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Research Article

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Continuing C3 Breakdown after Bilateral Nephrectomy in Patients with Membrano-Proliferative Glomerulonephritis

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ABSTRACT Serum levels of complement components and of C3 nephritic factor (C3NeF) were measured serially in two patients with membrano-proliferative glomerulonephritis who were subjected to bilateral nephrectomy and maintained by peritoneal dialysis for 2 wk before renal transplantation. In both patients, low levels of C3 and high levels of preformed alpha 2D, a C3 breakdown product, were present before nephrectomy and remained essentially unchanged during the anephric period. With transplantation, C3 levels rose towards normal and alpha 2D disappeared from the serum. The serum of both patients contained detectable amounts of C3NeF, a factor which has been shown to react with a cofactor found in normal serum to form an enzyme, designated C3 lytic nephritic factor (C3LyNeF), which will cleave C3 to form the breakdown products, β1A and alpha 2D. The level of C3NeF was high in one patient before nephrectomy, increased somewhat during the anephric period, and fell after transplantation. In the other patient, the C3NeF level was initially lower, remained relatively constant during the anephric period, and was not significantly affected by transplantation. In both patients, levels of C4 and C5 were either normal or elevated over the period of the study and bore no relationship to the C3 level. The following conclusions can be drawn from the data. The high levels of alpha 2D during the anephric period and the disappearance of this protein as C3 levels approach normal at the time of transplantation indicate that the low C3 levels were largely the result of C3 breakdown rather than diminished synthesis. The presence of C3NeF in detectable amounts in both patients suggest that C3LyNeF, formed by the reaction of C3NeF and cofactor, was responsible for the low C3 levels. Finally, the lack of effect of nephrectomy on C3, alpha 2D, and C3NeF levels indicate that the site of C3 breakdown was extrarenal and that C3NeF and cofactor are at least in large part of extrarenal origin.

INTRODUCTION

Membrano-proliferative glomerulonephritis, also designated progressive or hypocomplementemic persistent glomerulonephritis, is characterized by distinctive changes in glomerular morphology and, usually in children, by a marked and prolonged hypocomplementemia (1-3). Labeled antibody studies show that glomerular-bound C3 is consistently present and at times C1q and IgG can be demonstrated (4). The cause of the marked and prolonged hypocomplementemia is not established; it is often of such duration and severity that explanations commonly accepted for other types of hypocomplementemic glomerulonephritis appear inapplicable (1).

The hypocomplementemia in other types of nephritis is usually ascribed to in vivo activation of complement by glomerular-bound immune complexes (5-8). In several types of experimental nephritis in animals, the presence of such complexes in the glomeruli and their role in the production of glomerular inflammation has been well documented (9-13). In humans, hypocomplementemic nephritides in which glomerular immune complexes are demonstrable are those accompanying serum sickness, prolonged sepsis, and systemic lupus erythematosus, as well as the nephritis which follows a streptococcal infection (4, 14-16). That complement activation by an immune complex is instrumental in producing the glomerular inflammation in these diseases is supported by the observation that in disseminated lupus, the presence of immunologic glomerular injury can be correlated with the complement-fixing activity of the serum antinuclear antibody; nephritis occurred in those patients with

antinuclear antibody of high complement-fixing activity (17). Although these observations provide evidence that in these diseases the reaction of complement with glomerular-bound immune complexes is responsible for the glomerular inflammation, mediation of nephritis by non-complement factors cannot be excluded (18). Whatever the cause of the inflammation, the hypocomplementemia is best explained by the reaction of complement with immune complexes at intrarenal and possibly also extra-renal sites.

In membrano-proliferative nephritis, several factors would be eligible to account for the low serum levels of complement. One possibility is activation of complement by immune complexes as in the above hypocomplementemic nephritides. In support of this are the granular deposits of IgG and C1q which can at times be demonstrated in the glomeruli with labeled antibody and the electron-dense subendothelial deposits demonstrable by electron microscopy (4). A second possibility is decreased synthesis of C3. Diminished synthesis of C3 was indicated by the observation that the disappearance rate of radiolabeled C3 injected in hypocomplementemic patients with this disease was often normal (19). However, use of the same technique by others (20) as well as measurements of C3 breakdown products in serum (8) have given contrary results, providing no evidence for diminished synthesis. A third possibility is urinary excretion of complement components but significant excretion rates have not been demonstrable (3, 8, 19). Finally, complement-reactive factors present in the circulation could be responsible. An inactivator directed at one or more of the six terminal components of guinea pig complement has been demonstrated by Pickering, Gewurz, and Good (21) in the serum of patients with membrano-proliferative nephritis. More recently, work in this laboratory (22, 23) has indicated that a substance resembling closely the cobra venom factor (24, 25) in its reactivity with C3 can be demonstrated in the serum of these patients. This substance is a preenzyme designated the C3 nephritic factor or C3NeF. It has been shown to combine in the presence of Mg^{++} with a cofactor found in normal human serum, presumably to form a complex specifically reactive with C3 designated C3 lytic nephritic factor, or C3LyNeF. This complex will enzymatically cleave C3 to the breakdown products, alpha 2D and β1A.

The present study sheds some light on these alternatives by providing evidence that complement inactivation in the kidney contributes little or nothing to the hypocomplementemia in membrano-proliferative nephritis. Two patients with this disease rendered anephric before transplantation continued to have hypocomplementemia and evidence of C3 inactivation during the anephric period. The presence of C3NeF in the serum of both patients gave suggestive evidence that this factor was responsible for the hypocomplementemia.

METHODS

Serial serum samples obtained from two patients with membrano-proliferative nephritis and hypocomplementemia were allowed to clot at 25°C and the serum separated after centrifugation at 4°C. Serum specimens were stored at −70°C.

Measurement of C3. C3 and the other complement components were measured by the immunoelectrophoretic precipitin method (26). Details of the use of this method for measurement of C3 were described previously (8, 27). Results are expressed as the β1A globulin content of aged serum. The lower limit of the normal range for β1A globulin is 90 mg/100 ml and the average level, 150 mg/100 ml.

Measurement of C4. Antiserum used for measurement of C4 (formerly designated as β1E globulin) by the immunoelectrophoretic precipitin method was obtained from a goat immunized with beta and gamma globulins isolated from human serum by continuous flow electrophoresis. To remove antibody to other proteins, the antiserum was absorbed before use with a solution of Cohn fraction IV. This fraction was found to be devoid of C4. The precipitin arc resulting when the absorbed antiserum was reacted with whole serum was identified as representing C4 by reaction of identity with the arc produced by a monospecific rabbit anti-C4 serum kindly supplied by Dr. Hans J. Müller-Eberhard. To determine the end point in the quantitation, the supernatants obtained from the graded addition of serum to antiserum were reacted against the C4 contained in Cohn fraction III-2,3. A solution of this fraction was prepared by adding to each milliliter of a barbital buffer, pH 8.6, 25 mg of the Cohn fraction, incubating 30 min at 37°C, and centrifuging. The supernatant, diluted 1:10, was electrophoresed in agar. This method and that for C5 described below was calibrated in milligrams by use of a serum containing known concentrations of C4 and C5 kindly supplied to us by Dr. Peter J. Kohler.

Measurement of C5. The antiserum to C5 (formerly designated as B1F globulin) was obtained from goats immunized with an impure preparation of human C3 (28). Supernatants resulting from the graded addition of serum to this antiserum were reacted against C5 contained in a solution of human euglobulin which had been electrophoresed in agar. To eliminate interference by the C3 arc in reading the end point, the C3 in the euglobulin was removed before use by absorbing the preparation with a monospecific goat antiserum to C3. The absorbed euglobulin preparation was stored at −70°C.

Measurement of preformed alpha 2D. This C3 breakdown product was measured in aged serum and the results expressed in arbitrary units as described previously (8). As noted in that publication, the alpha 2D of aged serum comprises that formed from breakdown of C3 during aging and that which is preformed by in vivo breakdown of C3 and present in the blood at the time it is drawn. In normal serum there is no preformed alpha 2D (8) but it may be relatively abundant in the serum of patients with hypocomplementemic nephritis. To calculate the concentration of circulating preformed alpha 2D, use was made of the fact that when normal serum containing no preformed alpha 2D is allowed to age in vitro, breakdown of C3 causes formation of alpha 2D corresponding to approximately 21 U of D antigen per mg of β1A formed (8). This ratio, desig-

TABLE I

Method of Calculation of Circulating Preformed Alpha 2D
(Data from Case 2)

Serum specimens	(1) Total D antigen, aged serum	(2) A β1A, aged serum	(3) R (1)/(2)	(4) R×A (3)×(2)	(5) Preformed alpha 2D (1) − (4)
	U/100 ml	mg/100 ml			U/100 ml
Serum lacking preformed alpha 2D*					
4/20/68	1380	69	20	1380	0
5/2/68	1815	88.1	20.6	1815	0
Serum containing preformed alpha 2D					
4/3/68	930	29.7	20‡	594	336
4/18/68	930	38.6	20‡	772	158

* A previous study (8) indicated that the ratio, total D antigen/β1A, is constant at approximately 21 over a range of β1A concentrations from 90 to 324 mg/100 ml and the shape of the curve suggests that changes in the ratio do not begin to occur until levels are less than 60 mg/100 ml.

‡ The value of 20 was used in the calculation as representative in this patient of the ratio, total D antigen/β1A, in the absence of preformed alpha 2D.

nated R in the calculation, is independent of the C3 concentration of the normal serum. In the serum of patients with hypocomplementemic nephritis then, the circulating preformed alpha 2D, expressed as units of D antigen per 100 ml, can be calculated as follows:

Preformed alpha 2D = total D antigen − RA.

In this equation total D antigen is the D antigen concentration in units per 100 ml of the hypocomplementemic aged serum and A is the concentration of β1A in that serum in milligrams per 100 ml. In the present study the R value was determined separately for each patient. The value was calculated from the D antigen and β1A concentrations on specimens of serum which had been aged at 37°C for 1 wk and which had been obtained at times the C3 level approached or was in the normal range. The R value so determined in case 1 was 23 and in case 2, 20. Examples of the calculations of preformed alpha 2D are given in Table I.

Measurement of the C3 nephritic factor. The measurement of C3NeF in the hypocomplementemic serum was based on the ability of the serum to break down the C3 contained in normal human serum under standard conditions. Details of this reaction have been described earlier (23). In brief, the amount of C3 breakdown was quantitated by measurement of the loss of the B antigenic determinant of C3 after incubation of mixtures of 0.1 ml of the patient's serum and 0.3 ml of normal human serum for 20 min at 37°C. The initial B antigen concentration of the serum mixtures was always greater than 25 U/ml. The calibration curve used, shown in Fig. 1, was established by employing as a standard, various dilutions of serum from a hypocomplementemic patient with membrano-proliferative glomerulonephritis which contained C3NeF in high concentrations. A value of 100 U/ml was assigned to the undiluted standard serum. The relationship was linear but the line did not go through the origin. Therefore, concentrations of C3NeF below 15 U/ml were unmeasurable and between 15 and 25 U/ml were considered of questionable accuracy. In the present study during most of the period of observation of patient D. H. (case 2), C3NeF levels were in the range of 25 U/ml. To assess the reliability of these

values, aliquots of several of the specimens from this patient were reduced in volume by 50% by ultrafiltration and the C3NeF remeasured. C3NeF levels in this concentrated serum fell in the reliable range of the method and were very close to double those found in the original specimens.

RESULTS

Two patients with membrano-proliferative nephritis were subjected to bilateral nephrectomy and maintained by peritoneal dialysis for 2 wk before renal trasplantation. Their case histories and the results of serial measurements of several complement components and of C3NeF are reported below.

Case 1. M. F., a 12 yr old boy, had onset of persistant glomerulonephritis with depressed serum C3 levels in November, 1964, at the age of 8 yr. Renal biopsies performed 2 and 4 months after onset showed diffuse glomerular involvement with endothelial cell proliferation, lobulation, thickened capillary walls, and occasional crescents. By Jones methenamine silver stain, glomerular basement membranes were not detectable and capillary walls were thickened by a nonargyrophilic deposit typical of membrano-proliferative nephritis (1).

The subsequent clinical course of this patient has been partially reported elsewhere (2). Therapy included a 16 day course of cyclophosphamide and a 30 day course of azathioprine, both combined with prednisone, and prednisone alone, 0.6–1.0 mg/kg, once daily for 15 months. As noted elsewhere (2), serum C3 levels varied widely. Although he did well for some time, edema recurred and the BUN started to rise approximately 3 yr
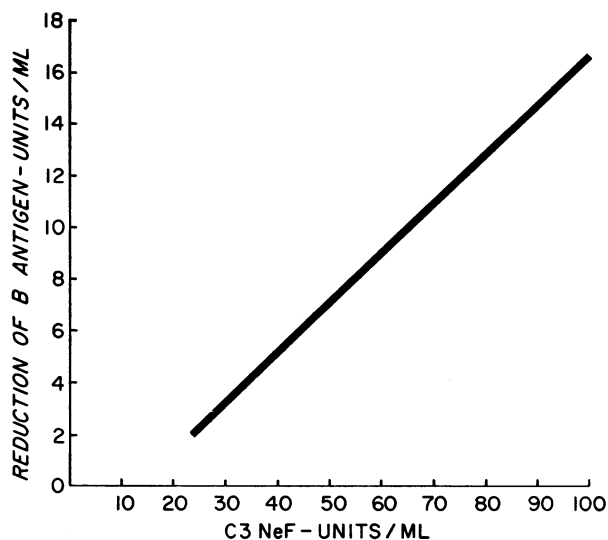

FIGURE 1 Calibration curve used for determination of C3NeF. Reduction in B antigen in mixtures of normal and nephritic serum under standard conditions is plotted against the C3NeF concentration in arbitrary units.

after onset. On 16 October 1968, bilateral nephrectomy was performed and for 16 days thereafter, before renal transplantation, daily peritoneal dialysis maintained the BUN below 60 mg/100 ml and the serum creatinine below 9.1 mg/100 ml. Therapy with azathioprine and prednisone was initiated 24 hr before transplant; the dosage regimen is shown in Fig. 2. No anti-lymphocyte globulin was used. The kidney donated by the mother (grade B match by lymphocyte typing) functioned immediately. The BUN became normal on the day of transplantation and the serum creatinine, the day after. No rejection episode has been detected. Recurrence of his original disease became manifest 22 days after transplantation. Excretion of protein at that time was 700 mg and of red blood cells, 574,000,000 per 12 hr, and the serum C3 level was at the lower limit of the normal range or slightly depressed.

Fig. 2 illustrates levels of complement components and of C3NeF before and during nephrectomy and also after renal transplantation. The C3 levels (β1A) were 27 mg/100 ml or less before nephrectomy and persisted at this level or lower during the 2 wk anephric period. After
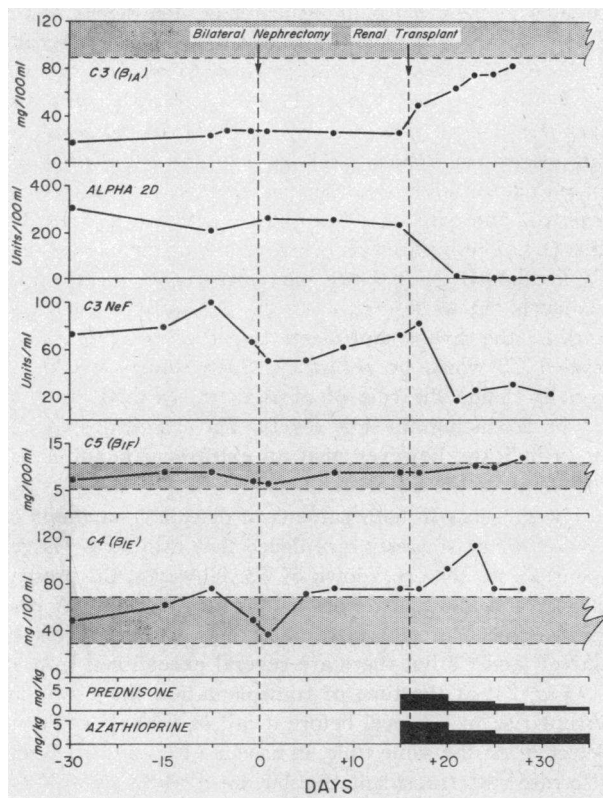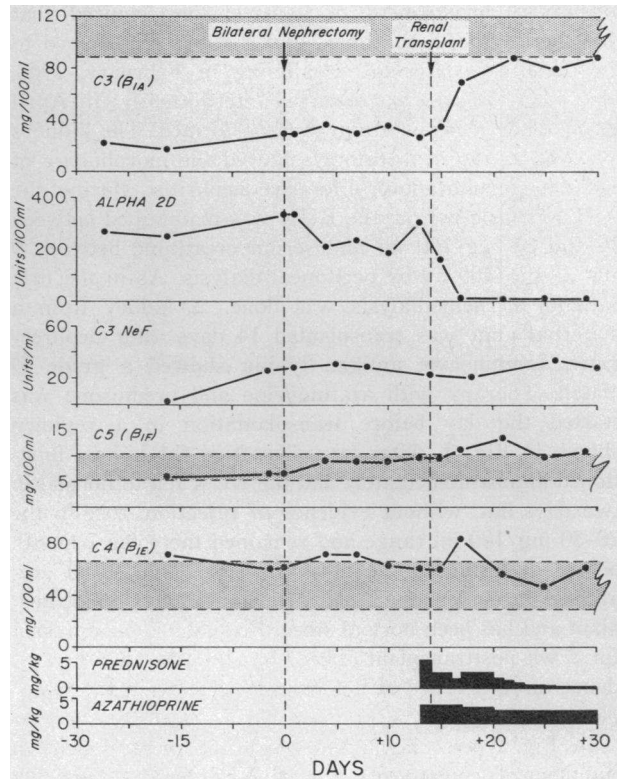

FIGURE 3 Effect of bilateral nephrectomy and renal transplantation on serum complement components, preformed alpha 2D, and C3NeF in patient D. H. (case 2). Shaded areas indicate the normal range of values.

transplantation, the C3 levels rose immediately and reached the lower limit of the normal range within 1 wk. Levels of preformed alpha 2D showed changes reciprocal to those of C3. Before and during the renoprival period they were markedly elevated, falling to zero immediately after transplantation as the C3 level rose.

Levels of C3NeF appeared to be falling at the time of nephrectomy but during the renoprival period, they stabilized and then rose from 48 to 82 U/ml. The levels remained elevated for 1 day after transplantation and then fell to the 16–30 U/ml range.

Reduced levels of C4 and C5 were never observed. In fact, levels of both of these components were at times elevated during the anephric and posttransplant periods.

Case 2. D. H., an 18 yr old female, had onset of edema and hematuria at the age of 5 yr. She was treated with penicillin and bed rest and clinical manifestations subsided but proteinuria and hematuria persisted. In February 1967, at the age of 17 yr, she became ill with pharyngitis. Examination revealed hypertension, a BUN of 80 mg/100 ml, and a serum C3 level of 6 mg/100 ml. In addition to supportive therapy, she received in October and November, 1967, a 52 day course of methylpredniso-

FIGURE 2 Effect of bilateral nephrectomy and renal transplantation on serum complement components, preformed alpha 2D and C3NeF in patient M. F. (case 1). The shaded areas indicate the normal range of values as determined in this laboratory.

lone. The development of hypertension required that this therapy be discontinued and she was transferred to the Children's Hospital. Beginning in February, 1968, peritoneal dialysis was required intermittently. In April 1968, bilateral nephrectomy was performed. The kidneys removed at the nephrectomy showed the morphology of end-stage membrano-proliferative nephritis. During the 2 wk anephric period, the BUN was maintained between 36 and 81 mg/100 ml and serum creatinine between 9 and 22 mg/100 ml by peritoneal dialysis. As in the first patient, no hemodialysis was done. A kidney from a maternal aunt was transplanted 14 days after nephrectomy. Lymphocyte antigen typing showed a grade D match. Therapy with azathioprine and prednisone was started the day before transplantation in a regimen shown in Fig. 3. After transplantation the kidney functioned almost immediately and the BUN fell to normal in two days but, without evidence of rejection, rose to the 20–30 mg/100 ml range and remained there for 1 month before declining to the normal range. The serum creatinine level remained elevated for 8 days posttransplant and has been normal since. Proteinuria was present for 2 wk posttransplant. There has been no definite evidence of recurrence of her original disease in the transplanted kidney.

In Fig. 3 are shown the results of measurement of complement components and C3NeF levels. The data are similar to those for the first patient. The serum C3 levels were consistently below 40 mg/100 ml before and during the anephric period and rose immediately after transplantation to approach closely the lower limit of the normal range 7 days posttransplant. Alpha 2D levels, on the contrary, were high before and during the anephric period and fell to zero immediately after transplantation. C3NeF levels had been high several months before nephrectomy but 18 days before had fallen to zero. Subsequently the level rose to 27 U just before nephrectomy and remained at this level during the anephric period, showing very little variation. As in the first patient, the levels of C4 and C5 were normal, tending to increase immediately after transplantation.

DISCUSSION

The present studies indicate that a C3-reactive substance which we have designated the C3 nephritic factor, or C3NeF, was present in the serum of these two patients. As shown previously (23), the characteristics of C3 breakdown by C3NeF are those of an enzymatic reaction. The similarities of the conditions and characteristics of this reaction to those for the cobra venom factor (24, 25) make the following scheme the most attractive to depict the total reaction:

$$C3NeF + Cofactor \xrightarrow{Mg^{++}} C3LyNeF$$
$$C3LyNeF + C3 \longrightarrow C3a(?) + \beta 1A + \alpha 2D$$
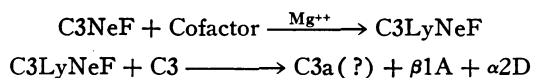
The first step of the reaction results in the formation of the C3 lytic nephritic factor or C3LyNeF, presumably a complex, which in turn rapidly cleaves C3. An alternative reaction sequence to that given above in which cofactor is an enzyme-inhibitor complex and C3NeF is an antagonist of the inhibitor which is generated in the nephritic patient, has been discussed elsewhere (23). The cleavage products of C3, $\beta 1A$, and alpha 2D, have been clearly identified as products of the reaction. Although C3a has not been identified immunochemically, preliminary work[1] indicates that a substance is liberated in the reaction mixture with biological properties like those of the C3a produced when other proteolytic enzymes react with C3 (29, 30).

Assuming the above mentioned reaction occurs in vivo, breakdown products of C3 should be found in the circulation. Previous studies (8) would indicate that $\beta 1A$ is lost rapidly from the circulation while alpha 2D continues to circulate for a time. No attempt has been made to detect C3a in vivo.

The observations on the two patients presented here render unlikely the possibility that the presence of reactive antigen-antibody complexes in the glomeruli contributes to their hypocomplementemia. The levels of C3 were virtually unaffected over a 2 wk period by the absence of kidneys. In addition, high levels of the C3 breakdown product, alpha 2D were present during the anephric period. These levels were also unaffected by the absence of kidneys and were of the same order of magnitude as seen in other patients with this type of nephritis and with a similar degree of hypocomplementemia (8). The high levels of alpha 2D give evidence that C3 breakdown is in large part responsible for the low C3 levels but to be certain of the mechanisms contributing to the hypocomplementemia, studies with radiolabeled C3 would be necessary. Such studies would especially define the role of changes in synthetic rate of C3 in producing the low levels. The conditions of the study indicate, however, that an extrarenal factor is responsible for the C3 breakdown.

The presence in both patients of detectable amounts of C3NeF gives suggestive evidence that this factor is responsible for the breakdown of C3. However, the present data do not allow final conclusions on this point. A certain degree of reciprocity exists between the levels of C3NeF and C3 but there are several exceptions. In case 1 (Fig. 2), at the time of transplantation there was an abrupt rise in C3 level before a fall in C3NeF could be detected. At the same time, in case 2 (Fig. 3), C3 levels also rose with transplantation but the already low C3NeF level showed no detectable change. Further, during the anephric period in case 2 the C3 level is almost as low

[1] Vallota, E. H., J. Forristal, N. C. Davis, and C. D. West. Unpublished observations.

as in case 1, but the level of C3NeF is considerably lower than in case 1. The out-of-phase changes in C3NeF and C3 in Case 1 and the failure of C3NeF to show a reciprocal relationship with C3 levels in case 2 are difficult to explain. It should be pointed out, however, that the combination of low levels of C3 and of C3NeF, seen in case 2, is commonly observed in patients encountered in our clinic with membrano-proliferative nephritis,[2] especially those who have received therapy with steroid in the recent past as had this patient.[3] Also it is possible that C3NeF serum levels as we presently measure them in vitro are not always an exact indicator of the activity of C3NeF in vivo.

Although the lack of exact inverse relationship between C3 and C3NeF levels in the present data precludes final conclusions, data from other sources (31, 32) indicates that C3NeF or a similar factor reacting directly with C3 must be responsible for the hypocomplementemia and the low C3 levels in patients with membrano-proliferative nephritis. Gewurz, Pickering, Mergenhagen, and Good (31) have shown that in patients with membrano-proliferative nephritis with low C3 levels, the levels of C1, C4, and C2 are, for the most part, normal, whereas in other hypocomplementemic nephritides the levels of these components are usually low. The normal C4 levels observed in the present study as well as measurements of the first three reacting components by other workers, summarized elsewhere (32), agree with the observations of Gewurz et al. (31). Thus, in membrano-proliferative nephritis, it would appear that the immune complex and the first three reacting components of complement are bypassed and that some factor, most logically C3NeF, is acting directly on C3 to produce the hypocomplementemia.

Attention should be directed again to the changes in levels of C3NeF and the presumably associated changes in C3 and alpha 2D occurring at the time of transplantation in both patients. The cause of the changes is not fully understood. In large part, the fall in C3NeF in case 1 may be the result of initiation of therapy with high doses of prednisone the day before transplantation. In some patients with membrano-proliferative nephritis with intact kidneys, studies in this laboratory[1] have shown that with intensive prednisone therapy C3NeF levels promptly fall and C3 levels rise. This change does not appear to be a direct result of inhibition of C3 breakdown by steroid since the addition of hydrocortisone to the reaction mixture in vitro had no effect on the rate of C3 breakdown. It thus appears that prednisone may

reduce the synthesis of C3NeF and, perhaps also, cofactor allowing the C3 levels to rise. The difficulty in ascribing the changes in the present patients solely to prednisone therapy is that the changes in levels in patients with intact kidneys did not occur as abruptly and dramatically as was seen here. Perhaps an additional factor in the present study is an absorption or inactivation of C3NeF by the transplanted kidney. Further study will be necessary to clarify this point.

The conclusions which may reasonably be drawn from the present observations may be summarized therefore as follows: (a) the low levels of C3 in these two patients results in large part, from C3 breakdown rather than diminished synthesis, (b) the site of C3 breakdown is extrarenal, (c) C3NeF, acting through C3LyNeF, appears most logically to be the factor responsible for the low C3 levels, although firm conclusions on this point cannot be drawn from the present data, and (d) C3NeF and cofactor are not produced in the kidneys.

Finally, it should be pointed out that the present studies provide no direct information as to the mechanism of production or maintenance of the glomerular inflammation in membrano-proliferative nephritis. It is obviously possible that if C3LyNeF, like the cobra venom factor (33), activates as well as inactivates C3, the disease could be the result of phlogistic action of the terminal components of the complement system. The observation that IgG, Clq, and electron-dense deposits may be present in the glomeruli, particularly early in this disease (4), suggests that immune complexes may play a role in its origin.

ACKNOWLEDGMENTS

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[3] Patient D. H. (case 2) received a 52 day course of steroid therapy terminating 105 days before transplant (1.5 mg/kg.). Two doses of 100 mg each were given 25 days before nephrectomy.

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