CHARACTERISTICS OF A NON-COMPLEMENT-DEPENDENT C3-REACTIVE COMPLEX FORMED FROM FACTORS IN NEPHRITIC AND NORMAL SERUM*

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An immune origin of glomerulonephritis, with complement playing a major role, was first suspected 50 yr ago when it was found that serum complement levels in nephritis were often low. Only recently however, with the identification of individual components, have details of the complement reaction become susceptible to investigation. It is already apparent that there are differences in the way in which complement is involved in nephritis. For example, serum levels of individual components have been shown to be variably reduced depending upon the type of nephritis (1–3). Furthermore, deposition of C3 in the glomeruli occurs in many cases in which C3 and total complement levels have remained normal during the course of the disease (4–6). In other types of nephritis, however, the configuration of C3 deposition correlates well with the supposed target of the immune reaction (4, 5).

The participation of complement in producing the type of nephritis designated hypocomplementemic persistent or membranoproliferative is of special interest. In this disease CH_{50} and C3 may remain at very low levels over long periods (6, 7) and C3 is usually found in the glomerular capillary walls by immunofluorescent techniques, often without the presence of identifiable IgG.[1] The persistently reduced serum levels of C3 have been explained by some as the result of diminished synthesis of C3 (8) and ascribed by others to an ongoing complement reaction in the glomeruli in which C3 is continuously being broken down (9). Recent observations suggest that a site of complement breakdown may be circulating plasma. Inactivators of guinea pig (10) and human (11) complement have been found in the serum of patients with acute, chronic hypocomplementemic, and lupus nephritis, and in a previous communication from this laboratory, Spitzer et al. (12) reported the presence of a factor in the

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[1] Spitzer, R. E., A. J. McAdams, and C. D. West. Unpublished observations.

serum of a patient with hypocomplementemic persistent (membranoproliferative) glomerulonephritis which was involved specifically in the breakdown of serum C3. This factor, designated C3 nephritic factor (C3NeF), appeared to combine with a cofactor present in normal human serum in the presence of Mg^{++} to form a complex which rapidly cleaves C3. The cleavage of C3 resulted in the loss of antibody reactivity of the B antigenic determinant of C3 and the formation of two breakdown products, β1A and α2D (13). This inactivator complex was named the C3 lytic nephritic factor (C3LyNeF). Neither the nephritic factor nor the cofactor was identifiable with components of the complement system. Details of the studies reported previously in brief form (12)

TABLE I

Levels of B Antigenic Determinant, β1A, C4, and C5 in HGS*

	B antigen‡	β1A§	C4‖	C5‖
	units/ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
HGS	2.9	17.6	33.6	6.5
Normal serum	>20	>90	27–67	5.5–11

* Measured by the immunoelectrophoretic precipitin method (15).

‡ See text for details on method of measurement.

§ Measured after sample had aged at 37°C for 1 wk (7).

‖ Methods standardized to express results in milligrams by means of a standard serum kindly supplied by Dr. Peter F. Kohler.

as well as the results of additional work characterizing these factors and their reaction are reported herein.

Materials and Methods

Serum from a Hypocomplementemic Patient with Membrano Proliferative Glomerulonephritis (HGS) Containing Nephritic Factor.—The studies to be reported were performed on serum obtained at one time in July 1968, from a patient with severe hypocomplementemic membranoproliferative glomerulonephritis in renal failure. The clinical course of the patient (MF) has been described previously (14). The blood was obtained by exchange transfusion with packed red blood cells. After clot formation at 25°C, the serum was immediately separated by centrifugation at 4°C and cooled. 2 ml portions were stored at −70°C until used. The levels of the B antigen of C3, of C3 (β1A), C4 (β1E), and C5 (β1F) in this serum specimen are given in Table I.

Normal Human Serum (NHS).—Blood was obtained from healthy donors and the serum separated as described above. The serum was either used on the day obtained or stored at −70°C.

Antiserum to A, B, and D Determinants of C3.—Antiserum to C3 determinants was produced by injection of highly purified C3 incorporated in complete Freund's adjuvant into goats as described previously (13). Disodium ethylenediaminetetraacetic acid (EDTA) was frequently added to the saline-antigen solution before incorporation in the adjuvant. The method of absorption of the antiserum to remove antibody to other proteins without removing that to specific C3 determinants was described earlier (13).

Euglobulin and Pseudoglobulin Fractions.—Serum was fractionated into euglobulin and pseudoglobulin by dialysis against 8×10^{-3} M EDTA, pH 5.4, with a conductance of 1.25 millimhos per cm as described by Polley et al. (17). After centrifugation, the pseudoglobulin was dialyzed overnight against 0.2 M phosphate buffer, pH 7.5, containing 0.9% NaCl. The euglobulin was washed three times with the same buffer as used for the initial dialysis and, after centrifugation, reconstituted with 0.3 M phosphate buffer, pH 8.1, containing 1% NaCl, 2×10^{-4} M EDTA and 1×10^{-3} M NaF. The final volume was ½ to ¾ that of the original serum. The solution was then dialyzed overnight against the same buffer. For experiments in which EDTA or NaF would interfere with the reaction, these were removed by a final dialysis against 0.02 M phosphate buffer, pH 7.5.

Quantitation of the B Antigen.—The concentration of the B antigen of C3 was determined by addition of increasing volumes of the solution to be quantitated to constant volumes of standardized antiserum with detection of the end point by double diffusion of the supernatant against antigen as described previously (15). The C3 contained in normal human plasma, obtained by drawing blood into EDTA, was used as antigen. The plasma was stored at −70°C. For part of the study the end point was determined by reacting the supernatants against EDTA plasma electrophoresed in agar as described in the original method (9, 15). However, for some determinations the original method was modified to determine the end point by double diffusion of supernatant against EDTA plasma without electrophoresis, essentially as in the method described by Wright (18). When this modification was employed, the EDTA plasma used as a source of antigen was diluted 1:25, whereas the dilution was 1:10 when the EDTA plasma was electrophoresed in agar. The modification also required restandardization of the antiserum. Samples of a pool of human EDTA plasma stored at −70°C were used to standardize modifications of the method or new batches of antiserum. Every effort was made to keep the arbitrary units used to express B antigen concentration constant throughout the study and representative of the same amount of antigen as in previous communications from this laboratory (9, 12).

RESULTS

In this as in previous studies (9, 12) the concentration of the B antigenic determinant has been used as a measure of intact C3 in the mixtures of NHS and HGS and disappearance of the B antigen used to quantitate C3 breakdown. The loss of the B antigen and the formation of the breakdown products β1A and α2D during incubation of mixtures of HGS and NHS is illustrated in the immunoelectrophoretic pattern in Fig. 1.

Velocity of the Reaction.—The velocity of C3 breakdown was estimated by B antigen disappearance in a number of experiments in which mixtures of equal volumes of NHS and HGS were incubated at 37°C at pH 7.6 for various intervals. As illustrated in Fig. 2, the concentration of B antigen rapidly diminished with incubation and the B antigen remaining after 20 min was between 10 and 20% of that initially present. When the period of incubation was extended beyond 20 min, to as long as 120 min, virtually no further breakdown occurred. The time curve of B antigen reduction is similar to that of substrate disappearance seen with most enzyme systems.

Effect of pH.—The effect of pH was assessed in mixtures of equal volumes of NHS and HGS as in the above experiments. To vary the pH, NaOH, 0.5 N, and

HCl, 0.5 N, were added to NHS and HGS before mixing. Between pH 6 and 9, as shown in Table II, the extent of breakdown of C3 remains constant.

It was found that below pH 6, acidity itself effects the B antigen or its measurement. At pH 4.1, the B antigen concentration of NHS was less than half its value at pH 7.7. Diminished reactivity of C3 with antibody at low pH has been noted by other investigators (19, 20).

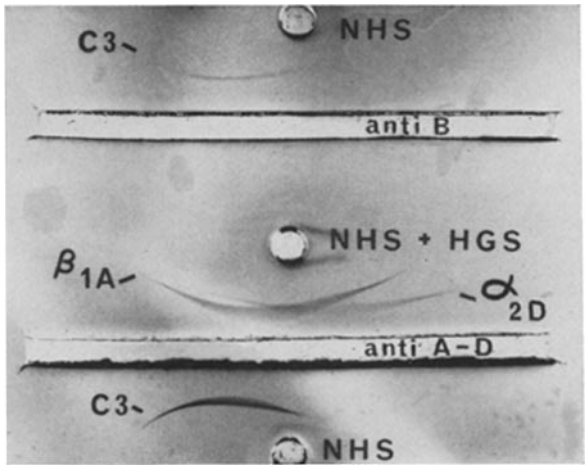

FIG. 1. Immunoelectrophoretic pattern of NHS and a mixture of NHS and HGS incubated at 37°C for 20 min. Top and bottom wells contain NHS which have undergone electrophoresis without incubation. In the middle well was placed the mixture of NHS and HGS after incubation. Antibody to the B determinant resulted in a typical C3 arc with NHS (top well) and no arc with the incubated mixture (middle well, top), indicating loss of the B determinant with incubation. Antibody to the A and D determinants reveals the presence of β1A and α2D in the incubated mixture (middle well, bottom), indicating breakdown of C3, and produces a typical C3 arc with NHS in the bottom well. Agar contained 0.01 M Na_2-EDTA. Anode is to the right.

Evidence for the Participation of a Cofactor.—Evidence that the reaction required a cofactor came from studies of pseudoglobulin and euglobulin fractions. Both fractions were dialyzed before use to rid them of EDTA and NaF. As seen in Table III, HGS caused rapid disappearance of the B antigen when added to NHS but had no effect on the B antigen when added to euglobulin made from NHS. For breakdown to occur, it was necessary to add NHS pseudoglobulin.

Further fractionation studies indicated that the active factor in HGS was also exclusively present in the pseudoglobulin fraction as shown in the last two experiments in Table III. We conclude from these observations (*a*) that a cofactor is present in normal serum which is necessary for the activation of

C3NeF found in HGS and (*b*) that both the cofactor and C3NeF are pseudoglobulins. Alone, C3NeF has no effect on C3 and it must be activated by reaction with cofactor, presumably to form a complex, the lytic nephritic factor designated C3LyNeF, before breakdown can be effected.

Evidence for Dependence on Mg^{++}.—When EDTA or NaF was added to NHS before mixing with HGS, breakdown of C3 was completely inhibited. The time of addition of EDTA and NaF was found to be important in the inhibition. As

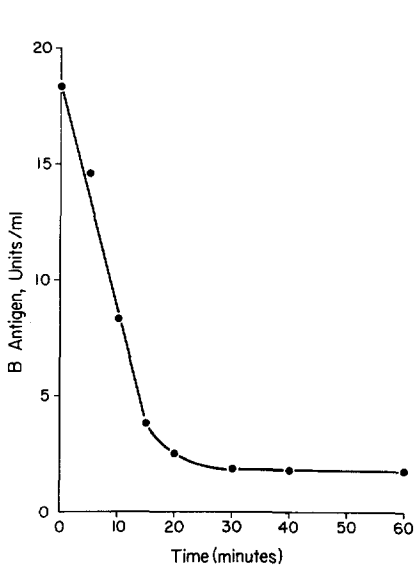

FIG. 2

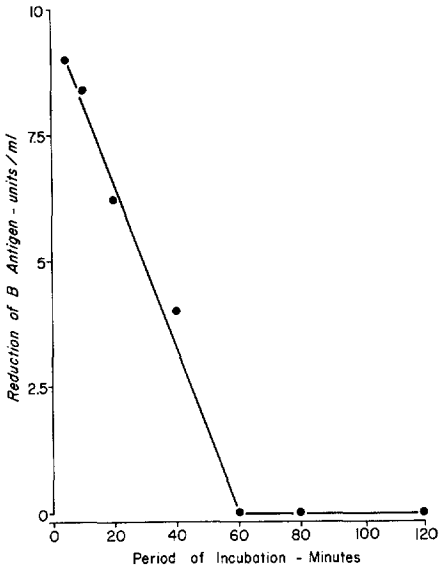

FIG. 3

FIG. 2. Rate of disappearance of B antigen in mixtures incubated at 37°C containing equal volumes of NHS and HGS.

FIG. 3. Effect of incubation on activity of C3LyNeF. Extent of breakdown of C3 produced by C3LyNeF which has been incubated at 37°C for periods varying from 5 to 120 min before reacting with C3. C3 breakdown occurred during a second incubation of 20 min duration.

illustrated in Table IV, when NHS and HGS were mixed in the presence of NaF, no breakdown occurred. However, when NaF was added after the serums were mixed, the reaction was not inhibited and the B antigen concentration diminished with further incubation at the same rate as in the control mixture in which NaCl was substituted for NaF. Other experiments showed that if only 15 sec elapsed before addition of NaF, breakdown was not significantly inhibited. These experiments are compatible with a two-step reaction as described above and indicate that the formation of C3LyNeF occurs rapidly and requires the presence of divalent cation.

To determine whether Ca^{++} or Mg^{++} was needed for the reaction, various

concentrations of each ion were added to reaction mixtures (Table V). Concentrations of Mg^{++} as low as $\frac{1}{6}$ that in serum allowed cleavage of C3 to occur; addition of Ca^{++}, even to concentrations four times that in serum, resulted in no C3 breakdown.

TABLE II

Effect of pH on Breakdown of C3 in Mixtures of Equal Volumes of NHS and HGS

pH	Initial B antigen concentration*	B antigen concentration after incubation at 37°C for 10 min
	units per ml	*units per ml*
9.0	22.7	5.3
7.5	23.1	5.0
6.0	22.0	5.0

* Mixture made at 0°C and immediately quantitated for B antigen.

TABLE III

Demonstration of Requirement for Cofactor

Mixture	B antigen after incubation for		Amount of C3 breakdown
	0 min*	20 min‡	
	units/ml	*units/ml*	%
NHS + HGS	23.8	3.2	87
NHS euglobulin + HGS	20.4	19.2	6
NHS euglobulin + HGS + NHS pseudoglobulin	12.9	2.2	83
NHS euglobulin + HGS pseudoglobulin + NHS pseudoglobulin§	16.1	3.8	77
NHS euglobulin + HGS euglobulin + NHS pseudoglobulin§	17.1	16.1	6

* Reactants mixed at 0°C and immediately quantitated.

‡ Incubation at 37°C.

§ Sufficient $MgCl_2$ added to produce final concentration of 1.25 mM/liter.

The reaction sequence can now be written as follows:

$$\text{C3NeF} + \text{cofactor} \xrightarrow{Mg^{++}} \text{C3LyNeF}$$

$$\text{C3LyNeF} + \text{C3} \longrightarrow \beta 1\text{A} + \alpha 2\text{D}$$

Evidence for a Non-Complement Origin of C3LyNeF.—Although the above experiments indicate that C3LyNeF resembles C3 convertase, $\overline{C4, 2}$ (21), in the conditions necessary for its formation and in its action on C3, there are a number of points of evidence indicating that convertase is not involved. Some of these were discussed in the previous publication (12). Further evidence is provided by an experiment designed to show that the formation of C3LyNeF could occur under conditions in which the formation of convertase was blocked. Serum

from a patient with hereditary angioneurotic edema (HANE serum) obtained during an episode of abdominal pain[2] was incubated for 3 hr at 37°C. As shown in Table VI, convertase could not be formed when the incubated HANE serum was the source of complement. Thus, when an immune precipitate was mixed

TABLE IV

Effect of Order of Addition of Reagents on C3 Breakdown by Nephritic Factor

Order of reagent addition*	B antigen after incubation at 37°C	
	0 min‡	2 hr
	units/ml	
NHS + NaF§ + HGS	15.5	15.0
NHS + HGS + NaF§	15.5	1.5
NHS + NaCl + HGS	15.5	1.6

* The final volume in all mixtures was constant.

‡ Reagents at 37°C when mixed; mixtures immediately reduced to 0°C.

§ Final concentration of NaF in reaction mixture was 7.5×10^{-3} M.

TABLE V

Effect of Various Concentrations of Mg^{++} and Ca^{++} on Formation of C3LyNeF

Ca^{++} or Mg^{++} concentration of pseudoglobulin mixture*	B antigen concentration after final incubation	Amount of C3 breakdown
mM/liter	*units/ml*	%
0	13.5	0
0.2 Mg^{++}	4.7	65
0.4 "	4.9	64
0.8 "	4.1	70
1.5 Ca^{++}	13.7	0
4.8 "	13.5	0
10.0 "	15.7	0

* Pseudoglobulin fractions from NHS and HGS from which Ca^{++} and Mg^{++} had been removed were mixed, Mg^{++} or Ca^{++} added as $MgCl_2$ or $CaCl_2$, 0.15 M NaCl added to bring all mixtures to a common volume, and the mixtures incubated at 37°C for 5 min. Euglobulin from NHS containing no Ca^{++} or Mg^{++} was then added and the mixtures incubated an additional 20 min at 37°C before measurement of B antigen.

with the HANE serum and with normal euglobulin as a source of C3, no breakdown of C3 occurred as compared with marked breakdown on addition of the precipitate to NHS. However, addition of either fresh or aged HGS to the incubated HANE serum resulted in marked breakdown of C3. Thus C3 breakdown by C3LyNeF is not dependent on the components or mechanisms responsible for convertase formation.

[2] HANE serum kindly supplied by Dr. Virginia Donaldson.

Effect of Storage and Heating on C3NeF, Cofactor, and C3LyNeF.—Incubation of HGS for 1 wk at 37°C had no effect on the activity of C3NeF. No diminution in activity was apparent when HGS so incubated was then stored at −20°C for as long as 17 months.

Prolonged storage of NHS at −70°C had no effect on the activity of cofactor. At 37°C, cofactor in NHS retained nearly full activity for 16 hr, but was completely destroyed after 24 hr. NHS lost 40% of its cofactor activity when stored at 4°C for 1 wk.

To determine the stability of C3LyNeF, normal human pseudoglobulin as a source of cofactor, and Mg^{++}, were added to HGS to form C3LyNeF and the mixtures incubated for varying periods at 37°C. NHS containing EDTA was

TABLE VI

C3LyNeF Activity in Mixtures Lacking Active C4 and C2

Reaction mixtures	B antigen after incubation at 37°C for		B antigen reduction
	0 min	20 min	
	units/ml		*units/ml*
Immune ppt. + HANE + NHS euglobulin	35.5	35.5	0
Immune ppt. + NHS	27.5	4.3	23.2
Fresh HGS + HANE + NHS euglobulin	35.8	19.0	16.8
Aged HGS‡ + HANE + NHS euglobulin	26.7	11.2	15.5

* Immune precipitate was formed at equivalence from bovine serum albumin (BSA)-goat anti-BSA.

‡ HGS incubated at 37°C for 1 wk.

then added to supply C3 and the mixtures again incubated at 37°C for 20 min. As seen in Fig. 3, there was a linear relationship between the disappearance of B antigen from the final reaction mixture and the time of incubation of C3LyNeF. C3LyNeF thus appears to be relatively unstable at 37°C with all activity lost in 60 min at this temperature.

Both C3NeF in HGS pseudoglobulin and cofactor in NHS pseudoglobulin were partially destroyed by heating at 56°C for 30 min and were completely destroyed after 1 hr at this temperature.

Effect of Temperature on the Reaction.—The temperature sensitivity of the total reaction was tested by mixing equal volumes of NHS and HGS at 0°C and then incubating the mixtures at other temperatures. The temperature sensitivity of each step of the two-step reaction was also determined.

The total reaction is markedly temperature sensitive. As seen in Table VII, there is virtually no breakdown of C3 in 90 min with incubation at 0°C and 25°C. At 32°C, breakdown occurs very slowly and is incomplete at 90 min as compared to the rapid and extensive breakdown at 37°C.

The temperature dependence of the first step of the reaction was tested by incubating mixtures of NHS pseudoglobulin and HGS pseudoglobulin in the presence of Mg^{++} at various temperatures to form C3LyNeF. After this incubation, NHS euglobulin containing EDTA was added as a source of C3 and the incubation continued at 37°C for 20 min. Table VIII shows that C3LyNeF

TABLE VII

*Effect of Temperature on Breakdown of C3 in Mixtures of Equal Volumes of NHS and HGS**

Temperature	Initial B antigen concentration	B antigen concentration after incubation	
		30 min	90 min.
°C	*units/ml*	*units/ml*	*units/ml*
0	16.9	16.9	16.9
25	17.2	14.2	14.2
32	16.9	14.4	8.0
37	17.1	2.2	1.3
37	16.9	2.9	1.4

* All mixtures were made at 0°C. For the initial B antigen quantitation, 0.075 M NaF was added and samples quantitated immediately after mixing.

TABLE VIII

*Effect of Temperature on C3LyNeF Formation**

Temperature	Initial B antigen concentration	B antigen concentration after second incubation	Amount of C3 breakdown
°C	*units/ml*	*units/ml*	%
4	19.2	19.2	0
25	22.7	22.2	3
32	22.7	19.2	15
37	19.2	13.1	32
37	22.7	16.0	30

* Mixtures of NHS pseudoglobulin and HGS pseudoglobulin containing Mg^{++}, made at 0°C, were incubated 5 min at the indicated temperatures to form C3LyNeF. After incubation, C3, as NHS euglobulin containing EDTA, was added and the mixtures incubated again at 37°C for 20 min.

formation was partially inhibited at 32°C and completely inhibited at 25°C and 0°C.[3] The results are similar to those for the total reaction shown in Table VII.

To test the temperature dependence of the breakdown of C3 by C3LyNeF, C3LyNeF was produced by incubating mixtures of NHS pseudoglobulin and

[3] The amount of C3 breakdown occurring in mixtures containing certain batches of pseudoglobulin and euglobulin was less than that seen in mixtures of NHS and HGS. Although the percentage breakdown with these batches was low, as in this experiment, the results were reproducible.

HGS pseudoglobulin at 37°C with Mg^{++}. After 5 min of incubation, NHS euglobulin containing EDTA was added and incubation continued at various temperatures as shown in Table IX. The results differed from those described above in that breakdown at 25°C and 32°C nearly equaled in magnitude that at 37°C.

It can be inferred from these results that the breakdown of C3 during the second step of the reaction is relatively insensitive to temperature and that formation of C3LyNeF is highly temperature sensitive, resulting in a high sensitivity of the total reaction.

Effect of Concentration of C3NeF and of C3 on the Rate of C3 Breakdown.— The effect of various C3NeF concentrations on the rate of C3 breakdown was assessed in a series of experiments in which the initial concentrations of C3 and cofactor were maintained constant and the amount of C3NeF, added as HGS,

TABLE IX

*Effect of Temperature on C3 Breakdown by C3LyNeF**

Temperature	Initial B antigen concentration	B antigen concentration after second incubation	Amount of C3 breakdown
°C	*units/ml*	*units/ml*	%
4	16.6	16.5	0
25	16.6	12.0	28
32	16.6	11.1	33
37	16.6	10.3	38

* Mixtures of NHS pseudoglobulin and HGS pseudoglobulin containing Mg^{++}, made at 0°C, were incubated at 37°C for 5 min. After incubation, C3, as NHS euglobulin containing EDTA, was added and the mixtures again incubated 20 min at the indicated temperatures

varied. The mixtures were brought to a common volume and incubated 20 min at 37°C. The results of three experiments in which the initial B antigen concentrations were 38, 19, and 9 units/ml are shown in Fig. 4 *a*, *b*, and *c*, respectively. In each experiment, C3NeF concentration in the reaction mixture was varied by including HGS in volumes of 0.033 to 0.1 ml. The final reaction mixture volume was kept constant at 0.4 ml. When the initial B antigen concentration was 38 units/ml (Fig. 4 *a*) the loss of the B antigen was linearly related to the concentration of C3NeF but at B antigen concentrations of 19 units/ml (Fig. 4 *b*) the curve levels off as C3NeF concentration is increased. Breakdown in mixtures containing 9 units/ml of B antigen (Fig. 4 *c*) was variable and was of approximately the same magnitude at all concentrations of C3NeF. At low C3NeF concentrations, the amount of breakdown tended to be about the same regardless of the initial B antigen concentration and in no experiment did the line relating C3NeF concentration to breakdown go through the origin.

In Fig. 5 are plotted the results of an experiment in which the concentration of C3NeF is kept constant at a high level and initial C3 concentration varied. At

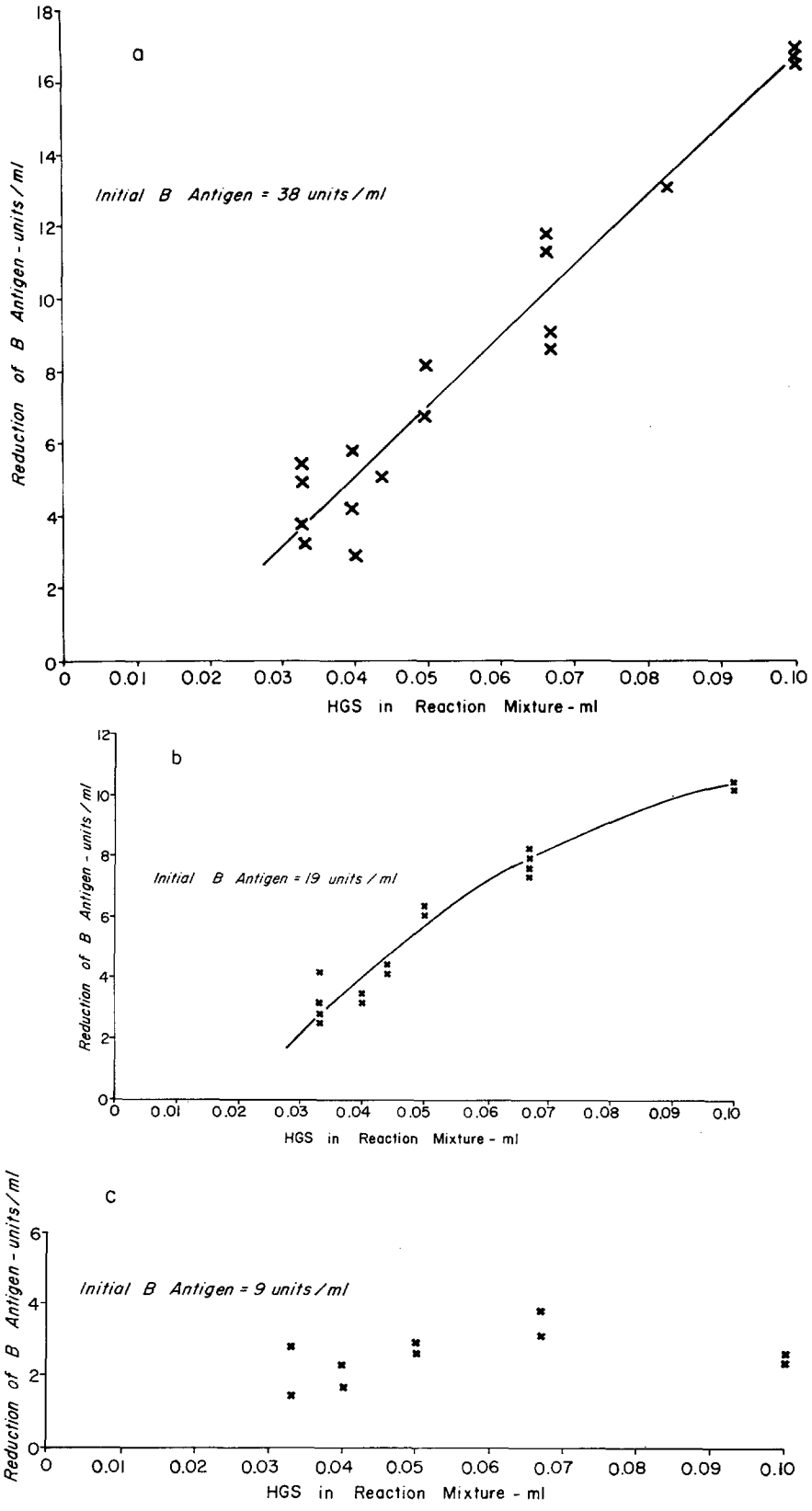
a
Initial B Antigen = 38 units / ml
Reduction of B Antigen - units / ml
HGS in Reaction Mixture - ml
b
Initial B Antigen = 19 units / ml
Reduction of B Antigen - units / ml
HGS in Reaction Mixture - ml
c
Initial B Antigen = 9 units / ml
Reduction of B Antigen - units / ml
HGS in Reaction Mixture - ml

FIG. 4 *a–c*

initial C3 concentrations giving B antigen levels between 24 and 40 units/ml, the rate of breakdown is constant, suggesting that C3:C3NeF ratios in this range are optimal and maximum velocity is obtained. As the initial B antigen concentration is lowered, C3 breakdown diminishes at a rate linearly related to initial C3 (B antigen) concentration.

The above data would suggest that the conditions for measuring C3NeF are optimum when the mixture has a B antigen concentration greater than 25

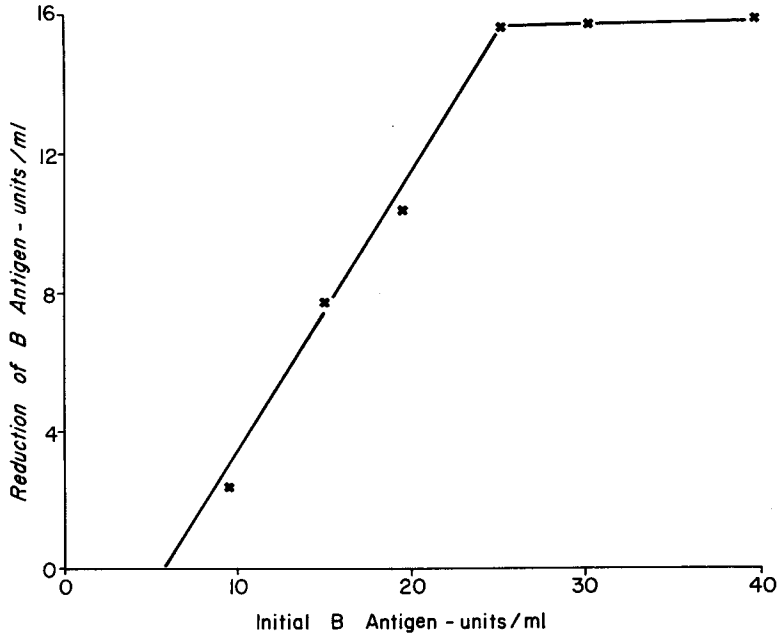

FIG. 5.—Breakdown of C3 versus initial B antigen (C3) concentration at constant concentrations of C3NeF. The reaction mixture consisted of 0.1 ml of HGS and 0.3 ml of NHS at various dilutions.

units/ml. At these high concentrations, the rate of breakdown is independent of the initial C3 concentration (Fig. 5), and changes in rate are linearly related to C3NeF levels (Fig. 4 *a*). These observations provide further evidence of the enzymatic character of the reaction.

Effect of Concentration of Cofactor on Rate of C3 Breakdown.—The effect of variable C3LyNeF concentrations, produced by varying the amount of cofactor in the mixture, was studied in mixtures containing constant amounts of HGS and Mg^{++} and with variable amounts of NHS as the source of cofactor. In

FIG. 4 *a, b, and c.* Breakdown of C3 versus amounts of C3NeF in reaction mixture when initial B antigen (C3) concentrations are 38, 19, and 9 units/ml, respectively. The concentration of cofactor in each series was optimum.

other experiments variable amounts of NHS pseudoglobulin were used as a source of cofactor. The mixtures were incubated at 37°C for 5 min to form C3LyNeF. Then, NHS euglobulin containing EDTA was added as a source of C3 and incubation continued. The amount of B antigen disappearing during the

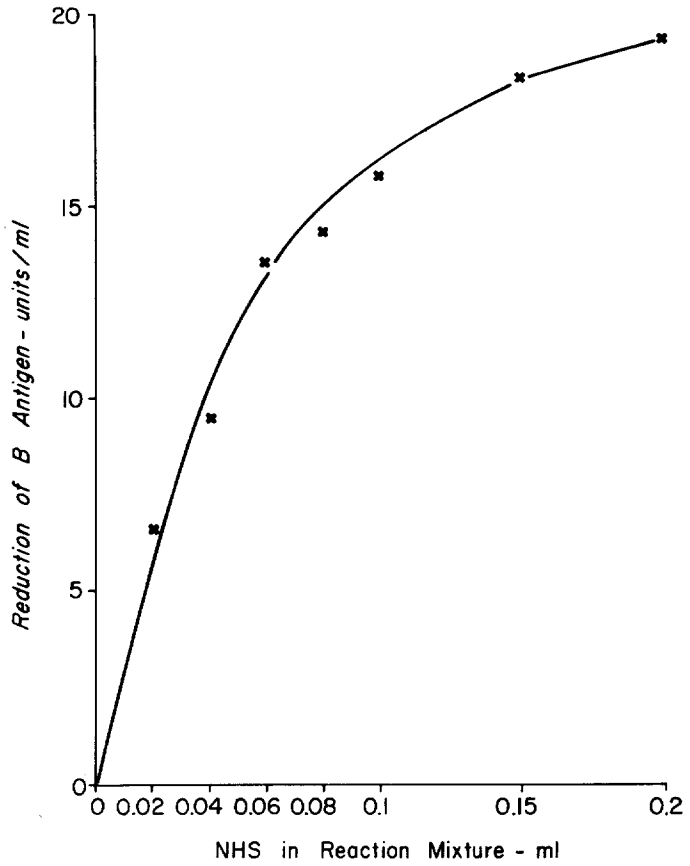

FIG. 6.—Effect of variable amounts of cofactor. Reaction mixtures of constant volume and HGS content but containing variable amounts of NHS as a source of cofactor (abscissa) were incubated. The C3 lytic activity of the C3LyNeF formed was tested by adding NHS euglobulin containing EDTA and continuing the incubation. The B antigen concentration in each tube at the start of the second incubation was always in the range in which the extent of C3 breakdown was independent of C3 concentration (Fig. 5).

second incubation in the experiment in which NHS was the source of cofactor is shown in Fig. 6. It is readily apparent that the reaction is very sensitive to C3LyNeF (cofactor) concentration. The line goes through the origin and as the C3LyNeF (cofactor) concentration increases, as expressed by the increasing

NHS in the mixtures, the amount of breakdown increases rapidly to level off as optimal concentrations are attained. The same relationship was observed when NHS pseudoglobulin was used as the source of cofactor.

DISCUSSION

The experiments presented in this paper are compatible with the presence of a preenzyme, designated C3NeF, in the serum of a hypocomplementemic patient with membrano-proliferative glomerulonephritis (HGS). Our studies reveal that in a mixture of fresh NHS and HGS, intact C3 rapidly disappears (Fig. 2) and two breakdown products are formed, both with a faster mobility than native C3 (Fig. 1). Failure to obtain breakdown when the euglobulin fraction of NHS is substituted for NHS as the source of C3 indicates that a cofactor present in the pseudoglobulin fraction of normal human serum is necessary for the formation of the active form of C3NeF, designated C3LyNeF. C3LyNeF appears to be an enzyme with C3 as substrate. The time course of C3 breakdown by C3LyNeF (Fig. 2), the temperature dependence of the reaction, and the dependence of C3NeF (Fig. 4 *a*, *b* ,and *c*), cofactor (Fig. 6), and C3 (Fig. 5) concentrations on rate of C3 breakdown are all consistent with an enzymatic reaction.

Inactivation of C3 by enzymatic cleavage and subsequent appearance of an inactive product with a faster electrophoretic mobility has been described in cell-free solutions (21). The inactivating enzyme in this study was a complement complex formed by C4 and C2, designated C3 convertase ($C,\overline{4, 2}$). This complex can also be bound to a cell surface and in this position produces both cytolytically active C3 and inactive C3 (22). The characteristics of formation and mechanism of action of C3LyNeF as described in the present paper are similar to those described for the formation and action of C3 convertase. However, several lines of evidence indicate that the mechanism of C3 breakdown does not depend on the presence of active C4 or C2 in the serum mixture or on the formation of convertase. (*a*) Serum from a patient with hereditary angioneurotic edema, incubated for 3 hr at 37°C, would not supply the first three reacting complement components (23, 24) necessary to produce C3 breakdown by an immune precipitate but was as effective as NHS in supplying cofactor. (*b*) Prolonged treatment of serum with ammonium sulfate, causing destruction of C4[4], does not affect its cofactor content. (*c*) Ancillary studies[5] have shown that cofactor does not lose its activity during its isolation by serum fractionation and column chromatography as is the case with C2 (17). (*d*) The half-life of convertase has been found to be 17 min (21), whereas half of C3LyNeF is inactivated in 30 min (Fig. 3). (*e*) The curve relating the activity of C3LyNeF (Fig. 3) to its time of incubation differs markedly from that for convertase (21). Thus,

[4] Davis, N. D. Unpublished observations.

[5] Vallota, E. H., N. C. Davis, J. Forristal, and C. D. West. Unpublished observations.

it seems highly improbable that C3LyNeF is dependent for its activity on any of the first three reacting components or on the formation of convertase.

The anticomplementary activity observed in this study can be differentiated from that classically described in the serum of patients with other chronic diseases, systemic lupus erythematosus particularly (25). Classical serum anticomplementary activity is usually ascribed to the presence of aggregated gammaglobulin and is not affected by heating the serum to 56°C for 1 hr. In fact, anticomplementary activity may be produced by this treatment (26). Similar heating of HGS, however, caused loss of C3NeF activity. Likewise, aggregated gammaglobulin in the NHS was ruled out by the fact that prolonged storage of NHS which would favor formation of aggregated gamma globulin (25) caused loss of cofactor activity. Thus, it is difficult to ascribe the breakdown of C3 to the presence of aggregated gamma globulin in the serum mixture. Further, aggregates as well as classical antigen-antibody or antibody-complement (27) complexes would breakdown C3 only through the participation of the first three reacting components of the complement sequence which, as has been shown above, need not be present in the reaction mixture.

The C3NeF inactivator system is not identifiable with the C3 inactivator (C3INA), nor with KAF (28) since both are heat stable and affect only cell-bound C3, whereas C3NeF is heat labile and affects fluid phase C3.

C3NeF and cofactor cannot be identified with factors liberated during clotting; heparinized plasma from patients with hypocomplementemic nephritis and from normal individuals have the same C3NeF and cofactor activity, respectively, as serum.

Recently, Pickering et al. (10) reported that the serum of hypocomplementemic patients with acute and chronic glomerulonephritis inhibited the hemolytic activity of guinea pig complement. The inhibition required the presence of divalent cation and was abolished by preheating the serum to 56°C for 30 min. It could be determined that the inhibition was directed at one or more of the six terminal complement components, since lysis not only of EA but also of EAC 1, 4, 2 was inhibited. To explain the inhibition, the authors postulated either (*a*) that large quantities of a normal or an abnormal inhibitor of complement activation was present in the nephritic serum or (*b*) that complement-inactivating complexes were either present in the nephritic serum or formed on mixing nephritic serum with guinea pig serum. The latter explanation was favored in view of the belief that the forms of nephritis studied were "complex" diseases.

The relation of this inactivator of guinea pig complement to C3LyNeF must remain conjectural. In preliminary studies in our laboratory it would appear that C3NeF is not present or present only in low concentration in the serum of patients with acute poststreptococcal nephritis. Since Pickering found high concentrations of inactivator in such serum it would appear that the phe-

nomenon he was observing was either dependent on a different mechanism or his method of measuring inactivator had a different sensitivity.

It should be noted that the results of the present study can be interpreted in a different way. One could speculate that an enzyme is normally present in serum which will cause rapid breakdown of C3 but is prevented from acting by being complexed to an inhibitor. In nephritis, an antagonist to the inhibitor could be generated which has the ability to activate the enzyme by reacting with the inhibitor. In this scheme C3NeF would be the antagonist, cofactor, the enzyme-inhibitor complex, and C3LyNeF, the activated enzyme. To comply with the data of the present paper, the enzyme and inhibitor could not exist separately in normal serum but would have to be present as a complex.

Rather than an enzyme-inhibitor complex and its antagonist, it seems more logical to explain the present data by combination of C3NeF with cofactor to form C3LyNeF because of the analogies of this system with that for the cobra venom factor. It was shown by Müller-Eberhard et al. (29) that when a fraction of cobra venom known as cobra venom factor (CoF) is added to normal serum in the presence of divalent cation, a substance with a sedimentation rate of 9S is formed which has the property of rapidly cleaving C3. CoF alone is inactive. Since CoF has a sedimentation rate of 7S and the precursor is 5S, it would appear that a complex formed of CoF and precursor is responsible for C3 lysis. Complex formation was confirmed by the observation that radio-labeled CoF was incorporated into the C3 inactivator. The several points of similarity between the cobra venom complex and the lytic nephritic factor makes it attractive to assume that they are analogous in the reaction sequence leading to their formation.

Recent studies (30) have indicated that the cobra venom complex not only inactivates but also activates guinea pig C3, producing lysis of sheep and guinea pig erythrocytes in the absence of antibody and in the absence of C1, C4, and C2. The many points of similarity of the two systems makes it possible that C3LyNeF could likewise activate the terminal components of complement and produce inflammatory reactions and lysis. Preliminary experiments in this laboratory have supported this assumption.

Assuming that the complement sequence can be activated by C3LyNeF starting with C3, the question arises as to whether C3LyNeF is responsible for the renal damage in nephritis. If C3LyNeF produced temporary activation of C3 and of the last five components at an appropriate receptor site in the renal glomerulus, it might be an important factor in the initiation and maintenance of glomerular inflammation. On the other hand, it is possible that C3NeF, like CoF (31), can prevent the inflammatory process initiated by antigen-antibody reactions in vivo by inactivating C3. Thus, by keeping C3 levels persistently low, C3NeF could act as a protective mechanism. As yet information is not available to allow a choice between these possibilities.

Whatever the role of C3NeF, it represents the first observation in man of a circulating, disease-associated, non-complement mechanism which causes inactivation of a specific complement component. It appears to represent the postulated analogue of the cobra factor in the mammal (29).

SUMMARY

When serum from a patient with membrano-proliferative glomerulonephritis and normal serum are mixed at 37°C, C3 is rapidly broken down to two more rapidly migrating components. In the mixture, a heat-labile pseudoglobulin, designated as the C3 nephritic factor or C3NeF, reacts with a pseudogolbulin in the normal serum, designated as cofactor, to form a C3 inactivator. By analogy with the cobra venom factor, the C3 inactivator is most likely a complex of the nephritic factor and cofactor. The complex has been designated as the C3 lytic nephritic factor or C3LyNeF. The reaction which results in the formation of C3LyNeF requires the presence of Mg^{++}, is highly temperature sensitive but occurs very rapidly at 37°C. In 20 min at 37°C, C3LyNeF can break down over 80% of the C3 in a mixture of normal and nephritic serum.

The two-step reaction which leads to C3 breakdown has an optimum pH ranging from 6.0 to 9.0. Experiments employing serum depleted of C4 and C2, as well as certain characteristics of the C3NeF system provide evidence that C3 breakdown with nephritic serum is not dependent on complement-inactivating immune complexes or on the action of convertase ($C\overline{4, 2}$). Data relating rate of C3 breakdown to the concentrations of C3NeF, C3, and C3LyNeF in the reaction mixture are similar to those for the reaction of enzyme with substrate. The biological significance of C3LyNeF in the production of glomerular inflammation has not been established.

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