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COMPLEMENT STUDIES IN MEMBRANO-PROLIFERATIVE GLOMERULONEPHRITIS

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SUMMARY

Detailed studies of the complement system were carried out in fifteen patients with membranoproliferative glomerulonephritis. The findings of reduced levels of C3 and C7 and of circulating breakdown products of C3 in fresh plasma suggested *in vivo* complement activation. Low C3 levels were associated with the presence of a serum factor (the C3 nephritic factor C3NeF) which was capable of breaking down C3 in normal serum *in vitro*. Metabolic studies using radioactive iodine labelled C3 showed no evidence of accelerated *in vivo* breakdown of parenterally administered C3 suggesting that hypocomplementaemia is either maintained by diminished C3 synthesis or that accelerated catabolism is occurring in a pool that does not freely exchange with parenterally given C3. The C3 nephritic factor has so far only been identified in patients with membranoproliferative nephritis and is therefore of major diagnostic significance in patients with glomerular disease.

INTRODUCTION

The widespread use of percutaneous renal biopsy has led to the separation of a group of patients with subacute or chronic glomerulonephritis, termed membranoproliferative glomerulonephritis (MPGN) or mesangiocapillary nephritis (Royer *et al.*, 1962; Churg, Habib & White, 1970; Habib, 1970; Cameron *et al.*, 1970). The distinctive histological appearances that define this disorder are a characteristic complex of thickening of the glomerular basement membranes, together with mesangial cell proliferation and sclerosis. Patients usually present in childhood or adolescence with a nephrotic syndrome, but some cases present as symptomless proteinuria or haematuria, and the same histological appearances are occasionally seen in patients who seemed originally to have post-streptococcal glomerulonephritis. A detailed review of the clinical and histological features of this condition was presented by Cameron *et al.* (1970). Apart from the characteristic histo-

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logical changes, the most distinguishing feature of these patients is that the majority show marked and prolonged reduction in the serum total haemolytic complement, due particularly to low levels of the third component, C3. This finding has led to the use of the term 'hypocomplementaemic persistent nephritis' in association with these patients (West *et al.*, 1965).

The cause of hypocomplementaemia in this condition remains uncertain. Turnover studies using radiolabelled C3 have given conflicting results, some suggesting impaired C3 synthesis as the major factor (Alper & Rosen, 1967), but others have shown accelerated C3 disappearance (Herdman *et al.*, 1970). In addition Spitzer *et al.* (1969) have described a serum factor (the so-called C3 nephritic factor) in a patient with this condition which breaks down C3 in serum *in vitro*. The purpose of the present paper is to describe detailed studies of the complement system, including turnover studies of radiolabelled C3, in a group of patients with membranoproliferative glomerulonephritis, and to confirm the finding of the nephritic factor, in some (but not all) patients with the characteristic histological appearance of this condition.

METHODS

Patients

Fifteen patients were studied. All had the characteristic changes of membranoproliferative glomerulonephritis on renal biopsy. Ten patients were included in the group published previously by Cameron *et al.* (1970). One patient was of particular interest because bilateral nephrectomy and then transplantation had been carried out over one year previously (see Fig. 8, Cameron *et al.*, 1970).

Complement components

The serum levels of C1q, C3 and C4 were measured by radial immunodiffusion against monospecific antisera. The anti-C1q was kindly donated by Professor John Hardwicke.

C6 levels were determined using a haemolytic technique (Tedesco & Lachmann, 1971). C7 levels were estimated by a haemolytic method based in the reactive lysis phenomenon (Thompson & Lachmann, 1970; Lachmann & Thompson, 1970). This system utilizes the principle that unsensitized guinea-pig red cells undergo lysis when mixed with a preparation of activated C56 and the remaining complement components. The degree of lysis is related to the concentration of C7 present in the test serum.

A similar system using unsensitized guinea-pig erythrocytes and purified C56 and C7 was employed to measure C8 and C9.

C3 breakdown in plasma

In three patients fresh EDTA plasma was tested for C3 breakdown products by carrying out crossed antibody electrophoresis in EDTA buffered agarose using a monospecific antiserum to C3.

C3 nephritic factor

The presence of a factor which would break down C3 in normal human serum was detected by incubation of the test serum with an equal volume of normal human serum (NHS) for 1 hr at 37°C. The incubation mixture was then assayed for C3 degradation

products by performing crossed antibody electrophoresis. In each assay the following controls were included—incubation of the same mixture at 4°C, incubation of the nephritic serum alone at 37°C, and incubation of normal human serum at 37°C for 1 hr. In preliminary experiments the observation of Vallota *et al.* (1970) that the reaction is inhibited by 0·01 M EDTA was confirmed, but the addition of the same concentration of EDTA after 10 min incubation did not prevent C3 conversion. This procedure minimized breakdown of C3 at 37°C and the addition of EDTA in this way was therefore carried out routinely in subsequent tests.

Ability of MPGN serum to generate cobra factor convertase (kindly performed by Miss P. Nicol, Department of Pathology, Cambridge).

The serum of one markedly hypocomplementaemic patient (with a high titre of C3NeF) was tested for C3 proactivator activity by incubation with highly purified cobra venom factor.

The convertase activity generated in the incubation mixture was tested by the addition of fresh plasma containing 0·01 M EDTA, and measuring the C3 breakdown using crossed antibody electrophoresis.

Glycine rich β glycoprotein (GBG) assay

The level of the glycine rich β glycoprotein in this patient's serum was measured by a Mancini technique using the commercially available rabbit antiserum to human β_2 glycoprotein II, (Behringwerke Ag.) Immunoelectrophoresis was also carried out against this antiserum to determine if the glycoprotein was in the form of the β glycoprotein (GBG) or the breakdown products which have α and γ electrophoretic mobilities.

Fractionation of nephritic serum

Fractionation of serum was carried out on sephadex G-200 in 0·2 M phosphate buffered saline pH 7·2 and on DEAE cellulose using 0·015 M phosphate buffer pH 8·0.

C3 metabolic studies

C3 was prepared from the euglobulin fraction of fresh serum by chromatography on DEAE cellulose, and purified on hydroxyapatite using sterile procedures throughout. Details of the procedures are given by Aston, Hobart & Lachmann (in preparation).

The protein was labelled with radioactive iodine by the iodine monochloride technique at a substitution ratio of less than 0·5 atoms of iodine to 1 molecule of C3. This procedure did not affect the haemolytic activity of the product. The radiolabelled protein was injected intravenously in a dose of approximately 30 μCi of ^{125}IC3 or 15 μCi of ^{131}IC3 and samples of blood and urine were collected over a period of 9 days. The protein-bound and free radioactivity were determined by precipitation of the samples with an equal volume of 20% trichloroacetic acid.

RESULTS

The results of the complement component assays are shown in Fig. 1. The levels of the early components (C1q and C4) were normal in all patients. C3 levels were markedly depressed in eight patients, but the remaining seven patients had C3 levels which were normal

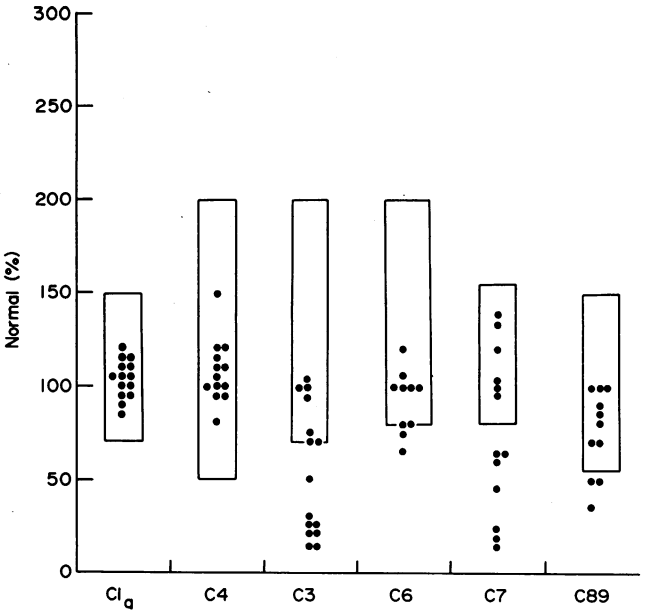

FIG. 1. Serum level of the complement components in MPGN. The boxed area indicates the normal range. The values are expressed as a percentage of a pooled normal standard.

or only slightly reduced. Levels of C7 were more often reduced in those patients with low C3 levels than in patients with normal C3 values, but there seemed to be no relationship between C3 levels and the levels of C6 or C8 and C9.

The results of the turnover studies using radiolabelled C3 are shown in Table 1. Analysis of the excretion of free radioactivity and the plasma protein-bound radioactivity curves suggested that approximately 20% of the injected protein was rapidly catabolized (in patients and normal controls) and this was presumed to reflect denaturation of the molecule during preparation or labelling. For this reason conventional compartmental analysis of the plasma protein bound radioactivity curves was not carried out. Instead, the results have been expressed in terms of the half-life of the final exponential slope of plasma protein-bound radioactivity, and as the fractional catabolic rate (FCR) using the excretion of free radioiodine in the urine after the first 48 hr. In one patient with end-stage renal

TABLE 1. C3 turnover studies

Patient	C3 (mg/100)	$T\frac{1}{2}$ (Hours)	FRC/hour	C3NeF
A.W.	15	52	0·020	+
M.L.	10	54	0·028	+
C.M.	85	57	0·024	−
A.S.	30	56	—	+
Normal	90–200	45–70	0·010–0·030	

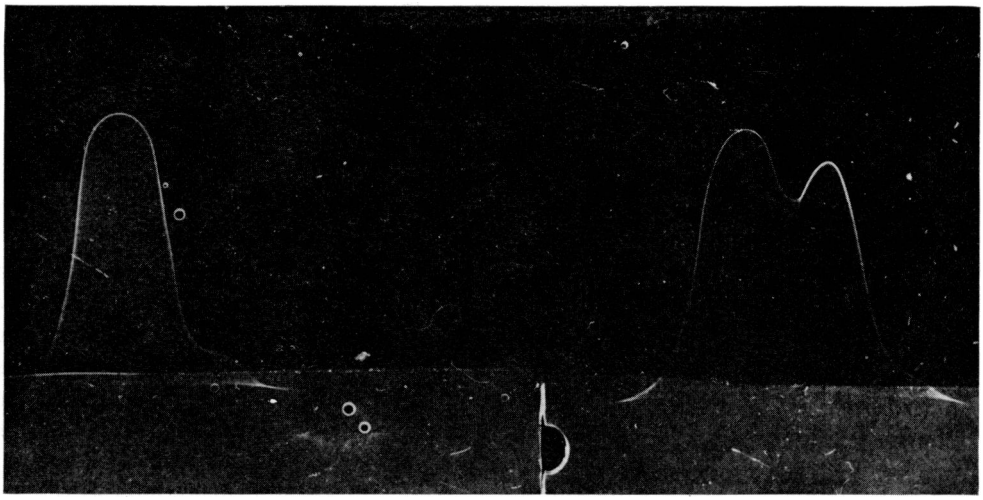

FIG. 2. Crossed antibody electrophoresis of normal serum, and normal serum after incubation with nephritic serum.

failure being treated by peritoneal dialysis, the turnover measurements were limited to the half-life. Table 1 shows that the half-lives and fractional catabolic rates were not significantly different from normal nor was there any difference between two patients (ML and CM) studied simultaneously (one of whom showed markedly depressed C3 levels and circulating C3 nephritic factor, and the other only slightly reduced C3 levels and no detectable C3 nephritic factor). The absolute catabolic rate (which is the product of the fractional catabolic rate and the intravascular mass of C3) was therefore markedly depressed in the hypocomplementaemic subjects. Since the patients were in a steady state, the absolute catabolic rate is equal to synthesis rate, and this therefore appeared to be markedly depressed

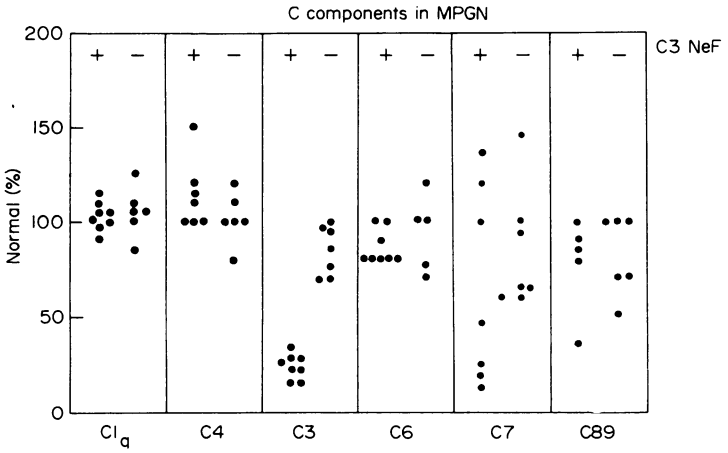

FIG. 3. Relationship between the presence of detectable nephritic factor (C3NeF) and the serum levels of the complement components.

in the three subjects with low C3 levels. Counting of urine protein-bound radioactivity showed that urinary C3 losses made no significant contribution to the overall turnover rates.

C3 breakdown products were detected in all three subjects in whom fresh plasma was available for testing.

The serum of fifteen patients was tested for the presence of C3 nephritic factor (C3NeF). An example of the effect of incubating serum from a patient with membranoproliferative nephritis with normal human serum is shown in Fig. 2. Significant breakdown of C3 could be detected when nephritic serum was incubated with normal human serum. The addition of nephritic serum to highly purified C3 did not result in C3 degradation. A C3NeF was demonstrable in the serum of seven of the fifteen patients. The relationship of this factor to the complement assays is shown in Fig. 3. The nephritic factor was only found in those patients with marked depression of C3 levels but there was no obvious correlation between the presence of C3NeF and the levels of the other complement components, although low levels of C7 were found in some patients with detectable C3NeF.

One hypocomplementaemic C3NeF positive serum showed a normal ability to generate cobra factor convertase. The GBG level was also normal in this serum, and immunoelectrophoresis showed that the material was of β mobility.

Fractionation of nephritic serum on Sephadex G-200 and DEAE cellulose suggested that the activity was associated with the second peak on G-200 and is eluted with IgG on DEAE cellulose.

DISCUSSION

Three features of interest emerge from these observations; namely evidence that the complement system is activated *in vivo*, the demonstration of a substance in the serum of these patients which breaks down C3 in normal human serum *in vitro* and the interpretation of the turnover studies.

The finding of reduced levels of C3 and of the later complement components such as C7 and C8 and 9, together with breakdown products of C3 in fresh plasma suggest that *in vivo* activation of the complement system is taking place, at least in some patients. Marked reduction of C3 levels was only observed in patients with demonstrable C3NeF suggesting that this factor is responsible for hypocomplementaemia.

The observation that C3NeF does not act on highly purified C3 confirms the suggestion of Vallota *et al.* (1970) that the production of a C3 lytic effect depends on more than one reaction. First a factor in nephritic serum reacts with a substance (or substances) present in normal human serum (termed co-factor(s)). It must be postulated that one at least of such co-factors is missing in the nephritic serum to account for its failure to convert added pure C3. The product of this reaction which can be called 'nephritic C3-convertase' then breaks down C3.

It is at first sight hard to reconcile these findings with the substantially normal disappearance rate of radiolabelled C3 found in the present study and reported by Alper & Rosen (1967). It is unlikely that the normal C3 turnover values can be explained by denaturation as a result of labelling. Labelling of C3 with radioiodine has been shown to be satisfactory *in vitro* (Müller-Eberhard, 1968) and the preparations used in our patients were shown to be biologically active by haemolysis and conglutination. The finding that

C3 breakdown products are cleared quickly (Alper & Rosen, 1967) also disposes of an alternative explanation, namely that the normal disappearance rates are the result of persistence of C3 breakdown products in the circulation. While small increases in catabolic rate might not be detected by the turnover technique, it is hard to accept that the marked depression of C3 levels (sometimes by a factor of 10 or greater) could result from accelerated breakdown of C3 without an obvious decrease in half-life, or an increase in the fractional catabolic rate of the labelled protein. The findings therefore suggest defective C3 synthesis as the major factor responsible for maintenance of the low C3 levels. Such impairment of synthesis may be the result of a secondary inhibition by C3 breakdown products or by another (as yet unidentified) mechanism.

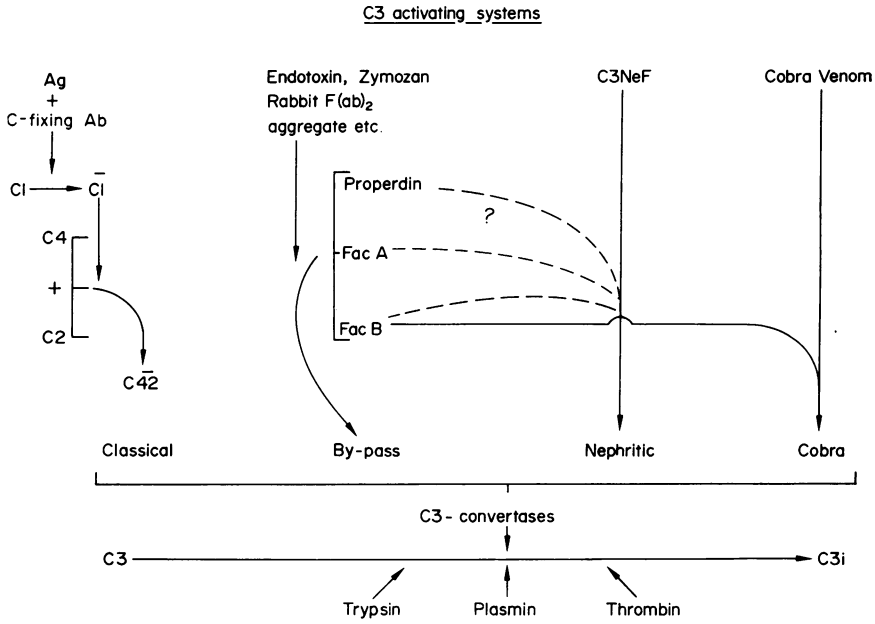

FIG. 4. Systems (including MPGN) by which C3 is known to be activated.

Another attractive hypothesis which would also reconcile the evidence for complement activation and the presence of C3NeF with normal disappearance rates of iodine labelled C3 is that accelerated catabolism is occurring in a pool not freely exchangeable with parenterally administered C3. This would require that the co-factor(s) C3 and C3NeF are generated in this pool. The reaction between C3, co-factor(s) and nephritic factor would occur using up all of at least one co-factor. Radiolabelled C3 administered intravenously would then have a normal survival but an excess of the C3NeF would also be detected in the serum.

Whichever hypothesis is correct the evidence suggests that the action of nephritic factor on C3 is not mediated through the early complement components. A number of substances have been identified which may activate the complement system at the C3 stage (the so-called alternate pathway or by-pass) (Fig. 4). These include bacterial endotoxin

(Gewurz *et al.*, 1970), a factor derived from cobra venom (Pickering *et al.*, 1969), the $F(ab)_2$ portion of the rabbit and guinea-pig IgG molecule (Oliveira *et al.*, 1970; Sandberg *et al.*, 1970; Reid, 1971) and IgA aggregates (Gotze & Müller-Eberhard, 1971). The alternate pathway is known to require the C3 proactivator (C3PA) as one component: this is believed to be identical with Factor B of the properdin system and may be identical with the glycine rich β_2 glycoprotein II (GBG) described by Haupt & Heide (1965), and independently isolated by Boenisch & Alper (1970). Abnormalities of the properdin system have been described in patients with MPGN: low serum levels of properdin were found by Gewurz *et al.* (1970) and Westburg *et al.* (1971) showed that properdin deposits were present in the glomeruli of patients with this disorder. The levels of C3PA and GBG were normal in one hypocomplementaemic patient. It is therefore evident that the depleted co-factor in nephritic serum is not C3PA and that C3NeF plus C3PA cannot alone give rise to a C3 convertase (i.e. it seems that C3NeF cannot be the C3PA convertase postulated by Gotze & Müller-Eberhard).

So far there is little information on the nature of the nephritic factor. Vallota *et al.* (1970) have reported that C3NeF is associated with the pseudoglobulin fraction of serum, and Thompson (1971) has evidence suggesting that the factor may be an IgG molecule of subclass 3. We similarly found maximum activity in the ascending region of the second peak on Sephadex G-200 and the activity was also eluted with IgG on DEAE cellulose.

Of major importance is the relationship between C3NeF and glomerular damage in MPGN. Immunofluorescent studies of the kidney in MPGN have shown that IgG and C3 are deposited in the glomeruli in a distribution similar to that of known immune complex disease (Berger, Yaneva & Hinglais, 1971; Herdman *et al.*, 1970; Kuyten, 1971; personal communication). It is therefore tempting to suggest that the factor is an immune complex; if so the fractionation experiments suggest that it is of unusually low molecular size. C3 breakdown is known to result in the liberation of a small molecular weight fragment (C3a) with chemotactic and anaphylatoxic activity (Bokisch, Müller-Eberhard & Cochrane, 1970). It is conceivable that altered vascular permeability produced by such a mechanism contributes to the glomerular localization of immune complexes such as has been shown in experimental immune complex disease (Cochrane, 1971). It seems unlikely that the C3NeF is the result of kidney disease; one of the patients had a bilateral nephrectomy and two others remained on haemodialysis without significant changes in low C3 levels. C3NeF has so far only been identified in the serum of patients with MPGN; the factor has not been detected in other hypocomplementaemic situations such as acute post-streptococcal nephritis or systemic lupus erythematosus.

A detailed review of the histological and clinical findings in our patients with MPGN did not show any differences between those patients with, and those without, detectable nephritic factor, but those with nephritic factor had more prolonged depression of C3 levels, and these were low at the time of study. The precise pathogenetic relationship of the C3 nephritic factor to MPGN is therefore not established. Of interest will be studies of individual patients relating the appearance and level of the C3NeF to C3 levels and disease activity, and to the development of nephritis in allografted kidneys. Whatever the pathogenetic significance of the C3 nephritic factor is, it is of importance in terms of its contribution to our understanding of the mechanisms by which the complement system may be activated. In addition the detection of this serum factor seems to be of major diagnostic significance in patients with glomerular disease.

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