The Mechanisms of Neutralization of Sensitized Equine Arteritis Virus by Complement Components

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(Accepted 24 July 1974)

SUMMARY

The mechanisms involved in the interaction of complement components with sensitized equine arteritis virus (EAV) were investigated. Virus neutralization and virolysis depended on both the concentration of the complement components and the concentration of the sensitizing antibody. High concentrations of C4, 2 and 3, with an optimal concentration of C1, were sufficient for neutralizing virus infectivity in the presence of excess antibody. The addition of the remaining five components (C5 to C9) of the complement system induced lysis of the previously neutralized virus particle. Lysis was initiated by C8 and was augmented by C9. Components C5 to C9 did not enhance neutralization produced by excess antibody and limiting concentrations of complement components. In contrast, addition of components C5 to C9 enhanced neutralization by means of lysis of the virus particle under conditions of low antibody concentration.

INTRODUCTION

In a previous report (Radwan, Burger & Davis, 1973) on the interactions between complement and sensitized equine arteritis virus (EAV), we proposed two sequential mechanisms: (1) neutralization of infectivity by early complement components, followed by (2) lysis of the virus particle induced by the remaining components of the complement system. Whole antisera and unfractionated equine or guinea pig serum as complement sources were employed in the previous studies. However, to determine more precisely the effect of the individual components of the complement system on sensitized EAV, quantitative studies were performed employing purified preparations of complement components, virus and IgG antibody.

The results reported here verify that the complement system enhances EAV neutralization by two different mechanisms; one, operating under conditions of excess antibody, neutralized infectivity apparently through stearic hindrance by C1, 4, 2 and 3. The other mechanisms increased the efficiency of neutralization under conditions of low antibody concentrations and involved lysis of the virus particle by the entire complement sequence.

METHODS

Cell culture and media. The NBL-6 line of equine dermal cells (American Type Culture Collection CCL57) was grown in Eagle's MEM with 10% foetal calf serum as described by Radwan & Burger (1973a). MEM with 2% calf serum was used as maintenance medium,
and MEM or phosphate-buffered saline, pH 8.0 (PBS) containing Ca++ and Mg++ was used as diluent.

**Virus.** Stocks of the Bucyrus strain of equine arteritis virus (EAV) (Doll et al. 1957) were prepared in NBL-6 cells as described by Radwan & Burger (1973a). Following clarification by low-speed sedimentation, the virus was sedimented at 131 000 g for 3½ h and the pellets resuspended in a small vol. of PBS. The virus was then banded on a continuous 20 to 60 % sucrose gradient at 131 000 g for 3½ h. The virus band was collected, diluted, sedimented at 220 000 g for 1 h, resuspended in PBS and stored in samples at -70°C. A sample of the EAV stock was radioactively labelled with [3H]-uridine (Radwan et al. 1973).

**Assay for virus infectivity.** The infectivity of EAV samples was measured by a plaque assay in NBL-6 cells as reported by Radwan & Burger (1973a).

**Antiserum.** Antiserum was obtained from a horse recovered from experimental infection by EAV. This infection was by inhalation from a nose bag of an aerosol of cell culture fluid containing 10^8 p.f.u./ml. Antiserum was collected two weeks following recovery. The stock IgG fraction containing 5 mg/ml was separated, purified and characterized as previously described (McGuire & Crawford, 1972). Anti-equine γ-globulin was prepared in goats as reported by McGuire & Crawford (1972).

**Sensitization of virus.** Equal vol. of appropriate dilutions of anti-EAV IgG and a known concentration of labelled or unlabelled EAV were mixed and incubated at 37 °C for 30 min immediately before use.

**Complement.** Normal guinea pig serum served as the source of the complete complement sequence. Purified guinea pig complement components were obtained from Cordis Laboratories, Miami, Florida. The activities quoted by the suppliers were 10 000 CH50/ml for C1 and 1000 CH50/ml for C2 to C9. These corresponded to \(7 \times 10^{11}\) and \(7 \times 10^{10}\) haemolytically effective molecules/ml (haem/ml) respectively, as determined by the ‘effective molecular titration’ method (Kabat & Mayer, 1961). All components except C1, C4, and C8 contained less than 1% contamination with other components. C1 and C4 contained from 0.8 to 2.4% contamination with C5. C8 contained 1.8% contamination with C9 (1000 CH50/ml C8 and 18 CH50/ml C9). The nomenclature used conforms to that proposed by the World Health Organization (1968). In this study, the concentrations of the complement components are expressed as the final concentrations in the reaction mixtures.

**Assay for virus particle lysis by complement components.** Radioactively labelled sensitized EAV (0.2 ml) at 26 °C was transferred into ultracentrifuge tubes. At 10 min intervals the appropriate tubes received 0.1 ml of each of the required complement components at a concentration of \(4.4 \times 10^6\) haem/ml to obtain the various combinations with sensitized virus. The mixtures were incubated at 37 °C for 15 min, then treated with diluent or ribonuclease (RNase) (Worthington Biochemical Co.) and incubated for an additional 15 min at 37 °C. Each mixture was then brought to a final vol. of 6.0 ml with PBS, centrifuged at 143 000 g for 3 h and the supernatant fluids collected. The pellets were resuspended to the original volume with PBS. Samples of 0.2 ml of supernatant fluids or sediments were digested with 1.0 ml NCS (Amersham/Searle Co.) and assayed for radioactivity on a Packard scintillation spectrometer (Radwan et al. 1973). Parallel samples of supernatant fluids and sediments were assayed for virus infectivity.

**RESULTS**

Preliminary experiments showed that no single component of guinea pig complement influenced the infectivity of sensitized or non-sensitized EAV. Undiluted components in the
EAV neutralization by complement components

Fig. 1. Effect of C1 concentration on neutralization of sensitized EAV. Samples of EAV-IgG containing $1 \times 10^9$ p.f.u./0.2 ml were incubated at 26 °C for 20 min with equal vol. (0.2 ml) of twofold serial dilutions of C1. The mixtures then received 0.2 ml of C4 ($2.3 \times 10^9$ haem/ml), and were incubated at 37 °C for 15 min prior to assay for virus infectivity. Samples of sensitized virus incubated with diluent, fresh whole guinea pig serum and anti-$\gamma$-globulin served as controls.

The following combinations also had no effect; C3, 5; C3, 5, 6; C3, 5, 6, 7; C3, 5, 6, 7, 8, and C3, 5, 6, 7, 8, 9. However, the mixture C1, 4, 2, 3, totally neutralized sensitized EAV but had no effect on non-sensitized virus. In the absence of C1, however, the mixture C4, 2, 3 was incapable of neutralizing sensitized virus, indicating that C1 was essential for neutralization by C4, 2, 3.

The following experiments demonstrate the relationship between the percentage of infectivity neutralized and the relative concentration of each of the first four components of the complement system.

**Effect of C1 concentration**

The results in Fig. 1 show the effect of different concentrations of C1 on the percentage of neutralization of EAV, previously sensitized with $2.5 \text{ mg IgG/ml}$, in the presence of $2.3 \times 10^{10}$ haem/ml of C4. The percentage of neutralization produced by C1, 4 depended upon the concentration of C1. The presence in the reaction mixture of a high concentration of C1 ($2.3 \times 10^{11}$ haem/ml) completely inhibited neutralization. In contrast, a combination of C1 at $1.6 \times 10^{10}$ haem/ml and C4 at $2.3 \times 10^{10}$ haem/ml produced about 70% neutralization.
Fig. 2. Effect of concentration of C4, C2 or C3 on neutralization of sensitized EAV. Sensitized virus (1 × 10^8 p.f.u./0.2 ml) was reacted for 15 min at 26 °C with (a) C1 (3 × 10^9 haem/ml) and serial two-fold dilutions of C4. Half of each mixture was then reacted with an equal vol. of C2 (3.5 × 10^10 haem/ml) (●●●) or with diluent (▲▲▲). C1 and sub-optimal C4 (2.2 × 10^8 haem/ml). Portions were then mixed with equal vol. of serial dilutions of C2. Half of each reaction mixture was then reacted with an equal vol. of C3 (1.8 × 10^10 haem/ml) (●●●) or diluent (▲▲▲). (c) Sub-optimal concentrations of C1 (1.8 × 10^8 haem/ml), C4 (3.5 × 10^8 haem/ml) and C2 (3.5 × 10^8 haem/ml). Samples then received equal vol. of serial twofold dilution of C3 (●●●) or diluent (▲▲▲). All mixtures were incubated for 15 min at 37 °C and assayed for virus infectivity as in Fig. 1. The dotted lines in (c) represents the average percent neutralization produced by C1, 4, 2 alone.

Effect of C4 concentration

To determine the effect of C4 concentration on neutralization, sensitized virus was reacted with C1 then incubated with serial twofold dilutions of C4 or C4 plus a constant amount of C2 (3.5 × 10^10 haem/ml). The percentage of neutralization increased up to about 70 % with increasing concentration of C4 up to 9 × 10^9 haem/ml (Fig. 2a). In another experiment, it was found that no significant additional neutralization was obtained by a fourfold increase in C4 concentration to 3.6 × 10^10 haem/ml. As seen in Fig. 2a, the addition of C2 to the system with C1 and C4 further enhanced neutralization, particularly at low C4 concentrations.

Effect of C2 concentration

Sensitized EAV was first incubated with C1 and a sub-optimal concentration of C4 (2.2 × 10^8 haem/ml), and then with various concentrations of C2 or with C2 plus 1.8 × 10^10 haem/ml of C3. Under these conditions, C2 augmented neutralization to a maximum of about 80 % (Fig. 2b). The addition of C3 to the system with C1, 4, 2 increased neutralization to 100 %.

Effect of C3 concentration

To study the effect of various concentrations of C3 on neutralization, sensitized EAV was incubated with sub-optimal concentrations of C1, C4 and C2. Samples of this mixture were then reacted with serial twofold dilutions of C3 or diluent. The percentage neutralization increased progressively and reached 100 % at a C3 concentration of 1.0 × 10^9 haem/ml (Fig. 2c).
**EAV neutralization by complement components**

![Diagram showing neutralization by complement components](image)

**Fig. 3. Effect of relative concentration of sensitizing antibody (IgG) on neutralization by early complement components.** Portions (0.5 ml each) of EAV (1 x 10^5 p.f.u./0.2 ml) were sensitized with equal vol. of serial twofold dilutions of IgG. Each mixture was reacted with sub-optimal C1 (7 x 10^8 haem/ml) and C4 (7 x 10^8 haem/ml). One sample of the mixture EAV-IgG-C1, 4 received diluent (▲——▲); a second sample received C2 (7 x 10^8 haem/ml) (●——●); and a third sample received C2 (7 x 10^8 haem/ml) and C3 (1.4 x 10^9 haem/ml) (■——■). All samples were adjusted to the same vol. with diluent. A sample from each EAV-IgG preparation received diluent and served as infectivity control. Undiluted antibody contained 5 mg IgG/ml.

**Effect of antibody concentration on neutralization by early components**

In the previous experiments, excess antibody (2.5 mg/ml) was used for virus sensitization. Therefore, it was of interest to determine the relationship between the concentration of sensitizing antibody and the percentage neutralization produced by the early complement components. A linear relationship was found to exist between these two parameters (Fig. 3).

**Neutralization and lysis of sensitized virus**

We reported earlier (Radwan et al. 1973) that interaction of sensitized EAV with fresh guinea pig or equine complement at 37 °C was followed by neutralization of virus infectivity and release of RNA, indicating structural damage to the virus particle. Also, it was shown
Table 1. Neutralization and virolysis of sensitized EAV by complement components

<table>
<thead>
<tr>
<th>Mixture assayed</th>
<th>Supernatant fluid</th>
<th>Sediment in original volume</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>p.f.u./ct/min/0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Nonidet + RNase</td>
<td>227 (84%)</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>26 (11%)</td>
<td>3</td>
</tr>
<tr>
<td>CI, 4, 2, 3 + RNase</td>
<td>29 (11%)</td>
<td>0</td>
</tr>
<tr>
<td>CI to 7 + RNase</td>
<td>23 (9%)</td>
<td>0</td>
</tr>
<tr>
<td>CI to 8 + PBS</td>
<td>42 (19%)</td>
<td>0</td>
</tr>
<tr>
<td>CI to 8 + RNase</td>
<td>68 (24%)</td>
<td>0</td>
</tr>
<tr>
<td>CI to 9 + PBS</td>
<td>76 (28%)</td>
<td>0</td>
</tr>
<tr>
<td>CI to 9 + RNase</td>
<td>154 (57%)</td>
<td>0</td>
</tr>
<tr>
<td>GPC + RNase</td>
<td>164 (61%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Equal vol. of [3H]-uridine-labelled sensitized EAV were incubated at 37 °C for 30 min with 1 % Nonidet P-40, diluent (PBS), complement components (C) in the combinations shown, and fresh guinea pig serum (GPC) with or without ribonuclease (1 mg/ml RNase). The mixtures were then centrifuged at 143,000 g for 3 h.

Table 2. Ability of C5 to C9 to enhance neutralization of sensitized EAV under conditions of limiting antibody and excess complement

<table>
<thead>
<tr>
<th>Mixture assayed</th>
<th>Neutralization as % reduction of infectivity (p.f.u.)</th>
<th>Significance of difference from CI, 4, 2, 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>GPC + PBS</td>
<td>93</td>
<td>---</td>
</tr>
<tr>
<td>GPC + RNase</td>
<td>100</td>
<td>---</td>
</tr>
<tr>
<td>CI, 4, 2, 3 + PBS</td>
<td>56 ± 10</td>
<td>---</td>
</tr>
<tr>
<td>CI to 9 + PBS</td>
<td>61 ± 2.6</td>
<td>0.1</td>
</tr>
<tr>
<td>CI to 9 + RNase</td>
<td>80 ± 8.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

EAV (2 × 10^8 p.f.u./0.2 ml) was sensitized with IgG antibody diluted 1:80. Equal portions (0.2 ml) were then mixed with diluent (PBS), fresh guinea pig serum (GPC), GPC and RNase, complement components (0.2 ml each) in the combinations shown (each mixture contained 7 × 10^8 haem/ml except C1 which was at 4.5 × 10^8 haem/ml) in the presence and absence of RNase (1 mg/ml). All mixtures were adjusted to the same vol. with diluent and incubated at 37 °C for 1 h before infectivity assay. Six replicates were run on each treatment and the difference compared by Student's t test.

that the interaction of sensitized virus with fresh complement at 2 °C resulted in neutralization but no nucleic acid release, suggesting that virus lysis was not an essential step for neutralization. In the present experiment, when radioactively labelled, sensitized EAV was reacted with different combinations of components, it was found that the mixture CI, 4, 2, 3, though sufficient for neutralization, did not cause release of labelled nucleic acid (Table 1). Neither was any label released following addition of components C5 to C7. In contrast, some release (24 %) occurred following reaction with C8. The presence of C9 increased the release to about 57 %. Thus, lysis was initiated by C8 and enhanced by C9.

Effect of components C5 to C9 on enhancement of neutralization

We then investigated the question of whether activation of the last five components of complement led to lysis of the virus particle and contributed to virus neutralization. Since antibody and the first four components were capable, in high concentration, of neutralizing 100 % of the sensitized virus, the degree of neutralization was restricted by performing the experiment in the presence of excess antibody, optimal concentration of CI and limiting
concentrations of components of C4, 2 and 3. Under these conditions, the addition of C5 to C9 did not enhance the percentage of neutralization produced by the first four components. In contrast, when the antibody, rather than the first four complement components, was made limiting, the addition of components C5 to C9 increased the percentage neutralized from 56 to 80% (Table 2).

**DISCUSSION**

The complement system, either the entire sequence or portions thereof, has now been shown to play an important role in the neutralization of the infectivity of a number of enveloped viruses including herpes simplex (Yoshino & Taniguchi, 1965; Hampar et al. 1967; Daniels, et al. 1969), Newcastle disease virus (NDV) (Linscott & Levinson, 1969), infectious bronchitis virus (IBV) (Berry & Almeida, 1968), C-type RNA virus (Oroszlan & Gilden, 1970), Rubella virus (Rawls, Desmyter & Melnick, 1967), and EAV (Radwan & Burger, 1973a). Few studies have attempted to dissect the roles of the various components in the neutralization process. The first four components have been shown to be essential and adequate for the neutralization by IgM antibody of the infectivity of Newcastle disease virus (Linscott & Levinson, 1969) and herpes simplex virus (Yoshino & Taniguchi, 1965). The importance of the proper relative concentrations of the interacting components has also been shown (Daniels et al. 1969).

In the present investigation, the first four complement components were found to be sufficient for the neutralization of EAV sensitized by antibody. With excess antibody, an optimal concentration of C1 and a high concentration of C4 produced a maximum of 70% neutralization. The subsequent addition to the reaction mixtures of C2 and C3 progressively augmented the percentage neutralization up to 100%. The observation that high concentrations of C1 inhibited subsequent action by C4 is in agreement with previous observations on the system with herpes simplex virus (Daniels et al. 1970) and may be a result of the fluid phase destruction of C4 by activated C1 (Müller-Eberhard & Lepow, 1965). The linear relationship between the concentration of the sensitizing antibody and the percentage of neutralization produced by the mixture of the first four complement components was also observed with herpes simplex virus (Daniels et al. 1970).

Several possibilities have been suggested for the mechanism by which antibody, with the first four complement components, brings about loss of virus infectivity. One is that the mass of the bound molecules stearically hinders attachment, penetration or uncoating of the virus particle (Ashe & Notkins, 1966; Notkins et al. 1968; Daniels et al. 1969; Radwan et al. 1973). Another is that the binding of complement components renders more stable the attachment of antibody to antigenic determinants on the virus particle surface (Adler, Walker & Fishman, 1971). The observations that neutralization of the infectivity of this virus is similarly amplified by anti-\(\gamma\)-globulin (Radwan & Burger, 1973a) is compatible with either of these mechanisms. However, the fact that the antibody bound to the EAV virus particle in the absence of complement could not be dissociated by dilution or sonication (Radwan et al. 1973) indicates that the role of complement is not merely to serve a stabilizing function.

We attempted to examine in neutralization the interaction with complement of IgM isolated from early convalescent horse serum. Using specific anti-IgM serum, we found binding of IgM to EAV. However, very small amounts of specific antibody were present in the IgM class, as compared with that of IgG, such that we were unable to obtain a preparation of IgM with sufficient activity and purity to enable a study of complement requirement (T. B. Crawford, T. C. McGuire & A. I. Radwan, unpublished data).
The terminal components of the complement system (C5 to C9) should be able to cause structural lesions to virus previously complexed with antibody and the first four components of complement. The addition of C5 to C9 was reported to have no damaging effect on neutralized Newcastle disease virus and herpes simplex virus (Linscott & Levinson, 1969; Yoshino & Taniguchi, 1969; Daniels et al. 1970). In these studies, however, the structural integrity of the virus particle was not confirmed. Ultrastructural lesions have been demonstrated in the virus envelope following interaction of infectious bronchitis virus with unheated antiserum (Berry & Almeida, 1968). In addition, lysis of the virus particle with loss of the internal components and neutralization of infectivity were reported for both C-type RNA virus (Oroszlan & Gilden, 1970) and EAV (Radwan & Burger, 1973b; Radwan et al. 1973). The present study indicates that lysis of the virus particle was initiated by C8 and augmented by C9. Apparently the mechanism of virolysis by complement is similar to that of immune cytolysis, since both processes involve enzymic action on a lipoprotein membrane (Ingram & Quinn, 1972; Tamura, Shimada & Chang, 1972).

Our further analysis of the functional role of complement components C5 to C9 required that the system be manipulated such that the first four components produced less than complete neutralization. The percentage of virus neutralized was first restricted by limiting the concentrations of C4, 2, 3 to leave antibody and C1 in excess. The subsequent additions of C5 to C9 showed no further neutralization of infectivity. It may be that these conditions resulted in C1, 4, 2, 3 active sites which were less than optimally efficient in activating the terminal components. However, when the percentage neutralization in the presence of C1, 4, 2, 3 was restricted by limiting the antibody rather than the complement components, some further neutralization was demonstrated in the presence of C5 to C9.

The relative efficiency of the two different mechanisms of neutralization of virus infectivity bears on their relative importance as protective mechanisms. In the early component mechanism, attachment of many antibody-complement sites to the virus particle surface hinders, probably through physical interference, the early stages of infection. This results in no permanent damage to the virus particle as shown by the fact that most of the infectivity neutralized by addition of anti-immunoglobulin antiserum to sensitized virus could be recovered by trypsin treatment, whereas only a small amount of infectivity neutralized by antibody and whole guinea pig serum was recoverable (Radwan et al. 1973). Action of the last 5 components causes irreversible damage to the virus particle itself, liberating the nucleic acid or permitting access of nucleases to the virus genome through envelope lesions. It is not known how many membrane lesions are necessary to render a virus particle non-infectious. One lesion may be sufficient if the one-hit theory of immune haemolysis (Mayer, 1961) holds for viruses as it does for mammalian cells (Tamura et al. 1972). Further, one active antibody site of bound IgM or a duplet of IgG molecules can give rise to many membrane lesions through the amplification mechanism (Frank, Dourmashkin & Humphrey, 1970). Thus, when there is a scarcity of antibody molecules, the lytic mechanism is likely to be much more efficient than the mechanism involving the first four components. This mechanism would seem to be most important in maintenance of long-term immunity against those viruses which are susceptible to complement-mediated neutralization.

This work was supported in part by NIH grants AI 09237 and AI 07471 and the Veterinary Science Research Division, Agricultural Research Service, U.S. Department of Agriculture.
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(Received 4 December 1973)