COMPLEMENT COMPONENTS REQUIRED FOR VIRUS NEUTRALIZATION BY EARLY IMMUNOGLOBULIN ANTIBODY*

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Communicated by K. F. Meyer, June 30, 1969

Abstract.—Virus neutralization by early antisera usually requires or is enhanced by the presence of complement. The particular components of the complement system which are needed for neutralization of Newcastle disease virus by early IgM antibodies were studied. It was found in this system that participation of only the first four of the nine complement components (C1, C2, C3, and C4) was necessary and sufficient for neutralization to occur. It was concluded that the role of complement in neutralization was most likely that of contributing bulk in the form of large protein molecules to the virus-antibody complex.

Although the precise mechanisms of virus neutralization by antibody are not known with certainty, it has clearly been established that neutralization of many viruses and bacteriophages by low-affinity early antibodies requires participation of the complement system.[1-7] This does not necessarily mean, however, that all the nine known components of complement are essential for neutralization. In recent years it has been found that many complement-dependent phenomena require the participation of only a limited number of components. Precipitation of immune complexes,[8] immune adherence,[9] phagocytosis enhancement,[10] chemotaxis,[11, 12] and anaphylotoxin generation[13] are some of these phenomena. Cytocidal and cytolytic effects, on the other hand, require all nine complement components.[14, 15] Virus neutralization in the presence or absence of complement does not result in destruction of the virus, since infectious activity can be recovered at low pH, by dilution, or by the use of proteolytic enzymes.[2, 3, 16, 17] Thus it was felt that neutralization might require the participation of fewer than nine complement components. This paper presents evidence that neutralization of Newcastle disease virus by early IgM antibody requires only the first four components of the complement system.

Materials and Methods.—*Virus:* A stock of Newcastle disease virus, strain "L-Kan," in chorioallantoic fluid, was used throughout this study. This material had a titer of 2×10^8 plaque-forming units/ml, and was stored at $-65°$C.

Antibody: Initial experiments took advantage of the presence, in fresh normal serum, of "natural" antibodies capable of Newcastle disease virus neutralization. For later work, rabbits were immunized four times intravenously with 1×10^8 plaque-forming units of this virus per injection, and bled periodically. After determining that the 6-day bleedings contained adequate amounts of complement-requiring neutralizing antibodies, the globulins of an unpooled 6-day serum were precipitated by 50% saturated ammonium sulfate, redissolved, and chromatographed on a 2.5×90 cm column of Bio-gel P-200. The clearly separated first protein peak was pooled and used as the source of IgM antibodies for subsequent experiments. When necessary, part of this pool was concentrated by negative pressure dialysis, and heated for 30 min at 56°C before use. The second protein peak was also pooled, and used as a source of IgG antibodies.

Complement components: Functionally pure guinea pig C1 (first component of complement) was prepared as previously described.[18] This material contained about 4 ×

10^{12} functional molecules of Cl/ml, and no detectable amount of any other complement component. Partially purified guinea pig C2 was prepared according to the method of Borsos *et al.*[19] For critical experiments this C2 was further purified by adsorption to EAC4 (sheep erythrocytes sensitized by antibody and C4), followed by washing and then elution at low temperature and high ionic strength, a process which yields functionally pure C2.[20] Purified C3 was prepared from guinea pig serum by repeated cellulose chromatography, with a final step of electrofocusing, and contained no other complement components. C4 was prepared by Shimada's method,[21] and contained no other components of complement except for a trace of C7 at a 1:2 dilution (C4 titer = 1000). (We are deeply indebted to Dr. Kusuya Nis[illegible]a of the Japanese National Cancer Center Research Institute in Tokyo for these C3 [illegible] preparations.) Serum from rabbits genetically deficient in C6 was kindly supplied by [illegible] Carlos Biro of the Instituto Nacional de Cardiología in Mexico City, and by Dr. Klaus Rother, presently at the Max Planck Institute of Immunobiology in Freiburg. The cobra venom factor used to inactivate C3 was generously provided by Dr. Charles Cochrane, Scripps Clinic and Research Foundation, La Jolla, Calif.

Virus neutralization assay: The tissue culture techniques and the assay system for Newcastle disease virus have been described.[22]

Results.—Fresh normal serum contains complement and sufficient "natural" antibody to neutralize some viruses and [illegible]iophages.[1, 2] Pooled normal rabbit and guinea pig sera were tested, and both showed strong Newcastle disease virus neutralizing activity. Other experiments indicated that neutralization was essentially complete within 10 to 20 minutes at 37°C.

Susceptibility to inactivation at 56°C is a classic but somewhat nonspecific attribute of the complement system. Other more specific properties include the blocking of complement function by chelation of Ca^{++} and Mg^{++}, and the specific inactivation of the C3 component by a factor isolated from cobra venom.[23, 24] The effect of these treatments on Newcastle disease virus neutralization by fresh guinea pig serum is shown in Table 1. Neutralization was prevented by heat treatment, by chelation with ethylenediaminetetraacetate (EDTA), and by destruction of C3 by the cobra venom factor. That the venom factor itself had no inherent destructive effect on Newcastle disease virus is shown by the fact that if the guinea pig serum was heated before treatment with the venom factor, no significant reduction in titer occurred.

Certain rabbits have been shown to have a complete genetically determined

TABLE 1. *Effect on Newcastle disease virus neutralization of blocking complement action by heat, EDTA, or cobra venom factor.**

Test material	Newcastle disease virus titer $\times 10^{-3}$	Per cent reduction
Buffer control	33	...
Unheated guinea pig serum	2.5	92
Heated guinea pig serum (56°C for 30 min)	25	24
Unheated guinea pig serum + EDTA	27	18
Unheated guinea pig serum + cobra venom factor	28	15
Heated guinea pig serum + cobra venom factor	28	15

* A reaction mixture composed of 0.1 ml of Newcastle disease virus, 0.1 ml of treated or untreated serum, and 0.8 ml of Medium 199 was incubated at 37°C for 30 min, after which the titer of Newcastle disease virus was determined by plaque assay. Venom factor treatment consisted of incubating guinea pig serum containing 0.1 volume of venom factor for 30 min at 37°C before adding to Newcastle disease virus. This treatment was shown in a separate assay to have destroyed 97% of the C3 activity in the serum. Control serum contained 0.1 volume of buffer, and was incubated the same way.

deficiency of the sixth component of complement (C6).[25] If Newcastle disease virus were to be neutralized by the serum of such rabbits, it would indicate that those complement components reacting in sequence after C5 are not essential for neutralization. This was indeed found to be the case: 76 and 90 per cent neutralization were brought about by two different C6-deficient serum samples, and heat and EDTA were both shown to block this neutralization. The fact that neutralization was not quite as complete with the C6-deficient sera as with fresh normal rabbit serum was attributed to the fact that these sera had been stored frozen for several years.

Thus far it had been established that neutralization was prevented by heating the serum at 56°C, by chelation with EDTA, or by the destruction of C3 with venom factor, but not by the lack of C6. These results indicated that neutralization required the participation of C1, C2, C3, and C4, but not C6, C7, C8, or C9. The importance of C5 was not testable by these methods. In order to establish the complement component requirements definitively, it became essential to work with purified components and with antibody fractions. Accordingly, rabbits were immunized with Newcastle disease virus, and sera were taken after six days, two weeks, four weeks, and nine weeks. In order to determine which sera contained antibodies capable of neutralization in the *absence* of complement (since such antibodies could interfere with interpretation of the experiments which were planned), an aliquot of each antiserum was heated at 56°C, and then tested for Newcastle disease virus neutralization. The six-day sera were the only ones lacking complement-*independent* neutralizing activity.

Accordingly a six-day serum was fractionated by gel filtration, and the IgG and IgM fractions were tested for neutralizing activity. In order to do this, it was necessary to have a source of complement which was free of anti-Newcastle disease virus antibodies. This was obtained by diluting fresh normal guinea pig serum 1:10 with a suspension containing 2×10^7 plaque-forming units/ml of Newcastle disease virus. This mixture was incubated 30 minutes at 4°C, then centrifuged at 30,000 rpm for 2.5 hours. The supernatant fluid, which contained an insignificant amount of residual virus, was used as absorbed guinea pig serum 1:10, to test for neutralizing activity in the 6-day IgG and IgM antibody frac-

TABLE 2. *Newcastle disease virus neutralization by IgG and IgM antibodies separated from a 6-day rabbit antiserum.**

Test material	Newcastle disease virus titer $\times 10^{-5}$	Per cent reduction
Buffer control	39	...
Absorbed guinea pig serum 1:10†	38	3
Absorbed guinea pig serum 1:10 + IgG undiluted	11	72
Absorbed guinea pig serum 1:10 + IgG 1:10	30	23
Absorbed guinea pig serum 1:10 + IgM undiluted	5	87
Absorbed guinea pig serum 1:10 + IgM 1:10	12	69

* A reaction mixture composed of 0.1 ml of Newcastle disease virus, 0.1 ml of IgG or IgM, 0.1 ml of unheated absorbed guinea pig serum 1:10, and 0.7 ml of Medium 199 was incubated at 37°C for 30 min, after which the titer of Newcastle disease virus was determined by plaque assay.

† Guinea pig serum was absorbed by diluting it tenfold in a suspension containing 2×10^7 plaque-forming units of Newcastle disease virus/ml, incubating at 4°C for 30 min, and centrifuging for 2.5 hr at 30,000 rpm.

tions. Table 2 shows that activity was present in both fractions, but was somewhat higher in the IgM, and other experiments confirmed that this activity was completely complement-dependent. The IgM fraction was concentrated 5-fold, in order to increase its activity for future experiments.

Several preliminary experiments with purified complement components and the concentrated IgM antibody failed to produce more than 30–60 per cent inactivation, until it became apparent that a dilution step was necessary after reaction of Newcastle disease virus with antibody and C1, to prevent the enzymatic degradation by C1 of the second and fourth complement components.[26, 27] Accordingly, the following method was adopted: virus was placed in three master tubes X, Y, and Z. IgM six-day antibody was added to tubes X and Z, and all were incubated 15 minutes at 30°C followed by 15 minutes at 0°C. Then purified guinea pig C1 was added to tubes Y and Z, followed by incubation for 30 minutes at 20°C. The contents of the three master tubes were then diluted 100-fold in veronal buffer, and aliquots were placed in secondary tubes (X1, X2, etc). Different combinations of highly purified C4 and C2 were then added to the appropriate tubes, followed by a second 30-minute incubation at 20°C. After the addition of highly purified C3 to certain tubes, all were held for one hour at 20°C. Finally, all reaction mixtures were brought to the same volume (0.35 ml), diluted, and assayed for residual virus. The results are shown in Table 3. It can be seen that neutralization took place only in the mixture (Z6) containing antibody and each of the first four components of complement, and that this neutralization was prevented by EDTA (Z5). Thus Newcastle disease virus neutralization by early IgM antibody required the participation of each of the first four components of the complement system.

Discussion.—The requirement for heat-labile serum factors in virus neutralization by early antibodies was intensively investigated by Taniguchi and Yoshino. These workers, using herpes simplex virus and the "R-reagent" methods then available, concluded that C1, C2, "C3," and C4 were all essential for neutralization.[28] With the subsequent resolution of "C3" into six separate components of complement (C3, C5, C6, C7, C8, and C9),[14] this question was reopened. The present report shows that C1, C2, C3, and C4 are both necessary and sufficient for

TABLE 3. *Complement component requirements for Newcastle disease virus neutralization by early IgM antibody.*

Master tube	Newcastle disease virus	IgM	C1	Secondary tube	EDTA	C4	C2	C3	Duplicate plaque	Counts	Per cent reduction
X	+	+	−	X1	−	−	−	−	90	130	. . .
				X2	−	+	+	+	86	80	24
Y	+	−	+	Y1	−	−	−	−	140	150	. . .
				Y2	−	+	+	+	160	130	0
Z	+	+	+	Z1	−	−	−	−	100	105	. . .
				Z2	−	+	+	−	120	116	0
				Z3	−	+	−	+	110	118	0
				Z4	−	−	+	+	109	122	0
				Z5	+	+	+	+	116	126	0
				Z6	−	+	+	+	2	3	97.6

neutralization of Newcastle disease virus by early IgM antibody. There is no reason to suppose that the complement requirements for early IgG antibodies would be any different, at least in a qualitative sense, although IgG antibodies interact less efficiently with the complement system,[29] and so are more difficult to work with.

It might be argued that components of complement subsequent to the first four could exert further effects on virus. In an independent study, Yoshino and Taniguchi very recently reported preliminary findings similar to those presented here, and showed that addition of the five remaining complement components beyond C4 (C5 through C9) was without additional effect.[30] Thus virus neutralization by early antibody can be grouped with immune adherence,[9] phagocytosis enhancement,[10] IgM-mediated adhesion to macrophages[31] and one type of anaphylotoxin generation,[13] as a reaction which requires the participation of antibody and only the first four components of the complement system.

Complement has been found to increase both the neutralization "titer" of early antisera, and the rate constant.[7, 32] Thus it allows a given degree of neutralization with fewer antibody molecules, and increases the efficiency with which they interact with virus. Even with late IgG antibodies, where complement produces no increase in titer, it may still increase the *rate* of neutralization.[7, 32] There probably is a significant relationship between the fact that antibodies produced early in the immune response invariably are of lower binding affinity than late antibodies,[33, 34] and the observation that the complement dependence of neutralization decreases with time after immunization.[3, 4, 7, 32] Since C1 alone is capable of increasing the binding affinity between antigen and very nonavid antibodies,[35] it was thought early in the present study that C1 might be the only component needed for neutralization by early antibody.

Early antisera are likely to contain antibodies in smaller quantities, of lower binding affinities, and directed against a narrower range of viral antigenic determinants than late antisera. All of these things would tend to reduce the amount of antibody bound to virus, and antibodies of low binding affinity would in addition be expected to compete poorly with host cell receptors for binding sites on the viral surface. Such antibodies should be rendered more effective by antiglobulin antibodies which would tend to cross-link adjacent molecules of antivirus antibody and make their dissociation more difficult, as well as to add more bulk to the virus-antibody complex. This probably explains the fact that the complement requirement for neutralization by early antisera can often be substituted for by antiglobulin antibodies directed against antigenic determinants on the antivirus antibodies.[7, 36, 37] This effect should be particularly pronounced with easily dissociable univalent antibody fragments or with isolated antibody light or heavy chains, as has been reported.[17, 37-39]

In view of the considerable heterogeneity of virus and host-cell types, and the various mechanisms of penetration, the existence of multiple modes of neutralization by antibody might well be expected. Agglutination of T2 phage so that the tail fibers and end-plates are unable to attach to bacterial receptors,[40] or the coating of virions with sufficient antibody so that adsorption to the host cell is blocked,[16, 41, 42] obviously will cause neutralization. Often, however, antibody

concentration is insufficient to prevent adsorption, yet quite adequate for neutralization.[16, 41–43] In such cases it has usually been suggested that antibody neutralizes by preventing penetration, probably through steric effects.[16,17,36,37,43,44] Certainly it does not seem unlikely that antibody and antibody-complement complexes could attain sufficient bulk to interfere with the proper orientation between virus and host-cell receptors, and so to block penetration.

Investigators working with vaccinia and Newcastle disease virus have found that antibody can also neutralize without affecting either adsorption or penetration, by blocking uncoating and the release of infectious subviral particles into the host cell cytoplasm.[42, 45] Here the role of antibody is somewhat more difficult to define, but it may interfere with interaction between host enzymes or co-factors and viral substrates, or with the physical disaggregation of capsomeres and release of nucleic acid. In this regard the "bridging" effect of bivalent or multivalent antibody molecules could be of great importance; numerous investigators have found that univalent antibodies or antibody fragments are far less effective in neutralization than bivalent molecules.[16, 36–39, 44, 46] The neutralizing activity of such univalent fragments or individual peptide chains is markedly enhanced by antiglobulin antibodies.[36, 37] which would have the effect of bridging between such fragments and thereby restoring their ligating properties. Compatible with this would be the finding that virus is reactivated when the antibody complexed with it is enzymatically cleaved to univalent fragments, and that this reactivation is blocked by antiglobulin antibodies.[17]

The precise role of complement in the neutralization process is still unclear, but is probably related to the mechanisms of neutralization by noncomplement-requiring antibodies. We think that the complement system probably functions by contributing additional large protein molecules (150,000 mol wt or greater) to the virus-antibody complex. While only a single molecule of C1 is bound by one molecule of IgM or two closely-associated molecules of IgG antibody,[29] such an antibody-C1 complex can form numerous activated C4-C2 complexes, several of which may be capable of binding nearby. This has clearly been shown for certain red cell antigens,[47] but has not been investigated with viruses. Each cell-bound C4-C2 activated complex is a highly efficient enzymatic center which is capable of activating thousands of molecules of C3.[48] In the case of red cell antigens, many of these activated C3 molecules become bound to the cell surface within a short distance of the C4-C2 complex.[48] If such binding can also occur to viral surfaces, or to gamma globulins complexed with the virus, then it would be possible for a relatively small number of antibody molecules, interacting with the complement system, to cause a great accumulation of large protein molecules at the viral surface, which could interfere with adsorption, penetration, or uncoating depending upon the particular system involved and the relative amounts of antibody and complement fixed to the virus.

Finally, these studies are of interest because of the possible role of antibody and complement in the limitation of the spread of virus early during the course of infection. Antibodies produced during the first few days after infection are, by themselves, incapable of neutralization, but in conjunction with the complement system they may very well be an important adjunct to the protective effect

of interferon. Recovery from viral infections by hypogammaglobulinemic individuals is not considered strong evidence against this concept, since such patients do produce significant, though markedly reduced, amounts of antibody.

Addendum: In a very recent paper (*Science*, **165**, 508 (1969)), Daniels *et al.* reported that neutralization of herpes simplex virus by early IgM antibody occurs in the presence of C1, C4, C2, and C3. In addition, however, they found that if a sufficiently high concentration of C4 was used, then C2 and C3 were no longer required. Their conclusions as to the mechanism of neutralization were similar to those presented here.

The excellent technical assistance of Miss Jean Jackson was greatly appreciated.

* Supported by USPHS grants AI 06464 and CA 10223, and by USPHS training grant 5 TO1 AI 299.

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