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ISOLATION AND CHARACTERIZATION OF FELINE C3 AND EVIDENCE FOR THE IMMUNE COMPLEX PATHOGENESIS OF FELINE INFECTIOUS PERITONITIS

HELEN E. L. JACOBSE-GEELS,[1] MOHAMED R. DAHA, AND MARIAN C. HORZINEK

From the Institute of Virology, Veterinary Faculty, State University Utrecht, The Netherlands; and the Department of Nephrology, University Hospital, Leiden, The Netherlands

Infections of cats with feline peritonitis (FIP) virus are usually inapparent but may lead to fatal polyserositis. We have recently advanced the hypothesis that immune complexes play an essential role in the pathogenesis of the condition. To support this hypothesis, the role of the third component of complement in FIP was investigated. In the present paper, the isolation of C3 from normal cat serum and some of its physical and immunologic properties are described. The final protein had an apparent m.w. of 185,000 and was composed of 2 polypeptide chains with m.w. of 128,000 and 71,000, respectively. When tested against whole cat serum, an antiserum raised in rabbits against purified C3 recognized only 1 protein whose identity with C3 was established. With the aid of this antiserum, depositions of C3 in renal glomeruli of FIP-affected cats were demonstrated by immunofluorescence. Their localization coincided with that of deposited IgG, thereby supporting the concept of an immune complex pathogenesis of FIP.

Feline infectious peritonitis (FIP)[2] is a viral disease of cats that is caused by a member of the Coronaviridae family (1). The infection is latent in most cases. When symptoms appear, they are the reflection of a polyserositis with accumulation of exudate in the body cavities and occasionally of neurologic and ocular damage. Once clinical symptoms have appeared, the disease usually has a fatal course. Upon post-mortem examination, pyogranulomatous organ lesions are prominent. Animals suffering from FIP possess high titers of anti-coronavirus antibodies (2, 3) and significantly decreasd levels of total hemolytic complement (C) (4). Some animals develop a proteinuria which is thought to be a reflection of a renal inflammation (5, 6).

These latter 2 conditions, together with our ignorance about feline C, caused us to study the involvement of the C system in FIP. Since it is known that activation of the third component of C by, e.g., immune complexes, may play a role in the development of inflammatory processes, we focused our attention on C3 and its presence in renal tissue of FIP-affected cats. The present paper supports the hypothesis of an immune complex pathogenesis of FIP expressed in a recent review (7).

MATERIALS AND METHODS

Chemicals and sera. The following chemicals were obtained as indicated between parentheses: polyethylene glycol 6000 (PEG; BDH, Amsterdam, The Netherlands), benzamidine (Aldrich-Europe, Beerse, Belgium), DE-52 cellulose (Whatman Biochemicals, Kent, England), hydroxyapatite, Biogel A1.5, dithiothreitol (DTT; Biorad Laboratories, Pleuger, The Netherlands), quarternary aminoethyl A50 Sephadex (QAE; Pharmacia Fine Chemicals Inc., The Hague, The Netherlands), XM50 diaflo ultrafiltration membranes (Amicon Corp., Lexington, MA), and zymosan (Nutritional Biochemicals Corp., Cleveland, OH).

Cat serum was obtained from healthy cats by cardiac puncture. The blood was allowed to clot at room temperature for 30 min. After 5 min centrifugation at 3000 rpm, the serum was collected and stored in aliquots at −70°C.

Assay of C3 hemolytic activity. The hemolytic activity of C3 was monitored during purification using the cellular intermediate $EAC1^{gp}\ 4^{hu}\ 2^{gp}$ (see Abbreviations) prepared as described (8). Normal human serum treated with hydrazine (9) and supplemented with functionally purified C5 served as a source for the C factors C5–C9. Half isotonic Veronal-buffered saline containing 2.5% dextrose, 0.1% gelatin, 5×10^{-4} M $MgCl_2$, and 1.5×10^{-4} $CaCl_2$ ($DGVB^{++}$) served as diluent in the hemolytic assay. One hundred-microlilter volumes of diluted C3 containing fractions were incubated with 1×10^7 $EAC1^{gp}\ 4^{hu}\ 2^{gp}$ cells and the C5–C9 mixture in 0.4 ml $DGVB^{++}$ for 1 hr at 37°C. The mixtures were subsequently brought to 2 ml with isotonic NaCl solution. After centrifugation, the average number of hemolytic sites generated was calculated using the formula $Z = -ln\ (1 - y)$ in which y represents the proportion of cells lysed.

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulphate (SDS) electrophoresis was performed as described (10) in 10% gels containing 1% SDS. Fifty microliters purified C3 fractions were mixed with 50 microliters 10 M urea-1.5% SDS solution and held at 37°C for 60 min. Reduction of C3 protein was done in 8 M urea containing 1% SDS and 0.02 M DTT at 56°C for 45 min. Thereafter, the samples were applied to the gels and subjected to electrophoresis at 10 mA/gel at 30°C until the bromophenol blue front marker had reached the end of the gel.

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[1] Correspondence: Dr. H. E. L. Jacobse-Geels, Institute of Virology, Veterinary Faculty, State University Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands.

[2] Abbreviations used in this paper: FIP, feline infectious peritonitis; $EAC1^{gp}\ 4^{hu}\ 2^{gp}$, sheep erythrocytes sensitized with rabbit antibody and bearing guinea pig C1 and C2 and human C4; $DGVB^{++}$, half isotonic Veronal-buffered saline containing 2.5% dextrose, 0.1% gelatin, 5×10^{-4} M $MgCl_2$ and 1.5×10^{-4} M $CaCl_2$; EDTA, ethylenediamine tetraacetate; PEG, polyethylene glycol; QAE, quarternary aminoethyl Sephadex; DTT, dithiothreitol.

Immunoelectrophoresis was carried out in 1.5% agarose (indubiose agarose, BDH, Amsterdam, The Netherlands), containing 0.025 M Veronal and 0.002 M EDTA[2] (pH 8.4) on 10 × 10 cm glass plates at 225 V for 35 min. Precipitation arcs were developed overnight with goat antiserum to human C3 and rabbit anti-feline C3.

Preparation of rabbit anti-feline C3 serum. Twelve micrograms of highly purified C3 from the post-QAE pool were suspended in 1.5 ml complete Fruend's adjuvant (CFA) and injected subcutaneously in a rabbit. After 2 wk, an equal dose in CFA was administered as a booster. Two weeks later the rabbit was bled and the serum was tested in double radial immunodiffusion against normal cat serum and purified feline C3.

Immune histology. Depositions of C3 were demonstrated using both direct and indirect immunofluorescence techniques. Unfixed feline kidney tissue from FIP cases and control animals was snap frozen in isopentane prechilled in liquid nitrogen and stored at −70°C until use. Cryostat sections (2 to 4 μm thick) were incubated with a fluorescein-conjugated IgG fraction of rabbit anti-feline C3 antiserum (diluted 1/4) for 30 min at room temperature in the direct technique. In the indirect technique, sections were incubated with an IgG fraction of rabbit anti-feline C3 (diluted 1/5) and subsequently stained with a fluorescein-conjugated goat anti-rabbit IgG antiserum (dilution 1/20). Before and after each incubation, the sections were rinsed 2 times for 10 to 15 min in phosphate-buffered saline (PBS) (pH 7.2). The sections were mounted in 10% glycerol in PBS and subsequently examined in a Leitz-Orthoplan fluorescence microscope. Photographs were taken on Kodak Tri-X-Pan film (Eastman Kodak Company, Rochester, NY).

RESULTS

Isolation. In order to prepare a specific antiserum against feline C3, C3 was isolated from fresh frozen cat serum. Therefore, 40 ml cat serum was stirred for 30 min at 0°C with 15.5 ml of a 25% PEG solution in 0.1 M phosphate buffer (pH 7.4) after the addition of 6 mg benzamidine and 0.930 ml of 0.086 M EDTA (pH 7.4). The precipitate was centrifuged for 15 min at 7000 rpm (5900 × G) and dissolved in 20 ml of 0.01 M Tris buffer (pH 8.0) containing 0.002 M EDTA, 1 mM benzamidine, and enough NaCl to yield a conductivity of 2.5 mS; it was then dialyzed against 1 liter of the same buffer.

The dialyzed material was applied to a 1.5 x 30 cm DE-52 column that had been equilibrated with dialysis buffer. After collection of 25 fractions of 4.3 ml each, a linear NaCl gradient in 500 ml starting buffer was applied to the column. The conductivity of every fifth fraction was determined at 0°C. Hemolytic C3 activity was measured in 1:50 dilutions of these fractions, and a 50-μl aliquot was used for protein detection by the Folin method (11).

C3 activity eluted between 3 and 7 mS (Fig. 1). Fractions 35 through 44, which contained the bulk of C3 activity, were pooled and dialyzed against 1000 ml phosphate buffer of pH 7.9 and a conductivity of 1.5 mS. The dialyzed pool was applied on a hydroxyapatite column (3.5 x 20 cm) and washed with 200 ml dialysis buffer, after which a linear phosphate gradient was applied. The eluate was collected in fractions of 4 ml each. C3 activity was measured in 1:25 dilutions and eluted between 3 and 7 mS (Fig. 2). The protein content of every fifth fraction was determined in 100-μl samples by Folin analysis. Fractions containing peak C3 activity (91 through 105) were pooled and concentrated to 5 ml by ultrafiltration on a Amicon XM-50 membrane.

The concentrated pool was then applied to a 2.5 x 95 cm Biogel A1.5 column and eluted with Veronal-buffered saline (pH 7.5) containing 0.15 M NaCl and 0.002 M EDTA. Fractions of 2.3 ml were collected, and 100-μl aliquots were tested for protein contents (Fig. 3). Fucntional C3 activity was measured in 1:20 dilutions of every fifth fraction and filtered at 66% of the

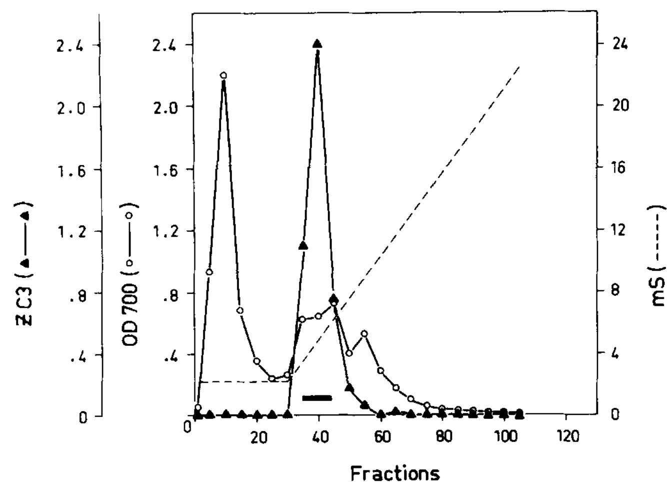

Figure 1. Chromatography on DE-52 cellulose of the 7% PEG precipitate of normal cat serum. Fractions were assayed for conductivity (-----), protein content (O——O) and C3 hemolytic activity in 1:50 dilutions (▲——▲). Fractions 35 to 44 were pooled.

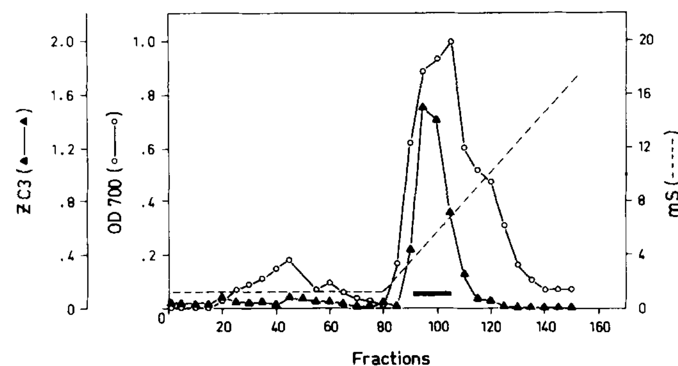

Figure 2. Hydroxyapatite chromatography of the post DE-52 pool containing C3. Fractions were tested for conductivity (-----), protein (O——O), and C3 hemolytic activity in 1:25 dilutions (▲——▲). Fractions 91 to 105 were pooled.

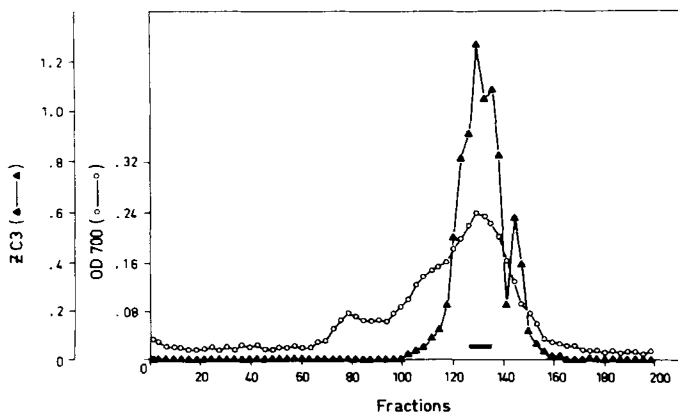

Figure 3. Gel filtration on Bio-Gel A1.5 of the concentrated hydroxyapatite pool. Fractions (2.3 ml) were tested for protein contents (O——O) and C3 hemolytic activity in 1:20 dilutions (▲——▲). Fractions 126 to 135 were pooled.

bed volume, which is comparable with the size of human C3. Fractions 126 through 135, containing the greater part of C3 activity, were pooled. Since the post-Biogel pool still contained contaminants, as assessed by SDS-PAGE, it was dialyzed against 0.01 M Tris buffer containing 0.002 M EDTA and enough NaCl to yield a conductivity of 2.5 mS. The dialyzed material was further purified on a QAE A50 column (1.5 x 10 cm). The column was washed with 120 ml starting buffer and eluted with a linear NaCl gradient that was collected in fractions of 3.2 ml each.

Fractions were tested for conductivity at 0°C, and the protein concentration was determined in 100 μl of the fractions (Fig. 4).

The protein profile consisted of 2 peaks, 1 of which contained C3 activity. Fractions that proved to contain no contaminants, as assessed by SDS-PAGE analysis, were pooled. Recovery of C3 protein in the QAE pool amounted to 17% of the total C3

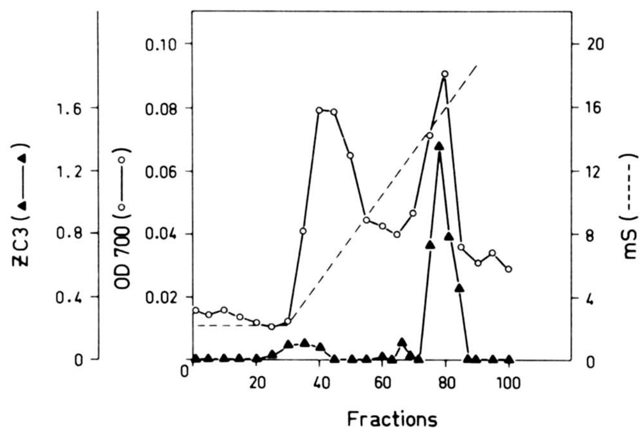

Figure 4. Anion exchange on QAE A-50 Sephadex of the post-Bio-Gel pool. Fractions were assayed for conductivity (-----), protein (○——○) and C3 hemolytic activity in 1:20 dilutions (▲——▲).

TABLE I

Yields of C3 protein during purification determined by radial immunodiffusion against rabbit anti-cat C3

	Volume	Total C3	Yield
	ml	*mg*	*%*
Starting serum	40	10.5	100
PEG precipitate	20	6.7	64
Post DE-52 pool	43	6.0	58
Post HAP pool	57	5.0	47
Post Bio-Gel pool	25	2.4	23
Post QAE pool	22	1.8	17

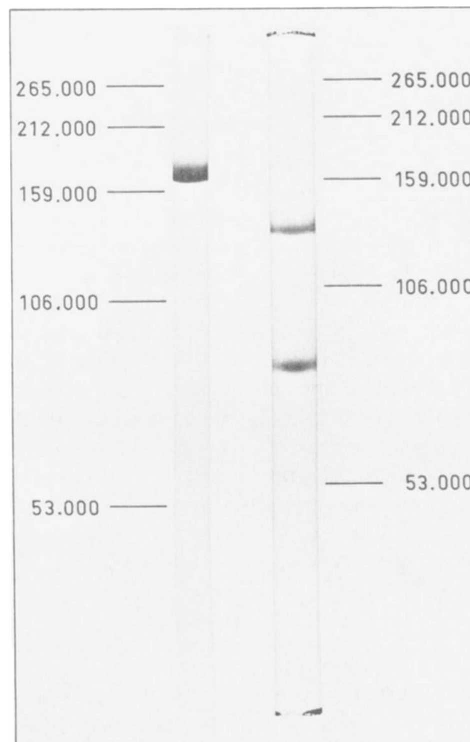

Figure 5. SDS-PAGE analysis of feline C3 in unreduced (*left*) and reduced (*right*) form.

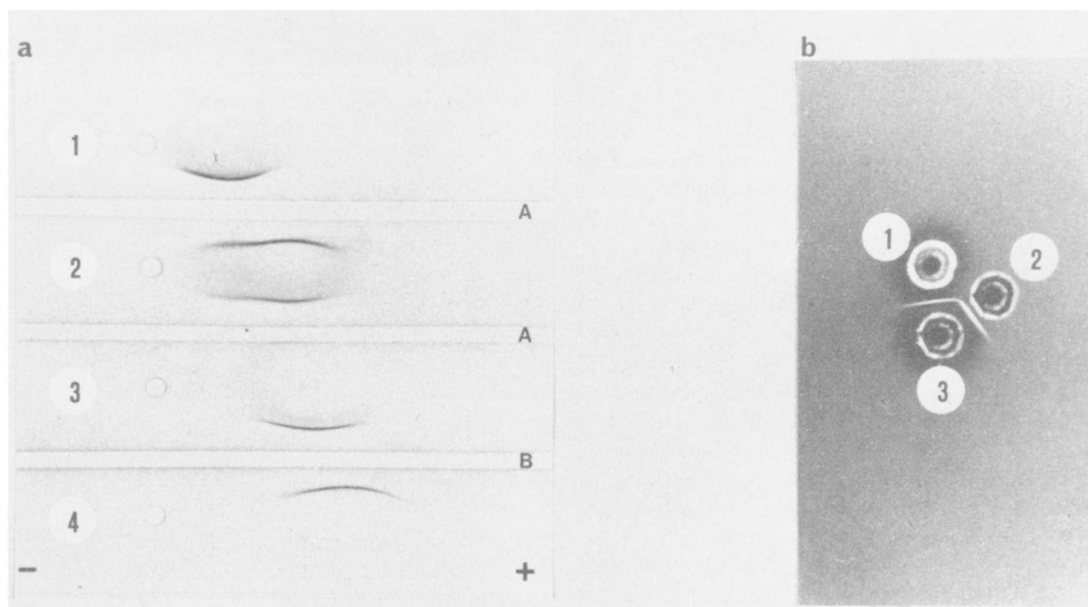

Figure 6. a, Immunoelectrophoretic analysis of human serum (*1*), zymosan-treated human serum (*2*), cat serum (*3*) and zymosan-treated cat serum (*4*). Precipitation arcs were developed with anti-human C3 (*A*) and anti-cat C3 (*B*). *b,* Radial immunodiffusion of normal cat serum (*1*) and purified C3 (*2*) against anti-cat C3 (*3*).

protein in the starting serum (Table I). The profile of hemolytic C3 activity in the pooled fractions of all chromatographic steps coincided with the C3 profile as assessed by radial immunodiffusion against rabbit anti-feline C3 antiserum.

Characterization. To investigate the homogeneity of the QAE-fractions, 50-μl samples were subjected to SDS-PAGE analysis. Unreduced samples migrated into the gels as 1 band, whereas reduction with DTT resulted in the appearance of 2 bands (Fig. 5). Their apparent m.w. were calculated by comparing the migration rates with those of m.w. markers (BDH biochemicals, Poole, England).

Unreduced C3 had a m.w. of 185,000 ± 7000 (mean ± 1 standard deviation); the 2 polypeptide chains encountered after reduction had m.w. of 128,000 ± 2300 and 71,000 ± 2100, respectively.

In order to obtain information on the cleavage of feline C3, normal cat serum was incubated with 2 mg/ml zymosan for 30 min at 37°C in parallel with normal human serum. After incubation, the residual hemolytic C3 activity in both feline and human serum was determined. Zymosan treatment of cat serum resulted in a decrease to 37% of the initial hemolytic activity, whereas 35% of the initial hemolytic C3 activity remained in the human serum. In immunoelectrophoresis, feline C3 showed a mobility slightly different from human C3. Zymosan treatment of feline serum resulted in only a minor change in electrophoretic mobility. Purified feline C3 gave a single continuous line of precipitation in double radial immunodiffusion when tested against whole cat serum and purified C3, respectively (Fig. 6).

Immune histology. Post-mortem examination of 6 experimental infected kittens and 8 spontaneous FIP cases revealed characteristic lesions for FIP on the surface of liver, kidney, and other internal organs. High anti-FIP virus antibody titers were present in the sera of these cats. When immunofluorescence with antibodies to feline C3 was performed on cryostat sections of kidney tissue of the field FIP-cats, fluorescence was found in the mesangial area of renal glomeruli in 6 of the 8 kidneys examined (Fig. 7*a*, Table II). Identical fluorescence was found in renal tissue of all of the experimental provoked FIP cases (Table II). Although not extensively studied, IgG depositions were found in a similar granular pattern as C3 depositions. The kidneys of the normal cats investigated showed a weak fluores-

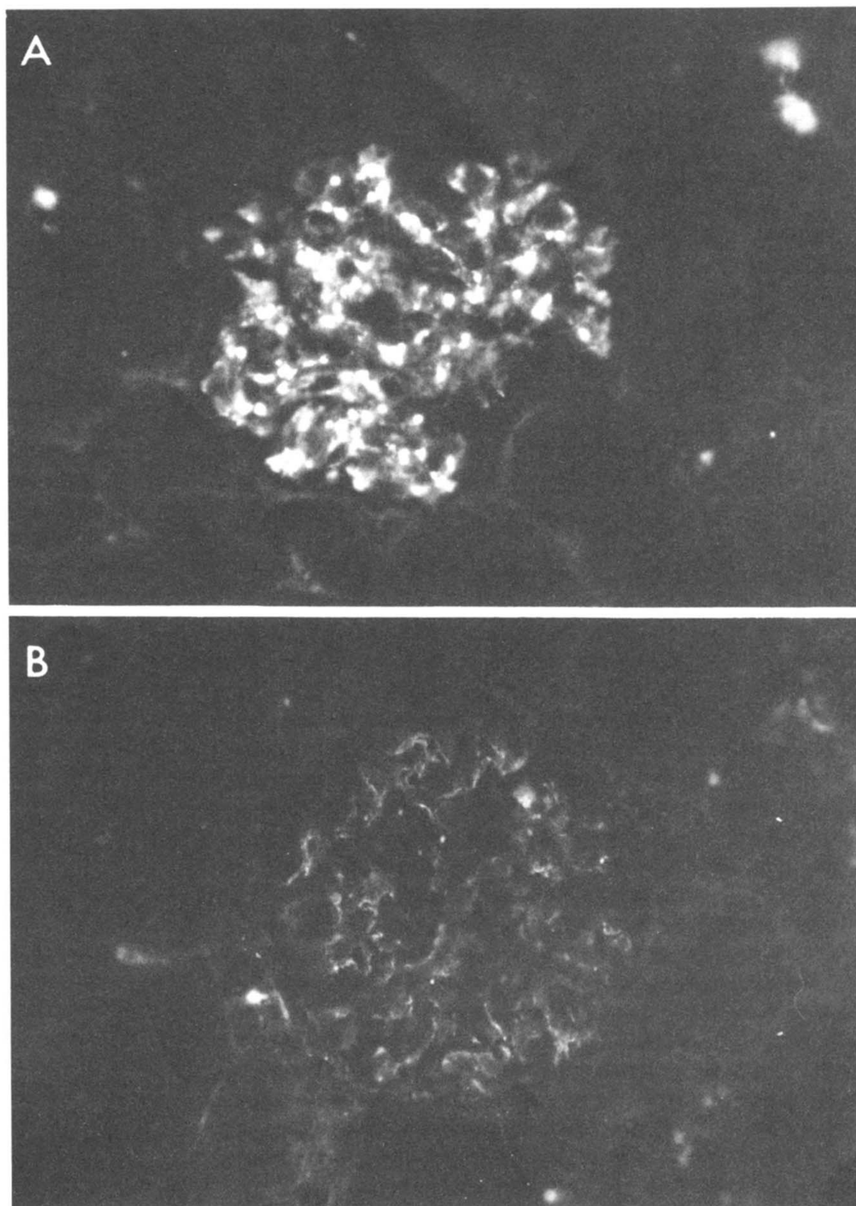

Figure 7. *a*, photomicrograph of a renal glomerulus from a FIP-affected cat stained with fluorescein-conjugated rabbit IgG anti-feline C3 (× 250); *b*, photomicrograph of a renal glomerulus from a control cat stained with fluorescein-conjugated rabbit IgG anti-feline C3 (× 250).

TABLE II

Deposition of C3 in renal glomeruli of FIP cats and normal cats

	Immunofluorescence of C3		
	No. of cats tested	No. of cats positive	No. of cats negative
FIP field cases	8	6	2
Experimental FIP cases	6	6	0
Controls	8	0	8

cence with anti-C3, both by the direct and by the indirect technique. Since this fluorescence (as depicted in Fig. 7*b*) was very weak, it was scored as negative.

DISCUSSION

Feline C3 was isolated from cat serum by 7% PEG precipitation and chromatographic purification in 4 steps, involving DE 52, hydroxyapatite, Biogel Al.5, and QAE A50. Yields of 11 to 17% were obtained in 3 experiments, 1 of which is summarized in Table I. The resulting material was homogeneous on SDS-PAGE analysis (Fig. 5) and gave only 1 precipitation arc with rabbit anti-whole cat serum in immunoelectrophoresis. Injection of the final preparation into a rabbit resulted in the formation of antibodies that reacted equally well with native C3 and its split products in untreated cat serum during immunoelectrophoresis. Treatment of cat serum with zymosan at a concentration of 2 mg/ml for 30 min at 37°C caused 63% C3 consumption but resulted in only a minor change in electrophoretic mobility. This finding is different from those found for rat C3 (13) and human C3 (Fig. 6*a*).

Proof that the isolated protein was C3 was provided by the capacity of the final material to react with the cellular intermediate $EAC1^{gp}\ 4^{hu}\ 2^{gp}$, resulting in lysis of these cells in the presence of the components C5-C9. The m.w. of feline C3 and its 2 disulfide-linked chains are comparable to human C3 (12) and rat C3 (13). SDS-PAGE analysis has revealed that storage of purified C3 at −20°C for 6 wk, and repeated thawing and freezing resulted in the appearance of a minor amount of a large protein, which is probably due to aggregation of C3.

No detectable depositions of C3 or IgG were found in the renal glomeruli of 8 control animals. Twelve out of 14 FIP cats, however, showed depositions of C3 and IgG in the mesangial area of the renal glomerulus (Fig. 7, Table II); negative results were obtained in 2 field cases, where no data on the length and severity of the disease process could be obtained.

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