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Significance of C3 nephritic factor (C3NeF) in non-hypocomplementaemic serum with membranoproliferative glomerulonephritis (MPGN)

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SUMMARY

C3NeF is an autoantibody of C3 convertase (C3bBb) and is often detected in the serum of hypocomplementaemic MPGN patients. Serum samples from 104 non-hypocomplementaemic MPGN patients (C3NeF) were studied. C3NeF, which cannot activate the alternative pathway, was found in the sera of 6 patients. We examined the C3NeF in purified IgG from five of the non-hypocomplementaemic serum samples (non-hypo C3NeF) and four hypocomplementaemic serum samples (hypocomplementaemic C3NeF) to determine why C3NeF does not induce C3 splitting and hypocomplementaemia. Purified IgG from non-hypo C3NeF stabilized EAC4b3bBb cells in a manner similar to IgG from hypocomplementaemic C3NeF in EDTA gelatin veronal buffer. However, the non-hypo C3NeF IgG did not stabilize C3 convertase (EAC4b3bBb cells) in the presence of control proteins (factors H and I), whereas the hypocomplementaemic C3NeF IgG did. The C3NeF in the hypocomplementaemic serum displayed two characteristics: (i) inhibition of intrinsic decay of Ce convertase (C3bBb); and (ii) inhibition of extrinsic decay by factors H and I. Although the C3NeF in the non-hypocomplementaemic sera did inhibit the intrinsic decay in a manner similar to the hypocomplementaemic C3NeF IgG, it did not inhibit the extrinsic decay. Due to the different characteristics of hypocomplementaemic C3NeF and non-hypo C3NeF in the serum samples, the non-hypo C3NeF did not activate C3. Therefore, we conclude that C3NeF exhibits a heterogeneity which is very important in relation to the pathogenesis of MPGN.

Keywords non-hypocomplementaemia C3 nephritic factor membranoproliferative glomerulonephritis

INTRODUCTION

It is known that MPGN is often associated with hypocomplementaemia [1]. C3NeF and C4 nephritic factor (C4NeF) are autoantibodies of C3 convertase formed by complement activation. Other undetermined factors are involved in hypocomplementaemia [2–7]. C3NeF also stabilizes C3 convertase (C3bBb) of the alternative pathway (inhibition of intrinsic decay) and inhibits the activity of control proteins, factors H and I (inhibition of extrinsic decay) [8,9]. Therefore, C3NeF forms a stable C3 convertase which, together with the amplification system, provides continuous activation of C3, resulting in hypocomplementaemia. However, the relationship between C3NeF and the pathogenesis of MPGN still remains to be elucidated. Although it has been reported that partial lipodystrophy (PLD) is a disease with the presence of both C3NeF and hypocomplementaemic MPGN simultaneously, its detailed characteristics have not been clarified [10]. Detection of C3 splitting factor in C3NeF has been attempted. The criterion for determination of whether this factor is an autoantibody of C3 convertase is its ability to stabilize C3bBb on sensitized sheep erythrocytes. Previously, C3bBb stabilizing activity (C3NeF) has been detected only in hypocomplementaemic sera. In a previous study, we reported a new assay which allowed direct detection of C3NeF without purifying IgG from the patient's serum [11]. Two-hundred and two MPGN patients were studied. Of these, 98 patients were hypocomplementaemic and 104 were non-hypocomplementaemic. We detected C3NeF in 27 of the 202 patients studied. Of these 27 patients, 21 were hypocomplementaemic and six were non-hypocomplementaemic. Therefore, to clarify why complement activation did not occur and the level of C3 did not clearly decrease even in the presence of C3NeF, we compared the C3bBb stabilizing factor which was detected in non-hypocomplementaemia (non-hypo C3NeF) with the C3bBb stabilizing factor in hypocomplementaemia.

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PATIENTS AND METHODS

Samples

From the original group of 202 previously studied MPGN patients, studies were performed on 104 non-hypocomplementaemic patients from 40 hospitals in whom the C3 levels were more than 40% above normal [11]. These patients were diagnosed by renal biopsy as MPGN. Four patients with hypocomplementaemic MPGN (hypocomplementaemic C3NeF) were also studied.

Buffers and reagents

This study was carried out according to the method of Harrison & Lachmann [12]. The following materials were used: isotonic veronal-buffered saline containing 0·1% gelatin (GVB); GVB containing 0·5 mM $CaCl_2$ and 0·5 mM $MgCl_2$ (GVB^{2+}); a mixture of equal volumes of GVB and 5% dextrose in water containing 0·1% gelatin; 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$ ($DGVB^{2+}$); GVB containing 0·04 M EDTA (EDTA-GVB); GVB containing 5% dextrose and 0·3 mM $CaCl_2$ (Ca^{2+} DGVB); and a mixture of equal volumes of 0·01 M TTHA and 5 mM $CaCl_2$ in GVB containing 5% dextrose (Ca^{2+} TTHA-GVB).

Complement components

Factors B and D were purified from fresh human sera according to the method of Harrison & Lachmann [12]. Factor H was purified from fresh human serum as previously described [12]. C2 and C3 were purchased from ChemoSero Therapeutic Research Institute and factor I was purchased from Cordis Co. C-EDTA (C3–C9 reagent) dissolved in guinea-pig serum was diluted 25-fold in 0·04 M EDTA-GBV. Factors H and I were diluted in 0·01 M EDTA-GVB to adjust their concentration so that 100 μl of EAC4b3bBb cells (1×10^8/ml) would decay in 10 min at 30°C. The volume used allowed the haemolytic activity to produce 100% inhibition.

Determination of complement components

Determinations were performed by single radial immunodiffusion using antisera of Clq, C4, C3, B, I (Behring Werke Co.) and C2 and H (Miles Laboratories) [13]. C5 was determined by the rocket immunoelectrophoresis method using antiserum from Behring Werke Co. [14]. The normal serum pool (NHS) value was considered 100% and the results were expressed as per cent normal values. C3d was determined by the Double-Decker Rocket method (15). Inulin-activated NHS incubated at 37°C for 30 min was considered the positive control, while pooled NHS was considered the negative control. The conversion of C3 in the sera from MPGN patients in 0·01 M EDTA was determined by antigen antibody cross-immunoelectrophoresis. The determination of CH_{50} was performed as described by Mayer [16].

Purified IgG preparations

Preparations containing C3NeF were obtained from the serum of each patient by fractionation of diethylaminoethyl (DEAE) Sephacel (Whatman) equilibrated with 20 M phosphate buffer, pH 8·0. The breakthrough fraction was pooled, concentrated to the same volume as the starting material, and dialysed against PBS, pH 7·4, before use. Normal human IgG was prepared from NHS by the same method.

Preparation of cell intermediates

EAC14b cells were prepared from sheep erythrocytes, rabbit anti-sheep erythrocytes and NHS as described by Nagaki *et al.* [17]. EA cells (1×10^9/ml) in Ca^{2+} TTHA-DGVB were incubated with half their volume of NHS for 5 min at 30°C, and then washed several times in Ca^{2+} TTHA-DGVB and CA^{2+} DGVB. They were subsequently incubated for 2 h at 37°C in DGVB.

EAC4b3b cells were prepared by incubating EAC14b cells with appropriate amounts of C2 and C3 for 20 min at 30°C. They were then washed in 0·01 M EDTA-GVB and incubated for 90 min at 37°C in 0·01 M EDTA-GVB. EAC4b3bBb cells were prepared by incubating EAC4b3b cells with limiting amounts of factors B and D for 5 min at 30°C. They were used immediately for EAC4b3bBb stabilization assay.

EAC4b3bBb stabilization assay (C3NeF assay)

This assay was carried out by stabilization of the alternative pathway C3 convertase and directly detects C3NeF without purifying the IgG from the patient's serum. First, 100 μl of EAC4b3bBb cells ($2{\cdot}5 \times 10^8$/ml) in DGVB were mixed with 25 μl of patient's purified IgG or serum, heat-inactivated at 56°C for 30 min and diluted 1:5 in EDTA-GVB for 15 min at 0°C. Then 100 μl of EDTA-GVB were added. Following incubation for 20 min at 30°C, 200 μl of guinea-pig complement diluted 1:25 with 0·04 M EDTA-GVB were added and the mixture incubated at 37°C for 1 h. After centrifugation, the degree of haemolysis was determined by measuring the optical density of the supernatants at 414 nm. The amount of lysis was expressed as a percentage of the control exhibiting complete lysis (100%) [11].

Effects of factors H and I on EAC4b3bBb and C3NeF

For the C3NeF assay, serial dilutions of C3NeF IgG in each patient were prepared. To equalize the stabilizing activities, the C3NeF IgG concentration was adjusted so that 80% of C3bBb sites were stabilized after incubation at 30°C for 20 min. Next, C3NeF IgG and EAC4b3bBb reacted in DGVB for 15 min and were washed twice with 0·01 M EDTA-GVB at 0°C. The resultant mixture was used as the EAC4b3bBb/C3NeF preparation. Then 0·01 M EDTA-GVB or 0·01 M EDTA-GVB with factor H (1 μg/ml) was added separately to the EAC4b3bBb/C3NeF cell preparation adjusted to a concentration of 1×10^8/ml. Each batch was incubated at 30°C and 100 μl were removed at timed intervals. Next 100 μl C-EDTA were added and further incubated for 1 h at 37°C. The haemolytic activity (OD_{414}) was then measured. EDTA-GVB (0·01 M) and 0·01 M EDTA-GVB with several concentrations of factor H were added to a similarly prepared complex of EAC4b3bBb/C3NeF cells to give a concentration 1×10^8/ml at 90 μl. After 15 min at 30°C, C-EDTA was added and the mixture was further incubated at 37°C for 60 min. The degree of haemolysis was then determined using the supernatant after centrifugation based on the OD_{414} using a method similar to the C3NeF assay. We then incubated 100 μl EAC4b3bBb/C3NeF cells similarly prepared in EDTA-GVB with factors H and I at 30°C for 15 min. Residual C3bBb sites were detected as stabilizing activities similar to the method of the C3NeF assay.

RESULTS

Of the 104 non-hypocomplementaemic patients with C3 studied, 40% had levels above normal. Of these, six patients

Table 1. Complement components in non-hypocomplementaemic and hypocomplementaemic MPGN

Patient	Clq	C4	C2	C3	C5	B	H	I	C3NeF
Non-hypo									
1	7	38	90	102	91	77	84	78	+
2	95	44	80	92	60	57	66	83	+
3	170	53	170	86	100	80	108	90	+
4	84	119	87	50	65	113	108	39	+
5	170	74	170	58	93	58	63	69	+
Mean±s.d.	105±68	66±33	119±46	78±22	82±18	77±23	86±22	72±20	
Hypo									
6	50	70	ND	5	8·3	ND	86	ND	+
7	111	96	112	5	51	66	80	86	+
8	100	93	ND	8·5	108·8	78·5	110	ND	+
9	98	22	90	5	30	37	82	81	+
Mean±s.d.	90±27	70±34	101	6±2	50±43	61±21	90±14	84	

Percent normal; ND, not done.

The C3 levels of the patients' sera were determined in five patients (1–5). Very low levels of C3 were not evident. This group is termed non-hypocomplementaemic (non-hypo). Four patients (6–9) in which very low levels of C3 were evident are regarded as hypocomplementaemic. In the non-hypo group, the levels of C3 varied from 50% to 102% with respect to that of pooled normal serum. In the hypo group, the levels of C3 were clearly low in all four patients.

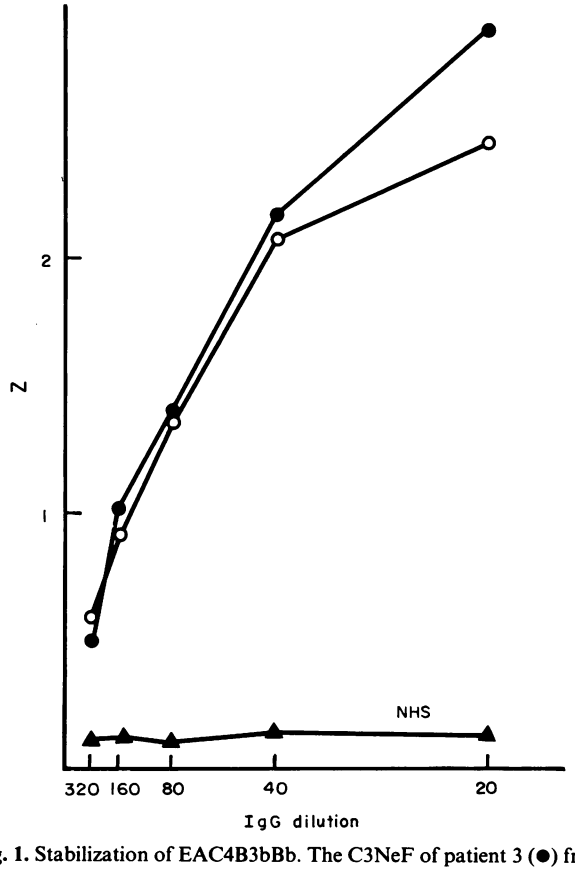

Fig. 1. Stabilization of EAC4B3bBb. The C3NeF of patient 3 (●) from the non-hypocomplementaemic group, who had 85% normal C3, and that of patient 6 (○) from the hypocomplementaemic group, who had less than 5% normal C3, were compared. The IgG fraction of the sera in both cases was collected by DEAE Sephacel ion exchange chromatography. Based on this dilution, the stabilizing activities were adjusted to the same activity for the subsequent studies.

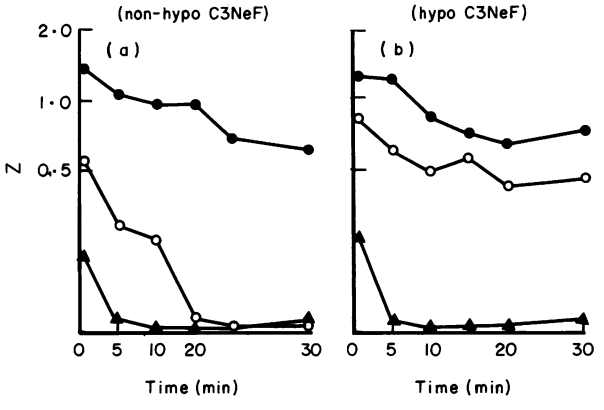

Fig. 2. Effect of factor H on EAC4b3bBb/C3NeF. In the two patients shown (patient 3 (a), patient 6 (b)), the decay of EAC4b3bBb cells in EDTA-GVB and in EDTA buffer with factor H was determined against time. In both patients, in EDTA-GVB alone the C3 convertase on the cell surface was stabilized even after incubation at 30°C for 30 min. But in the presence of factor H in EDTA-GVB the decay of C3 convertase was accelerated and no haemolysis was noted after incubation at 30°C for 20 min in patient 3 (a). As a control, the decay kinetics of cell-bound C3bBb in the presence of factor H is shown in EDTA-GVB+normal human IgG+factor H. EDTA-GVB+C3NeF IgG; EDTA-GVB+C3NeF IgG+factor H; EDTA-GVB+normal human IgG +factor H.

showed positive C3bBb stabilizing activity (C3NeF). The complement components in five of these six patients (the sixth patient had moved from the area and was unavailable for study) with C3NeF non-hypocomplementaemia demonstrated a C3 level from 50% to 102%. The level of CH_{50} was found to be within the normal range in each case. In these five patients, no C3 cleaving activity was observed in the fluid phase by cross-immunoelectrophoresis (data not shown). When compared with

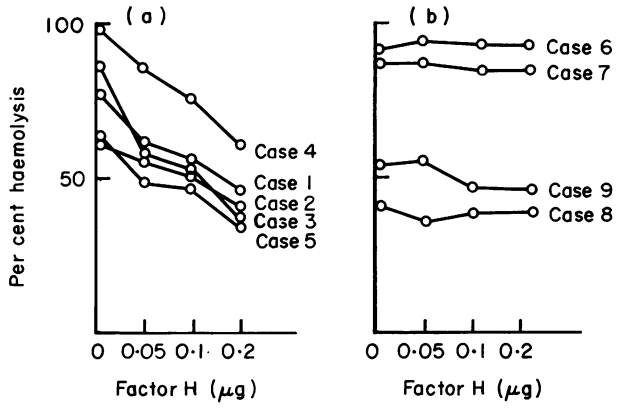

Fig. 3. Effects of various doses of factor H on EAC4b3bBb/C3NeF. (a) C3NeF from non-hypocomplementaemic MPGN. (b) C3NeF from hypocomplementaemic MPGN. All five non-hypocomplementaemic C3NeF and all four hypocomplementaemic C3NeF were tested by administering factor H for a time period of 15 min. A similar tendency was noted in all the non-hypocomplementaemic C3NeF. C3bBb sites were decayed dose-dependent on the amount of factor H. However, in the hypocomplementaemic C3NeF, the C3 convertase did not show any decay following the addition of factor H.

Table 2. Per cent inhibition by factor H on EAC4b3bBb/C3NeF in EDTA-GVB

	C3bBb stabilizing activity		
Patient	EDTA-GVB	H	Per cent inhibition
Non-hypo			
1	77	45·7	40·6
2	61	40·0	34·4
3	85·7	37·7	56
4	98	61·7	37·1
5	61·3	36·3	40·8
Hypo			
6	91·3	100	0
7	91·3	87	0
8	45·3	46·3	0
9	53·3	45·3	15·0

Patients 1–5 are the non-hypo patients with the per cent normal C3 values shown in Table 1. Patients 6–9 are the hypo patients with markedly low levels of C3. The per cent haemolysis values upon reaction in EDTA buffer and in the presence of factor H are expressed as the stabilizing activity. The inhibition of the activity in EDTA buffer with factor H in all patients was calculated as the per cent inhibition. As can be seen, 30–50% inhibition was observed in the non-hypo patients, while there was little or no inhibition in the hypo patients.

Table 3. Per cent inhibition by factors H and I on EAC4b3bBb C3NeF in EDTA-GVB

	C3bBb stabilizing activity				
Patient	EDTA-GVB	H	I	H+I	Per cent inhibition
Non-hypo					
1	76	56	77	35	55
2	86	56	86	50	43
3	75	57	76	49	35
Hypo					
6	98	95	99	86	12
7	96	86	92	79	17

The C3bBb stabilizing activities of three non-hypo patients and two hypo patients were further investigated following addition of factor I. Even when factors H and I were added simultaneously, the C3NeF of the non-hypo patients was inhibited, but that of the hypo patients was not.

fresh NHS and using Double-Decker Rocket immunoelectrophoresis, no increase in C3d was noted (data not shown). In the hypocomplementaemic patients, the C3 levels were all below 10% and the CH_{50} levels were below 10 units (Table 1).

We then compared the sera of patient 3 (non-hypo C3NeF) with the sera of patient 6 (hypo C3NeF). The IgG fractions of these samples were adjusted so that their C3NeF activities were similar (Fig. 1). The effect of C3NeF IgG on the decay of EAC4b3bBb cells in 0·01 M EDTA-GVB was investigated. In both samples (in EDTA buffer), the C3 convertase on the cell surface was stabilized and haemolysis was evident at 30°C for 30 min. Following addition of factor H, the decay of C3bBb sites was accelerated and after 20 min no haemolysis was noted for the C3NeF IgG in the non-hypocomplementaemic patient. Decreased haemolysis was observed after 20 min, but the decay of C3bBb sites was not accelerated for the C3NeF IgG in the hypocomplementaemic patient (Fig. 2). The effect of factor H on EAC4b3bBb/C3NeF complex formed from C3NeF of the other hypocomplementaemic and non-hypocomplementaemic patients was determined from the haemolytic ratio following addition of factor H at various doses and incubated for 15 min. No changes in haemolytic ratio were observed for the hypocomplementaemic patients, while the haemolytic ratio underwent a dose-dependent decrease with factor H in the non-hypocomplementaemic patients (Fig. 3).

We then estimated the percentage inhibition of the stabilizing activity after incubation of EAC4b3bBb/C3NeF complex at 30°C for 20 min in EDTA-GVB and in EDTA-GVB with factor H. In non-hypocomplementaemic patients, the per cent inhibition was 34–56%, but in the hypocomplementaemic patients, it was 0% except for one patient who showed a value of 15% (Table 2). Next, the per cent inhibition after addition of factors H and I was compared between three non-hypocomplementaemic patients and two hypocomplementaemic patients. Upon addition of factor I, no inhibition was observed, while upon addition of both factors H and I, 35–55% inhibition was noted in the non-hypocomplementaemic patients as compared with 12% and 17% in the hypocomplementaemic patients (Table 3).

DISCUSSION

We have devised a new method for detecting C3NeF directly [11]. This method allowed us to screen for the presence or absence of C3NeF in sera more easily than previous methods. As a result, even in the sera which did not show very low hypocomplementaemia, C3bBb stabilizing activity (C3NeF) was detected in some samples.

No increase in the level of C3d was observed in these non-hypocomplementaemic samples. Furthermore, no conversion of C3 was noted and their CH_{50} values were within normal limits. In spite of the presence of C3NeF, there was no evidence of complement activation in these samples. Therefore, we conducted a study to clarify why very low C3 levels and complement activation did not occur despite the presence of C3NeF. Even with purified IgG from these samples, a clear stability of C3bBb on the surface of sensitized erythrocytes was observed. To clarify the nature of the C3NeF in five patients with non-hypocomplementaemia, their C3NeF levels were compared with those in the hypocomplementaemic samples. No differences were observed in terms of the stabilization of EAC4b3bBb in EDTA. This indicated that they had identical characteristics in relation to the inhibition of the intrinsic decay of C3bBb.

First, we compared the C3NeF which possessed similar C3bBb stabilizing activities in very low hypocomplementaemic and non-hypocomplementaemic patients. In both instances, the IgG revealed almost the same level of activity. Following addition of factor H after the formation of EAC4b3bBb/C3NeF complex, the C3bBb stabilizing activity of C3NeF IgG was inhibited in the non-hypocomplementaemic patients, but no such inhibition was observed in the C3NeF IgG of the hypocomplementaemic patients and haemolysis occurred instead. These findings suggest that non-hypocomplementaemic C3NeF IgG does not inhibit the extrinsic decay of C3bBb, whereas hypocomplementaemic C3NeF IgG does possess this inhibiting function. Similarly, in other cases differences in characteristics were observed between non-hypocomplementaemic and hypocomplementaemic C3NeF.

Comparison of the effects of addition of factor H in varying amounts to non-hypocomplementaemic and hypocomplementaemic C3NeF revealed that in non-hypocomplementaemic patients, the C3bBb stabilizing activity was inhibited in a dose-dependent manner by factor H with a decrease in haemolytic activity. On the other hand, with hypocomplementaemic C3NeF, a stabilizing effect for EAC4b3bBb cells was observed independently of the concentration of factor H. By determining the per cent inhibition in hypocomplementaemic and non-hypocomplementaemic patients following addition of factor H in EDTA-GVB, distinct differences were observed. Even when both control proteins, factors H and I, were added, a difference in the per cent inhibition between the groups was evident. This phenomenon indicates that the C3NeF found in hypocomplementaemia can inhibit both the intrinsic and extrinsic decay, exhibiting the typical characteristics that C3NeF was previously known to possess. However, the C3NeF found in non-hypocomplementaemic patients can cause inhibition of the intrinsic decay in the same manner as the hypocomplementaemic C3NeF, but cannot inhibit the extrinsic decay of C3bBb. For this reason, since control proteins, factors H and I are present in sera even when C3bBb/C3NeF is formed, Bb is broken by the control proteins and activation of C3 does not occur. Also, we found that C3NeF from different patients caused varying degrees of protection from factor H inhibition (Table 2). The differences in the degree of susceptibility of various C3NeF-stabilized C3bBb to factor H are attributable to variations in the different populations of C3NeF C3bBb. Although very low inhibition was found in the hypocomplementaemic MPGN patients (Table 2), we hypothesize that there may be two or more types of C3NeF involved. These results are compatible with previous reports that C3NeF binds C3bBb not only to Bb but also to C3b in some cases [18].

There are forms of C3NeF which are either properdin-dependent or properdin-independent, and relationships with these factors need further investigation [19]. We conclude, therefore, that the C3NeF from hypocomplementaemic patients may include IgG which recognizes the Bb in the binding site of factor H, whereas the C3NeF from non-hypocomplementaemic patients may bind to Bb competitively with factor H. While C3NeF is the C3bBb autoantibody, there is more than one epitope in C3bBb and a variable degree of affinity to C3bBb which might produce variable characteristics in antibodies. As with hypocomplementaemic C3NeF, the relationship between MPGN and non-hypocomplementaemic C3NeF is still unclear. However, it is possible that under certain conditions non-hypocomplementaemic C3NeF may not be influenced by factors H or I locally in the kidney, contributing to complement activation. The C3NeF found in non-hypocomplementaemic MPGN, like the C3NeF found in hypocomplementaemic MPGN, needs further investigation. Further investigation of the detailed relationships between the pathogenesis of MPGN and autoimmunity is also necessary [20–24].

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