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

JAMES H. MATHEWS,* JOHN T. ROEHRIG, AND DENNIS W. TRENT

Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Fort Collins, Colorado 80522

Received 1 April 1985/Accepted 20 May 1985

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 (E2^c, E1^b, and E1^d) were assayed for *in vitro* complement activity. All three fixed murine complement to high titer. Limited pepsin digestion of the anti-E2^c 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 MAb, were protected until the Fc termini were cleaved at 3 h. Anti-E2^c F(ab')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-E2^c or anti-E1^b MAb. The results show that the anti-E2^c 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-E2^c 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-E1^d 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 Cγ2 region of the Fc, the site of C1q 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 *in vivo* immunity.

Our laboratory has been involved in long-term molecular, biochemical, and immunological studies with Venezuelan equine encephalomyelitis (VEE) virus, an alphatogavirus. 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 (E2^c, E2^f, E2^g, and E2^h), which mapped into the same critical site, expressed one or both of these biological functions. An MAb to another epitope (E1^b) with little or no *in vitro* biological activity had the same cross-reactivity pattern as the anti-E2^c. Competitive binding assays indicated that these epitopes have a close structural relationship on the virion spike. Recently, passive transfer of

* Corresponding author.

these MAbs into mice subsequently challenged with virulent VEE (TrD) virus showed not only that high-avidity MAb to the critical neutralization site (E2^c) was effective in protecting animals, but that non-neutralizing MAb to the E1^b was also protective (23). Nonneutralizing MAbs to vesicular stomatitis and Sindbis viruses have been found to be important in *in vivo* immunity (20, 42).

In this report, MAbs to the E2^c, E1^b, and E1^d epitopes of VEE (TC-83) virus as well as proteolytic digests of anti-E2^c were selectively investigated in passive transfer studies with complement-sufficient and de complemented 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 MAbs is Fc dependent, but not complement dependent.

MATERIALS AND METHODS

Mice and cells. BALB/c/AnNCrIBR 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/nSn 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 nonsecreting 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 ammonium sulfate precipitation, followed by chromatography over protein A-Sepharose. The MAbs used in this study were anti-E2^c (3B4C-4), anti-E1^b (3B2A-9), and anti-E1^d (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 DL-cysteine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). A time-course pepsin digestion of anti-E2^c 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 DL-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-E2^c 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-E2^c pepsin 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-E2^c 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 enhancement by a second antibody involved 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) MAbs

Antibodies ^a	Epitope	Isotype	ELISA ^b	Plaque reduction titer ^c		Complement fixation ^d	Mouse protection ^e
				Unenhanced	RAM IgG		
3B4C-4	E2 ^c	IgG1	25,600	40,960	40,960	25,600	1
3B2A-9	E1 ^b	IgG2a	25,600	80	2,560	6,400	5 to 10
3A5B-1	E1 ^d	IgG2a	25,600	<40	<40	6,400	100
VEE (TrD) HIAF ^f			25,600	80	5,120	25,600	+ ^g

^a All MAbs 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 ($n \geq 10$) 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.

dilution (1:200) of VEE hyperimmune ascitic fluid was mixed with various dilutions of murine complement samples. Controls that were extremely important in this ELISA system were diluent plus complement, antibody plus diluent, and antibody plus heat-inactivated or C3-depleted complement.

Passive antibody transfer and complement depletion. Known microgram concentrations of purified MAb or fragments diluted in phosphate-buffered saline were injected intravenously into the tail veins of either 3-week-old National Institutes of Health white Swiss mice (14 to 18 g) or adult (>16-week) C5-deficient mice (25 to 30 g). Twenty-four hours later, the Swiss and C5-deficient mice were challenged intraperitoneally (i.p.) with 10^2 and 10^3 i.p. 50% lethal doses of VEE (TrD) virus, respectively. Controls received phosphate-buffered saline intravenously and either virus or virus diluent i.p. Cobra venom factor (CVF) (Cordis Laboratories, Inc., Miami, Fla.) was used to deplete 3-week-old Swiss mice of C3. Ten units of CVF was inoculated i.p. into mice that had been passively immunized with VEE (TC-83) MAb 24 h earlier. Four hours later, the mice were challenged i.p. with VEE (TrD) virus; 48 h after that, the mice received another 10 U of CVF.

RESULTS

Complement activity of protective anti-VEE (TC-83) MAbs. On VEE (TC-83) virus, we originally defined with MAbs seven antigenic epitopes on the two major surface glycoproteins (E2 and E1), which make up the virion spike (39). The three most protective MAbs 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 (E2^c and E1^b) to alphavirus group reactive (E1^d). These MAbs 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 (E2^c) was also the most effective in protecting mice from virulent VEE (TrD) challenge. RAM IgG did enhance neutralization with the anti-E1^b MAb, but not the anti-E1^d MAb. The fact that RAM IgG did not enhance neutralization with the anti-E1^d MAb indicates that the E1^d epitope may not be expressed on the mature virion.

It is worthy to note that all three MAbs 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 sub-

stitution 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-E2^c 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 one that does not. More recently, some mouse IgG1 MAbs have been isolated that have high complement cytotoxicity titers (37, 51), and others show enhanced virus neutralization in the presence of complement (18).

Biochemical, immunochemical, and immunological analysis of anti-E2^c antibody fragments. To further investigate the association between complement fixation and protection, the high-affinity anti-E2^c MAb, with high-titered biological activity to the critical neutralization site on the virion, was selected for fragmentation 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')₂ 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-E2^c MAb 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 lowest concentration of antibody. A separate 1-h digest of the anti-E2^c 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-E2^c MAb fragments [Fab, Fab', and F(ab')₂], 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')₂ 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-E2^c MAb in a 10% reduced gel. Lanes: 1, molecular weight markers; 2, undigested anti-E2^c MAb; 3, 4, and 5, 1-, 2-, and 3-h unpurified pepsin digests of anti-E2^c 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.

ments would not fix guinea pig or rabbit complement in standard hemolytic and cytotoxic assays (data not shown).

Neutralization of VEE (TrD) virus with anti-E2^c MAb fragments. The bivalent F(ab')₂ neutralized VEE virus to titer with intact IgG; however, both of the monovalent fragments (Fab and Fab') were not very efficient in neutralization (Table 3). The generation of increasing amounts of Fab'-like fragments during the 3-h pepsin digestion period is reflected in the decreasing neutralization titers. The addition of RAM IgG [F(ab')₂ specific] increased the neutralization titer in all of the digests plus the purified monovalent fragments—possibly simulating the bivalent condition with subsequent cross-linking and neutralization. RAM IgG (Fc

TABLE 3. Neutralization of VEE (TrD) virus with anti-E2^c MAb fragments

Anti-E2 ^c MAb proteolytic digest	Plaque reduction neutralization titer ^a	
	Unenhanced	Second antibody enhancement with anti- F(ab') ₂
No pepsin	≥40,960 ^a	≥40,960
Pepsin, 1 h	10,240	20,480
Pepsin, 2 h	2,560	10,240
Pepsin, 3 h	640	10,240
Fab ^b	160	2,560
Fab' ^b	2,560	≥5,120
F(ab') ₂ ^b	40,960	≥40,960

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

^b Purified antibody fragments.

fragment specific) did not increase the neutralization titers of any of the purified fragments (data not shown).

Results of murine complement pathways in protection with anti-E2^c and anti-E1^b MAbs. 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 mice 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-E2^c and anti-E1^b 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-E1^b MAb, where 80% of the C3-depleted mice survived, compared with 100% of the untreated group. This difference was not significant (Fisher

TABLE 2. Effect of pepsin digestion on protection and complement activity with anti-E2^c MAb

Anti-E2 ^c MAb proteolytic digest	ELISA ^a		Mouse protection with the following amt (µg) of antibody:				Complement fixation
	Anti- Fc	Anti- F(ab') ₂	20	10	5	1	
No pepsin	25,600	12,800	ND ^b	10/10 ^c	10/10	8/10	25,600
Pepsin, 1 h	12,800	12,800	5/5	8/10	8/10	1/10	400
Pepsin, 2 h	6,400	12,800	5/5	7/10	5/10	0/10	<25
Pepsin, 3 h	1,600	12,800	1/10	1/10	3/10	0/10	<25
Fab ^d	ND	ND	0/5	ND	ND	ND	ND
Fab' ^d	<100	3,200	0/5	0/5	0/5	ND	ND
F(ab') ₂ ^d	ND	ND	0/5	1/5	0/10	0/10	<100

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

^b ND, Not done.

^c Survivors/total inoculated.

^d Purified antibody fragments. For purposes of comparison, the microgram concentrations of the MAb fragments were adjusted on a molar basis to compare directly with intact IgG. All reacted strongly in double immunodiffusion with anti-mouse F(ab')₂ antiserum, but were negative against anti-mouse IgG (Fc chain specific).

TABLE 4. Protection of C3-depleted mice with VEE (TC-83) MAbs

CVF	µg of antibody	Survivors/total inoculated with MAb:	
		Anti-E2 ^c	Anti-E1 ^b
+	20	10/10	ND ^a
-	20	5/5	ND
+	40	ND	8/10
-	40	ND	5/5

^a ND, Not done.

TABLE 5. Protection of VEE (TrD) virus challenged C5-deficient mice with VEE (TC-83) MAb^a

μ g of antibody	Survivor/total inoculated with MAb:	
	Anti-E2 ^c	Anti-E1 ^b
1	10/10	ND ^b
5	ND	9/10
10	10/10	ND
20	ND	10/10
50	5/5	5/5

^a All mice ($n = 10$, each group) survived VEE (TrD) virus challenge (10^3 i.p. 50% lethal doses) after passive transfer of rabbit polyclonal antiserum (0.2 ml of a 1:2 dilution) to the VEE E2 and E1 glycoproteins. C5-sufficient mice ($n = 5$, each group) were protected with 1 and 5 μ g, respectively, of anti-E2^c and anti-E1^b. All animals given PBS and then challenged with VEE (TrD) virus died.

^b ND, Not done.

exact two-tailed test, $P < 0.01$). The results of passive transfer with 1 to 50 μ g of anti-E2^c and anti-E1^b 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 antisera.

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-E2^c and anti-E1^b 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-E2^c MAb. The biological activities of purified anti-E2^c 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 E2^c 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 C γ 2 domain of BALB/c IgG1 (45). The binding site for Clq is also in the C γ 2 region of the IgG molecule (9, 49). Whereas limited pepsin digestion resulted in a decrease of both complement and Fc reactivity with our anti-E2^c 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-E1^b 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-E1^d MAb is different from protection mediated by the anti-E2^c and anti-E1^b 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 E1^d 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 E1^d epitope was further away from the E2^c than the E1^b, 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 (E2^c). The anti-E1^d 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-E1^d, like those described in this report for the anti-E2^c and anti-E1^b MAbs, were not performed here; 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-E1^d 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-E1^d 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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