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The immunogloblin nature of nephritic factor (NeF)

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SUMMARY

NeF was shown to be antigenically and structurally similar to IgG by the following experiments: (1) NeF activity in serum was absorbed by and, under acid conditions, could be eluted from (a) anti-myeloma IgG antibody coupled to Sepharose and (b) protein A–Sepharose. (2) Purified NeF could bind to anti-myeloma IgG–Sepharose and could be eluted with acid, and this binding was blocked by myeloma IgG. (3) An antibody to β_2 microglobulin, showing strong cross-reactivity with normal IgG, bound NeF activity before, but not after, absorption of the antiserum with IgG. (4) Sepharose-coupled antibodies to NeF could bind activity which was recovered in the acid eluate. This binding capacity was lost after absorption of the antibody with normal and myeloma IgG. (5) Structural similarity was demonstrated by pepsin and papain digestion, which resulted in NeF activity eluting with $F(ab')_2$ and Fab fragments from protein A–Sepharose and Sephadex G-150. (6) Autoradiography of PAGE–SDS of ^{125}I-labelled NeF eluted from EA4$\overline{3b}\overline{Bb}$ cells showed that NeF had a larger H chain than normal IgG, suggesting that NeF might be an abnormal IgG molecule.

INTRODUCTION

C3 nephritic factor (NeF) a factor found in sera from certain patients with hypocomplementaemic membranoproliferative glomerulonephritis (MPGN) (Spitzer *et al.*, 1969) and in patients with partial lipodystrophy (Sissons *et al.*, 1975) causes C3 cleavage in normal human serum by activation of the alternative pathway (Vallota *et al.*, 1970; Williams *et al.*, 1973). It has subsequently been established that NeF acts by binding to and stabilizing the alternative pathway convertases $\overline{C3bB}$ and $\overline{C3bBb}$ (Daha, Fearon & Austen, 1976; Amos *et al.*, 1976), probably by protecting the convertases from the effects of the naturally occurring inactivators: C3b inactivator and β1H (Whaley & Ruddy, 1976; Weiler *et al.*, 1976).

The nature of NeF has been controversial. Thompson (1972) showed, from chromatographic fractionation and immunoabsorption studies using anti-human IgG and subclass-specific antiserum, that NeF was an immunoglobulin, possibly IgG3. Work in this laboratory (Peters & Williams, 1972; and unpublished experiments in which $F(ab')_2$ anti-IgG absorbed NeF; Williams & Peters, 1972) also indicated that NeF was associated with IgG. However, Vallota *et al.* (1974) concluded from a series of experiments, using insolubilized anti-human IgG, that NeF could be separated from IgG. Schreiber, Gotze & Müller-Eberhard (1976a) also suggested that NeF was distinct from IgG and claimed that NeF consisted of a dimer of two polypeptide chains, each of 85,000 mol. wt. However, Davis *et al.* (1977) demonstrated placental transfer of NeF, providing further evidence that NeF might be an IgG immunoglobulin. In more recent studies in this laboratory, NeF could not be separated from IgG by a variety of immunochemical techniques (Amos *et al.*, 1976; Amos, Sissons & Peters, 1977).

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In the light of these discrepant claims, we have undertaken a series of experiments in order to clarify the relationship between NeF and IgG. We show that NeF is antigenically indistinguishable from IgG and that NeF activity is largely resistant to the effects of limited proteolytic digestion. The remaining NeF activity appears to be a property of the Fab portion of the antibody-like molecule. Gel filtration and elution of ^{125}I-labelled NeF from EA4$\overline{3bBb}$ cells further suggests that NeF might be an unusual IgG molecule.

MATERIALS AND METHODS

DEAE-cellulose and CM-cellulose. These were obtained from Whatmann Biochemicals Ltd, Springfield Mill, Maidstone, Kent, U.K.

Sephadex G-150 and G-75, CM-Sephadex, Sepharose 4B *and protein A-Sepharose.* These were all obtained from Pharmacia Fine Chemicals Ltd, Uppsala, Sweden.

Dithiothreitol, Iodoacetamide, acrylamide and NN'methylenebisacrylamide. These were supplied by BDH Chemicals Ltd, Poole, Dorset, U.K.

NNN'N'tetramethylethylene diaminoacetic acid (TEMED). This was from Koch-Light Ltd, Colnbrook, Bucks, U.K.

Cyanogen bromide. This was obtained from Eastman Kodak Ltd, Acornfield Road, Kirby, Liverpool, U.K.

Kodirex Autoprocess film. This was from Kodak Ltd, P.O. Box 33, Swallowdale Lane, Hemmel Hempstead, Herts, U.K.

Ethyleneglycol-bis(β-aminoethyl ether)NN'tetra-acetic acid (EGTA.) This was supplied by Sigma Chemical Co., P.O. Box 14508, St Louis, Mo 63178, U.S.A.

CFT and PBS. These were both from Oxoid Ltd, Wade Road, Basingstoke, Hants, U.K.

Sheep and guinea-pig erythrocytes. These were supplied by Tissue Culture Services, Henry Road, Slough, Berks, U.K.

Rabbit haemolytic serum and horse anti-whole human serum. These were from Wellcome Reagents Ltd, Wellcome Research Laboratories, Beckenham, U.K.

Normal human serum (NHS). This was obtained from normal healthy laboratory workers, of both sexes.

Human serum containing nephritic factor (NeF serum). This was obtained from several patients with partial lipodystrophy (PLD) and membrano-proliferative glomerulonephritis (MPGN). Purified NeF was isolated from the serum of a single patient with PLD.

The nomenclature of the classical complement components is in accordance with that agreed under the auspices of the World Health Organization (1968). The nomenclature for the alternative pathway is that adopted at the Second International Congress of Immunology, 1974, Brighton, U.K.

Purification of Human IgG. Myeloma IgG. This was purified from the 33% (saturated) ammonium sulphate precipitate of an IgG1 myeloma serum (kindly characterized by Dr David Catty, Department of Experimental Pathology, University of Birmingham, U.K.), by chromatography on DEAE-cellulose in 0·015 M phosphate buffer, pH 7·0. The breakthrough peak was applied to CM-cellulose in 0·02 M phosphate buffer, pH 6·0, and the IgG was eluted with a linear salt gradient, final concentration 0·3 M NaCl. The purified myeloma IgG gave a single line against anti-whole human serum when tested by immunoelectrophoresis.

Normal human IgG. This was prepared from pooled normal sera by three sodium sulphate precipitation steps of 18% (w/v), 12% and 12%. The final precipitate was dissolved in 0·01 M phosphate buffer, pH 8·0, and fractionated on DEAE-cellulose in this buffer. The breakthrough peak was applied to CM-Sephadex in 0·02 M phosphate buffer, pH 6·0, and eluted as described above. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (PAGE-SDS) showed that it contained 95–98% IgG.

Purification of NeF. This was according to the method of Vallota *et al.* (1974) as modified by Amos *et al.* (1976). PAGE-SDS indicated that more than 95% of the protein in purified NeF preparations was IgG.

Purification of complement components. Purification of functional assays of factors B and $\bar{D}$ were carried out as described previously (Amos *et al.*, 1976). C3 was purified essentially as described by Lachmann, Hobert & Aston (1973) with minor modifications in the initial salt precipitation steps (R.A. Harrison & P.J. Lachmann, in preparation).

Functional assay of NeF. Three assays were used.

(a) *C3 conversion in NHS.* The C3-splitting activity of NeF was assayed by incubating equal volumes of NeF and NHS alone or in the presence of 0·7 mM Mg^{2+} 10 mM EGTA for 30 min at 37°C. C3 conversion was measured by crossed immunoelectrophoresis (Laurell, 1965).

(b) *EA4$\overline{3bBb}$ stabilization.* The ability of NeF to stabilize the alternative pathway EA4$\overline{3bBb}$ convertase was tested by mixing two-fold dilutions of NeF with 5 μg factor B and 0·015 μg factor $\bar{D}$ (limiting dilutions) in a final volume of 30 μl. 50 μl 1% EA43b (prepared as described by Lachmann *et al.*, 1973) were added and the cells were incubated at 37°C for 5 min. 100 μl C-EDTA (NHS diluted 1:10 in PBS-0·04 M EDTA) was added and the cells were incubated at 37°C for 30 min. 0·6 ml CFD was added and lysis was measured from the optical density at 410 nm ($o.d._{410}$).

(c) *EA4$\overline{3bBb}$ agglutination.* The ability of NeF to agglutinate EA4$\overline{3bBb}$ cells was tested by incubating two-fold dilutions of NeF with 5 μg factor B and 0·015 μg factor $\bar{D}$ in a final volume of 30 μl. 50 μl EA43b cells were added and the plates were incubated at 37°C for 45–60 minutes until the cells had settled. The agglutination was estimated visually and scored as (+), (−) or (±).

Elution of NeF from EA4$\overline{3b}\overline{Bb}$ cells. This was essentially as described by Schreiber *et al.* (1976a). 50 μl ^{125}I-labelled NeF and ^{125}I-labelled IgG were mixed with 50 μg factor B and 0·045 μg factor $\bar{D}$ in a final volume of 180 μl in CFT. 200 μl 10% EA43b cells were added and the mixture was incubated at 37°C for 20 min. The cells were washed three times in cold CFT, resuspended in 100 μl CFT and incubated at 37°C for 60 min. The supernatants were counted and analysed by PAGE–SDS. In the control experiments, ^{125}I-labelled NeF and ^{125}I-labelled IgG were incubated with EA43b and EA without B and $\bar{D}$.

Purification of antisera. (a) *Rabbit antibody to myeloma IgG.* Animals were injected by i.m. injection of 1–2 mg purified myeloma IgG in FCA. After 14 days they were boosted by i.m. injection of 1–2 mg IgG in FIA, and the antisera were collected at weekly intervals. The IgG (20% Na_2SO_4 precipitated) fraction of the pooled antisera was further purified by passage down an immunoabsorbent column prepared from myeloma IgG coupled to Sepharose as described below. The specific anti-myeloma antibody bound to the immunoabsorbent and was then eluted with 0·2 M glycine–HCl buffer, pH 2·2. It gave a single line against a range of dilutions of NHS when tested by Ouchterlony double-diffusion.

(b) *Rabbit antibodies to normal human IgG.* These were gifts from Dr M.J. Hobart and Dr C. Stern (Department of Immunology, RPMS, Hammersmith Hospital, London). When tested in Ouchterlony double-diffusion both antisera gave a single line against NHS. $F(ab')_2$ anti-Fc was prepared from these antisera by the method of Lachmann (1971).

(c) *Rabbit antibody to 'NeF/IgG'.* This was prepared using partially purified NeF (Amos *et al.*, 1976), it gave a single line in Ouchterlony double-diffusion when tested against NHS and this showed identity with IgG.

(d) *Rabbit antibody to 'β_2 microglobulin/IgG'.* This was prepared using β_2 microglobulin which had been isolated from the urine of a patient with renal tubular dysfunction and who excreted large amounts of this protein in the urine. When tested in Ouchterlony double-diffusion (ODD), early antisera gave a strong line against NHS which was identical with IgG. Later bleeds gave a weaker line against IgG and after absorption with normal human IgG the resulting antiserum was monospecific for β_2 microglobulin.

Preparation of immunoabsorbents. The IgG antibody preparations of the myeloma serum and the rabbit antisera were coupled to Sepharose using the method of Cuatrecasas, Wilchek & Anfinsen (1968). After coupling, the immunoabsorbent columns were washed in 0·1 M $NaHCO_3$, PBS and 0·2 M glycine–HCl buffer, pH 2·8, and re-equilibrated in PBS. The efficiency of coupling estimated from the $o.d._{280}$ of the eluates from each washing step was usually between 85% and 95%.

Glutaraldehyde insolubilization. This was as described by Avrameas & Ternynk (1967).

Pepsin digestion of NeF and normal human IgG. This was carried out at low pH using the method described by Lachmann (1971). Briefly, 2·0 ml purified NeF (14 mg/ml) and IgG (15 mg/ml) were dialysed against 0·2 M sodium acetate buffer, pH 3·2, for 2 hr at 4°C. 1 ml samples were removed and neutralized with 1·0 M Tris (acid-treated control). The remaining 1 ml was incubated with 2% (w/w) pepsin at 37°C for 2 hr. The samples were then neutralized with 1·0 M Tris.

Papain digestion of NeF and normal human IgG. This was performed as described for rabbit IgG by Porter (1959). Two 1·0 ml samples of NeF and IgG in PBS containing 0·01 M cysteine and 0·002 M EDTA were incubated alone (control) and with 1% (w/w) mercuripapain (digest) at 37°C for 16 hr. The digestion was stopped by extensive dialysis of the digest against PBS at 4°C.

Sephadex G-150 chromatography of NeF. 0·9 ml samples of NeF and pepsin- and papain-digested NeF were applied to a column (1·5 × 100 cm) of Sephadex G-150 equilibrated in PBS. The column was run by upward flow at 12 ml/hr and 3 ml fractions were collected. The protein content was measured by $o.d._{280}$ and the NeF activity by stabilization of EA4$\overline{3b}\overline{Bb}$. Molecular weights were calculated by plotting of elution volume (V_E) against log mol. wt, using human IgG and serum albumin as standards.

Polyacrylamide gel electrophoresis. PAGE in buffers containing SDS (Weber & Osborn, 1969) was carried out by using the discontinuous buffer system described by Laemmli (1970). Preparation of the samples for the gel was described by Reid, Lowe & Porter (1972). Autoradiography was carried out using Kodirex Autoprocess plates.

^{125}I-labelling of NeF and normal IgG. This was according to the method of Morrison, Bayse & Webster (1971) as modified by Heusser *et al.* (1973).

RESULTS

Binding of NeF serum to anti-myeloma IgG–Sepharose and protein A–Sepharose

Separate serum samples (1 ml) from five patients (four with PLD and one with MPGN) were applied to a column (10 ml) of anti-myeloma IgG–Sepharose, equilibrated in PBS. The breakthrough peak was collected and the column was eluted with 0·2 M glycine–HCl buffer, pH 2·8. The results (Table 1a) indicate that there was no NeF activity in the breakthrough peak. The activity was bound to the anti-myeloma IgG–Sepharose and eluted with dilute acid.

Table 1b shows the results of the NeF assay of the sera from the same five patients after passage down a (5 ml) protein A–Sepharose column. In four of the patients most of the activity was retained on the column and eluted with acid. However, the serum from a fifth patient (patient 1, Table 1b) had significant NeF activity in the breakthrough peak.

TABLE 1. (a) Binding of C3 NeF to anti-myeloma IgG–Sepharose; (b) binding of C3 NeF to protein A–Sepharose

Patient	Percentage C3 conversion in NHS		
	Starting material	Breakthrough peak	Acid eluate
(a)			
(1) PLD	100	0	100
(2) PLD	100	0	95
(3) PLD	100	0	95
(4) MPGN	80	0	75
(5)PLD	100	0	100
NHS	0	0	10
(b)			
(1) PLD	100	60	75
(2) PLD	100	15	85
(3) PLD	100	5	90
(4) MPGN	80	5	60
(5) PLD	100	0	100
NHS	0	0	10

Binding of purified NeF to insolubilised anti-human IgG and anti-myeloma IgG–Sepharose

100 μl purified NeF was mixed with 2 ml rabbit anti-human IgG serum, insolubilized by precipitation with ethyl chlorofomate. 1 mg BSA was added to the mixture in a final volume of 1 ml and this was incubated overnight at 4°C with constant stirring. The precipitate was centrifuged at 2000 *g* for 10 min, and the supernatant was tested for NeF activity. All the NeF activity was absorbed by the precipitated anti-IgG. When normal rabbit serum was substituted for anti-IgG, no such absorption of NeF activity occurred.

We next examined the binding of purified NeF to an anti-myeloma IgG–Sepharose column. When 0·4 ml NeF (16 mg) was applied, all the protein and NeF activity was retained on the column, and was eluted with 0·2 M glycine–HCl (Fig. 1a). To demonstrate the specificity of the binding of NeF to anti-myeloma IgG–Sepharose, the column was then saturated with 20 mg of the myeloma IgG used to raise the antibody. When 12 mg purified NeF was applied the NeF activity was eluted in the 'breakthrough' peak and no NeF could be detected in the acid eluate.

Binding of purified NeF to rabbit antibody to 'NeF/IgG'–Sepharose

When tested in Ouchterlony double-diffusion against NHS, antibody to partially purified NeF gave a single line which showed a reaction of identity with normal IgG. This antiserum was absorbed with IgG and no longer gave a line against NHS, PLD serum, purified NeF or normal IgG. The unabsorbed and absorbed antibodies were coupled to Sepharose as described. The results (Table 2) indicate that whereas the IgG-absorbed anti-NeF could no longer bind NeF activity, the unabsorbed anti-'NeF/IgG'–Sepharose was capable of binding most of the applied NeF activity. The small amount of activity eluting in the breakthrough peak was probably due to overloading.

Binding of purified NeF to anti-'β_2 microglobulin/IgG'–Sepharose

The binding of NeF to an antibody raised to β_2 microglobulin (isolated from urine) which also reacted with IgG was tested before and after absorption of the anti-β_2 microglobulin with normal IgG. NeF activity was only bound to the unabsorbed anti-'β_2 microglobulin/IgG'–Sepharose column (Table 2).

In view of these findings, which clearly indicated an antigenic similarity between NeF and IgG, a series of experiments were designed to examine the effects of limited proteolytic digestion on the activity and subunit structure of NeF.

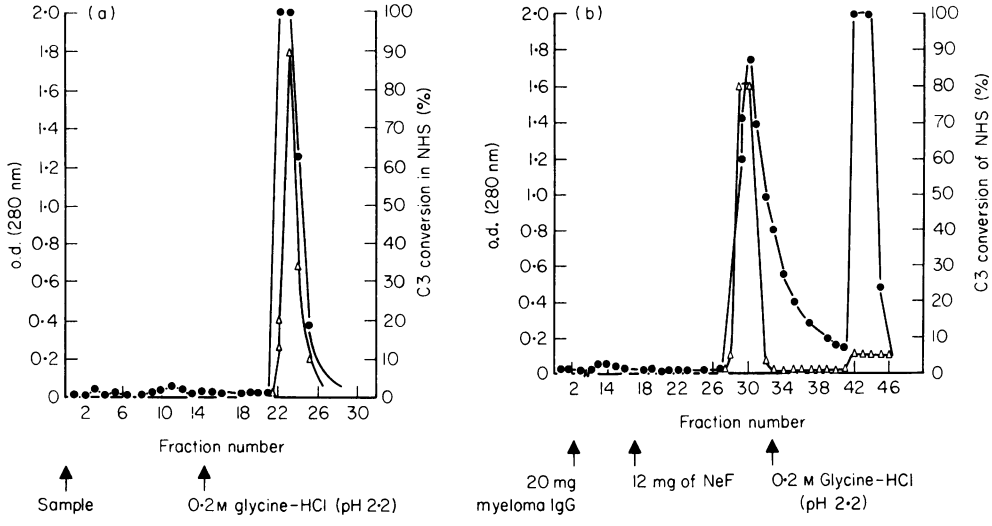

FIG. 1. (a) Binding of NeF to anti-myeloma IgG–Sepharose. 16 mg NeF was applied as indicated. After washing with PBS the column was eluted with 0·2 M glycine–HCl buffer, pH 2·2. (●) o.d., (△) percentage C3 conversion. (b) Blocking of binding of NeF to anti-myeloma IgG–Sepharose by Myeloma IgG. 20 mg Myeloma IgG was applied to the column, as indicated. After washing with PBS, 12 mg NeF was applied. The 'breakthrough' peak was collected and the column was eluted with 0·2 M glycine–HCl buffer, pH 2·2. (●) o.d., (△) percentage C3 conversion.

TABLE 2. Elution of NeF activity from immunoabsorbants of: (i) anti-'NeF/IgG'–Sepharose; and (ii) anti-Human 'β_2 microglobulin/IgG'–Sepharose, before and after absorption of the antisera with human IgG

Sample	Specific activity (lytic units EA4$\overline{3bBb}$/mg protein)	Total activity (lytic units EA4$\overline{3bBb}$)	Percentage activity*
(i) Anti-'NeF/IgG'–Sepharose			
(a) Breakthrough peak	0·9	2·7	17·4
(b) Acid eluate	3·2	12·8	82·6
IgG-absorbed anti-NeF–Sepharose			
(a) Breakthrough peak	3·7	17·8	100
(b) Acid eluate	0	0	0
(ii) Anti-'β_2 microglobulin/IgG'–Sepharose			
(a) Breakthrough peak	0	0	0
(b) Acid eluate	3·9	19·5	100
IgG-absorbed anti-β_2 microglobulin–sepharose			
(a) Breakthrough peak	3·4	13·6	100
(b) Acid eluate	0	0	0

* Percentage activity eluting in fraction compared with total activity eluted from column.

(a) *The effects of pepsin and papain digestion on NeF activity*

Pepsin digestion. Incubation of purified NeF and normal IgG with 2% pepsin at pH 3·2 for 2 hr caused virtually complete digestion of the IgG in both NeF and normal IgG preparations and the generation of F(ab')$_2$ and pFc' fragments. The assay of NeF before and after pepsin digestion showed that there was a slight increase in specific activity as measured by C3 conversion and by stabilization of EA4$\overline{3bBb}$ cells. However, pepsin digestion did cause a significant loss in the haemagglutinating titre (Table 3).

Papain digestion of NeF and normal IgG. This was done with 1% papain at pH 7·0 for 16 hr at 37°C, and resulted in the formation of Fab and Fc fragments (Fig. 2). NeF activity measured by C3 conversion

TABLE 3. Recovery of NeF activity after pepsin digestion and chromatography on protein A–Sepharose

Sample	Percentage protein	Specific activity C3 conversion (units/mg protein)	Percentage activity*	Specific activity EA4$\overline{3bBb}$ stabilization (CH_{50}/mg protein)	Percentage activity*	Specific activity EA4$\overline{3bBb}$ agglutination (units/mg protein)	Percentage activity*
NeF	100	0·41	100	9·3	100	6·04	100
Protein A–Sepharose							
Breakthrough peak	5·3	Nil	Nil	Nil	Nil	Nil	Nil
Acid eluate	65·6	0·35	47	7·0	42	6·5	69·6
Pepsin-digested NeF	100	0·68	100	10·8	100	2·2	100
Protein A–Sepharose							
Breakthrough peak	93·2	0·29	40	3·2	28	1·8	76
Acid eluate	7·4	0·25	2·7	12·9	8·8	5·3	18

* Percentage activity compared with material applied to column.

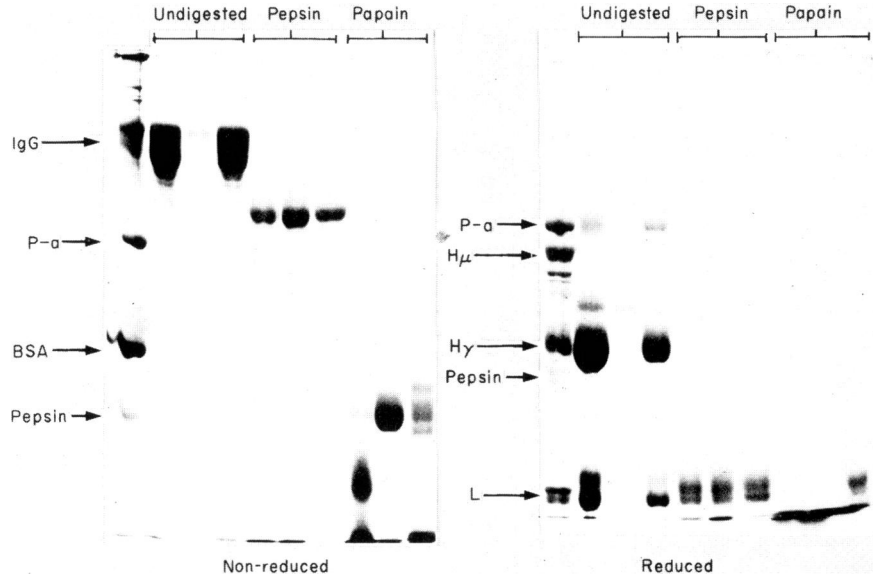

FIG. 2. Polyacrylamide–SDS gels of NeF before and after pepsin and papain digestion and chromatography on protein A–Sepharose. The following samples are shown, before and after reduction and alkylation: three samples of undigested NeF (Undig); of pepsin-digested NeF (Pepsin) and of papain-digested NeF (Papain). The three samples in each category, from left to right are: before fractionation; the breakthrough peak from protein A–Sepharose; and the acid eluate from Protein A–Sepharose. Markers: IgG, phosphorylase a (P-a), BSA and pepsin are shown non-reduced and reduced, together with the Hμ, Hγ and L chains of reduced IgM and IgG.

and EA4$\overline{3b}\overline{Bb}$ stabilization was largely retained (50–80% of the activity was recovered). Again there was a substantial loss in haemagglutination activity (Table 4).

The IgG control has no NeF-like activity before or after pepsin and papain treatment.

(b) *The effects of pepsin and papain digestion on NeF structure and binding to protein A–Sepharose*

(i) *NeF Structure.* PAGE–SDS of undigested NeF before reduction showed that it contained more than 95% IgG. After reduction three major protein components were found (Fig. 2). These corresponded with L chains (23,000 mol. wt), H chains (50,000 mol. wt) and a component of about 100,000 mol. wt, which was probably H-chain dimers.

After pepsin digestion only trace amounts of undigested IgG remained and most of the protein had a molecular weight of about 110,000. After reduction neither the H chain nor the H-chain dimer could be demonstrated and a single component of about 23,000 mol. wt was found (Fig. 2).

After papain digestion again only trace amounts of intact IgG remained. There was now a major component of 45,000 mol. wt with small amounts of material of about 20,000–30,000 mol. wt. After reduction one major component of approximately 23,000 mol. wt was detected (Fig. 2).

These experiments thus demonstrated that proteolytic digestion of NeF had been virtually complete. However, since it was not practicable to recover the activity from the gel, the possibility that the remaining NeF activity was due to a trace component which was resistant to proteolysis could not be excluded.

We therefore examined the effects of pepsin and papain digestion on the behaviour of NeF on Sephadex G-150. The results (Fig. 4) show that the NeF activity always eluted with, though slightly preceding, the major protein peak. These eluted with molecular weights of approximately 155,000, 110,000 and 43,000, which corresponded to undigested NeF, and pepsin- and papain-treated NeF respectively.

(b) *Binding of NeF to protein A–Sepharose.* Before digestion most of the protein in both the NeF and normal IgG preparations was retained on the protein A–Sepharose and eluted with dilute acid (Fig. 3).

TABLE 4. Recovery of NeF activity after Papain digestion and chromatography on protein A–Sepharose

Sample	Percentage protein	Specific activity C3 conversion (units/mg protein)	Percentage activity	Specific activity EA4$\overline{3b}\overline{Bb}$ stabilization (CH_{50}/mg protein)	Percentage activity*	Specific activity EA4$\overline{3b}\overline{Bb}$ agglutination (units/mg protein)	Percentage activity*
NeF	100	0·72	100	0·69	100	6·04	100
Papain-digested NeF	100	0·40	56	0·36	52	1·3	21·5
Papain-digested NeF Protein A–Sepharose	100	0·32	100	0·36	100	1·3	100
Breakthrough peak	50	0·46	80	0·45	89	1·3	50
Acid eluate	44	Nil	Nil	Nil	Nil	Nil	Nil

* Percentage activity compared with starting material.

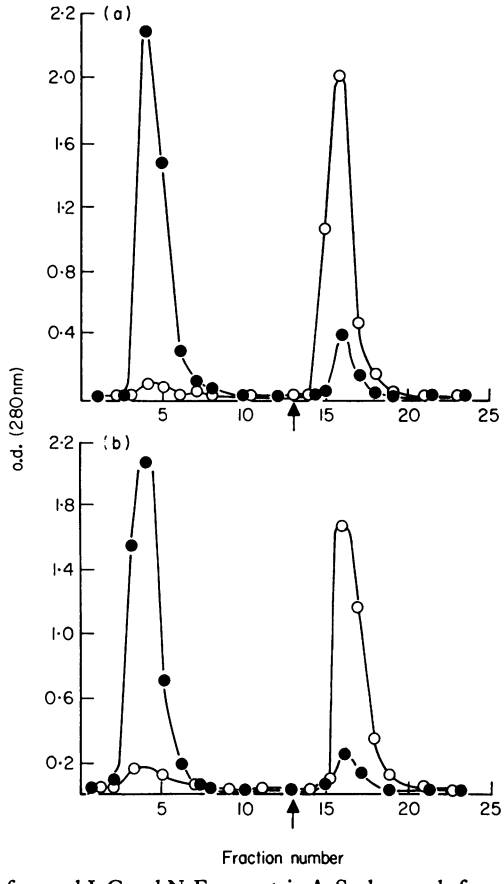

FIG. 3. Chromatography of normal IgG and NeF on protein A–Sepharose: before and after Pepsin digestion. After application of the samples of (a) IgG and (b) NeF the column was washed with PBS and eluted with 0·2 M glycine–HCl at 2·8. (○) o.d. of undigested sample, (●) o.d. of Pepsin-digested sample. Arrow shows application of glycine–HCl.

All eluted NeF activity was in the acid eluate: the breakthrough peak had no demonstrable activity (Table 3). Normal IgG had no NeF activity and none was recovered from the column.

After pepsin digestion, most of the protein in both the NeF and IgG preparations was now in the breakthrough peak (Fig. 3). Most of the NeF activity was also recovered in the breakthrough peak, and only a small proportion in the acid eluate. However, the specific activity of the acid eluate in this experiment was similar to that of the breakthrough peak, measured by the C3 conversion assay, and was increased in the EA4$\overline{3bBb}$ stabilization and agglutination assays. Another experiment (not shown in Table 3) showed similar increases in the specific activity in the acid eluate. The reason for the binding o this material to protein A–Sepharose is not known. It was not due to undigested Fc determinants, since this acid eluate did not react in Ouchterlony double-diffusion against monospecific $F(ab')_2$ anti-Fc. It may be due to residual specificities remaining in the hinge region after pepsin digestion.

After papain digestion approximately 50% of the protein was found in the breakthrough peak and about 40% was in the acid eluate (which contained the Fc fragments). All the eluted NeF activity was found in the breakthrough peak (Table 4). A surprising finding was that the putative Fab–NeF still had agglutinating activity. However, when the Fab fragment was further purified on Sephadex G-150, no haemagglutinating activity was found in the 3S fraction (43,000 mol. wt), although there was substantial recovery of EA4$\overline{3bBb}$ stabilization activity (Table 5).

The anomalous behaviour of NeF on Sephadex G-150, which showed that NeF activity eluted slightly before the major protein peak (Fig. 4), suggested that NeF might be larger than normal IgG. Further

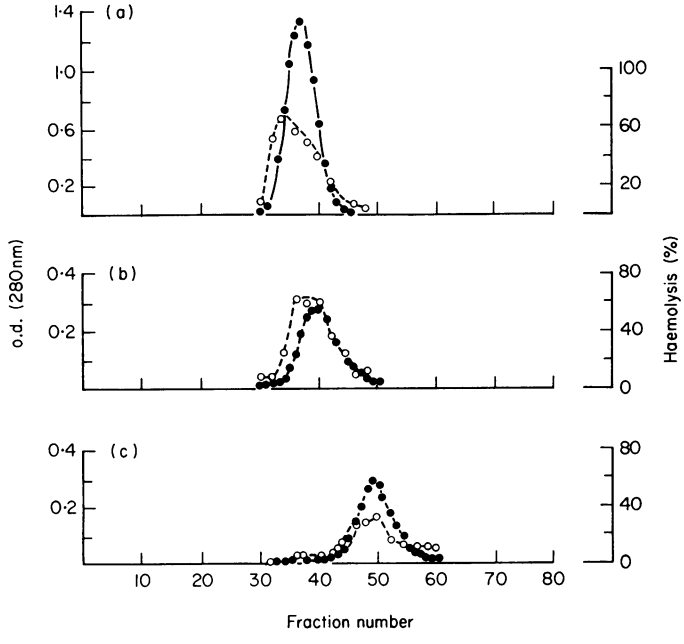

FIG. 4. Gel filtration on Sephadex G-150 of (a) NeF (b) pepsin-digested NeF and (c) papain-digested NeF. (●) o.d., (○) percentage haemolysis.

TABLE 5. Recovery of EA4$\overline{3b}\overline{Bb}$ stabilization and agglutination activities of papain-digested NeF and Fab NeF after chromatography on Sephadex G-150

Sample	Specific activity EA4$\overline{3b}\overline{Bb}$ stabilization (CH_{50}/mg protein)	Percentage activity*	Specific activity EA4$\overline{3b}\overline{Bb}$ agglutination (Units/mg protein)	Percentage activity†
Papain-digested NeF	0·18	100	1·9	100
Fab NeF (breakthrough peak from protein A–Sepharose)	0·35	100	0·62	24
Sephadex G-150 3S (of Fab NeF)	0·76	85	Nil	Nil

* With respect to material applied to column.
† With respect to starting material.

evidence for this came from experiments designed to investigate the subunit structure of NeF eluted from the cell-bound alternative pathway convertase (EA4$\overline{3b}\overline{Bb}$). In these experiments, ^{125}I-labelled NeF and ^{125}I-labelled IgG were incubated with EA43b cells in the presence of factors B and $\bar{D}$ and in control experiments ^{125}I-labelled NeF and ^{125}I-labelled IgG were incubated with EA43b and EA, without factors B and $\bar{D}$. The recoveries of ^{125}I-labelled NeF and ^{125}I-labelled IgG are summarized in Table 6. The overall recoveries were very low, between 0·2% (Table 6) and 2% (in experiments not shown here); however, the binding of NeF to the EA4$\overline{3b}\overline{Bb}$ cells was almost twice the binding of NeF to EA43b and three times greater than the binding of normal IgG to EA4$\overline{3b}\overline{Bb}$. PAGE–SDS analysis of the eluates showed that before reduction the major component of the ^{125}I-labelled NeF eluate from EA4$\overline{3b}\overline{Bb}$ cells had a molecular weight of 170,000. This was slightly larger than the starting material and eluates from the control cells (EA43b and EA) (Fig. 5). (The double banding pattern seen in the autoradiograph was also found in the normal IgG marker and was probably an artifact of the discon-

TABLE 6. Recovery of ^{125}I-labelled NeF and ^{125}I-labelled IgG after elution from EA43$\overline{3}$b$\overline{Bb}$, EA$\overline{43b}$ and EA

Cells	^{125}I-labelled NeF*	^{125}I-labelled IgG*
EA43$\overline{3}$o$\overline{Bb}$	0·23	0·07
EA$\overline{43b}$	0·15	0·20
EA	0·09	0·08

* Percentage eluted compared with counts offered.

tinuous buffer system used.) After reduction the ^{125}I-labelled NeF EA43$\overline{3}$b$\overline{Bb}$ eluate had two components, corresponding to H and L chains. The major H-chain component was slightly larger than normal H and had a molecular weight of 54,000 compared with 50,000 for the H-chain component of normal IgG, the ^{125}I-labelled NeF starting material and the eluates from EA43b and EA. The L-chain component migrated normally with a molecular weight of 22,000 (Fig. 5). PAGE–SDS analysis of the eluted ^{125}I-labelled IgG showed that all eluates had a molecular weight of 155,000 before reduction. After reduction the ^{125}I-labelled IgG eluates were made up of H and L chains of normal molecular weight.

DISCUSSION

The results presented here strongly suggest that NeF is an immunoglobulin. This conclusion was based on experiments demonstrating antigenic and structural similarities between NeF and IgG.

Antigenic similarity was suggested by the observation that NeF activity in serum bound to anti-

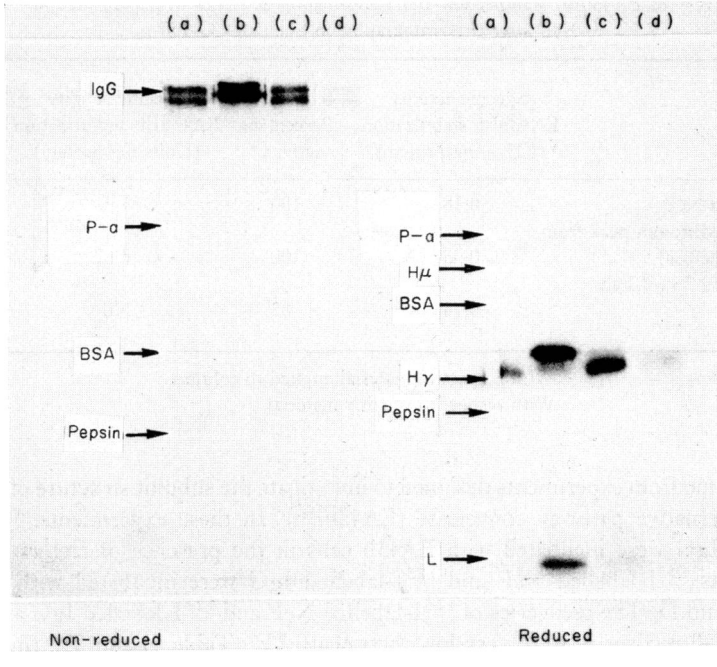

FIG. 5. Autoradiograph of PAGE–SDS of ^{125}I-labelled NeF eluted from EA43$\overline{3}$b$\overline{Bb}$ cells. The following samples are shown. Non-reduced: (a) ^{125}I-labelled NeF; (b) ^{125}I-labelled NeF EA43$\overline{3}$b$\overline{Bb}$ eluate; (c) ^{125}I-labelled NeF EA43b eluate; and (d) ^{125}I-labelled NeF EA eluate. The position of non-reduced markers IgG, phosphorylase a (P-a), BSA and pepsin are included. Reduced: (a) ^{125}I-labelled NeF; (b) ^{125}I-labelled NeF EA43$\overline{3}$b$\overline{Bb}$ eluate; (c) ^{125}I-labelled NeF EA43b eluate; and (d) ^{125}I-labelled NeF EA eluate. The position of reduced markers phosphorylase a, BSA, pepsin and the Hμ, Hγ and L chains of IgM and IgG are included for reference.

myeloma IgG–Sepharose, and to protein A–Sepharose, indicating the presence of Fc-like determinants. Similarly, purified NeF bound to immunoabsorbents made from several preparations of insolubilized antibody to normal IgG and to myeloma IgG. Binding to the latter was shown to be specifically blocked by myeloma IgG. NeF also bound to antibody raised against β_2 microglobulin, but this binding was abolished after the antiserum was absorbed with normal IgG. In all these experiments the NeF activity could be recovered by appropriate acid elution of the immunoabsorbents. Attempts to raise an antiserum recognizing specific determinants in NeF, not present in normal IgG, failed. In every instance such antiserum reacted strongly against normal IgG and after absorption with IgG the capacity to bind to and absorb NeF activity was lost.

The effects of limited proteolytic digestion indicated a structural similarity between NeF and IgG and showed that the NeF activity was a function of the Fab portion of the molecule. Thus after pepsin and papain digestion most of the NeF activity eluted in the breakthrough peak from the protein A–Sepharose, and no longer bound to the column, and gel filtration on Sephadex G-150 showed NeF activity eluting with molecular weights characteristic of $F(ab')_2$ and Fab after pepsin and papain digestion respectively.

These data provide strong evidence for the IgG nature of NeF and are in accord with our earlier work (Peters & Williams, 1972; Amos *et al.*, 1976) and the work of Thompson (1972). Davis *et al.* (1977) have lately reported transplacental transfer of NeF, providing further evidence for its similarity to IgG. Our results are, however, in complete contradistinction to those reported by Vallota *et al.* (1974) and Schreiber *et al.* (1976a), who claimed to have separated NeF from IgG by immunoabsorbent techniques. Schreiber *et al.* (1976a) further suggested that NeF was comprised of two identical chains of 85,000 mol. wt. It is possible that this component is identical to the 100,000 mol. wt. component which we observed in many reduced and alkylated NeF preparations and which we consider to be H-chain dimers.

Of considerable interest is the question of the relationship between NeF and the so-called 'Initiation Factor'. The existence of this factor was originally based upon the finding that antibody to NeF absorbed a component from normal serum which was necessary for initiation of the alternative pathway (Schreiber *et al.*, 1976a,b). Our findings that NeF is antigenically and structurally identical with IgG must therefore cast doubt on the nature and existence of 'Initiation Factor', at least as it was originally described. However, the role of IgG antibody in the initiation of activation of the alternative pathway deserves further consideration.

The questions still to be elucidated are the exact nature of NeF and its mode of action in stabilizing the alternative pathway convertases, $C\overline{bB}3$ and $C3\overline{bBb}$. One possibility is that NeF is an immunoconglutinin-like molecule, i.e. an autoantibody having specificity directed against newly formed determinants on the $C3\overline{bB}$ and $C3\overline{bBb}$ complex. Alternatively, NeF might be a small immune complex containing a pepsin- and papain-resistant antigen, such as carbohydrate or lipid. This might explain the observation that, on gel filtration, the peak of NeF activity always slightly preceded the protein peak. A further possibility is that NeF might be an abnormal IgG molecule. This view is supported by evidence that the H chain of NeF, eluted from $EA4\overline{3bBb}$ cells, was larger than the H chain of normal IgG, as judged by its mobility on PAGE–SDS. Further purification and analysis of the structure of this molecule should lead to the elucidation of its exact nature and the mechanisms of action of NeF.

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