A New Role for Complement in Experimental Membranous Nephropathy in Rats

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ABSTRACT The only established role for complement in mediating immunologic renal disease involves elaboration of leukochemotactic factors and neutrophil-dependent glomerular injury. In the passive Heymann nephritis (PHN) model of experimental membranous nephropathy, rats injected with sheep antibody to rat proximal tubular brush border antigen (Fx1A) form subepithelial deposits of sheep IgG and rat complement (C3), and develop heavy proteinuria after 5 d without glomerular inflammatory changes. To study the role of complement in mediating proteinuria in PHN, 16 rats were treated daily with cobra venom factor from before antibody injection to maintain C3 levels at <10% of pretreatment values and compared to 16 untreated controls. Proteinuria at 5 d was abolished in C3-depleted rats (4±1, controls 70±15 mg/d, $P < 0.001$), although renal deposition of ^{125}I-labeled antibody was the same in both groups (188±35 vs. 191±22 μg IgG/2 kidneys, $P > 0.5$). Nephritogenic doses of both the noncomplement-fixing $F(ab')_2$ portion and the γ2 subclass of anti-Fx1A IgG produced subepithelial deposits of immunoglobulin without C3, but proteinuria did not occur despite glomerular deposition of up to 70 μg/2 kidneys of γ2. However, glomerular deposition of as little as 60 μg of γ1 produced C3 fixation in vivo and heavy proteinuria. No neutrophil exudate could be detected histologically in PHN from the time of antibody injection through development of proteinuria. Proteinuria in five PHN rats depleted of neutrophils to <200/mm^3 with anti-neutrophil serum was not reduced compared to six controls with normal neutrophil counts (34±9.6 vs. 25±10.4 mg/d, $P > 0.5$).

These results demonstrate that proteinuria in the PHN model of membranous nephropathy is complement-dependent and strongly suggest a neutrophil-independent mechanism. Thus a new role for the complement system in mediating immunologic glomerular injury is identified.

INTRODUCTION

Immunopathologic studies in experimental animals and in man have now firmly established that glomerular antibody deposition mediates most forms of glomerular injury (1). At least three different mechanisms have been identified that may lead to immune deposit formation in glomeruli: antibody may bind to antigenic components of the glomerular basement membrane (GBM)[1] (2); antibody complexed to either exogenous or endogenous antigens in the circulation may form soluble immune complexes that are passively trapped by the glomerular filter (2); or antigens that are either glomerular constituents or glomerular tissue-bound exogenous macromolecules may bind free antibody to initiate *in situ* glomerular immune complex formation (3). Regardless of which of these mechanisms is involved, it is generally accepted that the resultant tissue injury, which manifests primarily as an increase in urine protein excretion, is usually effected by various mediator systems that are activated by the immune deposits (1, 4, 5). The best defined of these mediators is the complement system, components of which are

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[1] *Abbreviations used in this paper*: CVF, cobra venom factor; Fx1A, proximal tubular epithelial cell brush border antigen; GBM, glomerular basement membrane; IF, immunofluorescence microscopy; PHN, passive Heymann nephritis.

prominent in immune deposits in glomerulonephritis (6–8). Although certain anti-GBM antibodies may induce heavy proteinuria independently of the complement system (5, 9–11) most studies in nephrotoxic nephritis models have shown that the complement system plays an essential role in this process (8, 9, 12–14). Further, it has been established that complement-dependent glomerular injury involves the influx of neutrophils and local inflammation induced by the leukochemotactic complement fragment C5a and can be prevented by neutrophil depletion (4, 5, 8, 9, 15). Numerous other biologic activities of various products of complement activation have been identified but there is presently no direct evidence that complement mediates glomerular injury by any mechanism other than that involving neutrophils (5, 8, 16).

We (17, 18) and others (19) have recently studied the mechanisms of subepithelial immune deposit formation in the passive Heymann nephritis (PHN) model in rats, a model that closely resembles membranous nephropathy in man. These studies showed that deposits may form locally by binding of free antibodies to a fixed glomerular antigen rather than from circulating complex trapping as previously believed. Careful, sequential studies of the development of this lesion during the 5 d between antibody injection and appearance of proteinuria revealed that complement was a prominent early constituent of the subepithelial immune deposits, but that proteinuria occurred in the absence of any detectable neutrophil infiltrates (17, 18). These findings are similar to those in the related autologous immune complex nephropathy (Heymann nephritis) model and in membranous nephropathy in man (20, 21). In the present report our studies of the role of complement and neutrophils in PHN are described in detail. The results strongly suggest a previously unrecognized role for the complement system that is independent of neutrophils in this unique form of glomerular injury. These findings may be important in understanding the pathogenesis of several noninflammatory glomerular diseases in which complement deposits are prominent.

METHODS

All studies were carried out during the heterologous phase of PHN induced in rats by a single intravenous injection of sheep antibody to rat proximal tubular brush border antigen (Fx1A) as described in detail previously (17, 18, 22). To clarify the studies performed, the experimental protocol is outlined first followed by the specific details of each procedure.

Experimental design

ROLE OF AUTOLOGOUS PHASE

PHN was induced in two groups of rats immunosuppressed to prevent a host antibody response. This was done to eliminate any role for an autologous phase reaction involving glomerular deposition of rat IgG in mediating the proteinuria which occurs at 4–5 d in PHN. 1 d before administration of a proteinuric dose of 30 mg of anti-Fx1A IgG i.v.: one group of nine rats received 860 rad of total body irradiation administered from a dual ^{137}Cs source small animal irradiator (Gammocell 40, Atomic Energy of Canada Ltd., Toronto, Canada) at a dose rate of 140 rad/min. A second group of seven rats was given a single dose of cyclophosphamide (Cytoxan, Mead Johnson & Co., Evansville, Ind.), 150 mg/kg i.p. A control group of six rats received 1 ml of saline i.p. Urine protein excretion of all rats in the three groups was measured on days 4–5 after antibody administration, and renal biopsies were obtained and studied for deposition of sheep IgG, rat C3, and rat IgG by direct immunofluorescence (IF).

IN VIVO DEPOSITION OF RAT C3

In vivo deposition of the third component of rat complement (C3) in PHN was assessed by direct IF of renal biopsies from four rats obtained 20 min, 4 h, and daily for 5 d after anti-Fx1A injection using the fluorescein-conjugated IgG fraction of an anti serum to rat C3.

IN VITRO COMPLEMENT FIXATION

The ability of glomerular immune deposits to bind human C3 was assessed in vitro by incubating sections of kidney from PHN rats with fresh or heat-inactivated human serum and staining for human C3 by direct IF as described below. In addition, the various anti-Fx1A reagents used in the study were tested for in vitro complement fixing ability before injection by layering them on sections of normal rat kidney to produce proximal tubular brush border staining followed by exposure to fresh human serum and staining for human C3.

SERUM COMPLEMENT LEVELS

Serum C3 levels were measured during the development of PHN in six rats on serum samples obtained from the tail vein 30 min, 3 h, and 24 h after antibody injection and then daily for 5 d. C3 levels were determined by Mancini single radial immunodiffusion (23) and expressed as a percentage of the base-line C3 level in each animal measured before antibody injection. Control rats received injections of normal sheep IgG.

COMPLEMENT DEPLETION STUDIES

The role of complement in the development of proteinuria in PHN was assessed by depleting rats of C3 for 5 d by repeated injections of purified cobra venom factor (CVF) prepared as described below. After obtaining a base-line serum sample for C3 level, 11 rats were complement depleted by an initial injection of 300 U/kg of CVF i.p. in four divided doses starting 24 h before injection of 25 mg of anti-Fx1A antibody i.v. followed by daily injections of CVF, 100 U/kg i.p., for 5 d as described by Cochrane et al. (14). Daily serum C3 levels were measured as described above. A control group of 11 age-matched rats were injected in parallel with the same dose of anti-Fx1A but were not complement depleted. Urine protein excretion was measured in both groups on days 4–5. This study was repeated using five rats, complement depleted with CVF that had been treated as described below to inactivate phospholipase A_2 and five control rats injected with saline.

Effect of complement depletion on antibody deposition

To ensure that the effect of complement depletion on the development of proteinuria in PHN was not due to altered glomerular antibody deposition, in vivo glomerular antibody binding was evaluated by IF and electron microscopy of renal biopsies and by quantitative techniques using ^{125}I-labeled antibody as described below. 18 rats, 9 of which were treated with CVF as described above and 9 of which received daily injections of saline, were given 24 mg anti-Fx1A IgG-^{125}I i.v. At killing on day 5 the kidneys were perfused *in situ*, tissue was obtained for IF and electron microscopy, and antibody deposition was measured from the kidney radioactivity.

Effect of CVF on proteinuria in other models

To determine whether the inhibition of proteinuria by CVF in PHN was indeed due to C3 depletion and absence of C3 from glomerular immune deposits, or to some other unidentified effect of CVF on glomerular permeability, CVF was administered to PHN rats with established proteinuria and to rats during the development of nonimmunologic glomerular injury.

Of 11 rats with fully developed PHN and proteinuria 11 d after anti-Fx1A administration, 5 were complement-depleted for 3 d with CVF as described above and the other 6 received no treatment. Urine protein excretion was measured in both groups before and after CVF. In a second study eight rats were given aminonucleoside of puromycin (ICN Pharmaceuticals, Inc., Covina, Calif.) 100 mg/kg i.v. Four were treated as above with CVF from the day before administration of aminonucleoside and for 6 d thereafter and the remaining four were given daily injections of normal saline on the same schedule. Urine protein excretion was measured in both groups on days 6–7.

Studies with noncomplement-fixing antibodies

To further establish the dependency of proteinuria upon in vivo fixation of complement, PHN was induced by two types of noncomplement-fixing anti-Fx1A antibodies. Gamma 1 (complement-fixing) and gamma 2 (noncomplement-fixing) subclasses of sheep anti-Fx1A IgG prepared, trace labeled with ^{125}I, and characterized as described below, were injected into two separate groups of six rats per group. Urine protein excretion was measured in both groups on days 4–5. At killing on day 5, renal tissue was obtained for IF and electron microscopy, and glomerular antibody deposits were quantified as described below. The dose of γ1 anti-Fx1A IgG (15 mg) was shown in a pilot experiment to produce proteinuria on days 4–5. The doses of γ2 anti-Fx1A IgG, 18 mg (three rats) and 22 mg (three rats), were chosen to ensure that the amount of glomerular binding of γ2 antibody was at least equivalent to that of γ1 antibody. F(ab')$_2$ fragments of anti-Fx1A prepared, trace labeled with ^{125}I and characterized as described below, were given intravenously to nine rats. Urine protein excretion was measured on days 4–5 before killing on day 5 when renal tissue was obtained for IF and electron microscopy, and glomerular antibody deposits were quantified as described below. The doses of anti-Fx1A F(ab')$_2$, 20–40 mg (the molar equivalents of 35–70 mg of IgG), were purposely chosen to be greater than the dose of anti-Fx1A IgG (20 mg) that regularly produces proteinuria on days 4–5 in an attempt to ensure that the amount of glomerular bound F(ab')$_2$ exceeded the proteinuric threshold previously defined in this model (18).

Role of neutrophils

The role of neutrophils in the pathogenesis of proteinuria in PHN was evaluated in two experiments. Histologic evidence for participation of neutrophils in the glomerular lesion of PHN was sought in biopsies obtained from at least five rats 10 min, 1, 4, 12, and 24 h, and 2, 3, 4, and 5 d after injection of 25 mg of anti-Fx1A. Five control rats were injected with 25 mg of normal sheep IgG. Tissue for light microscopy was processed as described below. Neutrophils were counted in at least 10 glomeruli in two micron-coded sections from each biopsy stained with periodic acid-Schiff and expressed as neutrophils/10 glomeruli. (Neutrophil counts were generously provided by Dr. Magda Stilmant of the Mallory Institute of Pathology, Boston City Hospital.)

A group of five rats was depleted of neutrophils by injection of a specific rabbit anti-rat neutrophil globulin, 0.8 ml i.v., which was prepared as described below and given every 12 h from the morning before PHN induction and for 5 d thereafter. A group of six littermates with PHN were given normal rabbit globulin on the same schedule. Urine protein excretion was measured in both groups on days 3–4 and on days 4–5. Circulating neutrophils were measured on blood obtained from the tail vein before each morning dose of antiserum. Total peripheral leukocyte counts were measured on a Coulter counter model ZB1 (Coulter Electronics, Inc., Hialeah, Fla.), and the percentage of neutrophils obtained by counting 200 cells on a smear of peripheral blood stained with Wright's stain. Only rats with less than 200 neutrophils/mm^3 throughout the 5-d period of the study were considered to be neutrophil depleted.

Technical details

Production and characterization of anti-rat Fx1A IgG, γ1 and γ2 subclasses, and F(ab')$_2$ fragments

Sheep were hyperimmunized with rat Fx1A prepared as described by Edgington et al. (24) and emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). After three to four monthly injections of 75–100 mg of Fx1A, IgG antibody was isolated from a 50% ammonium sulfate precipitate of heat-inactivated (56°C, 30 min) whole serum by ion-exchange chromatography on DEAE-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and absorbed with rat serum, erythrocytes, leukocytes, and platelets.

The γ1 and γ2 subclasses of sheep IgG were separated from a 50% ammonium sulfate precipitate of whole antiserum by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Inc.) using 0.0175 M sodium phosphate buffer, pH 7.4, to elute the more cationic fraction (γ2) followed by application of a linear NaCl gradient to 0.3 M to obtain the more anionic (γ1) fraction. The two major IgG peaks were pooled separately, concentrated in an Amicon PM 30 ultrafilter (Amicon Corp. Scientific Sys. Div., Lexington, Mass.) to ~10 mg/ml and stored at −70°C until used.

F(ab')$_2$ fragments were prepared from IgG isolated from anti-Fx1A antiserum as described above by digestion with pepsin according to the method of Nisonoff et al. (25). Approximately 100 mg of IgG, at a concentration of 10–20 mg/ml, was incubated with pepsin (crystallized twice, Worthington

Biochemical Corp., Freehold, N. J.) at a protein:enzyme ratio of 100:1 in 0.2 M acetate buffer, pH 4.5, at 37°C for 18 h. Digestion was terminated by raising the pH to 8.0 with 1.0 N sodium hydroxide. $F(ab')_2$ fragments were separated from undigested IgG (<10% of total protein) and smaller fragments by gel filtration chromatography on Sephadex G-150 (Pharmacia Fine Chemicals, Inc.). Samples of 50 mg in 1.5 ml were applied to a 1.5 × 90-cm column and eluted with 0.01 M sodium phosphate–0.2 M sodium chloride buffer, pH 8.2, at room temperature. The $F(ab')_2$ peak was concentrated to 8.5 mg/ml, dialyzed against 0.02 M phosphate-buffered saline, pH 7.2, and stored at −70°C until used. The molecular size of $F(ab')_2$ fragments of sheep IgG (96,000 daltons) was calculated from the elution volume from the Sephadex G-150 column precalibrated with blue dextran, aldolase, ovalbumin, chymotrypsinogen, and ribonuclease A.

In addition to the chromatographic elution profiles obtained in preparing IgG, γ1 and γ2 subclasses and $F(ab')_2$ fragments, the following procedures were performed to further characterize each reagent. To assess purity and specificity, immunoelectrophoresis and micro-Ouchterlony reactivity in 1% agarose was determined against whole rat serum and to sheep IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) (26, 27). The isoelectric points of each reagent were determined by gel isoelectric focusing (generously performed by Dr. Helmut Rennke of the Peter Bent Brigham Hospital) using a model M-150 slab electrofocusing apparatus (MRA Corp., Clearwater, Fla.) and techniques described in detail elsewhere (28, 29). Antibody reactivity in vitro with glomeruli and proximal tubular brush borders was determined by indirect IF on cryostat sections of normal kidney stained with fluorescein-conjugated antiserum to sheep IgG (N. L. Cappel Laboratories Inc.) (17, 18). In vitro complement-fixing ability was assessed by injecting rats with IgG or $F(ab')_2$ antibodies and studying kidneys 5 d later by IF and electron microscopy as described below. In addition, the in vivo glomerular binding activity of each preparation was quantitated as outlined below using reagents labeled with ^{125}I. The protein concentrations of purified preparations of sheep antibody were measured by the Biuret method.

Induction of PHN and Quantitation of Antibody Deposits

PHN was induced in male Sprague-Dawley rats weighing 140–200 g (CD, Charles River Breeding Laboratories, Wilmington, Mass.) by intravenous injection of various doses of sheep anti-Fx1A IgG, γ1, γ2 or $F(ab')_2$. All studies of proteinuria and glomerular antibody deposition were performed 5 d later. This time was selected on the basis of previous studies in this model demonstrating that proteinuria occurs 4–5 d after injection of a nephritogenic quantity of antibody, and that autologous phase deposition of rat IgG in glomeruli is minimal or absent at 5 d (18, 22). Urine protein excretion was measured on 24-h urine specimens collected from animals in individual metabolic cages with free access to water. Protein content was measured by a sulfosalicylic acid method (30) using a commercial whole serum standard (Lab-Trol, Dade Div. American Hospital Supply Corp., Miami, Fla.). The urine protein composition was determined by agarose gel electrophoresis and densitometry of urine samples concentrated 20-fold (11).

The amount of antibody deposited in kidneys and glomeruli of individual rats injected with various ^{125}I-labeled preparations was measured by methods described in detail elsewhere (18). For the purposes of this study, total kidney bound antibody of each rat was determined 5 d after radiolabeled antibody injection by counting perfused whole kidneys and correcting for background, decay, and nonspecific binding as described (18). Antibody specifically bound in glomeruli was measured from the radioactivity of glomeruli isolated from kidneys of individual rats using differential sieving techniques (18).

Complement and Neutrophil Studies

Preparation of anti-rat C3. Anti-rat C3 was prepared by immunizing rabbits three times with a repeatedly washed precipitate of boiled zymosan (Sigma Chemical Co., St. Louis, Mo.) and fresh rat serum as described by Mardiney and Müller-Eberhard (31). After absorption with lyophilized plasma from rats decomplemented with CVF as described above, anti-C3 made a characteristic bimodal arc in the β1 globulin region on immunoelectrophoretic analysis reacting primarily with the faster moving arc in fresh serum and with the slower moving arc in aged or heat-inactivated (56°C, 30 min) rat serum (31, 32). Anti-C3 stained deposits of human antinuclear antibody on cryostat sections of normal rat kidney after exposure to fresh rat serum, as described below in the in vitro complement fixation test, but failed to stain antibody-treated sections exposed to heat-inactivated rat serum. Fluorescein conjugation of antibody and subsequent characterization was carried out as described in detail elsewhere (20, 22).

In vitro C3 fixation. Washed, unfixed cryostat sections of PHN kidneys and normal rat kidney that had been previously incubated with the various anti-Fx1A reagents were tested for in vitro complement-fixing ability by incubating them with the appropriate dilutions of fresh or heat-inactivated human serum in veronal buffer at 20°C for 45 min followed by washing and staining for human C3 by direct IF (11, 33). Human antinuclear antibody-containing serum was used as a positive control for the in vitro complement-fixation procedures (33).

CVF. CVF obtained from *Naja naja* (Cordis Laboratories Inc., Miami, Fla.) was isolated by ion-exchange chromatography on DEAE-cellulose (Whatman, Inc., Clifton, N. J.) or DEAE-Sephadex followed by gel filtration chromatography on Sephadex G-200. The final product was free of contamination with lecithinase (phospholipase A_2) as judged by failure to lyse guinea pig erythrocytes incubated with CVF and egg yolk (34). To further ensure that phospholipase A_2 contamination was not responsible for the results observed, complement-depletion studies were also carried out using CVF that had been incubated with 0.1 mM para-bromophenacyl bromide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 37°C and pH 8.0 for 30 min as described by Shaw et al. (34), to inactivate any remaining phospholipase A_2.

Preparation of anti-rat neutrophil serum. Rat neutrophils were isolated by administering 10 ml of 0.1% oyster glycogen (Sigma Chemical Co.) in saline or Hanks' balanced salt solution containing 1 U/ml of heparin i.p. on two successive days to 12 300–400 g Sprague-Dawley rats as described by Cochrane et al. (9). Peritoneal exudates were collected 3 h after the second injection. The washed cell pellet, which contained >90% neutrophils, was emulsified in incomplete Freund's adjuvant (Difco Laboratories) and used to immunize three rabbits. Antiserum, collected 3 wk later, was absorbed with lyophilized whole rat serum and neutrophil-free rat erythrocytes obtained from 18 rats 24 h after administration of nitrogen mustard (Mustargen hydrochloride, Merck, Sharp & Dohme Canada Ltd., Quebec, Canada), 2 mg/kg i.p. Additional absorptions were carried out with rat lymphocytes and thymocytes obtained from mesenteric lymph nodes and thymuses of 10 rats. The globulin fraction of the absorbed antiserum was isolated by a single 50% ammonium sulfate

precipitation. It was reconstituted to half the original volume and dialyzed extensively against phosphate-buffered saline, pH 7.2, and stored at −70°C until used.

Tissue processing and IF procedures

Renal tissue obtained by wedge biopsy of kidneys under ether anesthesia was processed for light microscopy, IF, and electron microscopy as described in detail elsewhere (20). Tissue fixed in 10% neutral formalin, sectioned at 2 μm and stained with periodic acid-Schiff reagent was examined for glomerular neutrophil counts as described above as well as other histological changes.

Direct and indirect IF procedures were performed on tissue snap-frozen in dry ice-isopentane, and cryostat sections fixed in ether-alcohol were studied using techniques and controls described previously (17, 20). All biopsies were stained with the IgG fraction of monospecific antisera to sheep IgG and rat IgG, C3 and albumin (N. L. Cappel Laboratories) conjugated with fluorescein isothiocyanate (Bio-Quest, BBL Falcon Products, Div. of Becton, Dickinson and Co., Cockeysville, Md.) by a dialysis method (35). Antibody to rat IgG was not cross-reactive with sheep IgG by immunoprecipitin or IF analysis.

Antisera to human C3 (N. L. Cappel Laboratories) and human Clq (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) were used in the in vitro complement-fixation studies. IF was evaluated and photographed on a Leitz Ortholux II microscope equipped with a Ploempak 2.2 vertical fluorescence illuminator (E. Leitz Inc., Rockleigh, N. J.). Tissue for electron microscopy was immersion-fixed in glutaraldehyde and postfixed in osmium followed by en bloc staining in uranyl acetate for 30 min before embedding in Epon 812 (Shell Chemical Co., Houston, Tex.).

Statistical analysis

Student's t test for unpaired data was used to analyze the results of renal binding of labeled antibody and urine protein excretion (36). Analysis of variance was used to evaluate the changes in daily serum C3 levels (36). Differences were regarded as significant when $P < 0.05$. All values are expressed as mean±1 SD unless otherwise stated.

RESULTS

Characteristics of the heterologous phase of PHN. The glomerular lesion induced by anti-Fx1A was identical to that described in detail (17, 18, 22). Granular deposits of sheep IgG were seen within minutes by IF and increased in amount until day 5. Trace deposits of rat IgG were detectable in some glomeruli of proteinuric rats on days 4 and 5. Proteinuria was first apparent on days 3–4 in some rats, but on days 4–5 virtually all rats were proteinuric. The urine protein excretion on day 5 was nonselective with only 75% representing albumin.

Suppression of the host response to heterologous IgG. The results of immunosuppression studies demonstrated that the development of proteinuria in PHN is not dependent on an autologous phase reaction since all rats given total body irradiation or cyclophosphamide became proteinuric in the absence of rat IgG deposits in glomeruli. Lethally irradiated rats (n = 9) excreted 47±37.1 (range: 10–117) mg/24 h, which was not significantly different from control rats (n = 6), which excreted 83±44.9 (range: 25–123) mg/24 h ($P < 0.2$). Rats given cyclophosphamide (n = 7) excreted 42±13.5 (range: 24–61) mg/24 h which was significantly less than controls ($P < 0.05$) but still clearly abnormal. The upper 99% confidence limit of protein excretion in 20 normal age-matched rats was 3.5 mg/24 h. There were no glomerular deposits of rat IgG in the renal biopsies on day 5 in any of the rats in the two immunosuppressed groups. In contrast, all rats in the control group had trace-1+ granular capillary wall deposits of rat IgG by IF on day 5.

In vivo deposition of rat C3. Sequential biopsies of four rats injected with unfractionated anti-Fx1A IgG showed that C3 was first detectale by IF in glomerular deposits at about 24 h. By day 5, C3 deposits were identical in pattern and distribution to the deposits of sheep IgG, though of slightly less intensity.

In vitro C3 binding. When sections of kidney obtained on day 5 from four rats with PHN were incubated with fresh or heat-inactivated human serum and stained for human C3, glomerular deposits bound C3 in fresh, but not in heat-inactivated, serum. Similarly, when stained for human C1q, finely granular deposits were also detected. When normal rat kidney sections were first incubated with anti-Fx1A to produce proximal tubular epithelial brush border deposits, followed by incubation with fresh or inactivated human serum and then stained for C3, positive brush border staining was seen with fresh, but not with inactivated serum. Proximal tubules and glomeruli of normal rat kidney incubated with normal human serum without prior incubation with anti-Fx1A did not fix C3 or C1q.

Serum C3 in PHN. Fig. 1 shows the changes in serum C3 in six rats injected with a proteinuric dose of anti-Fx1A. Each rat served as its own control. The serum C3 level at each time interval is expressed as a percentage of the level obtained before antibody injection. The initial rise in serum C3 at 30 min and 3 h (Fig. 1) coincided with a transient rise in hematocrit to 57±1.9% at 30 min and 46±3.3% at 3 h from a normal level of 42±1.4%. The hematocrit returned to the preinjection level by 24 h. When corrected for this change in hematocrit, a slight but insignificant fall in C3 levels was found at 30 min and 3 h (Fig. 1). Serum C3 levels then rose significantly above base line at 2 d ($P < 0.02$) and remained elevated for the remainder of the study (Fig. 1).

Effect of complement depletion on proteinuria. Serum C3 measured daily in 13 rats treated with CVF from the day before anti-Fx1A antibody injection until day 5 was reduced to <10% of the pretreatment level throughout the study period in all but one rat. Serum C3

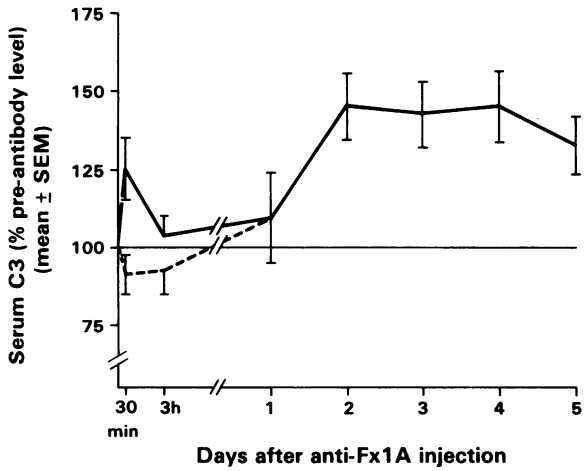

FIGURE 1 Serum C3 levels measured for 5 days after injection of a proteinuric dose of anti-Fx1A antibody. The dashed line represents values corrected for a transient decrease in plasma volume 30 min and 1 h after antibody injection.

values, measured just before induction of PHN and 1 and 5 d thereafter in CVF and saline-treated rats are shown in Table I. A marked inhibition of proteinuria on days 4–5 was found in CVF-treated rats as compared to saline-treated controls (Table I) ($P < 0.001$). CVF, treated to inactive phospholipase A_2, was as equally effective as untreated CVF in depleting five PHN rats of C3 (6±2.1% of base line on day 5) and in preventing the development of proteinuria (7±5.5 mg/24 h). These results are included in those shown in Table I.

Glomerular IgG and C3 deposition in complement-depleted PHN rats. Renal biopsies on day 5 in six CVF treated rats with PHN showed diffuse, granular deposition of sheep IgG identical to that of saline-treated controls but deposition of C3 in vivo on glomerular capillary walls was absent. When sections of these biopsies were incubated with fresh, normal, human serum followed by staining for human C3, in vitro fixation of human C3 was found that was identical to that found in kidneys from noncomplement-depleted rats. By electron microscopy CVF-treated PHN rats had discrete, subepithelial electron dense deposits identical to those previously demonstrated in noncomplement-depleted PHN rats (17, 18, 22).

When the amount of ^{125}I-labeled anti-Fx1A deposition was measured in kidneys from nine rats treated with CVF to deplete complement and compared to the amount deposited in kidneys of nine saline-treated controls, the amount of specific antibody deposition was not significantly different in the two groups (Table I) ($P > 0.5$).

Effect of CVF on established proteinuria in PHN. 11 rats with fully developed PHN on day 11 and established proteinuria (196±35.1 mg/24 h) were randomly divided into two groups. Five rats injected daily with CVF excreted 217±86.6 mg protein/24 h after treatment for 3 d, whereas six untreated controls excreted 242±32.5 mg protein/24 h ($P > 0.5$).

Effect of CVF on the development of proteinuria in nonimmunologic glomerular injury. Eight rats given aminonucleoside of puromycin to induce nonimmunologic glomerular injury were randomly divided into two groups of four rats each. One group was treated with CVF from the day before aminonucleoside administration and for 6 d thereafter. The other group was treated with saline according to the same schedule. Proteinuria was present in all animals on days 6–7, and there was no significant difference between the groups (CVF: 205±61.2 mg/24 h; saline: 249±46.4 mg/24 h, $P > 0.25$).

Noncomplement-fixing antibodies in the induction of PHN. By immunoelectrophoresis, the subclasses of sheep IgG each made a single arc in the gamma region with antiserum to whole sheep serum and sheep IgG, but the γ1 subclass migrated more rapidly than the γ2 subclass. This finding was confirmed by isoelectric focusing that showed no overlap in isoelectric point (pI) (γ1: 5.3–7.8; γ2: 8.1–9.6). The presence of antibody activity against Fx1A in both subclasses was confirmed by micro-Ouchterlony, and reactivity with proximal tubular brush border was demonstrated by indirect IF

TABLE I
Serum C3, Urine Protein Excretion, and Renal Binding of ^{125}I-labeled Anti-Fx1A in PHN Rats Treated with CVF or Saline

Group	Serum C3			Urine Protein	Kidney-bound antibody*
	Day 0	Day 1	Day 5	Days 4–5	
		% base line		*mg/d*	*μg/2 kidneys*
Saline	100 (6)	109±35.4 (6)	133±21.0 (6)	70±15.0 (16)	188±35 (9)
CVF	5±0.8 (6)	7±1.5 (5)	6±4.2 (13)	4±1.0 (16)	191±22 (9)

All values are mean±SEM, number studied is in parentheses.
* ^{125}I-labeled anti-Fx1A IgG measured in kidneys on day 5.

on normal rat kidney. When injected to produce glomerular deposits, the γ1 subclass fixed heterologous C3 in vitro (Fig. 2c) but the γ2 subclass did not (Fig. 2f).

The results of urine protein excretion on days 4–5 and glomerular antibody binding on day 5 in two groups of rats given either γ1 or γ2 anti-Fx1A that had been trace-labeled with ^{125}I are shown in Fig. 3. All six rats given γ1 anti-Fx1A were proteinuric on days 4–5 (41±22 mg/24 h) (Fig. 3). Urine protein excretion in the six rats injected with γ2 anti-Fx1A did not exceed 7.4 mg/24 h (mean: 5±1.6 mg/24 h) despite the fact that in four rats the amount of glomerular-bound antibody was greater than that in three rats given γ1 anti-Fx1A, which were proteinuric (Fig. 3). Glomerular antibody binding (γ1: 91±32.1 μg/2 kidneys; γ2: 66±12.8 μg/2 kidneys) was not significantly different in the two groups ($P < 0.2$), whereas the difference in urine protein excretion on days 4–5 was highly significant ($P < 0.005$) (Fig. 3). IF of renal tissue obtained at sacrifice on day 5 showed diffuse, granular, glomerular capillary wall staining for sheep IgG in rats injected with either γ1 or γ2 anti-Fx1A (Fig. 2a and 2d). Tubular brush border staining was found only in proteinuric rats given γ1 anti-Fx1A. Rat IgG was found in trace amounts in both groups. Glomeruli of rats given γ2 anti-Fx1A had minimal or no deposits of rat C3 (Fig. 2e), whereas those of rats given γ1 anti-Fx1A had diffuse, granular capillary wall staining for rat C3 (Fig. 2b). Glomerular deposits of γ1 anti-Fx1A fixed human C3 in vitro (Fig. 2c), whereas those of γ2 anti-Fx1A did not (Fig. 2f).

By immunoelectrophoresis against antiserum to whole sheep serum and to sheep IgG, anti-Fx1A $F(ab')_2$ fragments made a single arc of precipitation in the same region as native sheep IgG and the pI range was 6.1–9.6 as compared to 4.8–9.6 for native sheep IgG anti-Fx1A. Micro-Ouchterlony revealed the same three lines of reactivity against Fx1A as previously shown with native IgG anti-Fx1A (17, 24). By indirect IF, $F(ab')_2$ fragments of anti-Fx1A produced intense proximal tubular epithelial cell brush border staining and faint, diffuse glomerular staining for sheep IgG on sections of normal rat kidney but failed to fix C3 in vitro. The results of urine protein excretion on days 4–5 of seven rats given 20–40 mg of radiolabeled anti-Fx1A $F(ab')_2$ antibodies was not significantly different from that of normal, age-matched rats (Fig. 3). The maximum glomerular antibody binding achieved on day 5 was, however, less than the minimum amount of γ1 IgG that caused proteinuria (Fig. 3). IF of the kidneys obtained at sacrifice on day 5 showed diffuse, discrete, very fine, granular staining of sheep IgG on the glomerular capillary wall, and intense brush border staining. Staining for rat C3 was negative. Electron microscopy showed numerous, small subepithelial electron-dense deposits located mainly in the region of filtration slit pores.

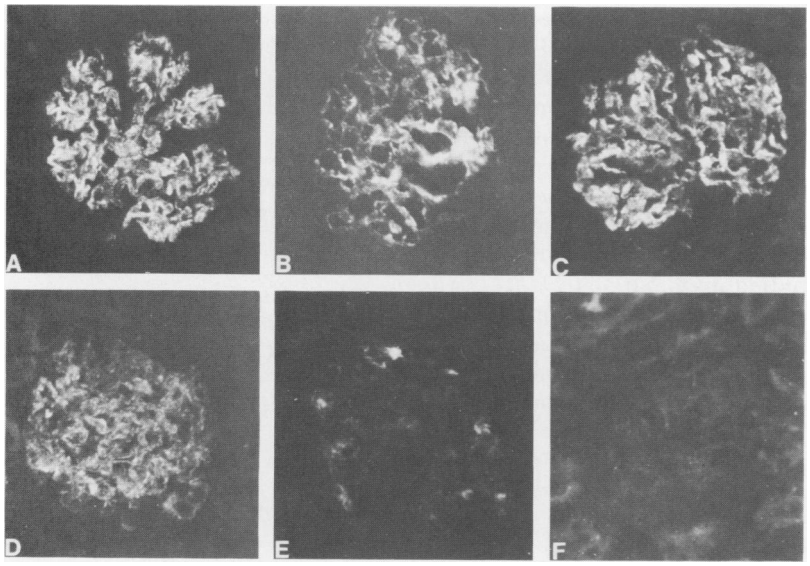

FIGURE 2 Immunofluorescent photomicrographs 5 d after injection of γ_1 (a–c) or γ_2 (d–f) anti-Fx1A IgG. Both subclasses of antibody form typical finely granular subepithelial deposits on glomerular capillary walls (a and d). Rat C3 is deposited in vivo in a pattern similar to that of IgG in rats with γ_1 deposits (b) but is absent in rats with γ_2 deposits (e). Exposure to fresh human serum in vitro results in C3 fixation by γ_1 deposits (c), but γ_2 deposits do not fix heterologous C3 in vitro (f). (Original magnifications 450×).

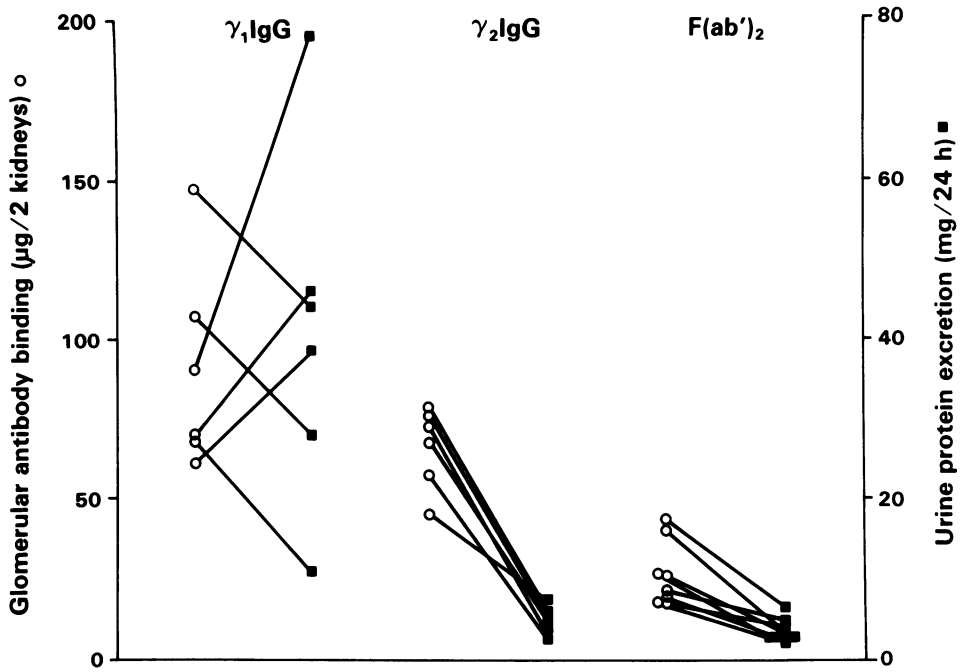

FIGURE 3 Glomerular antibody binding (open circles) and urine protein excretion (closed squares) 5 days after injection of rats with various doses of the γ_1 and γ_2 subclasses and the F(ab')$_2$ fragment of anti-Fx1A IgG. All rats injected with complement-fixing γ_1 were proteinuric. Rats injected with noncomplement-fixing γ_2 did not become proteinuric, although glomerular binding of γ_2 antibody in several animals exceeded the amount of antibody deposition in several proteinuric rats in the γ1 group.

Neutrophils in the pathogenesis of proteinuria in PHN. Sequential renal biopsies obtained from rats injected with a proteinuric dose of anti-Fx1A were examined by light microscopy for the presence of neutrophils within the glomeruli. Biopsies from four rats at each of nine time intervals from 10 min to 5 d revealed <3 neutrophils/10 glomeruli, which was not different from values in four control rats injected with normal sheep IgG. No histologic abnormalities were noted in PHN rats studied up to 5 d.

Five rats injected with specific anti-neutrophil serum had neutrophil counts of <200/mm^3 from before anti-Fx1A administration until day 4. Smears from some rats were totally devoid of neutrophils. The value of <200 neutrophils/mm^3 frequently represented a single abnormal looking neutrophil in a smear of peripheral blood. On day 5, neutrophil counts exceeded 200 cells/mm^3 in some rats. Therefore protein excretion, measured from days 3–4 rather than days 4–5, was compared to controls. The results of neutrophil counts and urine protein excretion of rats treated with anti-neutrophil serum and saline-treated controls are shown in Table II. Despite marked neutrophil depletion in the experimental group, there was no difference in urine protein excretion between the groups on days 3–4. In addition, four cyclophosphamide-treated PHN rats that were proteinuric on days 4–5 (range: 24–50 mg/24 h) also had neutrophil counts of <200/mm^3 on day 5.

DISCUSSION

The results of this study establish that the complement system is an essential mediator of proteinuria in the PHN model of membranous nephropathy in rats. They

TABLE II
Neutrophil Counts and Urine Protein Excretion of PHN Rats Treated with Specific Anti-Neutrophil Globulin or Normal Rabbit Globulin

Group	Neutrophil counts	Urine protein Days 3–4
	cells/mm^3	*mg/d*
Normal rabbit globulin	1,100–5,000 (6)*	25±10.4 (6/6)‡
Anti-neutrophil globulin	0–200 (5)	34±9.6 (5/5)§

* Range of daily neutrophil counts, number studied is in parentheses.
‡ Mean±SEM, number proteinuric/number studied in parentheses.
§ Not significantly different from control ($P < 0.5$).

also strongly suggest that this effect is independent of neutrophils and therefore different from previously described mechanisms of complement-induced glomerular injury (1, 4, 5, 8, 16). The essential findings can be summarized as follows: Injection of rats with sheep antibody to rat Fx1A results in immediate deposition of sheep IgG along the subepithelial surface of the glomerular capillary wall, which increases progressively in amount and granularity over 5 d in keeping with the kinetics of glomerular antibody deposition in this model defined in previous quantitative studies (18). Within 24 h rat C3 is detectable by IF deposited in a pattern and intensity similar to that of IgG. Despite this active C3 fixation in vivo, careful sequential studies reveal no neutrophil (or mononuclear cell) infiltrate in glomeruli and no inflammatory histologic changes. After 4–5 d nephritogenic quantities of antibody are deposited along with complement resulting in an increase in glomerular permeability and a marked and nonselective increase in urine protein excretion. Although some host IgG deposition is detectable in glomeruli by IF at the onset of proteinuria, our observations in rats immunosuppressed with cyclophosphamide or lethal irradiation suggest that proteinuria at 5 d is caused almost entirely by the heterologous antibody.

The critical role of complement in mediating proteinuria in PHN is clearly demonstrated by the finding that C3 depletion with purified CVF prevented glomerular C3 deposition and completely inhibited the development of proteinuria, without reducing the quantity of antibody that deposited in glomeruli or the ability of such deposits to fix complement in vitro. Additional evidence for the complement-dependent nature of glomerular injury in this model was provided by studies using noncomplement-fixing antibodies. We have previously shown that the development of proteinuria on days 4 and 5 in rats given unfractionated anti-Fx1A IgG is dependent upon the amount of antibody deposited (18). The threshold of glomerular-bound antibody required to produce proteinuria, about 90 μg IgG/2 kidneys, was presumably a mixture of γ2 (noncomplement-fixing) and γ1 (complement-fixing) subclasses (18). From the present study, the proteinuric threshold of glomerular-bound γ1 anti-Fx1A is clearly <60 μg IgG/2 kidneys, and four of six rats given noncomplement fixing γ2 antibodies had more than 60 μg IgG in their kidneys at 5 d but did not become proteinuric. Thus, it is reasonable to conclude that the failure of γ2 antibodies to produce proteinuria is due to some property other than the amount of antibody deposited. While supramolar doses of $F(ab')_2$ anti-Fx1A antibodies similarly failed to cause proteinuria, we cannot be certain that the amount of $F(ab')_2$ found in glomeruli exceeded the threshold for proteinuria. Coupled with the observation that C3-depletion prevented the appearance of proteinuria, it seems likely that the inability of γ2 (and perhaps also $F(ab')_2$) anti-Fx1A antibodies to alter glomerular permeability in the doses used is due to their inability to activate complement.

The essential requirement for complement deposition to induce proteinuria in this model was shown to be independent of neutrophils by the complete absence of any detectable neutrophils in glomeruli from the time of antibody injection until after proteinuria developed and by the failure of sustained neutrophil depletion to <200/mm^3 with specific antiserum to significantly alter proteinuria in rats with normal C3 levels. Severe neutropenia was noted also in cyclophosphamide-treated rats in which it similarly failed to prevent proteinuria.

These findings provide convincing evidence that the complement system, from C3 and perhaps through the terminal components, is an essential mediator of glomerular injury induced by subepithelial immune deposits in PHN. What is unusual, in the light of previous studies of complement-dependent experimental glomerular injury, is the total absence of inflammation during the genesis of the lesion, and the lack of effect of neutrophil depletion. In contrast to our findings in PHN, in nephrotoxic nephritis induced by heterologous anti-GBM antibodies, complement-dependent glomerular injury has been shown, in numerous studies, to be mediated by the action of neutrophils (4, 8, 9, 15), whereas the less common form of noninflammatory glomerular lesion has been shown to be complement independent (5, 10–12). Thus, in complement-dependent nephrotoxic nephritis neutrophils are prominent in the glomerulus before the onset of proteinuria (9, 10, 15) and neutrophil derived proteases, along with GBM fragments, are found in the urine (4, 8, 37). Neutrophil involvement in complement-mediated tissue injury is believed to be effected largely through generation of chemotactic products of complement activation, primarily C5a (4, 8). There is no known explanation for the absence of neutrophils from glomeruli despite complement activation in this study, but it might be the result of a barrier to immune adherence provided by the endothelium and GBM that separates the immune deposits from the capillary lumen. The direction of ultrafiltration might also prevent chemotactic factors from reaching the circulation. While neutrophils have recently been shown capable of inducing glomerular damage in the autologous phase of nephrotoxic nephritis without prior complement activation, (38, 39), the present study is the first to suggest that the complement system may injure glomeruli without the participation of neutrophils. In the only other study of complement in experimental "immune complex" glomerular disease, complement depletion ameliorated the vasculitis of

acute serum sickness but had no apparent effect on proteinuria (4, 40).

These studies do not elucidate the mechanism of complement-dependent glomerular injury in this model and, since noninflammatory, complement-induced organ damage has not previously been described, any theories of pathogenesis must be speculative. Amelioration of proteinuria in rats depleted of complement or given noncomplement fixing antibodies is clearly not the result of reduced glomerular antibody deposition, which tends to rule out any effect of putative glomerular complement receptors (41) or other factors, such as enhanced reticuloendothelial system clearance of antibody (42) which might have influenced the amount of antibody deposited. Similarly, based on these findings, as well as our previous studies using vasoactive amine blockers in PHN (18, 22), it is apparent that immune deposit formation (and consequent proteinuria) is not dependent upon anaphylatoxin (C3a and C5a) or complement-induced vasoactive amine release from mast cells as has been demonstrated in other models (43–45). There is some evidence from tracer studies in Heymann nephritis that electron-dense deposits located in the subepithelial space in the region of the slit diaphragm represent areas of increased permeability to macromolecules (46). It is therefore conceivable that alterations in the solubility of immune precipitates during incorporation of complement, as has been described in vitro (47), might give rise to areas of increased permeability resulting in loss of glomerular capillary barrier function.

Finally, it is possible that the terminal C5b-C9 membrane attack complex might, in a way analogous to its cytolytic effect (48), damage the integrity of the glomerular filter. Although there is no evidence that GBM is composed of lipid bilayers, immune deposits in PHN develop in the subepithelial space adjacent to the epithelial cell membrane and membranous slit-pore diaphragm (17, 22), which provide the final barrier to filtration of some proteins (49) and might be susceptible to attack by the C5b-C9 complex. Activation of C5b-C9 has been shown to expose hydrophobic peptides which insert into the lipid bilayer of membranes and result in membrane damage (48). This process may result in the release of membrane lipid and some of the inserted peptides are believed to form transmembrane channels that cause osmotic swelling and cell lysis (48). It is of interest in this regard that in complement-dependent nephrotoxic nephritis, an immediate loss of GBM lipid phosphorous occurred after anti-GBM antibody injection that preceded the influx of neutrophils (50), and that less proteinuria occurred in mice deficient in C5 (51).

This study provides new insight into the role of complement in immunologic renal injury and suggests a possible explanation for the noninflammatory nature of certain human glomerular diseases, such as membranous nephropathy and membranoproliferative glomerulonephritis, in which glomerular complement fixation occurs.

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