960</td>
<td>25,600</td>
<td>1</td>
</tr>
<tr>
<td>3B2A-9</td>
<td>E1b</td>
<td>IgG2a</td>
<td>25,600</td>
<td>80</td>
<td>6,400</td>
<td>5 to 10</td>
</tr>
<tr>
<td>3A5B-1</td>
<td>E1d</td>
<td>IgG2a</td>
<td>25,600</td>
<td>&lt;40</td>
<td>6,400</td>
<td>100</td>
</tr>
<tr>
<td>VEE (TrD)</td>
<td></td>
<td></td>
<td>25,600</td>
<td>80</td>
<td>5,120</td>
<td></td>
</tr>
<tr>
<td>HIAF</td>
<td></td>
<td></td>
<td>25,600</td>
<td>5,120</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All MAbIs were purified and adjusted to 1 mg/ml.
b Antibodies were reacted VEE (TrD) antigen (2 μg per well) and assayed for endpoint titers with goat anti-mouse IgG (Fc fragment specific), alkaline phosphatase conjugate, and substrate.
c Plaque reduction neutralization test titers were based on 90% plaque reduction. The final concentration of IgG RAM used for neutralization enhancement was 1:50.
d Fixation of murine complement was determined by ELISA with goat anti-mouse C3 peroxidase conjugate.
e Amount (micrograms) of antibody required to protect >50% of mice (n ≥ 10) inoculated intravenously with antibody and then challenged 24 h later with 100 i.p. 50% lethal doses of VEE (TrD) virus.
f Polyclonal hyperimmune ascitic fluid.
g All mice survived VEE (TrD) virus challenge after 0.2 ml of 1:5 dilution of antibody was passively transferred.

RESULTS

Complement activity of protective anti-VEE (TC-83) MAb.

On VEE (TC-83) virus, we originally defined with MAbIs seven antigenic epitopes on the two major surface glycoproteins (E2 and E1), which make up the virion spike (39). The three most protective MAbIs selected for this study, as defined in passive transfer experiments against the virulent VEE (TrD) virus, had specificities for epitopes ranging from VEE subtypes 1A-1D and II (E2a and E1b) to alphavirus group reactive (E1d). These MAbIs were evaluated for their ability to fix murine complement in vitro (Table 1). All three fixed complement to high titer. The MAb to the critical neutralization site (E2a) was also the most effective in protecting mice from virulent VEE (TrD) challenge. RAM IgG did enhance neutralization with the anti-E1d MAb, but not the anti-E1b MAb. The fact that RAM IgG did not enhance neutralization with the anti-E1d MAb indicates that the E1d epitope may not be expressed on the mature virion.

It is worthy to note that all three MAbIs fixed guinea pig complement in a standard hemolysis assay, and lysed VEE (TrD) virus infected P815 (DBA/2) mastocytoma cells in the presence of rabbit complement (data not shown). The substitution of mouse complement for rabbit complement in the cytotoxic assays resulted in cell lysis, albeit at levels lower than those with rabbit complement (data not shown). It is of particular interest that the anti-E2c N MAb (IgG1 isotype) fixed complement, since it has been reported and generally accepted that mouse IgG1 does not fix complement (13, 29). However, Ey et al. (11) have shown that there are at least two different subclasses of IgG1; one that fixes complement efficiently and the other that does not. More recently, some mouse IgG1 MAbIs have been isolated that have high complement activity titers (37, 51), and others show enhanced virus neutralization in the presence of complement (18).

Biochemical, immunochemical, and immunological analysis of anti-E2c antibody fragments. To further investigate the association between complement fixation and protection, the high-affinity anti-E2c MAbs, with high-titered biological activity to the critical neutralization site on the virion, was selected for fragment analysis with proteolytic enzymes. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of 1- to 3-h pepsin digests is shown in Fig. 1. During this time, the digestion profile in the reduced gel showed an increase in the degradation of the heavy chains from 55 to 28 kilodaltons while the light chains remained constant. Analysis of the digests by immunodiffusion with anti-Fc and anti-F(ab')2 specific antisera showed decreasing Fc reactivity, but the anti-F(ab')3 reaction was similar in all digests.

Protective capacity of pepsin digests as related to complement and Fc activity. The 1- to 3-h pepsin digests of the anti-E2c MAbIs were inoculated into mice to ascertain protective capacity; the digests were also evaluated for complement fixation with murine complement and their capacity to bind to intact VEE virions (Table 2). At 1 h, the complement fixation titer had decreased 64-fold, but the MAb still protected mice at all but the highest concentration of antibody. A separate 1-h digest of the anti-E2c MAb also had a murine complement fixation titer of 1:400, but protected 6 out of 10 mice with 1 μg of antibody. The diminution in the protective capacity of the digests over the 3-h period did not appear to correlate with the decrease and disappearance of complement fixation activity or the ability of the digested fragments to bind to virions, but was associated with diminishing Fc activity (Table 2, Fig. 1). Purified anti-E2c MAb fragments [Fab, Fab', and F(ab')3], devoid of any Fc reactivity, did not protect mice even when the equivalent of 20 times the amount of antibody normally needed for protection was used. The F(ab')3 did not fix murine complement. All frag-
FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of 1- to 3-h pepsin digests of anti-E2c MAb in a 10% reduced gel. Lanes: 1, molecular weight markers; 2, undigested anti-E2c MAb; 3, 4, and 5, 1-, 2-, and 3-h unpurified pepsin digests of anti-E2c MAb, respectively. Notice that the silver stain differentiates between the light chains and heavy chain remnants. Immunodiffusion results ranging from +++ (strong precipitant band) to ± (weak precipitant band) with anti-Fc and anti-F(ab')2 mouse antisera are shown at the bottom of the lanes.

TABLE 2. Effect of pepsin digestion on protection and complement activity with anti-E2c MAb

<table>
<thead>
<tr>
<th>Anti-E2c MAb proteolytic digest</th>
<th>ELISA*</th>
<th>Mouse protection with the following anti (μg) of antibody:</th>
<th>Complement fixation</th>
<th>Anti-Fc</th>
<th>Anti-F(ab')2</th>
<th>Anti-E2c MAb proteolytic digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pepsin</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>1 h</td>
<td>2,560</td>
<td>2,560</td>
<td></td>
<td>2,560</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>2 h</td>
<td>6,400</td>
<td>6,400</td>
<td></td>
<td>6,400</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>3 h</td>
<td>1,600</td>
<td>1,600</td>
<td></td>
<td>1,600</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>Fab*</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Fab'</td>
<td>&lt;100</td>
<td>3,200</td>
<td></td>
<td>3,200</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Fab(ab')2</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Fab(ab')2*</td>
<td></td>
<td></td>
<td></td>
<td>ND</td>
<td>1/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Titers represent the mean endpoint values of triplicate samples; standard errors were within 10% of the mean.

** ND, Not done.

TABLE 3. Neutralization of VEE (TrD) virus with anti-E2c MAb fragments

<table>
<thead>
<tr>
<th>Anti-E2c MAb proteolytic digest</th>
<th>Plaque reduction neutralization titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unenhanced</td>
<td>Second antibody enhancement with anti-Fab'</td>
</tr>
<tr>
<td>No pepsin</td>
<td>≥40,960*</td>
</tr>
<tr>
<td>Pepsin, 1 h</td>
<td>10,240</td>
</tr>
<tr>
<td>Pepsin, 2 h</td>
<td>2,560</td>
</tr>
<tr>
<td>Pepsin, 3 h</td>
<td>6,400</td>
</tr>
<tr>
<td>Fab*</td>
<td>160</td>
</tr>
<tr>
<td>Fab'</td>
<td>2,560</td>
</tr>
<tr>
<td>Fab(ab')2</td>
<td>40,960</td>
</tr>
</tbody>
</table>

* Titers read as reciprocal of last antibody dilution showing 70% reduction in VEE (TrD) virus plaques.

** Purified antibody fragments.

Results of murine complement pathways in protection with anti-E2c and anti-E1b MAb. We have shown that the most sensitive passive transfer system for assaying the protective capacity of VEE MABs was to vary the antibody concentration while holding the VEE (TrD) virus 50% lethal challenge dose constant (23). We have also shown that when 3-week-old nonimmune mice are challenged peripherally with 100 i.p. 50% lethal doses (approximately 20 PFU) of VEE (TrD) virus, there is rapid virus replication in both extraneural and neural tissues resulting in death at 5 to 6 days (23). Therefore, in our complement depletion experiments, it was important to know whether the active complement levels remained depressed for at least 4 days. Mice were inoculated i.p. with 10 U of CVF at 0 and 48 h. Four mice in each group were bled on days 1 to 4 and day 7. Complement activity was monitored in the murine complement fixation assay. Untreated control group had an endpoint complement fixation titer of 1:320 in the presence of 1:400 VEE (TrD) polyclonal hyperimmune ascitic fluid, whereas all of the CVF-treated mice through day 7 had a titer of <1:10. Therefore, the complement levels did remain depressed for at least 7 days. In C3-depleted mice, both complement pathways are blocked; however, some involvement of the classical pathway must be considered, since deposition of early complement components on virions has been found to play a role in virus neutralization (1, 33). Doses of 20 and 40 μg, respectively, of anti-E2c and anti-E1b MABs were inoculated into both C3-depleted and untreated mice (Table 4). The MABs protected mice in both groups. The only indication of a difference was with anti-E1b MAB, where 80% of the C3-depleted mice survived, compared with 100% of the untreated group. This difference was not significant (Fisher fragment specific) did not increase the neutralization titers of any of the purified fragments (data not shown).
TABLE 5. Protection of VEE (TrD) virus challenged C5-deficient mice with VEE (TC-83) MAb

<table>
<thead>
<tr>
<th>µg of antibody</th>
<th>Anti-E2c</th>
<th>Anti-E1b</th>
<th>Survivor/total inoculated with MAb:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10/10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5/5</td>
<td>5/5</td>
<td></td>
</tr>
</tbody>
</table>

* All mice (n = 10, each group) survived VEE (TrD) virus challenge (101, i.p., 50% lethal doses) after passive transfer of rabbit polyclonal antisemurum (0.2 ml of a 1:2 dilution) to the VEE E2 and E1 glycoproteins. C5-deficient mice (n = 5, each group) were protected with 1 and 5 µg, respectively, of anti-E2c and anti-E1b. All animals given PBS and then challenged with VEE (TrD) virus died.

a ND, not done.

exact two-tailed test, \( P < 0.01 \). The results of passive transfer with 1 to 50 µg of anti-E2c and anti-E1b MAB into C5-deficient mice are shown in Table 5. In C5-deficient mice, both complement pathways are intact, but there are no terminal lytic events. Mice were protected from VEE (TrD) virus challenge, even at low concentrations of antibody. Mice were also protected with polyclonal rabbit E2 and E1 antiserum.

DISCUSSION

For several viruses, we and others have mapped with MAbs the function of neutralization and protection to biologically active critical site(s) on the virion surface; MAbs to other epitopes will bind to the virion, but will not neutralize, although some will protect animal hosts from virulent virus challenge; still other MAbs will not bind to intact virions, but will protect (20–24, 39, 40–42). The antigenic epitopes involved in this latter phenomenon are cryptic on the mature virion, but expressed on the infected cell membrane (42). Several immunological investigations have suggested that antibody-dependent classical complement pathway-mediated in vitro neutralization of virus infectivity by either lysis of infected cells or virions may be logically extrapolated to the protective effect these antibodies might have in vivo (5, 12, 33, 36, 42). Other reports have stressed the importance of the Fc part of the immunoglobulin molecule in protection (4, 7, 30). In the present study, we have used our well-defined protective MAbs, which fix murine complement to ascertain the role of complement and Fc in protection from VEE (TrD) virus infection by (i) investigating the ability of our MAbs to protect mice with impaired complement pathways and (ii) investigating the ability of antibody fragments, devoid of complement-fixing activity, to protect complement-sufficient mice.

Decomplemented mice and mice deficient in C5 have been suitable models to evaluate aspects of immunity and pathogenesis with Sindbis virus (15). With our anti-E2c and anti-E1b MAbs, both groups of mice were protected from VEE (TrD) virus challenge. This indicated that neither the terminal membrane attack complex (C5 through C9) nor C3, which is essential for alternate complement pathway activity, was necessary for protection. These results were similar to those in a study with herpes simplex virus, in which C5-deficient mice recovered from a herpes simplex virus infection after passive transfer of anti-herpes simplex virus IgG (30).

To rule out the possibility that the deposition of early complement components—C1, C4, and C2 in decomplemented mice of C1, C4, C2, and C3 in C5-deficient mice—may have contributed to the protective response and to further define the role of the Fc and Fab in protection, we prepared antibody fragments by enzymatic cleavage of the anti-E2c MAb. The biological activities of purified anti-E2c MAb fragments [Fab, Fab', and F(ab')2] were compared with those of the intact parent IgG. The order of in vitro neutralization was F(ab')2 > Fab' > Fab, with the divalent F(ab')2 being as efficient in neutralization as the intact E2c MAb. No fragment fixed complement and no fragment conferred protection from virus infection after passive transfer. These results are consistent with those of studies with western equine encephalitis virus and murine leukemia virus, in which F(ab')2 fragments would neutralize virus in vitro, but not protect animals from virus challenge (4, 7).

Another study with herpes simplex virus found that F(ab')2 would confer protection when inoculated before or just after virus, but not once the infection was established (30). A more recent study with vesicular stomatitis virus showed that F(ab')2 from neutralizing MAb, but not from nonneutralizing MAb, was protective; protection for the latter MAb required an intact Fc (20). In most of these studies, it was postulated that the Fc, albeit not at the level of complement fixation, was important in protection. The fact that our F(ab')2 did not protect complement-sufficient animals indicated that activation of the alternate complement pathway by intact virions or virus-infected cells, with subsequent lysis in the presence of F(ab')2, was not operable in our murine systems.

It is known that pepsin cleaves between residues 245 and 246 in the Cγ2 domain of BALB/c IgG1 (45). The binding site for Clq is also in the Cγ2 region of the IgG molecule (9, 49). Whereas limited pepsin digestion resulted in a decrease of both complement and Fc reactivity with our anti-E2c MAb (IgG1 isotype), the protection profile suggested that the Fc, independent of complement, was necessary for protection (Fig. 1, Table 2). This differentiation of Fc effector functions by enzymatic digestion indicated that antibody-dependent complement activity via the classical complement pathway was not necessary for protection in our VEE system. Similar pepsin digestion of the anti-E1b MAb (IgG2a isotype) resulted in loss of complement activity proportional only to the digestion of the intact molecule to Fab' (our unpublished observation). This difference in enzyme susceptibility between isotypes in their complement-binding regions may be related to interchain disulfide bonding differences as well as hinge range flexibility, which may sterically impede or accelerate proteolytic activity (J. L. Winkelhake, personal communication). A recent study has shown that the rotational movement of the Fab arms of a monoclonal IgG1 appeared to be restricted (38). Also, the angle of the Fab arms in complement activation is important (3).

The mechanism of protection mediated by the anti-E1d MAb is different from protection mediated by the anti-E2c and anti-E1b MAbs. In terms of preexisting immunity, the latter two MAbs can protect by directly binding to the virion. The failure of RAM IgG to enhance in vitro neutralization indicated that the E1d epitope was cryptic on the mature virion; therefore, protection by this MAb may be at the level of the cell membrane. From a structural point of view, the E1d epitope was further away from the E2d than the E1d, but still overlapped with the latter (39). The amount of MAb required for protection (Table 1) was inversely related to the epitope's structural proximity to the critical neutralization site (E2d). The anti-E1d MAb may protect by affecting a
crucial step in virion morphogenesis after binding to its respective epitope on infected cell membranes. Studies with the anti-E1d, like those described in this report for the anti-E2a and anti-E1b MAbs, were not performed herein; however, in our laboratory the E1 glycoprotein of western equine encephalitis has been mapped with MAbs (17). An MAb was isolated that mapped to the same epitope as our anti-E1b MAb; this epitope was largely cryptic on the western equine encephalitis virion. This MAb did not fix complement, but did protect mice from VEE (Trd) virus challenge at microgram concentrations of MAb similar to those of our anti-E1d MAb, indicating that complement may not play a role in protection mediated by these antibodies.

In our laboratory we have determined the sequences of the nucleic acids that code for the E2 glycoproteins of both VEE (Trd) and TC-83 viruses (Trent et al., manuscripts in preparation). This should enable us to locate the sequence, as defined by MAb, to be used in a subunit or synthetic vaccine. We are currently investigating the role of the VEE glycoprotein epitopes in cell-mediated immunity.

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LITERATURE CITED


