Clin. exp. Immunol. (1986), **65**, 73–79.

Effect of nephritic factor on C3 and on the terminal pathway of complement *in vivo* and *in vitro*

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(Accepted for publication 5 February 1986)

SUMMARY

Plasma samples from patients with nephritic factor (NeF) were examined for their C3 converting activity. C3, C3dg, C5 and the fluid phase terminal complement complex (TCC) were quantified. All patients had evidence of C3 activation with low plasma C3 and high C3dg. Some patients had normal C5 and normal TCC levels, and thus no evidence of terminal pathway activation *in vivo*; others, with slower C3 conversion *in vitro*, had low C5 levels with TCC either elevated or in the upper normal range, suggesting *in vivo* activation of the terminal pathway. These observations were confirmed by *in vitro* experiments using purified NeFs. It is concluded that considerable activation of C3 may occur *in vivo* without a simultaneous activation of the terminal pathway, and that NeF is heterogeneous with regard to its ability to activate complement.

Keywords nephritic factor complement activation C3 terminal complement complex.

INTRODUCTION

C3 nephritic factor (NeF) is an IgG molecule (Thompson, 1972; Amos, Sissons & Peters, 1977) reacting via its antigen binding part (Scott *et al.*, 1978) with the alternative pathway C3 convertase, C3bBb, which is stabilized and rendered resistant to the physiological inactivators, factor I and H (Daha, Fearon & Austen, 1976). Consequently, in patients with NeF a continuous activation of the alternative pathway takes place.

The human terminal complement complex (TCC) is composed of the components C5b, C6, C7, C8 and C9. It exists in two forms depending on the site of activation. The C5b-9(m) complex (Tranum-Jensen *et al.*, 1978, Bhakdi & Tranum-Jensen, 1983) is the mediator of complement lysis, whereas the SC5b-9 complex (Podack, Kolb & Müller-Eberhard 1977; Podack & Müller-Eberhard, 1980) is the nonlytic, fluid phase analogue, in which the terminal components are associated with the S-protein (Podack & Müller-Eberhard 1979).

Recently, the TCC has been detected in normal human EDTA-plasma (Mollnes, Lea & Harboe, 1984), and in higher amounts in patients with pathologically increased complement activation (Mollnes *et al.* 1985c). We have observed that in most cases with evidence of increased complement activation, the content of both C3dg and TCC has been elevated. However, in several cases only one of these indicators was increased, suggesting that the early and the late phase of the complement cascade to some extent may act independently. A recent study (Mollnes, 1985a) demonstrated

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different activation patterns and elimination rates for C3dg and TCC *in vivo*, indicating that both the early and the late phase should be examined to get a more complete evaluation of complement activation *in vivo*.

It has been observed that in some patients with NeF, C3 turnover was markedly increased, whereas turnover of C5 was normal (Sissons *et al.*, 1977). This led to the suggestion that the terminal pathway may not be activated despite considerable activation of C3. The aim of the present study was therefore to examine the effect of NeF on the terminal pathway. Exposure of neoantigens (Kolb & Müller-Eberhard 1975) in C9 when the terminal five components assemble into the TCC, is the most direct evidence for activation of the whole terminal pathway. In this study, TCC was quantified using a monoclonal anti-neoantigen antibody, and used as indicator of terminal pathway activation.

MATERIALS AND METHODS

Nephritic factor (NeF) assay. The NeF activity was measured as described previously (Peters *et al.*, 1972, Williams *et al.*, 1974). Briefly, the C3 splitting capacity was anlaysed by incubating equal volumes of patient serum with a pool of normal human serum (NHS) alone or in the presence of 5 mM Mg^{2+} − 10 mM EGTA (ethylene glycol-bis ($\frac{1}{2}$ − aminoethyl ether) NN′ tetraacetic acid) or 10 mM EDTA (ethylene diamine tetraacetic acid) at 37°C for 30 min, or 3 h. C3 conversion was measured by crossed immunoelectrophoresis. In addition, NeF-containing IgG was purified by collecting the breakthrough peak obtained by fractionation of patient serum on DEAE (diethyl aminoethyl)-Sephacel (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) with 15 mM phosphate buffer, pH 7·6. This IgG was tested for its stabilization activity of EAC43bBb (Scott *et al.*, 1978).

Complement components. C3 and C5 were quantified by radial immunodiffusion and referred to a standard pool (100%). Sheep-anti-human C3 was produced at Hammersmith Hospital, London, and sheep-anti-human C5 was obtained from Seward Laboratories, (UAC House, Blackfriars Rd, London SE1).

C3dg assay. C3dg was measured as described in detail elsewhere (Mollnes, 1985b). Briefly, the C3dg containing supernatants obtained after precipitation with polyethyleneglycol were used in a biotin-avidin enzyme-linked immunosorbent assay (ELISA). The results were expressed in arbitrary units referring to a zymosan activated serum pool as standard.

The terminal complement complex (TCC). An ELISA was performed as described in detail elsewhere (Mollnes *et al.*, 1985c). Briefly, polystyrene plates (Nunc, Copenhagen, Denmark) were coated with a mouse monoclonal antibody (MCaE11) specific for a neoantigen of the C9 moiety of human TCC (Mollnes *et al.*, 1985d). EDTA-plasma samples, diluted 1 : 10, either freshly drawn or stored at −70°C, were tested. Binding of TCC was detected by a rabbit anti-human C9 antiserum (Behringwerke AG, Marburg, FRG., lot 104303E) in the second antibody layer. Finally, peroxidase conjugated sheep anti-rabbit Ig (Amersham, UK., lot 7) was added. Substrate was ABTS (2,2-azino-di-3-ethylbenzthiazoline sulphonic acid; Boehringer Mannheim, GmbH, FRG). Coating of the plates with MCaE11 was performed in PBS (phosphate buffered saline) for 48 h at 4°C whereas all other incubations were made for 45 min at 37°C in PBS containing 0·1% Tween-20. Optical density was read at 405 nm and the results were referred to a standard curve constructed by a two-fold dilution of a zymosan activated human serum pool, which was defined to contain 1000 AU (arbitrary units)/ml undiluted. The normal range (2·2–6·6 AU/ml) was defined after examination of EDTA-plasma samples from 40 healthy blood donors.

Complement activation in vitro. Purified NeF-containing IgG was added to NHS. C3 conversion and TCC generation were measured after 30 and 90 min incubation at 37°C. Normal human IgG was used as a negative control and cobra venom factor (CVF) as a positive control.

Patients. Twelve plasma samples from six patients with membranoproliferative glomerulonephritis (MPGN) or partial lipodystrophy (PLD) were examined. All these patients had NeF defined by EA3bBb stabilization. Serial samples from a patient with acute poststreptococcal glomerulonephritis (AGN) with no evidence of NeF were also included.

EDTA plasma. Samples were separated immediately after sampling and stored at −70°C.

Table 1. *In vitro* C3 conversion (30 min incubation) and EDTA-plasma concentration of C3, C3dg, C5 and TCC are shown for one patient with acute glomerulonephritis (A) and six patients with nephritic factor (No. 1–6). C3 conversion is expressed as per cent above baseline consumption of NHS incubated with PBS. For most patients several samples drawn at different times were examined. The TCC/C5 ratios were calculated. The normal limits are indicated at the top of the table.

Patient	Sex/Age	*In vitro* C3 conversion (%)	C3 60–135%	C3dg 20–45 AU/ml	C5 80–130%	TCC 2·2–6·6 AU/ml	TCC/C5 <8·2	Diagnosis
A	M 56	Neg	21	261	56	13·0	23·2	AGN†
		Neg	24	146	49	11·5	23·4	
		Neg	21	121	52	5·9	11·3	
		Neg	30	69	114	4·0	3·5	
		Neg	80	28	56	3·3	5·8	
1	F 21	75	13	250	155	2·8	1·8	MPGN‡
		95	<10	350	100	3·6	3·6	
2	F 36	70	23	275	100	6·0	6·0	PLD§
3	F 20	30	70	51	104	6·0	5·7	MPGN
		40	68	112	80	17·3	21·6	
		60	40	299	120	6·3	5·2	
4	M 10	20*	<10	107	25	5·2	20·8	MPGN
		30*	<10	120	23	5·9	25·6	
		25*	11	105	12	5·2	43·3	
5	F 33	100*	<10	222	32	6·7	20·9	MPGN
		65*	<10	195	54	6·3	11·6	
6	F 6	60*	<10	240	5	10·3	206·0	MPGN

* 3 h incubation (slow conversion).
† Acute glomerulonephritis (post-streptococcal).
‡ Membranoproliferative glomerulonephritis.
§ Partial lipodystrophy.

RESULTS

Complement activation in vivo

In vitro C3 conversion and EDTA-plasma concentration of C3, C3dg, C5 and TCC were measured in seven patients. The data is presented are Table 1. Serial samples of patient A were obtained during the course of an acute post-streptococcal glomerulonephritis with evidence of considerable activation of both the early and the late phase, with low C3 and C5 values, and generation of high amounts of C3dg and TCC (Fig. 1). There was a close positive correlation between C3dg and TCC values, and thus between early and late phase activation. Spearman's rank correlation coefficient was +1·0 and the two-tailed *P*-value was <0·01. NeF was not detected in this patient.

The *in vivo* activation pattern in patients with NeF varied considerably. In patient no. 1 and 2 *in vitro* conversion of C3 was rapid and easily detectable. Plasma concentration of C3 was low, and C3dg high, but there was no evidence of terminal pathway activation since both C5 and TCC concentration was normal.

In patients nos 4, 5 and 6 their serum contained C3 conversion activity that was slow. Significant C3 conversion was detectable only after prolonged incubation with NHS. Time course experiments showed virtually no conversion after 30 min incubation but conversion increased with incubation time to a maximum at 3 h. Such C3 conversion persisted in Mg EGTA and was abolished in EDTA (<5% conversion with either patient serum or PBS incubated with EDTA-NHS). Purified IgG from these patients similarly showed C3 conversion after prolonged incubation and were capable of stabilizing EAC3bBb, and hence were shown to contain NeF activity. In these patients the *in vivo* C3 concentration was low and C3dg was high. C5 values were low and the TCC either elevated or at the

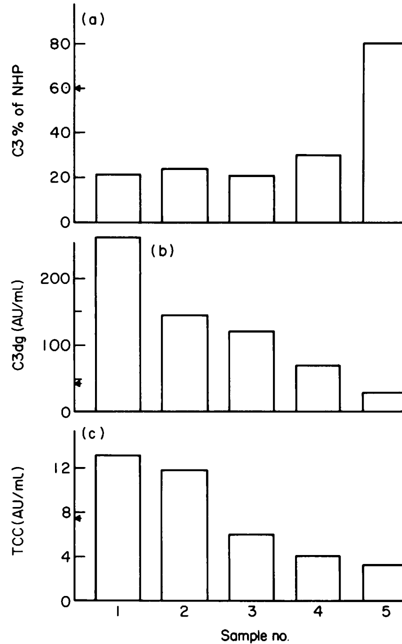

Fig. 1. C3 (a), C3dg (b) and TCC (c) values in EDTA-plasma samples from a patient with acute poststreptococcal glomerulonephritis. C3 increased, whereas C3dg and TCC decreased to normal values during the course of the disease. Horizontal arrow heads indicate limits between normal and pathological values. The columns represent means of triplicates.

upper normal limit, which is consistent with pathologically increased activation of both the initial and the terminal pathways. Since an elevated TCC level combined with a lowered C5 should be regarded as a stronger indication of activation than a similar TCC value combined with normal or increased C5, the ratio TCC/C5 may be a better indication of terminal pathway activation than each individual value. This ratio is included in Table 1, and the highest normal limit was found to be 8·2 by dividing the upper normal limit of TCC (6·6) by the lower normal limit of C5 (0·80). Values higher than 8.2 may indicate pathologically increased activation of the terminal pathway.

Patient no. 3 shows both patterns of activation. The samples tested were taken at 2 monthly intervals. The first sample shows NeF activity occurring together with a normal C3, a slightly increased C3dg and no evidence of terminal pathway activation. The two subsequent samples show C3 activation *in vivo*, one with a raised TCC and the other with a normal TCC.

Thus, all NeF patients showed signs of increased *in vivo* C3 activation, whereas only those with a slow *in vitro* C3 conversion had increased terminal pathway activation *in vivo*. This suggested that patients with NeF were heterogenous with respect to *in vivo* activation of the terminal pathway.

Complement activation in vitro

To further investigate the extent to which different patterns of complement activation observed in plasma were due to different properties of the NeFs, the complement activation *in vitro* by purified NeF from patient no. 1 was compared with patient no. 5. C3 conversion and TCC generation were measured after incubation of the NeFs with normal human serum for 30 and 90 min at 37°C. Fig. 2 shows induction of considerable C3 conversion by both NeF preparations. NeF from patient no. 5 eventually produced a comparable degree of C3 conversion to NeF from patient no. 1 after 90 min incubation. Normal IgG gave minimal background conversion, whereas an extensive, although somewhat slow, conversion was obtained with CVF.

Increased generation of TCC was observed only with NeF from patient no. 5 and with CVF.

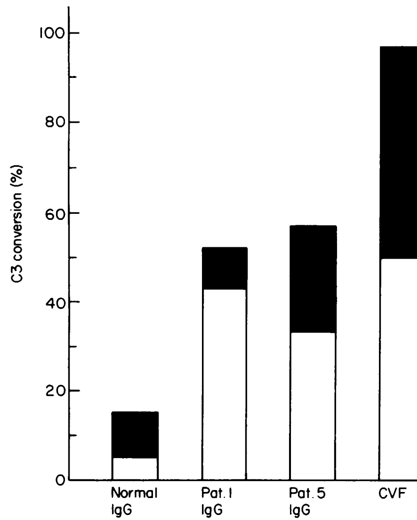

Fig. 2. C3 conversion measured by crossed immunoelectrophoresis after incubation of NHS with normal IgG, IgG fractions from patients no. 1 and 5, and CVF (cobra venom factor). (□) C3 conversion after 30 min incubation; (■) additional conversion obtained after a total incubation time of 90 min.

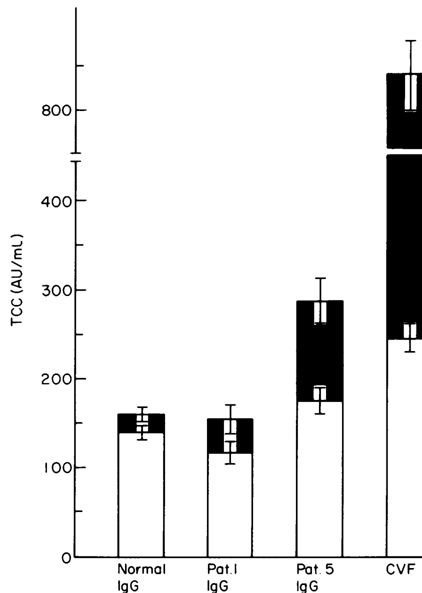

Fig. 3. Generation of TCC in the same experiment as shown in Fig. 2. The columns represent means ± s.d.

NeF from patient no. 1, gave TCC values comparable to or slightly lower than those obtained with addition of normal IgG. These results are shown in Fig. 3.

These *in vitro* experiments are consistent with the observations in plasma and demonstrate that NeFs differ in the pattern of complement activation generated *in vitro*.

DISCUSSION

We have used a sensitive method for measuring terminal complement pathway activation *in vivo* and have shown that patterns of complement activation differ with nephritic factor; some patients show evidence of C3 activation *in vivo* with no terminal pathway activation, whereas others show activation of both initial and terminal pathways. The different patterns of complement activation *in vivo* were reproduced *in vitro* with NeF preparations from two patients.

In the patient with poststreptococcal glomerulonephritis C3 activation was associated with activation of the whole terminal pathway. A similar pattern was observed when complement was activated with CVF or zymosan *in vitro*. It has been shown (Müller-Eberhard & Schreiber 1980) that CVF binds to Bb, thereby forming a C3 convertase which is able to bind C3b, resulting in a functionally active C5 convertase. These convertases are resistant to the regulatory protein Factor I (Alper & Balavitch 1976). Thus, CVF leads to a continuous activation of both the early and the late phase of the cascade.

The heterogeneity of NeFs seen in this study may explain the discrepant results from two previous studies. Sissons *et al.* (1977) found NeF patients to have normal C5 turnover associated with increased C3 turnover and Arroyave *et al.* (1974) showed terminal pathway activation by NeF *in vitro*.

It has been suggested by several recent reports that regulation mechanisms may arrest the activation at certain levels, in contrast to the previous view that early phase activation would inevitably lead to late phase activation. Iida & Nussenzweig (1981) have postulated complement receptors as inhibitors of the complement cascade, and Salama *et al.* (1983) have shown that the cascade may be arrested at the C4b/C3b level in autoimmune haemolytic anaemia.

NeF is an IgG autoantibody that stabilises the alternative pathway C3 convertase, C3bBb, and hence causes continued cleavage of C3. We have shown that this C3 cleavage is not necessarily accompanied by increased C5 convertase by the addition of further C3b molecules to give (C3b) nBb. The results of our *in vitro* experiments suggest that ineffective C5 convertase formation is not due primarily to lack of C3b since different NeFs caused different degrees of terminal pathway activation for the same degree of C3 conversion. Daha, Kok and van Es (1982) have found that the stabilization of C3bBb by different NeFs is inhibited to varying degrees by CR1, which could be expected if NeFs recognize different epitopes and react with different affinities against the convertase. It was later shown (Daha Deelder & van Es, 1984) that two monoclonal antibodies against Factor B stabilized C3bBb by rendering it resistant to Factor H, whereas a third monoclonal antibody showed no stabilization but rather caused accelerated decay of the convertase. Similar mechanisms may be responsible for the heterogeneity among patients with NeF with respect to terminal pathway activation. Some NeFs may bind to the C3 convertase in a way that inhibits generation of C5 convertase, and thus explain the dissociation between early and late activation in these patients.

The difference of NeF with regard to the activation of the late complement components may have its impact in the pathogenesis of complement induced tissue damage, but the significance of these findings is at present unclear. Split products generated from activation of the initial part of the cascade participate in a variety of biological functions related to inflammation. Activation of the terminal pathway may lead to membrane damage by the terminal complement complex, which has been implicated as an important mediator of glomerular injury in experimental nephritis (Groggel *et al.* 1983). Immunohistochemical studies of C3 and TCC deposition in the kidneys of patients with NeF will be required to confirm which complement mechanisms may contribute to the tissue lesion, and may indicate whether the plasma values reflect disease processes in the kidneys.

In conclusion, we have demonstrated that NeFs are heterogeneous with regard to their complement activating properties. Some NeFs activate both the early and the late phase of the complement cascade, whereas others activate C3 strongly without any activation of the terminal pathway, thus illustrating a situation of complete dissociation between early and late phase activation of complement both *in vivo* and *in vitro*. These observation demonstrate the advantage of independent examination of early and late phase activation with regard to NeFs in particular, and to complement activation in general.

This study was financially supported by the Norwegian Women's Health Organization and by the Norma and Leon Hess Foundation. Y.C. Ng holds a Training Fellowship from the Medical Research Council. Excellent technical assistance was performed by Ms Lise Utheim and Ms Deirdre Grennan. Ms Hilde Schwensen typed the manuscript. They are all gratefully acknowledged.

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