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Neutralization of Sensitized Virus by Purified Components of Complement*,†

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Abstract. Herpes simplex virus which had been sensitized with immunoglobulin M antibody was neutralized by serum deficient in the fifth and sixth components of complement (C) but not by serum deficient in the fourth component C (C4). The sequential addition of the functionally purified components of C showed that the activated first component of C ($C\bar{1}$) failed to neutralize sensitized virus. However, in the presence of an optimal concentration of $C\bar{1}$, the addition of C4 resulted in neutralization. The amount of virus neutralized was dependent upon the concentration of immunoglobulin M used to sensitize the virus and the concentration of $C\bar{1}$ and C4. The addition of the second component of C (C2) to reaction mixtures containing an optimal concentration of $C\bar{1}$ and a limiting concentration of C4 resulted in increased neutralization and the amount of virus neutralized was dependent upon the concentration of C2. The addition of the third component of C (C3) to reaction mixtures containing an optimal concentration of $C\bar{1}$ and limiting concentrations of C4 and C2 also resulted in increased neutralization and the amount of virus neutralized was dependent upon the concentration of C3.

Under certain conditions, antisera against herpes simplex virus (HSV) produce little or no neutralization unless complement (C) is present.[1–4] Little is known, however, about the mechanism by which complement neutralizes virus. Recent studies on the mechanism of action of anti-γ-globulin on virus sensitized with antiviral antibody suggested that attachment of the anti-γ-globulin to the antiviral antibody on the sensitized virion resulted in neutralization by more extensive coverage of the surface of the virion than occurred with antiviral antibody alone.[4–6] This pointed to the possibility that the mechanism of neutralization of sensitized virus by C also might be due to increased coverage of the virion surface by the attachment of C. Extensive studies on the mechanism of action of C on erythrocytes sensitized with anticell antibody showed that there are at least nine components of C and that these components act in a definite sequence.[7] Furthermore, some of these components are capable of attaching to the anticell antibody and to the cell surface. Most of the components of C are now available in functionally pure form and this made it possible to study the individual

reaction steps of the C sequence as applied to virus neutralization. An earlier study showed that the first ($C\bar{1}$) and fourth (C4) components of C were necessary for neutralization of sensitized virus.[8] The present investigation was undertaken to study quantitatively the effect of the sequential addition of the functionally pure early components of C ($C\bar{1}$, C4, C2, and C3) on virus neutralization.

Materials and Methods. Virus: Herpes simplex virus, strain CHR-HSV-3, was grown in primary rabbit kidney cells and was assayed by its ability to form plaques of monolayers of primary rabbit kidney cells.[4] All dilutions were made in glucose-gelatin-barbital buffer containing Ca^{++} and Mg^{++} (GGBB).[9]

Antibody to virus: Anti-HSV was prepared by immunizing New Zealand white rabbits with HSV as described previously.[4] Serum was collected 9 days after immunization and immunoglobulin M (IgM) was separated by fractionating the serum on G-200 Sephadex.[4] All sera were heated at 56°C for 30 min.

Human serum containing antibody to HSV[8] was kindly supplied by Dr. Herbert S. Heineman, University of Pittsburgh School of Medicine.

Sensitization of virus: HSV was sensitized with anti-HSV as described earlier.[8, 10] Except where indicated, the IgM fraction of anti-HSV was used to sensitize the virus (HSV-IgM). Titration experiments showed that sensitization with the IgM fraction of undiluted rabbit anti-HSV or in a 1 in 2 dilution of human anti-HSV serum failed to produce significant neutralization (0–20%).

Complement: Whole guinea pig serum served as the source of C. Functionally pure guinea pig C components were prepared by Cordis Laboratories, Miami, Florida, based upon the methods of Nelson *et al.*[9] The titer of the C components was reported earlier[8] and was determined by "effective molecular titration,[11] based on the principles of the one-hit theory of immune hemolysis.

Sera deficient in complement: Mouse serum deficient in C5[12] was obtained from B 10. D2/Sn "old" male mice and normal mouse serum was obtained from B 10. D2/Sn "new" male mice. The C deficiency of the old mice was confirmed using the methods of Terry *et al.*[13]

Rabbit serum deficient in C6 was kindly supplied by Drs. Klaus and Ursula Rother.[14]

Guinea pig serum was made deficient in the fourth component of complement by treating fresh guinea pig serum with C4 inactivator kindly supplied by Dr. Joerg Jensen.[15] The treated serum contained less than 2 CH50/ml of C and less than 1.0×10^9 effective molecules/ml of C4. The matched control serum contained 500 CH50/ml of C and 1.4×10^{12} effective molecules/ml of C4.

Results. Neutralization of sensitized virus by sera deficient in components of complement: The first experiment was designed to see whether serum deficient in C6 was capable of neutralizing sensitized virus. The data in Table 1 show that both normal rabbit serum and serum deficient in C6 were capable of neutralizing sensitized virus. Neither of these sera, however, neutralized unsensitized virus.

The second experiment shows that serum deficient in C5 also was capable of neutralizing sensitized virus. Neither C5-deficient serum nor normal mouse serum had any effect on unsensitized virus.

The third experiment shows that serum made deficient in C4 failed to neutralize sensitized virus. Addition of purified C4, however, resulted in the neutralization of 88 per cent of the sensitized virus.

The above experiments indicated that the terminal components of C (C5 through C9) were not needed for neutralization and suggested that $C\bar{1}$ alone (contained in the C4 deficient serum) was not sufficient to produce neutraliza-

TABLE 1. *Neutralization of sensitized and unsensitized virus by sera deficient in components of complement.*

Sera tested	Virus Neutralized (%)	
	Sensitized	Unsensitized
Normal rabbit*	90	0
C6-deficient rabbit	60	0
C6-deficient rabbit plus C6	76	0
Normal mouse†	76	0
C5-deficient mouse	80	0
Normal guinea pig‡	84	0
C4-deficient guinea pig	0	0
C4-deficient guinea pig plus C4	88	0
GGBB	0	0

* Portions of 0.1 ml of HSV which had been sensitized with the IgM fraction of rabbit anti-HSV were mixed with 0.1 ml of C6-deficient rabbit serum or 0.1 ml of normal rabbit serum. Then 0.1 ml of either C6 or GGBB was added to each reaction mixture and incubated at 37°C for 1 hr.

† Portions of 0.1 ml of HSV which had been sensitized with human anti-HSV serum were incubated at 37°C for 1 hr with 0.2 ml of C5-deficient mouse serum or normal mouse serum.

‡ Portions of 0.2 ml of HSV which had been sensitized with the IgM fraction of rabbit anti-HSV were incubated for 30 min with equal volumes of undiluted C4 or GGBB. The reaction mixtures then were diluted with 2.0 ml of GGBB and 0.2 ml portions were removed and incubated for 30 min with equal volumes of a 1 in 10 dilution of C4-deficient serum, undiluted guinea pig C, or GGBB.

tion. These findings also suggested that neutralization of sensitized virus was dependent upon the presence of at least C4. To study the effect of C4 and the possible effect of C2 and C3 on the neutralization of sensitized virus, the following experiments were performed with functionally pure C components.

Neutralization of sensitized virus as a function of $C\bar{1}$ concentration: Preliminary experiments showed that individually the purified C components ($C\bar{1}$, C4, C2, and C3) were incapable of neutralizing sensitized or unsensitized virus. In combination, however, $C\bar{1}$ and C4 were capable of neutralizing sensitized virus but had no effect on unsensitized virus.[8] The effect of different concentrations of

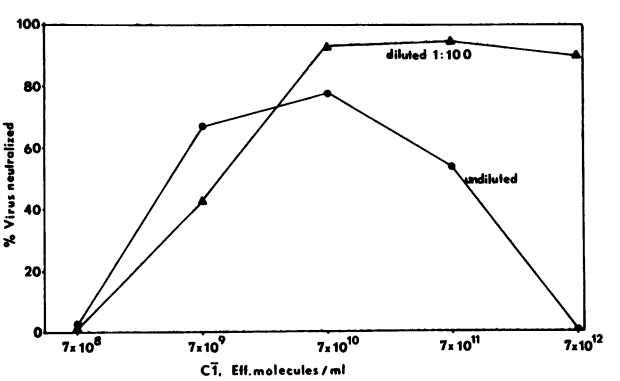

FIG. 1.—Effect of different concentrations of $C\bar{1}$ on the neutralization of sensitized virus by C4. Portions of 0.3 ml of HSV-IgM containing $10^{5.6}$ PFU/ml were incubated with an equal volume of different concentrations of $C\bar{1}$. Incubation of HSV-IgM with GGBB and whole guinea pig C served as controls. At the end of 40 min, 0.2 ml was removed from half of the reaction mixtures and incubated for 30 min with an equal volume of C4 containing 6.0×10^{11} effective molecules/ml. The other half of the reaction mixtures were diluted 1 in 100 in GGBB before incubation with C4. All incubations in this and subsequent experiments were performed at 30°C.

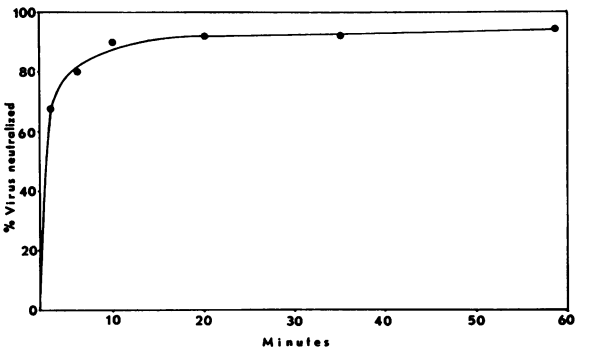

FIG. 2.—Kinetics of interaction of $C\bar{1}$ with sensitized virus. HSV-IgM was incubated with optimal $C\bar{1}$ (3.5×10^{10} effective molecules/ml) or GGBB. At timed intervals samples were removed and diluted 1 in 100 in C4 containing 6.0×10^{11} effective molecules/ml. These reaction mixtures were incubated for another 30 min and samples were assayed for surviving virus.

$C\bar{1}$ on the neutralization of sensitized virus by C4 is illustrated in Figure 1. The data indicate that the amount of neutralization produced by C4 was dependent upon the concentration of $C\bar{1}$. The presence of more than 7×10^{10} effective molecules/ml of $C\bar{1}$ in the reaction mixture inhibited neutralization by C4. If, however, the $C\bar{1}$ in the reaction mixture was diluted 1 in 100 prior to the addition of C4, neutralization was not inhibited. These findings support the hypothesis that excess $C\bar{1}$ in the fluid can destroy C4,[16] thereby rendering it ineffective for viral neutralization. In addition, these experiments suggest that $C\bar{1}$ binds to HSV-IgM and that this HSV-IgM-$C\bar{1}$ complex is relatively stable upon dilution. In all subsequent experiments HSV-IgM was incubated at 30°C for 40 minutes with optimal $C\bar{1}$ (3.5×10^{10} effective molecules/ml).

Kinetics of interaction of $C\bar{1}$ with sensitized virus: The data in Figure 2 show that incubation of HSV-IgM with optimal $C\bar{1}$ for one minute allowed C4 to neutralize 68 per cent of the virus and that incubation of HSV-IgM with $C\bar{1}$ for ten minutes resulted in neutralization of 90 per cent of the virus by C4. Incuba-

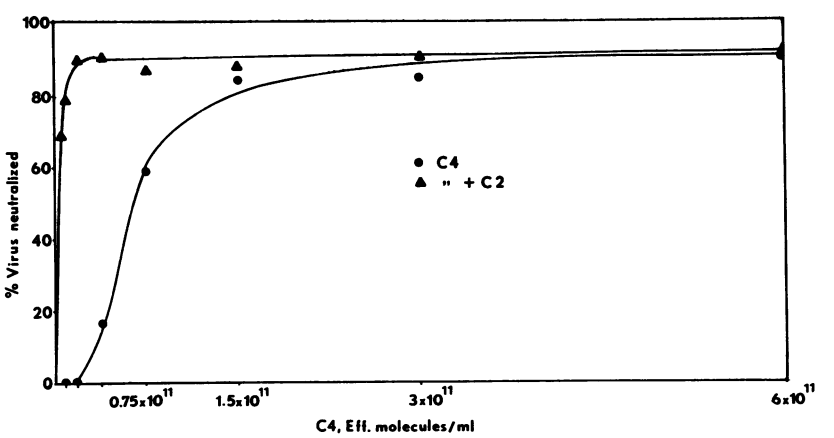

FIG. 3.—Neutralization of sensitized virus as a function of C4 concentration. HSV-IgM was incubated with optimal $C\bar{1}$ or GGBB. The reaction mixtures then were diluted 1 in 5 in GGBB and 0.4 ml samples were removed and incubated for 30 min with equal volumes of GGBB or serial twofold dilutions of C4. Then 0.2 ml was removed from each reaction mixture and incubated for 30 min with an equal volume of either GGBB or C2 containing 3.0×10^{11} effective molecules/ml.

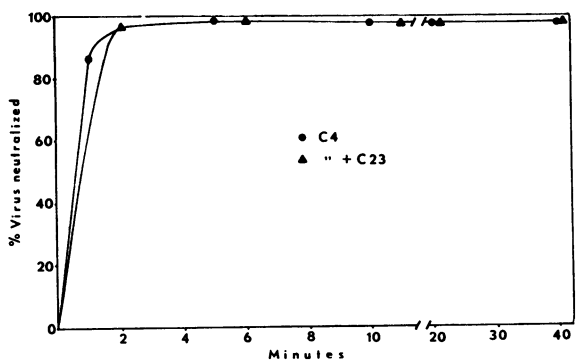

FIG. 4.—Kinetics of neutralization of sensitized virus by C4 in the presence and absence of C2 and C3. HSV-IgM was reacted with optimal $C\bar{1}$. Portions of 0.2 ml were removed and incubated with 0.2 ml of C4 containing 6.0×10^{11} effective molecules/ml. At timed intervals, 0.02 ml portions were removed and incubated for 30 min with 0.18 ml of either GGBB or a mixture containing 6.0×10^{11} effective molecules of C2/ml and 3.0×10^{11} effective molecules of C3/ml.

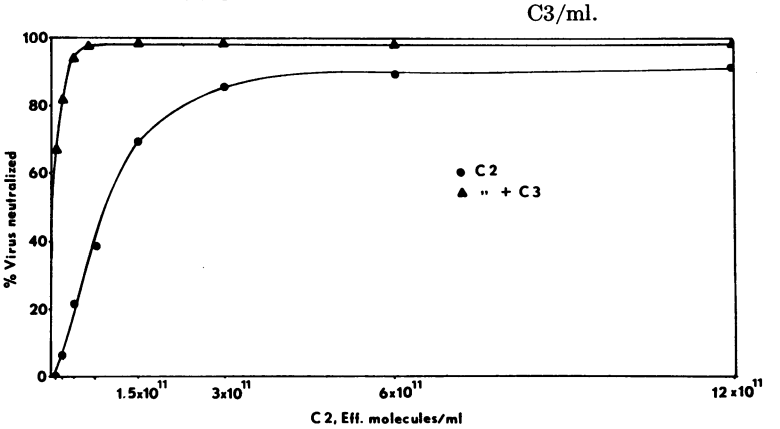

FIG. 5.—Neutralization of sensitized virus as a function of C2 concentration. HSV-IgM was reacted with optimal $C\bar{1}$. The reaction mixtures were diluted 1 in 5 and portions were removed and incubated for 30 min with equal volumes of either GGBB or C4 containing 0.37×10^{11} effective molecules/ml. Portions of 0.2 ml then were removed and mixed with equal volumes of different dilutions of C2 or GGBB. Then 0.2 ml of GGBB or C3 containing 3.0×10^{11} effective molecules/ml was added to the appropriate tubes and incubated for 30 min.

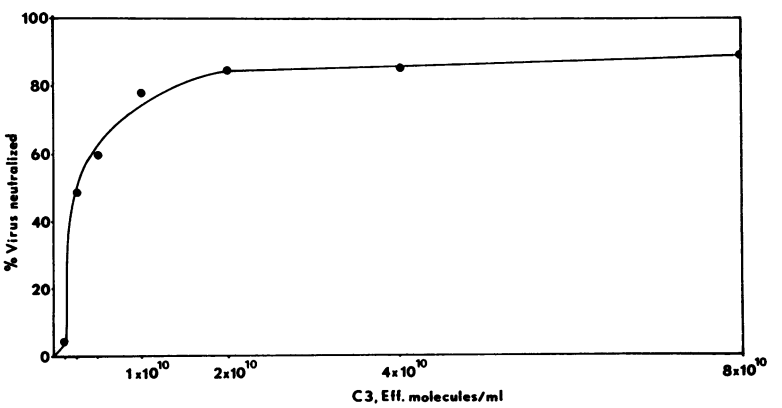

FIG. 6.—Neutralization of sensitized virus as a function of C3 concentration. HSV-IgM was reacted with optimal $C\bar{1}$. Portions of 0.5 ml were removed and mixed with 0.5 ml of C4 containing 0.37×10^{11} effective molecules/ml and 0.5 ml of C2 containing 6.0×10^{11} effective molecules/ml. The reaction mixtures were incubated for 30 min and diluted with 2.0 ml of GGBB. Portions of 0.2 ml were removed and incubated for an additional 30 min with qual volumes of serial twofold dilutions of C3 or GGBB.

tion for more than ten minutes did not result in any measurable increase in the amount of virus neutralized.

Neutralization of sensitized virus as a function of C4 concentration: Sensitized virus was reacted with an optimal concentration of $C\bar{1}$ and then incubated with different concentrations of C4 or C4 plus C2. The data in Figure 3 show that the amount of virus neutralized was dependent upon the concentration of C4. Furthermore, at low concentrations of C4, the addition of C2 resulted in increased neutralization. At high concentrations of C4, however, the addition of C2 did not increase neutralization. Controls showed that in the absence of either $C\bar{1}$ or C4, the addition of C2 did not produce neutralization.

Kinetics of neutralization of sensitized virus by C4: A concentration of C4 (6.0×10^{11} effective molecules/ml) which produced maximum neutralization (see Fig. 3) was used to study the kinetics of neutralization of sensitized virus by C4. The data in Figure 4 show that C4 neutralized over 87 per cent of the sensitized virus within one minute. Because of the rapidity of the reaction, further neutralization could not be demonstrated by the addition of C2 and C3.

Neutralization of sensitized virus as a function of C2 concentration: HSV-IgM was preincubated with an optimal concentration of $C\bar{1}$ and a concentration of C4 (0.37×10^{11} effective molecules/ml) which on the basis of the data from Figure 3 did not produce maximum neutralization. Figure 5 shows that the amount of virus neutralized was dependent upon the concentration of C2. Furthermore, at low concentrations of C2, the addition of C3 increased neutralization. At high concentration of C2, however, the addition of C3 did not significantly increase neutralization. Controls showed that in the absence of C2, the addition of C3 did not produce neutralization.

Neutralization of sensitized virus as a function of C3 concentration: To study the effect of different concentrations of C3 on the neutralization of sensitized virus, HSV-IgM was preincubated with $C\bar{1}$, C4, and C2. A concentration of C4 and C2 was used, which in this experiment did not produce maximum neutralization. As seen in Figure 6, the amount of virus neutralized was dependent upon the concentration of C3. Control experiments showed that virus which had not been exposed to C4 or C2 was not neutralized by C3.

Relationship between the concentration of antibody used to sensitize the virus and the amount of neutralization produced by whole guinea pig C and purified C components: The data in Figure 7 show that the amount of virus neutralized by whole guinea pig C or the C components was dependent upon the concentration of anti-HSV used to sensitize the virus. At low concentrations of anti-HSV, whole guinea pig C produced more neutralization than the C components. C2 and C3 did not increase the amount of neutralization produced by $C\bar{1}$ and C4 but a limiting concentration of C4 was not used in these experiments.

Discussion. The work of Berry and Almeida[17] with homotypic antisera suggested that heat-labile serum factors (presumably complement) were capable of attaching to and neutralizing avian infectious bronchitis virus without producing "holes" in the virion. Taniguchi and Yoshino[1] reported that all four of the "classical" components of C were required for neutralization of HSV; however, they used R reagents[11] rather than purified C components. Our work with sera

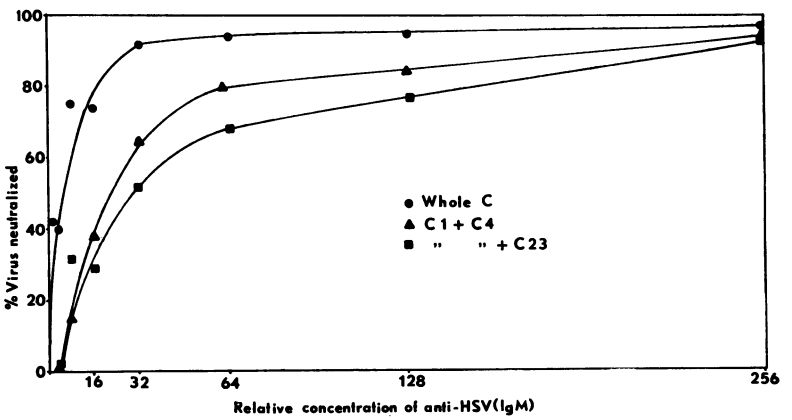

FIG. 7.—Relationship between the concentration of antibody used to sensitize the virus and the amount of neutralization produced by whole guinea pig C and purified C components. Virus was sensitized with serial twofold dilutions of the IgM fraction of rabbit anti-HSV. Each preparation of HSV-IgM was reacted with optimal $C\bar{1}$. Portions of 0.2 ml were removed and incubated for 30 min with equal volumes of C4 containing 6.0×10^{11} effective molecules/ml. The reaction mixtures were diluted with 0.4 ml of GGBB and 0.3-ml portions were removed from each reaction mixture and incubated for 30 min with 0.3 ml of either GGBB or a mixture containing 6.0×10^{11} effective molecules of C2/ml and 3.0×10^{11} effective molecules of C3/ml. HSV-IgM which had been put through the same incubation and dilution steps but not exposed to $C\bar{1}$, C4, C2, or C3 was incubated with undiluted whole guinea pig C.

deficient in C5 and C6 suggested that the terminal C components were not required in the neutralization of HSV. Experiments with the functionally pure C components showed that in the presence of an optimal concentration of $C\bar{1}$, sensitized virus was neutralized by C4 and that neutralization was increased by C2 and C3 when the concentration of C4 was limiting. Furthermore, our studies showed that C components reacted with sensitized virus in a definite sequence and that the order of this sequence was the same as that for the attachment of C components to sensitized erythrocytes.[7] In addition virus-antibody (VA) complexes can be produced which contain intermediate reaction products of the C sequence. The data suggest that virus-antibody complexes can exist in combination with $C\bar{1}$ as an infectious virus-antibody $C\bar{1}$ complex, in combination with C4 as an infectious virus antibody $C\bar{1}4$ complex, and in combination with C2 as an infectious virus antibody $C\bar{1}42$ complex.

The demonstration that the sequential addition of the functionally pure C components can neutralize sensitized virus supports the hypothesis that C produces neutralization by the piling up of components on the surface of the virion.[8] The ability of the early C components to neutralize sensitized virus represents a hitherto unrecognized biological function for these components. The fact that $C\bar{1}$ alone failed to neutralize sensitized virus suggested that not enough of this component became firmly attached to the virion so as to cover the surface of the virion. From the studies on sensitized erythrocytes it is known that for each bound molecule of $C\bar{1}$ more than one molecule of C4 can attach to the cell surface.[18, 19] A similar C4 amplification step may be involved in the neutralization of virus antibody $C\bar{1}$ by C4. Although the experiments presented here showed that

the terminal C components (C5 through C9) were not required for neutralization, they do not exclude the possibility that these components attach subsequent to neutralization by $C\bar{1}$, C4, C2, and C3. Whether the terminal C components can neutralize virus exposed to amounts of antiviral antibody and $C\bar{1}$, C4, C2, and C3 that fail to neutralize has not yet been determined.

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