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The cofactors required by C3 nephritic factor to generate a C3 convertase *in vitro*

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SUMMARY

The mechanisms by which a C3 convertase is generated by C3 nephritic factor (NeF) were investigated using purified NeF, C3, C3b, factor B and factor $\bar{D}$ of the alternative pathway of complement activation.

NeF could generate a C3 convertase with C3 and B in the absence of $\bar{D}$, and without cleavage of B. At lower concentrations of NeF the addition of $\bar{D}$ was required to generate a C3 convertase, and B cleavage now occurred. The generation of both the $\bar{D}$-independent and $\bar{D}$-dependent C3 convertases with NeF was inhibited by preincubation of the C3 source with C3b inactivator (KAF); isolated C3b was more efficient than the C3 preparations used in generating the $\bar{D}$-independent C3 convertase with NeF. These experiments indicate that C3b is required for the formation of both convertases, and that the reaction occurring with apparently native C3 is due to trace amounts of C3b.

It is concluded that the C3 convertase generated by NeF in the absence of $\bar{D}$ is $C3\overline{bB}$ (NeF), and that generated in the presence of $\bar{D}$ is the feedback convertase $C3\overline{bBb}$. The relevance of these experiments to reactions which may occur *in vivo* is discussed.

INTRODUCTION

The serum of many patients with hypocomplementaemic mesangiocapillary nephritis (MCGN) contains a factor, C3 nephritic factor (NeF), (Spitzer *et al.*, 1969; Vallota *et al.*, 1970), which is capable of causing C3 cleavage in whole human serum by a mechanism independent of the classical pathway of complement activation (Peters *et al.*, 1972; Williams *et al.*, 1973); NeF and hypocomplementaemia are found more commonly in those patients with MCGN whose renal biopsies show electron dense deposits within the glomerular basement membrane ('dense deposit disease') (Williams *et al.*, 1974; Habib *et al.*, 1974). Patients with partial lipodystrophy have an unusually high incidence of MCGN, usually of the 'dense deposit' type, and many patients with partial lipodystrophy who do not have overt nephritis also have low serum C3 concentrations accompanied by NeF (Sissons *et al.*, 1975). The observation that the complement abnormalities can occur without nephritis in some patients suggests that these complement changes may precede and predispose to the development of MCGN (Peters & Williams, 1974).

The structure of NeF and the mechanism of the reaction by which it cleaves C3 are as yet incompletely characterized, and its role in the pathogenesis of MCGN unresolved. NeF acts independently of the classical pathway, as shown by its ability to cleave C3 in C2-deficient serum (Williams *et al.*, 1973). We have previously reported that NeF does not require properdin (P) to form a C3 convertase, but does require C3b as an essential co-factor to form a C3 convertase in whole serum from patients with MCGN (Williams *et al.*, 1973); however, Schreiber *et al.* (1975) have recently reported that it is native C3, rather than C3b, that is required for formation of the NeF C3 convertase *in vitro*.

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This paper describes the results of experiments performed, in consequence of these discrepant findings, to determine the co-factors required by NeF to form a C3 convertase *in vitro*.

MATERIALS AND METHODS

Purified human complement components. C3 was purified from the euglobulin of a 20% Na_2SO_4 precipitate of whole serum by DEAE–cellulose and hydroxylapatite chromatography (Lachmann, Hobart & Aston, 1973). Functionally active factors B and $\overline{D}$* were purified from the supernatant of a 20% Na_2SO_4 precipitation of whole serum by CM cellulose chromatography (Lachmann *et al.*, 1973). B was further fractionated by DEAE cellulose chromatography (Götze & Müller-Eberhard, 1971), and $\overline{D}$ by Sephadex G-75 chromatography. Each preparation was free of the other complement proteins used in the experiments to be described, by functional and antigenic assays. The B and $\overline{D}$ contained no other proteins, and the C3 one trace protein contaminant, on immunoelectrophoresis and double diffusion in gel against anti-whole human serum (Wellcome). Functionally purified C3b inactivator (KAF) was prepared from the euglobulin fraction of whole serum by DEAE–cellulose and Sephadex G-200 chromatography (Lachmann *et al.*, 1973). The KAF used in these experiments had a titre of 1/600 in the conglutination assay (Lachmann *et al.*, 1973), and caused no C3 conversion in whole human serum.

C3b was prepared by incubation of purified batches of 2·5 mg/ml C3 with 1% w/w trypsin (Sigma) for 1 min at room temperature and inactivation of the trypsin with twice this amount of soya bean trypsin inhibitor (Sigma).

Cobra venom factor. Cobra venom factor (CVF) was purified from *Naja naja* venom (Sigma) by DEAE–cellulose and Sephadex G-200 chromatography and contained 640 units of CVF activity/ml (Ballow & Cochrane, 1969).

Measurement of C3 splitting activity (NeF). Equal volumes of NHS and test material were incubated at 37°C for 10 min, EDTA was then added to a final concentration of 0·01 M and the mixture incubated for a further 30 min at 37°C. C3 conversion was then assessed by crossed immunoelectrophoresis (Laurell, 1965) in 1% agarose (BDH) in barbitone/glycine–tris buffer, I = 0·08, pH 8·6, using a goat anti-human C3 antiserum.

Functional assays for factor B, factor $\overline{D}$, *C3.* Factor B and factor $\overline{D}$ were functionally assayed by a haemolytic plate technique as described by Martin *et al.* (1975). Functional C3 was assayed by haemolytic titration (Lachmann *et al.*, 1973).

Purification of NeF. The serum from two patients with PLD, without overt nephritis, but with high NeF activity, was fractionated by a method similar to that described by Vallota *et al.* (1974). Serum was initially applied to DEAE cellulose equilibrated with 0·02 M PO_4 pH 7·0. The C3 splitting activity was found in the breakthrough peak.

This was further fractionated on CM Sephadex equilibrated with 0·02 M PO_4, pH 6·0, and eluted with a linear gradient rising to 0·3 M with NaCl. C3 splitting activity was eluted at a conductivity of 11–15 mmhos. The C3 splitting fractions were pooled and after concentrating to a total protein concentration of 5 mg/ml and ultracentrifugation this pool was found to be free of factor $\overline{D}$ (by functional assay), factor B (by functional and antigenic assays), and of Properdin and C3 by double diffusion in agarose gel against specific antisera; on IEP and double diffusion in agarose gel against anti-whole human serum (Wellcome) IgG was the only identifiable protein. For these experiments IgG was not removed from this final pool. 2 μl of this preparation of NeF was required to cause 50% C3 conversion in a reaction mixture containing 24 μg of C3 and 6 μg of B with 0·5 mM Mg^{2+} in a volume of 20 μl.

Co-factor requirements of NeF. The co-factors required by NeF to form a C3 convertase *in vitro* were determined by incubating NeF with purified C3 and components of the alternative pathway (B, D and Mg^{2+}).

Two different types of reaction mixture were employed in these experiments. In one the formation of the convertase was not separated from its action ('one-step reaction'); in the other the formation of the convertase (which was found to be Mg^{2+}-dependent) was separated from its action (which is Mg^{2+}-independent) by the addition of EDTA followed by a source of C3 ('two-step reaction'). Details of these two different types of reaction mixture are given in Fig. 1. The concentrations of the reagents are expressed as final concentrations—the total volume of all reaction mixtures was 20 μl. C3 conversion was always assessed by crossed immunoelectrophoresis and quantitated by planimetry, and in some experiments C3 consumption was also assayed by haemolytic titration. B cleavage was assessed by immunoelectrophoresis using a monospecific rabbit anti-human factor B.

RESULTS

B requirements of NeF

B was required for the formation of a C3 convertase in all the following experiments. The minimum amount of B required for the formation of a C3 convertase in the presence of 8 μl of NeF and the absence of $\overline{D}$ (see below) was 20 μg ml, using the one-step reaction.

* The components of the alternative pathway are designated by capital letters; a bar over the letter(s) indicates the enzymatically active form.

Factor B = glycine-rich β-glycoprotein (GBG) or C3 proactivator (C3PA): Bb is the larger of two fragments produced when B is cleaved.

Factor $\overline{D}$ = activated factor D, GBGase or C3 proactivator convertase (C3PAse). D is a putative precursor form of $\overline{D}$.

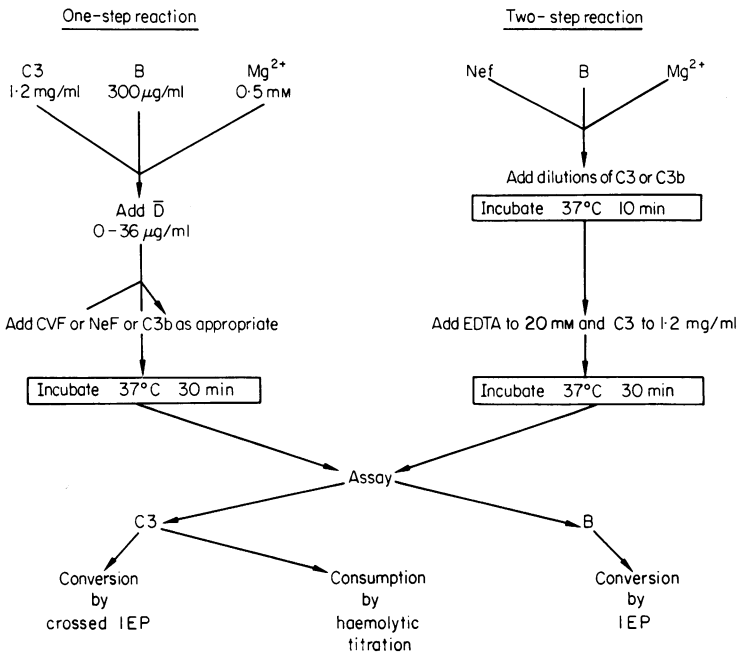

FIG. 1. Details of the two types of reaction mixture employed in determining mechanisms of C3 convertase formation.

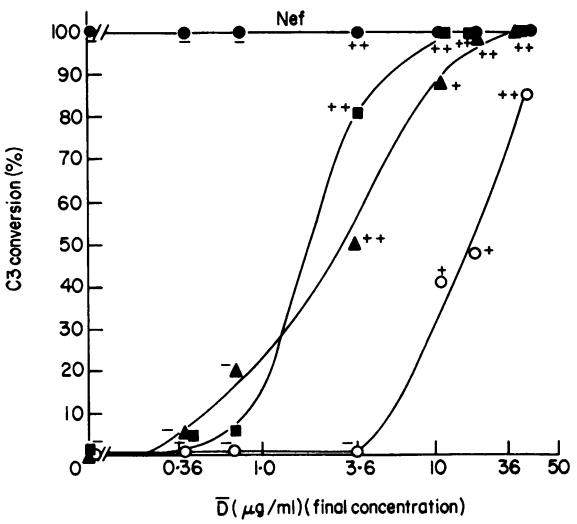

FIG. 2

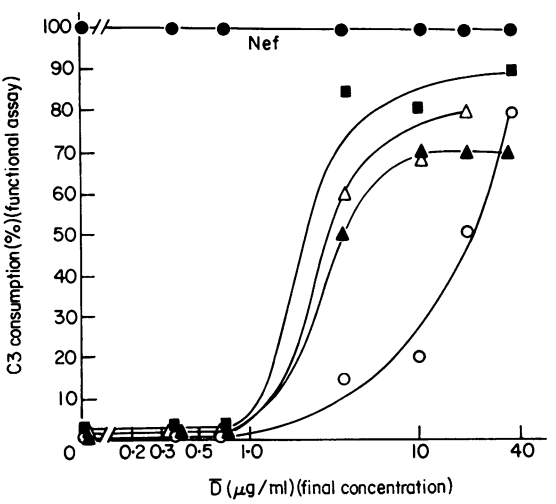

FIG. 3

FIG. 2. D̄ requirements of NeF (●), CVF (■) and C3b (▲) for formation of a C3 convertase. 8 μl of NeF, 8 μl of CVF or C3b in a final concentration of 125 μg/ml were added to C3 and B in the one-step reaction. (○), C3, B and D̄ alone. + = B cleavage; − = no B cleavage.

FIG. 3. D̄ requirements of NeF (●), CVF (■), and C3b1 and C3b2 (△ and ▲) for formation of C3 convertase. (○) D̄ alone. Experimental details as for Fig. 2 but C3 consumption assessed by haemolytic titration. B conversion was as shown in Fig. 2.

The $\bar{D}$ requirements of NeF, C3b and CVF, C3 convertases (Figs 2 and 3)

The amount of $\bar{D}$ required by NeF, C3b and CVF respectively to form a C3 convertase was assessed using the one-step reaction and six different concentrations of $\bar{D}$ from 0 to 36 μg/ml (see Figs 2 and 3). C3b and CVF were found to have similar $\bar{D}$ requirements for the formation of a C3 convertase, and the resultant C3 conversion was always accompanied by B cleavage. By contrast, the amount of NeF used in this experiment (8 μl) caused 100% C3 conversion in the absence of any added $\bar{D}$; moreover this C3 conversion was not accompanied by B cleavage until a $\bar{D}$ concentration sufficient to allow the generation of a C3 convertase by C3b or CVF was attained (3·6 μg/ml or greater). At high concentrations of $\bar{D}$ (10·8 μg/ml or greater) C3 conversion and B cleavage occurred in the absence of any added activator (i.e. NeF, C3b or CVF); when 10·8 μg/ml of $\bar{D}$ was incubated with C3 alone no C3 conversion occurred. C3 consumption was also assessed by haemolytic titration in these experiments and similar curves obtained (Fig. 3).

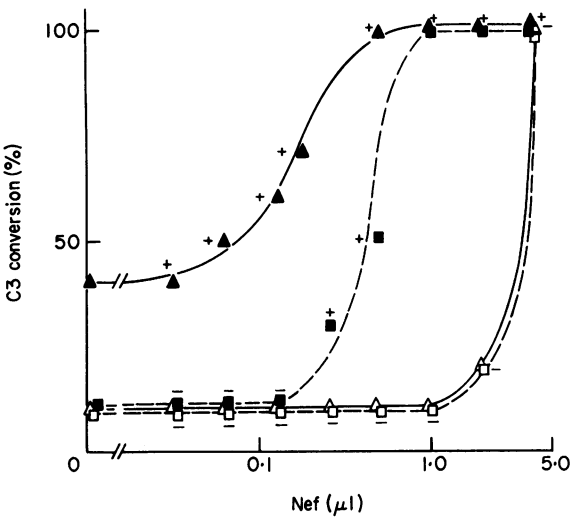

FIG. 4

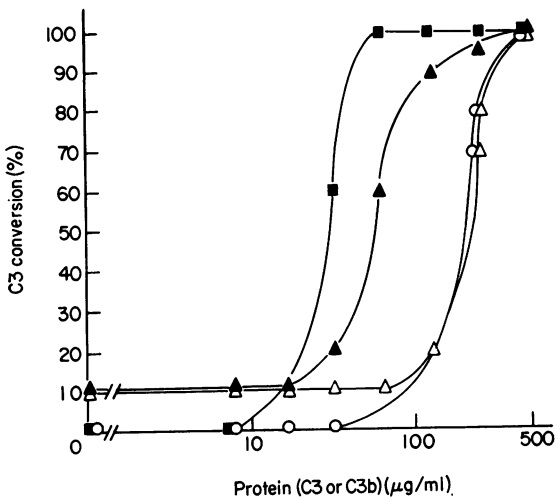

FIG. 5

FIG. 4. Effect of increasing $\bar{D}$ concentrations on the ability of varying concentrations of NeF to generate a C3 convertase. C3 conversion assessed in one-step reaction. + = B cleavage; − = no B cleavage. (▲) $\bar{D}$ = 10·8 μg/ml; (■) $\bar{D}$ = 3·6 μg/ml; (△) $\bar{D}$ = 0·36 μg/ml; (□) $\bar{D}$ = 0 μg/ml).

FIG. 5. Comparison of C3 and C3b in generation of a C3 convertase with NeF and B in the absence of $\bar{D}$. C3 conversion assessed in two-step reaction. 'C3b1' (■) and 'C3b2' (▲) = two separate batches of trypsin generated C3b. 'C31' (○) and 'C32' (△) = two separate C3 preparations.

The effect of varying $\bar{D}$ and NeF concentrations on the generation of the C3 convertase by NeF (Fig. 4)

The effect of $\bar{D}$ on the formation of the C3 convertase by NeF was studied using the one-step reaction, with $\bar{D}$ added at three different concentrations and NeF in amounts ranging from 0 to 4 μl. At high NeF concentrations (4 μl) 100% C3 conversion occurred in the absence of $\bar{D}$ and without cleavage of B. The addition of 0·36 μg/ml of $\bar{D}$ did not increase the C3 conversion caused by this amount of NeF, nor did B cleavage occur. Higher concentrations of $\bar{D}$ (3·6 and 10·8 μg/ml) caused increased C3 conversion by a given amount of NeF and this was now always accompanied by B cleavage.

At this point these experiments appeared to have defined three C3 converting enzymes: one generated in the presence of NeF, C3 and B without B cleavage; a second with NeF, C3, B and $\bar{D}$ accompanied by B cleavage; and a third with high concentrations of $\bar{D}$, C3, B and also accompanied by B cleavage.

Comparison of C3 and C3b in the generation of C3 convertase by NeF in the absence of $\bar{D}$ (Fig. 5)

A comparison was made between using C3 or C3b to form the NeF dependent C3 convertase in the absence of $\bar{D}$, employing the two-step reaction. Two separate preparations of C3 and of C3b were used and it can be seen that on a weight for weight basis C3b was more effective than C3 in forming the NeF dependent C3 convertase (approximately 7 times more C3 than C3b being required to achieve 50% C3 conversion).

This experiment led us to investigate whether C3 was simply less efficient than C3b in forming the NeF C3 convertase or whether the reaction occurring with the apparently native C3 was in fact due to the presence of trace amounts of C3b. We therefore pretreated the C3 with KAF to destroy any contaminating C3b.

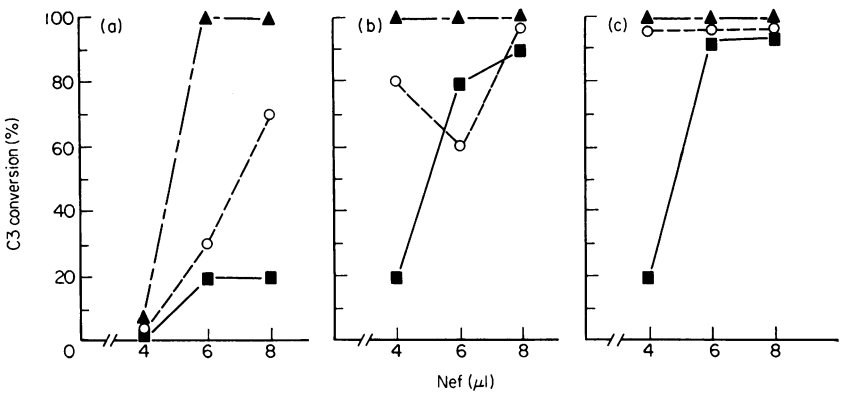

FIG. 6. Effect of preincubation of C3 source with KAF on its subsequent ability to form a C3 convertase with NeF in the absence and presence of $\bar{D}$. C3 was preincubated with 4 μl (▲), 8 μl (○) and 12 μl (■) of KAF at 37°C for 20 min. The C3 was then incubated with B, NeF (4, 6 or 8 μl) and $\bar{D}$: (a) 0; (b) 3·6; (c) 10·8 μg/ml; and C3 conversion assessed in the one-step reaction.

The effect of using KAF-treated C3 to form the NeF-dependent C3 convertase (Fig. 6)

C3 was first incubated with three different concentrations of KAF at 37°C for 20 min. This KAF-treated C3 was then mixed with B and Mg^{2+}, in the presence of different concentrations of $\bar{D}$ (0, 3·6 and 10·8 μg/ml) and three differing amounts of added NeF (4, 6 and 8 μl); C3 conversion was assessed in the one-step reaction.

Preincubation of C3 with KAF inhibited the formation of the C3 convertase by NeF in the absence of $\bar{D}$, the degree of inhibition being proportional to the amount of KAF used (Fig. 6). This inhibition of C3 conversion could be overcome in a dose-dependent fashion either by increasing the concentration of NeF, or of $\bar{D}$ in the reaction mixture.

The time of preincubation of C3 and C3b with KAF was then extended to a maximum of 4 hr to ensure inactivation of all fluid phase C3b. NeF was also preincubated with KAF to exclude any effect of KAF on NeF.

Effect of preincubation of C3, C3b or NeF with KAF for different times (Fig. 7)

C3 or C3b was incubated with KAF (using 2, 4 and 8 μl of KAF) for 4 hr at 37°C and was then used as the source of C3 or C3b in the two-step reaction in the absence of $\bar{D}$, as for the experiments in Fig. 5. KAF treatment of both C3 and C3b caused inhibition of C3 conversion by NeF. The longer period of preincubation with KAF resulted in greater inhibition of C3 conversion, whereas preincubation of NeF with KAF had no effect on its subsequent ability to generate a C3 convertase.

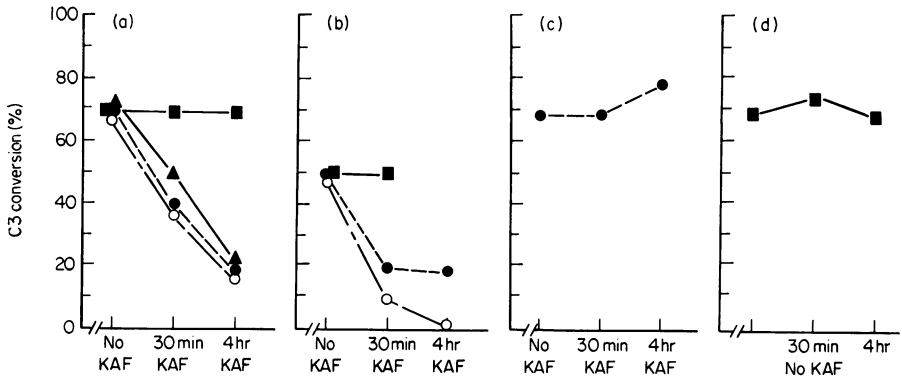

FIG. 7. Effect of preincubation of (a) C3b, (b) C3 or (c) and (d) NeF with KAF for varying times. C3, C3b and NeF were preincubated at 37°C in the absence of KAF (■) and in the presence of 2 μl (▲), 4 μl (●) and 8 μl (○) of KAF, for 30 min or 4 hr. The ability of these reagents to generate a C3 convertase with NeF in the absence of D was then assessed in the two-step reaction.

Effect of preincubation of C3 source with KAF on the C3 convertase formed at high $\bar{D}$ concentrations

As the C3 convertase formed with C3 and B in the presence of NeF was inhibited by pretreating the C3 with KAF, as shown above, we next examined the C3 convertase generated by C3, B and high concentrations of $\bar{D}$ (as shown in Fig. 2) to see if this too was KAF inhibited. Preincubation of the C3 source with 2 and 6 μl of the KAF preparation previously used resulted in complete inhibition of formation of the C3 convertase with C3, B and high $\bar{D}$ concentrations.

DISCUSSION

These experiments have shown that NeF generates a C3 convertase by at least two mechanisms. In the first, which has no requirement for $\bar{D}$, a convertase was generated by incubation of C3 and factor B in the presence of NeF; B was not cleaved in this reaction. However, when $\bar{D}$ was added to this reaction mixture B cleavage could be obtained when the amount of $\bar{D}$ was that required for the generation of the feedback convertase by C3b, or its analogue CVF, suggesting that the feedback convertase $C3\overline{bBb}$ had been generated.

When the dose–response relationships between NeF, $\bar{D}$ and C3 conversion were examined it was evident that the presence of $\bar{D}$ permitted the generation of a C3 convertase at much lower concentrations of NeF than were required in its absence. Thus the $\bar{D}$ concentrations (3·6 μg/ml) permitting efficient feedback activation by CVF or C3b allowed an approximately ten-fold reduction in the amount of NeF required to initiate C3 conversion.

In view of our earlier finding that NeF-induced C3 conversion in whole serum was dependent on the presence of C3b (Williams *et al.*, 1973) we now undertook a series of experiments to re-examine the role of C3b in the formation of the non-$\bar{D}$-requiring C3 convertase. We first compared purified C3 and trypsin-generated C3b in the generation of this convertase in the absence of $\bar{D}$, and found that C3b was approximately seven times more efficient than native C3. This result, together with our earlier report that prior treatment with KAF caused reduction in the conversion of C3 added to MCGN serum (Williams *et al.*, 1973), led to the series of experiments in which the C3 source (C3 or C3b) was pre-incubated with purified KAF; dose and time-dependent inhibition of C3 conversion was observed with C3b and apparently native C3.

These experiments thus show that both enzymes require C3b and not native C3 for their generation. We therefore propose that the non $\bar{D}$-dependent C3 convertase generated by NeF, without B cleavage, is $C3\overline{bB}$ (NeF) and that the $\bar{D}$-dependent C3 convertase generated in the presence of NeF, with B cleavage, is the feedback convertase $C3\overline{bBb}$ (see Fig. 8).

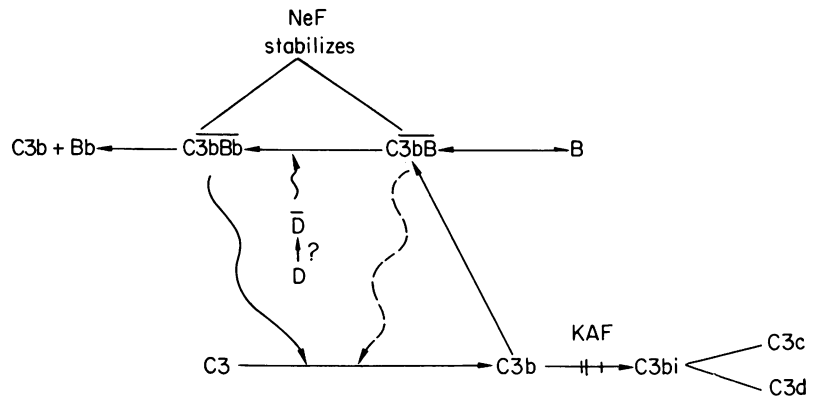

FIG. 8. Diagram showing the postulated C3 convertases generated in the presence of NeF and the absence and presence of D. - - ► = relatively weak enzyme.

We also found that a C3 convertase could be generated by high concentrations of $\bar{D}$ with C3 and B; $\bar{D}$ had no effect on C3 alone and the reaction could be inhibited by pretreating the C3 source with KAF, indicating a requirement for C3b. However, this experiment does not exclude the possibility that at high concentrations of $\bar{D}$ a weak C3 convertase is formed with native C3 and B (with B cleavage), generating a small amount of C3b—this could then form the feedback convertase $C3\overline{bBb}$. The formation of this feedback convertase would have been inhibited by the KAF remaining in the reaction mixture.

Our findings are at variance with those of Schreiber *et al.* (1975). These workers also found that NeF could generate a C3 convertase in the presence of relatively high levels of $\bar{D}$ (similar to those required by C3b) accompanied by B cleavage, and that at lower concentrations of $\bar{D}$ (less than required for the feedback enzyme) C3 convertase generation was not accompanied by B cleavage. However, these workers were unable to generate a C3 convertase with NeF in the absence of $\bar{D}$. The possibility that our findings were due to contamination of reagents with $\bar{D}$ was excluded using a sensitive haemolytic plate assay technique (this assay was readily capable of detecting quantities of $\bar{D}$ (0·36 μg/ml) that had no B-cleaving effect in the NeF-reaction). Schreiber *et al.* also claimed that the generation of a C3 convertase by NeF required C3 rather than C3b; indeed, that the reaction was actually inhibited by C3b, a result completely in opposition to those of our experiments.

Daha, Fearon & Austen (1975) have shown that one effect of NeF is stabilization of the feedback convertase $C3\overline{bBb}$, prolonging its half-life from approximately 3 to 40 min. Our experiments (taken in conjunction with those of Daha *et al.*) suggest that the C3 convertase generated by NeF is $C3\overline{bB}$—and that this (as suggested for $C3\overline{bBb}$ by Daha *et al.*) is also stabilized by NeF. We propose that the generation by NeF of the C3 convertase in the reaction mixtures apparently containing only native C3 is in fact due to trace contamination with C3b: trace amounts of the enzyme $C3\overline{bB}$ would be generated which in the presence of the stabilizing influence of NeF would lead to further C3b generation, which in turn would accumulate in sufficient amounts to generate more of the nephritic factor C3 convertase, $C3\overline{bB}$,

The problems to be resolved in relating these reactions to events *in vivo* are at least two: the first, the mechanism by which the initial C3b necessary for the convertase formation is generated; the second, the relative roles of $\bar{D}$-dependent and $\bar{D}$-independent reactions *in vivo*.

The mechanisms by which C3b might be made available to start the reaction are obscure. Two types of explanation have been advanced; the first is that there is low grade turnover and cleavage of C3 *in vivo* (e.g. by normal catabolism, or by other proteolytic plasma enzymes, or even by activation of the classical pathway)—these explanations fall within the concept of the 'tickover' hypothesis of Lachmann & Nicol (1973). The second explanation is that there is some specific reaction sequence leading to the generation of this initial C3b; for instance it is claimed (Stitzel & Spitzer, 1974) that activated properdin can directly cleave C3 in the absence of any other serum factors. Indirect support for some initial C3b generating

system comes from the observation that NeF was detectable in the serum of two normocomplementaemic patients with MCGN; these sera had normal concentrations of C3, B, properdin and generated a C3 convertase normally with cobra-venom factor (Williams *et al.*, 1974). A similar situation may be created *in vitro*: it was a comparatively early observation that NeF containing MCGN serum does not convert added purified C3 (Vallota *et al.*, 1970). This is difficult to explain if C3 is required for the generation of the NeF C3 convertase, as described by Schreiber *et al.*, but is easily explained if C3b must be generated, as we infer from our observations.

The second question, namely the extent to which the $\bar{D}$-independent reaction contributes to *in vivo* C3 breakdown, is also incompletely resolved. Some indirect evidence for an *in vivo* role for this reaction is the lack of relationship between the presence of NeF and serum concentrations of B in patients with MCGN, the only significant difference between NeF-positive and NeF-negative patients being low C3 levels in those with NeF (Williams *et al.*, 1974). Furthermore, a patient with evidence of *in vivo* C3 conversion and B activation without any cleavage of B has been reported by Day & Müller-Eberhard (1974); we have also observed (Charlesworth *et al.*, 1974) one NeF-positive patient with MCGN and PLD, who had a serum C3 of 6 mg/100 ml with increased C3 turnover, but normal serum B concentrations and normal B turnover, favouring non-feedback dependent activation of complement in this patient.

It might be imagined that, as $\bar{D}$ is presumably usually present *in vivo*, the $\bar{D}$-dependent (feedback) C3 convertase generated by NeF (or at least stabilized by it) would be the more significant enzyme in the *in vivo* situation, since the *in vitro* observations showed that this system caused C3 conversion at much lower concentrations of NeF that the $\bar{D}$-independent convertase. However, this cannot be assumed to be the case until more is known of the form in which D exists *in vivo*, whether as the putative precursor (D) or the active form ($\bar{D}$), and if D does exist, of the factors causing its activation.

There is no immediately apparent cause for the discrepancy between our results and those of Schreiber *et al.* (1975), in particular of their finding that C3b inhibited, and ours that it enhanced, the generation of a NeF C3 convertase. Further work is required to explain this discrepancy, and to relate this type of *in vitro* dissection of the mechanism of action of NeF to the more complex situation *in vivo*.

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