Human C4-Binding Protein

ASSOCIATION WITH IMMUNE COMPLEXES IN VITRO AND IN VIVO

JULIO SCHARFSTEIN, ELIZABETH B. CORREA, GLORIA R. GALLO, and VICTOR NUSSENZWEIG, *Department of Pathology, New York University School of Medicine, New York 10016*

ABSTRACT C4-binding protein (bp), a glycoprotein with specific binding affinity for the activated form of C4 (C4b), has recently been isolated from human serum and partially characterized. This report demonstrates that C4-bp is incorporated into soluble immune complexes after complement activation in vitro. The reaction requires Ca^{++} ions and the presence of C4 in serum.

Immunopathological studies of various forms of glomerulonephritis revealed intense C4-bp deposition in glomeruli from patients with immune-complex type of pathogenesis. C4-bp deposition was in close correlation with that of C4. These observations, together with the in vitro association of C4-bp to immune complexes, support the notion that the deposits in glomeruli represent the local accumulation of immune complexes.

INTRODUCTION

Recent studies in our laboratory have described the isolation from human (1) and mouse (2) serum of a protein with binding affinity for fluid phase-activated C4 (C4b). This protein, named C4-binding protein (bp)[1] was purified to homogeneity from human plasma; it is a glycoprotein, with a sedimentation coefficient of ≅11S, and consists of several disulfide-bonded subunits of mol wt 75,000. Stoichiometric analysis of the composition of C4-bp-C4b complexes revealed that C4-bp is multivalent in relation to C4b.

C4-bp is an essential cofactor in the proteolysis of C4b by C3b-inactivator (3), and may be related to the high molecular weight C3b-C4b-inactivator cofactor described by Nagasawa and Stroud (4).

This report deals with the detection of C4-bp in association with immune complexes prepared in vitro and as found in vivo in human glomerular disease.

METHODS

Diluents

Veronal-buffered saline, pH 7.4, ionic strength 0.15 M containing 0.1% gelatin (gelatin veronal buffer $[GVB]^{--}$), and GVB^{--} with 0.00015 M $CaCl_2$ and 0.005 M $MgCl_2$ (GVB^{++}) were used as diluents as described (5). By mixing equal volumes of GVB^{++} with 5% dextrose in water containing the above concentrations of divalent cations, dextrose GVB $(DGVB)^{++}$ was prepared. $DGVB^{--}$ was prepared as described for $DGVB^{++}$, but divalent cations were omitted. DGVB-EGTA-Mg^{++} consisted of $DGVB^{--}$ containing 0.01 M of EGTA and 0.002 M $MgCl_2$. DGVB-EDTA consisted of $DGVB^{--}$ containing 0.01 M of Na_2H-EDTA. All the above buffers were adjusted to pH 7.4. Barbital-EDTA buffer, pH 8.6, contained 0.023 M sodium barbital, 0.0037 M barbituric acid, and 0.002 M EDTA. When indicated in the text, ovalbumin (Sigma Chemical Co., St. Louis, Mo.), or rabbit serum albumin (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ill.) were added to these buffers to a concentration of 1 mg/ml. Dulbecco's phosphate-buffered saline was from Grand Island Biological Co., Grand Island, N. Y.

Purified complement components and reagents

C4-bp was purified from human plasma and characterized as described (1). Analysis of the purified preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed two major bands which were identified as C4-bp by affinity chromatography on Sepharose 4B-(Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) C4b and by immunoprecipitation with antibodies monospecific to C4-bp. In addition, two minor contaminants were detected. After radiolabeling of the purified preparation, 60% of the radioactivity was specifically bound to Sepharose 4B-C4b par-

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[1] *Abbreviations used in this paper*: AgAbC, complement-treated immune complexes; bp, binding protein; BSA, bovine serum albumin; DGVB, dextrose gelatin veronal buffer; GVB, gelatin veronal buffer; Ova, ovalbumin; Staph A, Staphylococcus aureus.

ticles, or immunoprecipitated by anti-C4-bp. The two C4-bp variants differ slightly in molecular weight and net charge.

Purified human C4, prepared as in (6) was a gift from Dr. I. Gigli, New York University School of Medicine, New York. C4b was prepared by incubating 10 mg of C4 with 0.4 mg of C1-esterase ($\overline{C1s}$) (7) for 24 h at 4°C. C3 was prepared by the method of Tack and Prahl (8). Serum from a patient with a genetic deficiency of C4 was given to us by Dr. H. Ochs, University of Washington School of Medicine, Seattle, Wash. Formaldehyde-fixed Staphylococcus aureus (Staph A, New England Enzyme Center, Tufts University, Boston, Mass.) was washed as described by Kessler (9). Human and rabbit gamma globulin were purchased from Miles Laboratories, Inc., and the immunoglobulin (Ig)G fractions were prepared by DEAE-cellulose chromatography. Human C4b and human IgG were coupled to CNBr-Sepharose 4B as previously described (10).

Antisera

Rabbit antisera to C4 and C3, fluorescein isothiocyanate-conjugated goat anti-human IgG, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins were purchased from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Antiserum to bovine serum albumin (BSA) was obtained by injection of BSA emulsified in Freund's complete adjuvant into 5- to 6-mo-old female rabbits. Rabbit antiserum to ovalbumin (Ova) was a gift from Dr. Z. Ovary, New York University School of Medicine, New York. Rabbit antiserum to human C4-bp was prepared as described (1). By crossed immunoelectrophoresis it reacted strongly with C4-bp, and weakly with C4. The antiserum was made monospecific to C4-bp by repeated absorption with Sepharose beads conjugated to purified C4b. After absorption, the presence of remaining antibodies to C4 was verified by a radioimmunoassay carried out by mixing 0.01 μg of purified, radiolabeled C4 in a volume of 10 μl with 75 μl of serially diluted antiserum to C4-bp. Then, 25 μl of 10 mg/ml anti-human C4 immuno-beads (Bio-Rad Laboratories, Richmond, Calif.) were added, and after 20 min incubation at room temperature the beads were washed in phosphate-buffered saline and counted for radioactivity. Standards consisted of dilutions of an antiserum to C4 whose antibody content was determined by quantitative precipitation reaction. The radioimmunoassay was positive in samples of the standard containing >5 ng of antibodies to C4. No contamination with anti-C4 was detected in the absorbed antiserum to C4-bp.

Gamma globulin from the various antisera was obtained by ammonium sulfate precipitation followed by DEAE-cellulose chromatography.

Radiolabeling

BSA, IgG-anti-BSA, and C4 were radiolabeled with ^{125}I or ^{131}I, by a modification (1) of the method of Hunter and Greenwood (11). The specific activity was between 1 and 2×10^6 cpm/μg protein.

Immune precipitation of complement-treated immune complexes (AgAbC) with antibodies to complement components

Preparation of AgAbC. Immune complexes were prepared in antigen excess, by mixing 480 μg of radiolabeled anti-BSA (IgG), containing 37% antibody as determined from quantitative precipitation reactions with 96 μg of BSA. The mixture was incubated at 37°C for 1 h, and stored at 4°C for 48 h. The precipitate was collected after centrifugation at 10,000 *g* for 20 min, washed three times in DGVB^{++}-Ova, and resuspended to 1 ml in the same buffer. This stock suspension was used to prepare soluble AgAbC as described (12–14), mixing 0.1–0.2 μg of antibody in a volume of 0.1 ml with 0.3 ml of fresh human serum diluted 1:3 in DGVB^{++}-Ova. The mixture was incubated at 37°C for 1 h. The reaction was stopped by addition of cold barbital-EDTA buffer containing rabbit albumin and centrifuged at 2,000 *g* for 10 min. The supernate was collected and used on the same day as the source of soluble AgAbC. 65–80% of the precipitates were recovered in the supernate as complement-solubilized complexes. In control tubes, in which the immune complexes had been treated with heat-inactivated human serum, only 15–25% of the complexes were recovered in the supernate.

Immune precipitation of AgAbC. The assay for detecting complement-bound immune complexes is based on the findings that after solubilization by complement, immune complexes display reduced Fc-dependent binding activity for Staph A, as shown in the experiment described in the legend of Fig. 1. It is very likely that this effect is a consequence of the binding of large amounts of C3, C4, and other complement molecules onto the antibody molecules which are part of the complexes (13). The assay described in detail below consists of the incubation of the complexes with rabbit antibodies to selected complement components, followed by treatment of the newly formed complexes with Staph A. The

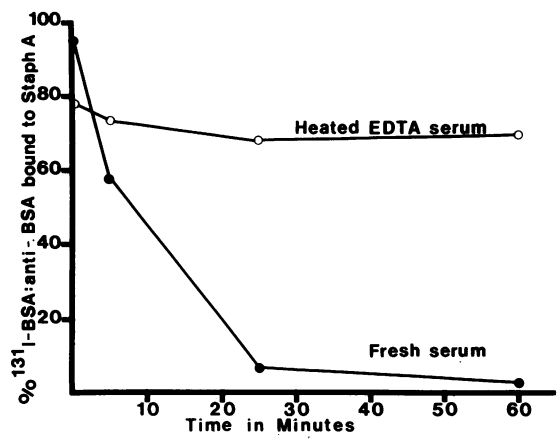

FIGURE 1 Diminished avidity for Staph A of AgAbC. In this experiment, soluble antigen-antibody complexes were prepared by mixing 3 μg of ^{131}I-BSA with IgG-anti-BSA in slight antibody excess. The mixture was incubated at 37°C for 30 min, diluted to 2.5 ml with DGVB^{++}, and cleared of large aggregates by centrifugation at 10,000 *g* for 20 min. 200 μl of this supernate, containing 90% of ^{131}I-BSA as part of the soluble immune complexes, was mixed with 200 μl of either (*a*) fresh human serum, or (*b*) heated serum (1 h at 56°C) containing 10 mM EDTA. 25-μl samples were removed at different times (triplicates), and added to 0.3 ml of a 10% suspension of Staph A in barbital-EDTA buffer, pH 8.6, containing 1 mg/ml Ova. The samples were vigorously mixed in a vortex mixer, diluted to 1 ml in the above buffer, and centrifuged at 2,000 *g* for 10 min. The supernates and pellets were counted for radioactivity, and the results expressed as percent of total immune complexes bound to Staph A. When complexes were treated with fresh serum for 25 min, they displayed very reduced avidity for Staph A under the experimental conditions described.

recognition by the rabbit antibodies of the appropriate moieties on AgAbC, provides new and nonhindered Fc-binding sites for Staph A. This assay provides a sensitive method for detection of complement-bound immune complexes which previously depended upon direct immune precipitation by specific antisera (13). Direct immune precipitation, in contrast to the present assay, requires prolonged periods of incubation to insure maximal precipitation, and, in many cases, the addition of purified carrier protein if the complement component studied is present in low concentrations.

The procedure adopted in all experiments was as follows: 10- to 50-μl samples of soluble ^{125}I-AgAbC containing 0.5–1 ng of antibody were mixed with IgG fractions of rabbit antibodies to C3, C4, C4-bp, or Ova (30 μl, containing 78 μg of IgG). In all cases this represented a large excess of antibody. Then, 30 μl of normal rabbit IgG (1 mg/ml) was added to the samples, followed by 30 μl of a 10% suspension of Staph A in barbital-EDTA buffer. After mixing, the samples were rapidly diluted to 0.6 ml with cold barbital-EDTA buffer containing rabbit albumin and centrifuged at 2,000 g for 15 min. The supernate was carefully aspirated with the aid of a Pasteur pipette connected to a vacuum pump. The pellets were counted and the results expressed as percent of total immune complexes bound to Staph A. Replicates (4–6) were used in all experiments. Control tubes included soluble immune complexes mixed with Ova, followed by anti-Ova and complexes mixed with normal rabbit IgG. The values obtained only depict the distribution of the different complement components among the complexes, but may not reflect the absolute amounts of complement components present in these AgAbC.

Immunofluorescence

Studies were performed on frozen sections of renal biopsies done on patients admitted to New York University Medical Center for diagnostic work-up of renal disease. Pathologic studies routinely include 2-μm sections for light microscopy, direct immunofluorescence microscopy, and, usually, electron microscopy. In this investigation, additional sections were examined with the indirect immunofluorescence technique employing antisera raised in rabbits, followed by fluoresceinated goat anti-rabbit immunoglobulins (polyvalent). The latter preparation, found to be cross-reactive with human immunoglobulins, required successive treatments with Sepharose 4B-human IgG until all cross-reacting antibodies were removed.

Specificity of staining was tested in different ways. The following antisera, absorbed with specific antigen to achieve abrogation of fluorescent staining, or treated with nonrelated complement components, were used in sections from many patients: anti-C4 absorbed with C4; anti-C3 absorbed with C3; anti-C4-bp absorbed with C4-bp; anti-C4-bp absorbed with IgG; anti-C4-bp absorbed with C4; and anti-C4-bp absorbed with C3. In addition, renal biopsies were incubated with nonimmune rabbit serum followed by fluoresceinated anti-rabbit immunoglobulins.

RESULTS

Table I shows that C4-bp and, as expected, C3 and C4 (13) are incorporated into immune complexes incubated with fresh serum to activate the complement system. Treatment of AgAbC with the immunoglobulin fractions of anti-C3, anti-C4, and anti-C4-bp, results in the precipitation of 64, 44, and 29% of the complexes, respectively. Control tubes were prepared by mixing the AgAbC with Ova, followed by anti-Ova in antibody excess, to determine the extent of trapping of AgAbC into an unrelated lattice. The amounts of Ova:anti-Ova complexes were in excess of those calculated to be present in the experimental tubes. The extent of nonspecific trapping was small because, in the presence or absence of Ova:anti-Ova, 11 and 7% of AgAbC were precipitated, respectively.

The specificity of the antisera used is shown in the same table. Preincubation of anti-C4, or anti-C3 immune reagents with purified C4-bp, did not affect their precipitating activity, although anti-C4-bp activity was significantly inhibited after treatment with C4-bp. Conversely, the addition of pure C4 to antiC4-bp was without effect, but it markedly inhibited the activity of the anti-C4 reagent.

The incorporation of C4-bp into complexes depends upon the activation of the classical complement pathway (Table II). When AgAbC complexes were prepared with serum diluted in buffers containing Ca^{++} and

TABLE I

Immunoprecipitation of AgAbC by Monospecific Antisera to Complement Components

Reagents used to immunoprecipitate the complexes		AgAbC complexes immunoprecipitated (±SD)	
Antibodies to:	Mixed with*		
		%	
C4-bp	Buffer	28.9±4.7	(1*a*)
	Purified C4-bp	17.8±2.3	(1*b*)
C4	Buffer	44.0±3.4	(1*c*)
	Purified C4-bp	38.1±5.4	(1*d*)
C3	Buffer	64.5±6.1	(1*e*)
	Purified C4-bp	64.4±3.1	(1*f*)
Ova‡	Buffer	11.1±4.5	
C4-bp	Buffer	29.6±2.1	(2*a*)
	Purified C4	28.0±1.4	(2*b*)
C4	Buffer	33.6±3.7	(2*c*)
	Purified C4	14.6±1.9	(2*d*)

Significance between differences in experimental and control groups was determined by one-tailed t tests. 1*a* vs. 1*b*, 2*c* vs. 2*d*, $P < 0.005$; 1*c* vs. 1*d*, 1*e* vs. 1*f*, 2*a* vs. 2*b*, NS. Four to six replicates were included in each experiment.

* 30 μl of anti-C4, anti-C4-bp, or anti-C3 were mixed with 10 μl of purified C4-bp (350 μg/ml), C4 (400 μg/ml), or buffer before the addition of AgAbC. C4-bp and C4 were in antigen excess as determined from quantitative precipitation reactions.

‡ Control for nonspecific trapping of AgAbC into an unrelated lattice. The corresponding antigen, Ova, was present in the diluent of AgAbC.

TABLE II

Requirements for C4-bp Incorporation into Immune Complexes: Activation of Complement by the Classical Pathway and Presence of C4

Immune precipitation of AgAbC with antibodies to	Serum treatment of ^{125}I-anti-BSA:BSA complexes*				
	Serum-DGVB^{++}	Serum-Mg-EGTA	Serum-EDTA	C4D-serum	C4D-serum + C4
C4-bp	31.4±5.6 (*a*)	13.3±0.2 (*b*)	—	14.7±1.3 (*c*)	22.7±0.8 (*d*)
C4	32.1±4.4 (*e*)	13.7±0.1 (*f*)	—	15.2±0.9 (*g*)	30.0±1.5 (*h*)
C3	67.3±4.5	68.1±4.8	9.3±4.0	50.3±2.0	50.3±6.2

Controls for nonspecific trapping of AgAbC into an unrelated lattice as in Table I. Significance between differences among experimental groups was determined by one-tailed *t* tests. The differences between a vs. b, c vs. d, e vs. f, and g vs. h, were significant, $P < 0.001$.

* Serum diluted 1:4 in the various buffers indicated. The results represent the mean percentages (±SD) of AgAbC immunoprecipitated. Four to six replicates were included in each experimental group.

Mg^{++}, 67, 32, and 31% were immunoprecipitated by antibodies to C3, C4, and C4-bp, respectively. When AgAbC complexes were prepared with serumEGTA-Mg^{++} to prevent activation of the classical pathway, the incorporation of C4 and C4-bp was inhibited, while that of C3 was unaffected. If complexes were treated with serum-EDTA, C3 deposition was inhibited.

The experiments shown in Table II demonstrate that the presence of C4 in serum is required for the incorporation of C4-bp into immune complexes. In these studies, complexes were incubated either with C4-deficient serum or with the same serum after reconstitution with pure C4. In the absence of C4, only 14.7% of the complexes were precipitated by antibodies to C4-bp. This level of precipitation is nonspecific, as 15.2% were precipitated by anti-C4, and these values do not differ significantly from those obtained when complexes treated with Ova were treated with anti-Ova (Table I). In contrast, when the C4-reconstituted serum was used, 30 and 22.7% of complexes were immunoprecipitated by antibodies to C4 and C4-bp. Therefore, incorporation of C4-bp into immune complexes is C4-dependent. In agreement with previous results (12, 13) a large proportion of complexes incubated with C4-deficient serum incorporated C3, as a result of the activation of the alternative pathway.

The immunofluorescence findings in 43 renal biopsies of patients with a variety of glomerular diseases are shown in Table III. Some, such as lupus nephritis, membraneous nephritis, membranoproliferative glomerulonephritis, and acute poststreptococcal glomerulonephritis, are currently held to be immune complex types of glomerulonephritis. In the remaining, the pathogenesis is less certain or unknown.

Of the patients with lupus nephritis, five had diffuse proliferative, one focal proliferative, three membranous, and one mesangial lupus glomerulonephritis. In all of these, regardless of morphologic type, C4-bp was detected in glomeruli in the same distribution as C3, C4, and immunoglobulins (Fig. 2). Similarly, C4-bp was detected in idiopathic membranous glomerulonephritis (six of seven cases), in membranoproliferative glomerulonephritis (six of six cases) and in Berger's nephropathy (one case). Often, the staining for C4-bp was more intense and diffuse than that of C4; and in four instances, C4-bp, but not C4, was detected in glomeruli.

In contrast, in only one of four cases of acute poststreptococcal glomerulonephritis were C4-bp, C4, and IgG detected, although all showed the presence of C3 deposits.

Finally, only one of eight assorted diseases without known immune complex pathogenesis (i.e., minimal change disease, idiopathic hematuria, amyloidosis, diabetes, idiopathic focal glomerulonephritis, and nephrocalcinosis) showed deposits of both C4-bp and C4. Thus, a very good correlation exists between the absence of C4-bp deposits and nonimmune-complex-mediated glomerulonephritis.

DISCUSSION

The main findings of this study are that, as in the case of complement components C3 and C4, C4-bp is incorporated into serum-treated immune complexes, and is found in glomerular lesions from patients with immune-complex types of glomerulonephritis.

The antiserum used in this study was specific for

TABLE III

Immunofluorescence Findings in Renal Biopsies of Patients with Glomerular Diseases

Diagnosis	No. of patients	Positive immunofluorescence with antisera to			
		Ig	C3	C4	C4-bp
Presumed immune complex glomerulonephritis					
Lupus glomerulonephritis	10	10/10*	10/10	10/10	10/10
Membranous glomerulonephritis (idiopathic)	7	7/7	6/7	3/6	6/7
Membranoproliferative glomerulonephritis	6	5/6	6/6	4/6	6/6
Acute poststreptococcal glomerulonephritis	4	1/4	4/4	1/4	1/4
Berger's nephropathy	1	1/1	1/1	0/1	1/1
Unclassified					
Proliferative and sclerosing glomerulonephritis	2	1/2	1/1	0/2	2/2
Glomerular sclerosis	4	1/4	0/4	0/4	0/4
Presumed coagulopathy					
Crescentic glomerulonephritis	1	0/1	1/1	0/1	0/1
Nonimmune complex					
Minimal change disease	1	0/1	0/1	0/1	0/1
Idiopathic hematuria	2	0/2	0/2	0/2	0/2
Amyloid	2	0/2	1/2	1/2	1/2
Diabetes	1	1/1	0/1	0/1	0/1
Idiopathic focal proliferative glomerulonephritis	1	0/1	0/1	0/1	0/1
Nephrocalcinosis	1	0/1	0/1	0/1	0/1

* The fractions represent the number of positive samples over the number of patients tested.

C4-bp by several criteria. By crossed immunoelectrophoresis it reacted strongly with C4-bp and with no other serum or plasma protein. Its activity in the immunoprecipitation assays (Table I) or in immunofluorescence was not inhibited by purified human C4, C3, or IgG, although it was markedly diminished or abolished after absorption with purified C4-bp (Table I and Fig. 1). Conversely, the latter preparation did not inhibit the activities of the antibodies to C3 and C4, or to IgG. More recently, other antisera have been prepared against one of the two variant forms of C4-bp purified to homogeneity.[2] These monospecific antisera were also used to detect C4-bp in kidney sections with similar results.

Our in vitro studies indicate that the incorporation of C4-bp into immune complexes is C4-dependent and depends upon the activation of complement via the classical pathway. This is shown in the experiments (Table II) in which complexes were treated with serum in the presence of EGTA to chelate Ca^{++} ions, or with serum from patients with a genetically determined C4-deficiency. Under these conditions, only the alternative pathway is activated, and neither C4 nor C4-bp were bound to the immune complexes. Addition of purified C4 to the C4-deficient serum resulted in incorporation of C4-bp and C4. The C4-dependence of C4-bp association to immune complexes is explained by the earlier findings that C4-bp forms stable complexes with the activated form of C4 (C4b) (1).

These findings led us to examine kidney sections of patients with various forms of nephropathy for the presence of C4-bp in glomeruli. We observed that in most cases in which immune complex pathogenesis was suspected, abundant glomerular deposition of C4-bp was found, in close correlation with that of C4.

The notion that C4-bp deposits reflect the local accumulation of circulating immune complexes is supported by several observations. Such deposits are absent in cases of glomerular disease not known to be associated with immune complex pathogenesis. On the other hand, in systemic lupus erythematosus, a prototype of immune complex glomerulonephritis, deposits invariably contained C4-bp as well as C4, C3, and IgG. In a limited survey of biopsies of patients with acute poststreptococcal glomerulonephritis, C3 was uniformly present, while IgG, C4, and C4-bp were absent in some. This point requires further observation in more patients, but suggests that, as in other biopsies from patients with poststreptococcal glomerulonephritis in which properdin was found, the alternative pathway of complement activation may be operative (15).

In some patients with idiopathic membranous glomerulonephritis we did not detect C4, while heavy C4-bp deposition was observed. The discrepancy may result from hindrance of C4b moieties by associated

[2] Fujita, T., and V. Nussenzweig. Manuscript in preparation.

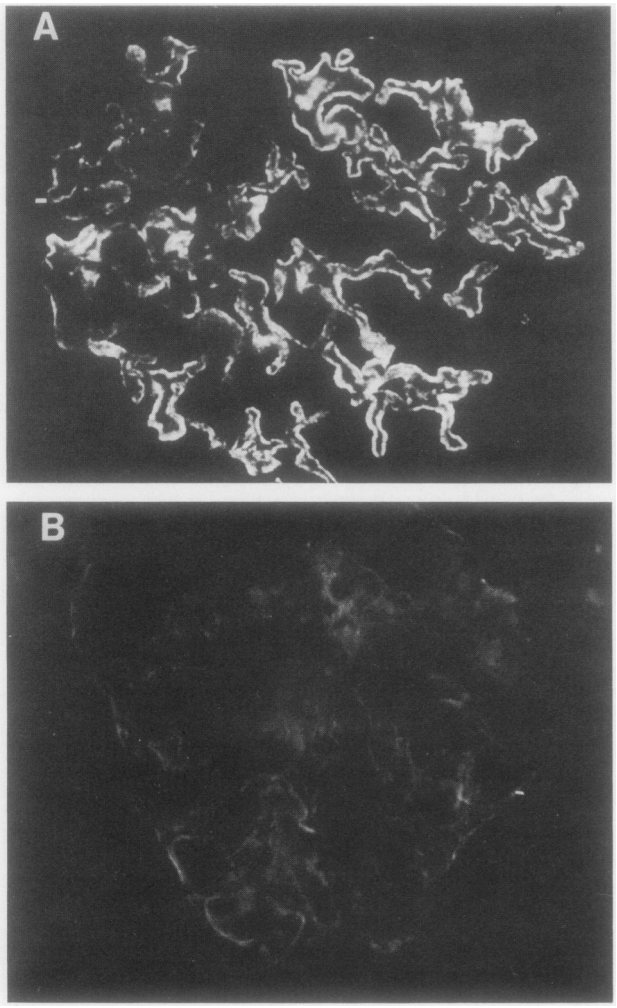

FIGURE 2 Immunofluorescence microscopy (×280) of a renal biopsy of a patient with membranous lupus nephritis. (A) Frozen section incubated with rabbit anti-human C4-bp followed by fluorescein-conjugated goat anti-rabbit gamma globulins shows bright diffuse granular staining of deposits in glomerular capillary tufts. Similarly, there was also staining for IgG, C3, C1q, and C4. (B) Frozen section incubated with rabbit anti-human C4-bp after absorption with C4-bp at slight antigen excess, as determined by quantitative precipitation reaction.

C4-bp, degradation of C4 by C4-bp and C3b-inactivator (3), or higher affinity of the anti-C4-bp antibodies as compared with anti-C4. Whatever the reason, the presence of C4-bp in glomeruli appeared to be a more sensitive indicator of classical pathway activation than the presence of C4.

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