A SERUM FACTOR IN CHRONIC HYPOCOMPLEMENTEMIC NEPHRITIS DISTINCT FROM IMMUNOGLOBULINS AND ACTIVATING THE ALTERNATE PATHWAY OF COMPLEMENT*, ‡

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A form of chronic nephritis characterized by mesangial proliferation and thickened capillary walls is often accompanied by low serum complement levels. Hypocomplementemia is primarily attributable to the low serum concentration of C3 (1, 2). The first three reacting components of the classical complement pathway, C1, C2, and C4, are usually within normal range (3).

We have previously postulated that the hypocomplementemia is caused by the C3 nephritic factor (C3NeF),[1] which may be found in the serum of these patients and which activates the complement system through an alternate pathway (4–6). In fact, it has recently been demonstrated that the action of C3NeF in vitro requires the C3 proactivator (C3PA) (7). Evidence indicating that activation of complement does occur in vivo includes the demonstration of C3d (α_{2D}) (8), an enzymatic cleavage product of C3, and the finding of low levels of properdin and C3PA in the serum of these patients (7, 9). Further, C3 metabolic studies have revealed an increased fractional catabolic rate, and in some patients also a decreased synthetic rate (10).

We now wish to report the method of isolation of C3NeF and the delineation of its mechanism of action. C3NeF isolated from the plasma of three different patients with hypocomplementemic chronic glomerulonephritis (HCG) was found to be distinct from immunoglobulins, and to effect C3 cleavage through recruitment of the entire C3 activator or properdin (P) system.

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[1] *Abbreviations used in this paper:* AI, anaphylatoxin inactivator; AT, anaphylatoxin; CM, carboxymethyl; CII, Cohn fraction II; C3NeF, C3 nephritic factor; C3PA, C3 proactivator; C3PAse, C3 proactivator convertase; GSHE, glutathione-treated human erythrocytes; HCG, hypocomplementemic chronic glomerulonephritis; IF, initiating factor; NHS, normal human serum; P, properdin.

Materials and Methods

Serum and Plasma.—Serum and plasma were obtained from three patients with HCG at the Children's Hospital Research Foundation, Cincinnati, Ohio.

Method of Isolation of C3NeF. Triethylaminoethyl (TEAE)-cellulose chromatography: 25 ml of serum were dialyzed against 0.02 M phosphate buffer, pH 7.0, overnight. The serum was applied to a 4 × 18-cm column containing TEAE-cellulose which had been equilibrated with the same buffer. The C3-converting activity was found in fractions corresponding to the first protein peak. This material was concentrated by ultrafiltration to approximately 2 ml in an Amicon concentration device using a XM 50 ultrafilter (Amicon Corp., Lexington, Mass.). At this stage a radiolabel was introduced in order to obtain protein elution profiles after subsequent separation procedures. A portion of the material was labeled with ^{125}I by the chloramine-T method according to McConahey and Dixon (11). The specific radioactivity was approximately 3.236 cpm/μg of protein.

Carboxymethyl (CM) Sephadex C50 chromatography: The active pool from the TEAE-cellulose column containing 30 mg of protein was dialyzed against 0.02 M phosphate buffer, pH 6.0, and then applied to a 1.7 × 20 cm CM Sephadex C50 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with the same buffer. The C3-converting activity was found in the early effluent, and the fractions were pooled and concentrated. The adsorbed protein was eluted with a NaCl concentration gradient from a conductance of 15.0–30 mmho/cm.

Molecular sieve chromatography using Sephadex G-200: The active pool from the CM Sephadex step was concentrated to 1 ml containing 13 mg protein and applied to a 1 × 30 cm Sephadex G-200 column (Pharmacia Fine Chemicals, Inc.) equilibrated with phosphate buffer, pH 7.5, ionic strength 0.2. The active material was eluted together with 7S γ-globulin.

Immune adsorption of immunoglobulin: 10 ml of polyvalent goat antiserum to γ-globulin was mixed with 10 ml of 0.2 M acetate buffer, pH 5.2, and the protein was insolubilized by addition of 1.6 ml of ethyl chloroformate (12). After thorough washing the solid adsorbent was suspended in 2 ml of the active C3NeF pool from the gel filtration step, containing 0.05 M EDTA. The suspension was kept overnight at 4°C and then subjected to centrifugation at 20,000 rpm for 1 h in a no. 40 rotor and a Spinco L2 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The recovered supernatent was dialyzed against 2 × 10 liters of phosphate buffer, pH 7.0, ionic strength 0.05.

Elution of adsorbed γ-globulin: The immune adsorbent was washed with 2 × 50-ml phosphate buffer, pH 7.0, ionic strength 0.05, and washed once with 50 ml of 1 M NaCl. The adsorbed protein was then eluted with 3 × 10 ml of 4 M potassium iodide in 0.01 M Tris buffer, pH 8.2, and 0.1% bovine serum albumin (13). The eluted protein was concentrated in an Amicon concentration device using a XM 50 ultrafilter (Amicon Corp.).

C3NeF Assay.—Presence of C3NeF in serum or serum fractions was ascertained by three different criteria: (*a*) Detection of C3 and C3PA conversion by immunoelectrophoresis in mixtures of 25 μl of the C3NeF-containing sample with 100 μl of normal human serum (NHS) incubated at 37°C for 30 min; (*b*) measurement of whole human complement hemolytic activity present in the same mixtures (14); and (*c*) the ability of C3NeF to initiate lysis of glutathione-treated human erythrocytes (GSHE) in presence of normal serum according to a method to be described.[2] To prevent C3 cleavage due to contaminating endotoxin or γ-globulin aggregates, the C3NeF samples were subjected to ultracentrifugation for 1 h at 49,000 rpm before the assay.

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[2] Arroyave, C. M., E. H. Vallota, and H. J. Müller-Eberhard. Hemolysis due to activation of the alternate complement pathway by C3 nephritic factor (C3NeF). *J. Immunol.* Manuscript submitted for publication.

Immunochemical Analysis.—Immunoelectrophoresis was performed using 2% agar in barbital buffer, pH 8.6, with 0.02 M EDTA. For the analysis of the C3NeF preparations by the double diffusion-in-gel method, rabbit antisera to IgG3, Fc, kappa, lambda, and C3NeF were used.

Zone Electrophoresis.—1.5 ml of plasma from a patient with chronic nephritis and hypocomplementemia were separated by electrophoresis on Pevikon blocks in barbital buffer, pH 8.6, ionic strength 0.05, for 16 h at 4°C and 3.5 V/cm. Microzone electrophoresis was performed on cellulose acetate strips in a Beckman microzone electrophoresis apparatus, Model R101, using Beckman barbital buffer B-2 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), pH 8.5, ionic strength 0.075 and 250 V for 20 min at 20°C.

Sucrose Density Gradient Ultracentrifugation.—Linear sucrose density gradients (7-31%) were produced with the Buchler automatic density gradient device (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.). Ultracentrifugation was performed for 17 h at 35,000 rpm. and 4°C in a Spinco L-2 machine employing an SW 39 rotor (Beckman Instruments, Inc., Spinco Div.). Fractions were obtained using a Buchler gradient fractionation device (Buchler Instruments Div., Nuclear-Chicago Corp.). For *s*-rate determinations C1q (11S) and bovine serum albumin (4.4S) were used as reference substances.

Sera Deficient in Various Serum Proteins.—C2-deficient serum was kindly supplied by Dr. Vincent Agnello, The Rockefeller University, New York. Serum reagents deficient in P, C3PA or C3 proactivator convertase (C3PAse) were prepared according to Götze and Müller-Eberhard (15). Nephritic serum was rendered deficient in C3NeF using a solidified antiserum to C3NeF. The same immune adsorbent was used for treatment of NHS.

Generation and Measurement of Anaphylatoxins (AT).—48 μg of a C3NeF preparation was incubated at 37°C for 30 min with NHS depleted of the anaphylatoxin inactivator (AI) by immune adsorption (16). C3a and C5a activity was demonstrated by the guinea pig ileum test as described (17).

RESULTS

Purification of C3NeF.—Serum from patients with HCG having high C3NeF activity was used. The starting material (25 ml) was chromatographed on a TEAE-cellulose column using 0.02 M phosphate buffer, pH 7.0 (Fig. 1). The C3-converting activity was found in the early column fractions together with

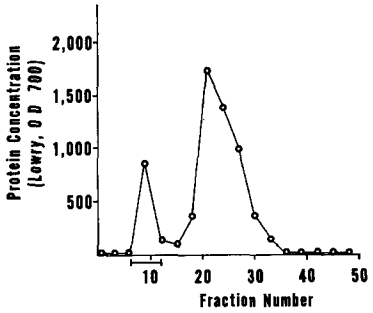

FIG. 1. First step of isolation procedure of C3NeF. TEAE-cellulose chromatography of serum from a patient with HCG using 0.02 M phosphate buffer, pH 7.0. Only the elution profile of the nonadsorbed protein is shown. The bar indicates the pool of C3NeF activity containing fractions.

IgG, C1q, and P. The protein content of these fractions was 1.8% of the starting material. The active material was further purified by chromatography on a CM Sephadex C50 column (Fig. 2). The C3-converting activity was eluted together with IgG, but preceding elution of C1q and of P. The next step consisted of molecular sieve chromatography as shown in Fig. 3. Again, the activity was eluted together with IgG which accounted for 75% of the protein contained in the active fractions. In order to ascertain as to whether the C3-cleaving activity was a property of IgG, the pool was treated with insolubilized polyvalent antihuman immunoglobulin. After immune adsorption the active material remained in the supernate. The immunoglobulins eluted from the adsorbent with 4 M KI were inactive in the C3NeF assay. The yield, purification, and specific activity are listed in Table I. The absolute increase in C3NeF activity during the first fractionation procedure may be due to an inhibiting effect of unknown nature in whole serum. The degree of purification was approximately

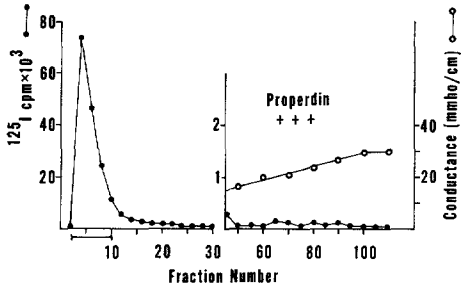

FIG. 2. Second step of isolation of C3NeF. CM Sephadex chromatography of C3NeF containing pool of the first chromatographic step (Fig. 1). The C3NeF activity was eluted with 0.02 M phosphate buffer, pH 6 (left panel). Bar indicates C3NeF containing fractions whice were pooled for further separation. The right panel shows elution of the adsorbed protein, including P, by sodium chloride concentration gradient. P was detected immunochemically.

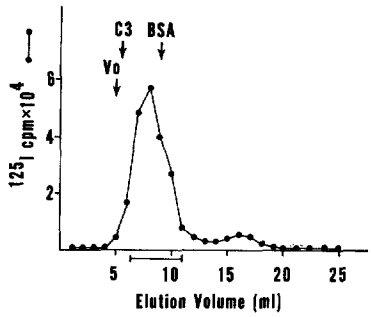

FIG. 3. Third step of isolation of C3NeF. Molecular sieve chromatography on Sephadex G-200 of C3NeF containing pool from the second chromatographic step (Fig. 2). Phosphate buffer, pH 7.5, ionic strength 0.2, was used.

1,000-fold, and the final yield 15–25%. Assuming that 90% of the isolated protein represents C3NeF, its concentration in the original serum may be calculated to be 55–95 μg/ml.

Immunochemical and Physicochemical Characterization of C3NeF.—The isolated active material before and after the immune adsorption step was subjected to double diffusion-in-gel analysis using antiwhole human serum and monospecific antisera to IgA, IgM, IgG3, the Fc and $F(ab)'_2$ fragments, and kappa and lambda chains. As shown in Fig. 4, precipitin lines formed between anti-IgG3, -Fc, -kappa, and -lambda only with the material before immune adsorption (st) and with the reference proteins, Cohn fraction II (CII) (Mann Research Laboratories Inc., New York), IgG3, or IgG2. The active material after immune adsorption failed to develop a precipitin line with these antisera and with antisera to IgA, IgM, and $F(ab)'_2$. Two of three antisera to whole

TABLE I

Purification of C3NeF from Serum

Material	Volume	Protein	Activity	Purification
	ml	*mg*	*U**	*U/mg*
Serum	25	1,625	250	0.15
TEAE pool	2	30	416	13.86
CM pool	1	13	371	28.54
G200 pool	1	1.6	83	51.86
After IA	0.5	0.4	62.5	156.25
Final Purification:				1,041 ×
Yield:			15–25%	

* 1 U, C3NeF activity required for 100% inactivation of hemolytic activity in 100 μl NHS in 30 min at 37°C.

human serum (Behringwerke AG, Marburg, West Germany) gave a negative precipitin reaction under the conditions used. The third antiserum, made in this laboratory, permitted detection of one or more weak precipitin lines.

The nonidentity of C3NeF with IgG was further demonstrated by Ouchterlony analysis with an antiserum raised with one of the preparations of C3NeF. When a preparation of C3NeF containing IgG was analyzed with anti-C3NeF and anti-IgG the resulting precipitin lines clearly crossed (Fig. 5). Antiserum produced to C3NeF of one patient also reacted with C3NeF of the other two patients.

Isolated C3NeF behaved as γ-globulin on cellulose acetate electrophoresis (Fig. 6). When whole nephritic serum was subjected to Pevikon block electrophoresis, C3NeF activity was also detected in the γ-globulin fraction. Both C3NeF activity and the antigen detected by anti-C3NeF had similar distributions (Fig. 7).

Dose-Response of C3NeF Activity.—C3NeF activity was measured by determining the degree of reduction of hemolytic activity in NHS after incubation with C3NeF. When increasing quantities of isolated C3NeF were incubated with a constant volume of serum, a linear dose response was obtained. Fig. 8 shows the plot of micrograms of C3NeF/100 μl of NHS vs. the percent of residual hemolytic activity in the NHS sample.

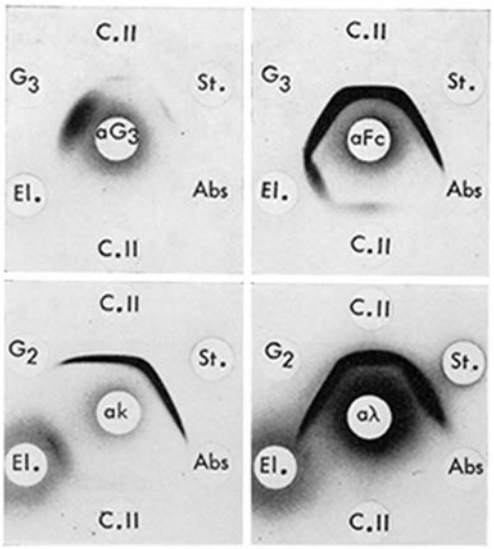

FIG. 4. Immunochemical analysis of C3NeF before (St.) and after (Abs.) absorption with solidified antihuman immunoglobulin. The center well contains antiserum to IgG3 (aG3), Fc fragment (Fc), kappa chain (ak), and lambda chain (aλ). Cohn fraction II (C.II) was used in two different concentrations, 1 mg/ml and 0.1 mg/ml, respectively. Protein eluted from the immune adsorbent with 4 M KI:EL.

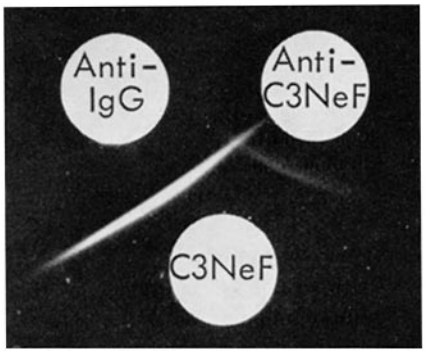

FIG. 5. Analysis of crude C3NeF with anti-IgG and an antiserum prepared with highly purified C3NeF. The antigen recognized by anti-C3NeF is distinct from the IgG contaminant.

Mechanism of Complement Inactivation by C3NeF.—Addition of isolated C3NeF to genetically C2-deficient serum which had an unimpaired function of the alternate pathway resulted in efficient cleavage of C3 and C3PA. The immunoelectrophoretic patterns shown in Fig. 9 are representative for normal

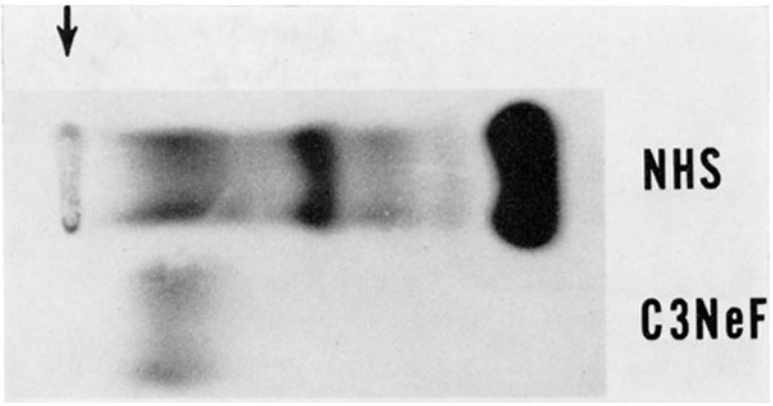

FIG. 6. Electrophoretic behavior of isolated C3NeF after immune adsorption on cellulose acetate at pH 8.5. For comparison, the pattern of whole human serum is shown.

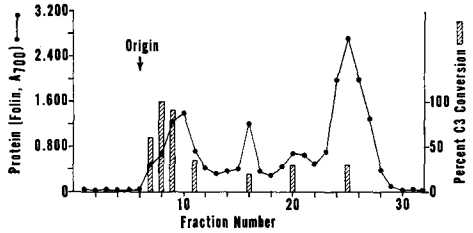

FIG. 7. Localization of C3NeF in fractions of whole human serum after zone electrophoresis on a Pevikon block at pH 8.6.

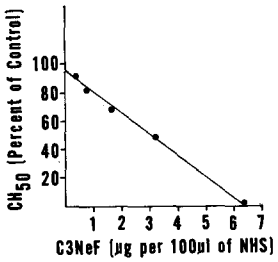

FIG. 8. Quantitative relation between the amount of isolated C3NeF added to NHS and the resultant reduction in hemolytic activity. 6.4 μg of C3NeF caused 100% loss of the activity of 100 μl serum.

and C2-deficient serum. The results indicate that C3NeF action of C3 does not require C2 but involves C3PA. Since C3PA was affected by C3NeF in normal and in C2-deficient serum, we tested whether it forms a complex with a normal serum protein such as C3PA in analogy to cobra venom factor. ^{125}I-radiolabeled C3NeF, before immune adsorption, was added to NHS and the mixture incubated for 5 min at 37°C. Marker substances for *s*-rate determinations were added and the mixtures subjected to sucrose density gradient ultracentrifugation. For comparison, C3NeF not in mixture with NHS, was examined under the same conditions. Fig. 10 shows that C3NeF alone and C3NeF added to

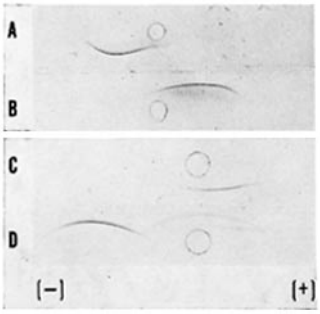

FIG. 9. Immunoelectrophoretic demonstration of C3 and C3PA conversion by C3NeF. Pattern is representative for normal and C2-deficient human serum. A and C, untreated serum; and B and D, serum incubated with C3NeF. A and B were developed with anti-C3, and C and D with anti-C3PA.

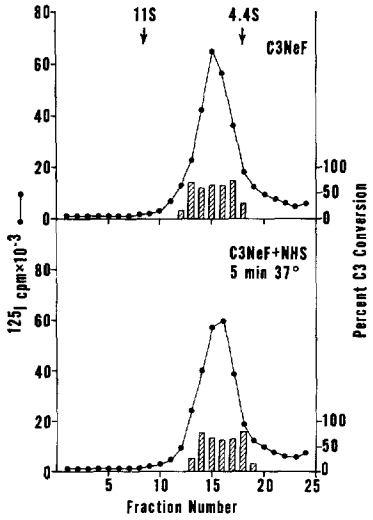

FIG. 10. Zone ultracentrifugation of C3NeF in sucrose density gradient. Upper panel, C3NeF alone; lower panel, C3NeF in NHS. Comparison of velocity of C3NeF under both conditions indicates lack of complex formation with normal serum protein.

serum sediment with an identical velocity, approximately 7S. The result indicated that the factor does not enter into a complex with C3PA. Isolated C3NeF was unable to cause C3 conversion in serum depleted of either P, C3PA, or C3PAse. Similarly, it failed to cause C3PA conversion in serum depleted of P, C3, or C3PAse. The requirement of C3 in the C3PA cleavage reaction was demonstrated using serum containing less than 5% of the normal amount of C3. Whereas isolated C3NeF had no effect on C3PA in this serum, it caused C3PA conversion after addition of isolated C3 to restore a normal C3 level. The results are summarized in Table II. According to these data C3NeF acts exclusively through the alternate pathway of complement activation.

Generation of AT by C3NeF in Serum Depleted of the AI.—Incubation of isolated C3NeF with NHS, which had been rendered deficient in AI, invariably resulted in appearance of AT activity. The active material appeared to consist of both C3a and C5a since the guinea pig ileum became resistant to challenge by isolated human C3a and C5a (Fig. 11).

Occurrence of a C3NeF Analogue in Normal Serum and its Possible Function.—Three different HCG sera were treated with solidified antiserum to highly purified C3NeF. This treatment resulted in removal of C3NeF activity and antigen. Ouchterlony analysis of multiple normal sera invariably revealed a

TABLE II

C3 and C3PA Conversion by C3NeF in Sera Deficient in Various Serum Proteins

Serum depleted in	addition	% conversion	
		C3	C3PA
0	0	0	0
0	C3NeF*	85	70
C2	C3NeF	70	50
P	C3NeF	0	0
C3PA	C3NeF	0	0
C3PAse	C3NeF	0	0
C3	C3NeF	0	0

* C3NeF preparation no. 5, 6 μg of C3NeF/100 μl of serum.

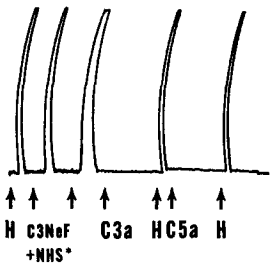

FIG. 11. Demonstration of AT activity in serum previously depleted of the AI (NHS) and then treated for 30 min at 37°C with isolated C3NeF. H, response to histamine.

weak but definite precipitin reaction with anti-C3NeF. Treatment of several different normal sera with the solid anti-C3NeF immune adsorbent led to disappearance of the antigen detected by anti-C3NeF and to an impairment of the function of the alternate pathway. Lysis of glutathione-treated human erythrocytes by inulin-activated serum became negative. When a fraction of normal serum containing the C3NeF antigen was added to the anti-C3NeF-depleted serum, the lytic capacity was restored.

DISCUSSION

At present the origin and nature as well as the possible role of the nephritic factor in the pathogenesis of renal disease is not well understood. Evidence obtained in other laboratories appears to relate the anticomplementary factor found in HCG to the immunoglobulins.

Thompson (18) prepared a fraction from HCG serum which contained C3-cleaving activity when added to normal serum and which was rich in IgG3. The notion that IgG3 might have nephritic factor activity was strengthened by the observation that the activity was removable with insolubilized rabbit antihuman IgG.

Day et al. (19) described a patient with focal glomerulonephritis associated with an in vitro conversion of C3 which was found to be dependent on cold, metal ions, and IgA. These authors postulated that C3 inactivation was effected by the patient's IgA and by activation of the alternate pathway. In kidney biopsies C3, P, and IgA were detectable, whereas IgG, IgM, and C3PA were not observed.

The above observations are consistent with the hypothesis that the anti-complementary activity in nephritic serum may reside in immunoglobulins. In contrast, the present study indicates the clear distinctness of isolated nephritic factor from immunoglobulins. This was shown for nephritic factor from three different patients. Adsorption of partially purified C3NeF with solidified anti-Ig removed all immunochemically detectable immunoglobulins without removing C3NeF activity. In fact, the specific activity in terms of units per milligram increased threefold by the adsorption step. Further, an antiserum prepared to highly purified C3NeF did not react with human Ig, but gave a positive precipitin reaction with C3NeF and C3NeF-containing serum fractions. This antiserum also removed C3NeF activity from nephritic sera. Although it was prepared to an individual C3NeF, it reacted with that of other patients, suggesting that C3NeF is an entity which is qualitatively similar or identical in different patients. These observations need not contradict the work of other investigators, because other nephritides may well be associated with anticomplementary serum factors which constitute immunoglobulin.

When C3NeF was first described, the possibility was considered that it might constitute a mammalian analogue to cobra venom factor. As such it should enter into a complex with C3PA when added to normal serum. The

present experiments have precluded this possibility, however, they have shown a dependence of C3NeF action on the presence of C3PA, as was also shown by others (7). In addition, we demonstrated the requirement of P, C3, and C3PAse.

Since anti-C3NeF recognized an antigen in NHS, the possibility was raised that normal serum contains an inactive C3NeF analogue which may be the precursor of C3NeF. Because removal of this antigen impaired the alternate pathway, it probably constitutes one of the heretofore unrecognized components of the P system. The dependence of nephritic factor on P, C3, C3PAse, and C3PA and the failure of inulin to activate normal serum, treated with anti-C3NeF, strongly suggest that the normal C3NeF analogue precedes P in the molecular events of the alternate pathway. As such it may represent the component which directly interacts with activating substances and which triggers the P pathway. Fig. 12 presents our current concept of the alternate pathway which must be regarded as tentative. Work is underway to establish the function of the normal C3NeF analogue.

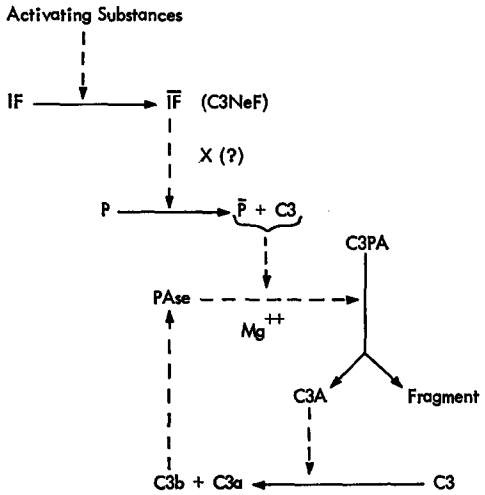

FIG. 12. Schematic representation of hypothesis linking nephritic factor (C3NeF) to a normal component of the P pathway (IF). According to this hypothesis, activating substances confer activity on IF ($\overline{IF}$) which resembles that of C3NeF. Probably in cooperation with an as yet unidentified serum factor (X), $\overline{IF}$ activates P and thus sets in motion the events involving C3, C3PAse, and C3PA that have been described in detail elsewhere (15).

SUMMARY

Nephritic factor (C3NeF) has been isolated from plasma of patients with hypocomplementemic chronic glomerulonephritis (HCG) by ion exchange and molecular sieve chromatography. This material was further treated with solidified anti-Ig antiserum. The purified material failed to react with antiserum to human IgG, IgG3, Fab, Fc, and kappa and lambda chains, but retained full

C3NeF activity. The nonidentity of C3NeF with IgG was further demonstrated by Ouchterlony analysis using anti-IgG and anti-C3NeF. Isolated C3NeF was found to be a protein with a sedimentation coefficient of 7S and a mol wt of 150,000 daltons, which on microzone electrophoresis and gel electrophoresis at pH 8.6 behaved as a γ-globulin. C3NeF is not a C1q precipitin and does not activate the classical complement pathway. Unlike cobra venom factor, it failed to enter into a complex with C3 proactivator (C3PA) when incubated with normal human serum (NHS) and then subjected to sucrose density gradient ultracentrifugation. The action of isolated C3NeF on C3 requires C3PA, C3PA convertase (C3PAse), and properdin (P). Similarly, C3PA conversion by C3NeF requires P, C3PAse, and C3. Total hemolytic activity was lost by incubation of 64 μg of C3NeF/1 ml NHS at 37°C for 30 min. Both C3a and C5a anaphylatoxin could be generated by C3NeF in serum previously depleted of anaphylatoxin inactivator. Anti-C3NeF was found to detect an antigen in all NHS tested. Treatment of NHS with solidified anti-C3NeF caused impairment of the alternate complement pathway. It failed to sustain lysis of glutathione-treated human erythrocytes initiated by inulin. It is conceivable that the normal serum constituent which is removed by anti-C3NeF constitutes the inactive precursor of C3NeF, and a heretofore unrecognized component of the alternate pathway.

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