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IN VITRO ALTERNATIVE PATHWAY ACTIVATION OF COMPLEMENT BY THE BRUSH BORDER OF PROXIMAL TUBULES OF NORMAL RAT KIDNEY[1]

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The purpose of this study was to investigate which structures of the nephron, if any, are capable of directly activating the complement (C) system. To this end, two sets of experiments were performed. First, activation of C was assessed on sections of frozen kidney tissue, using the indirect immunofluorescence technique for the demonstration of C fixation. Second, glomerular or tubular fractions of kidney were incubated with normal fresh serum, and subsequent C consumption was measured. The data obtained support the interpretation that the brush border of proximal tubules activates the alternative pathway of the C system. This phenomenon may have pathogenic significance in conditions of aselective proteinuria.

Deposition of complement (C) components, indicating either classical or alternative pathway activation of the C system, or both, is commonly observed in kidneys of patients with glomerulonephritis (1, 2). In most cases the fixation of C appears to be secondary to antigen-antibody reaction (1, 2). However, sometimes—for example, in patients with basement membrane dense deposit disease—evidence for immunologically mediated C fixation is lacking (3). In such instances the possibility may be entertained that the C system is directly activated by glomerular or tubular structures.

The aim of the present study was to investigate whether the C system may be activated directly by one or more of the various structures of the nephron. It was found that the brush border of proximal tubules of rat kidney fixes C by activating the alternative pathway. This phenomenon may have pathogenic significance in conditions of aselective proteinuria.

MATERIALS AND METHODS

Fixation of C by cryostat sections of rat kidney. To determine which kidney structures, if any, fix C, we used unfixed or fixed 4-μm thick sections of normal Lewis rat kidney snap-frozen in liquid nitrogen. The methods of fixation, carried out at 4°C, were the following: acetone for 15 min, absolute ethanol for 5 or 15 min, methanol for 5 or 15 min, and 3.5% paraformaldehyde for 6 or 15 min. After washing in phosphate-buffered saline, pH 7.3 (PBS), the sections were incubated at 37°C for 40 min with 20 μl of the following reagents: three different fresh normal human sera (HS)[3]; three different fresh normal rat sera (RS); EDTA[3]-treated HS and RS (100 μl 0.1 M EDTA in PBS was added to 1 ml of HS and RS); Mg EGTA[3]-treated HS and RS (100 μl EGTA buffer, pH 7.4, containing 0.05 M $MgCl_2$, was added to 1 ml of HS and RS); C-inactivated HS and RS (heating at 56°C for 60 min); the enzyme inhibitors Aprotin (4) (Sigma, St. Louis, MO) or phenylmethylsulfonyl fluoride (PMSF; Sigma) (5), followed by HS and RS (preceding the incubation with HS or RS, the kidney sections were first incubated for 5 min with PBS containing 15 "trypsin-inhibitor units" of Aprotin/ml or with 10% ethanol in PBS containing 2.5 mg of PMSF/ml); HS and RS preincubated with Fx1A (see below; 20 mg/ml of serum) for 1 hr at 37°C; HS and RS preincubated with baker's yeast spores, as described by Vallota and Mueller-Eberhard (6); HS from which C1q, C3, or properdin had been removed by precipitation at equivalence with the IgG fraction of anti-human C1q, C3, and properdin antisera, respectively (7). After washing with PBS, the sections were incubated with fluorescein isothiocyanate- (FITC) conjugated antisera to rat immunoglobulins, IgG, or C3 (Cappel Laboratories, Cochranville, PA) or to human IgG, IgM, IgA, C3 (Hyland Diagnostics, Deerfield, IL), Clq (Kent Laboratories, Bellevue, WA), C4 (Cappel Laboratories), or properdin (Kent Laboratories). These antisera were found to be monospecific when tested by immunoelectrophoresis as well as by immunodiffusion. Cryostat sections of normal rat liver and normal rat jejunum were processed in the same way as the kidney sections. The sections were examined with a Leitz Ortholux microscope equipped with an HBO-200 high-pressure mercury-vapor lamp, using a GB12 exciter filter and a KV490 barrier filter. The intensity of fluorescence staining was semiquantitatively evaluated as follows: 0, negative; +, slight; ++, moderate; +++, marked. Reproducibility of the results was assessed by repeating each individual experiment at least three times.

Preparation of a glomerular and tubular kidney fraction. Fx1A, a cortical fraction containing brush border of proximal tubules, was prepared from rat kidneys according to the method described by Edgington *et al.* (8). A glomeruli-rich fraction obtained during the preparation of Fx1A was further purified by repeated centrifugation at 400 × G for 5 min. As judged by phase-contrast light microscopy, the final glomerular preparation contained less than 1% of tubular structures. Both tubular and glomerular preparations were lyophilized. Endotoxin was not detectable in either preparation when tested by the limulus assay (9).

C assays. C consumption in HS or RS after incubation for 1 hr at 37°C with the tubular (20 mg Fx1A/ml of serum) or glomerular preparation (20 ng/ml of serum) was determined by measuring total hemolytic C activity (CH_{50}) (10). To differentiate classical from alternative C pathway activation, Mg EGTA and EDTA treated sera were used (11, 12). Effective inhibition of either pathway in these Mg EGTA- or EDTA-treated sera was shown by measuring CH_{50} values after preincubation of these sera with sheep red blood cells sensitized with rabbit antiserum (EA cells; 5×10^8/ml of serum) or with inulin (25 mg/ml of serum) (Sigma).

C3 activation in serum was studied by a modification of the bidirectional immunoelectrophoresis technique (13). Electrophoresis was performed in 0.4 M Tris-glycine buffer, pH 9.5, on a gelatinized cellulose acetate sheet (Cellogel, Chemetron, Milan, Italy) measuring 14 x 14 x 0.03 cm. Five-tenths microliter of undiluted serum, previously incubated for various periods of time with 2 mg/ml of Fx1A, was submitted to electrophoresis for 55 min at 200 V (14). Then, 20 μl of rabbit anti-human C3 antiserum (Behringwerke, AG, Marburg-Lahn, West Germany) diluted with 120 μl of PBS were spread with a volumetric distributor, measuring 6 x 2 x 0.1 cm. The second electrophoresis, performed after a 90-degree turn of the sheet and electrode

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[3] Abbreviations used in this paper: HS, fresh normal human serum; RS, fresh normal rat serum; EDTA, (ethylenedinitrilo)-tetraacetic acid; Mg EGTA, Mg ethyleneglycol-*bis*-(β-amino-ethyl ether)-*N,N'*-tetra-acetic acid; PMSF, phenylmethylsulfonyl fluoride; Fx1A, a kidney cortical fraction containing components of the brush border of proximal convoluted tubules; CH_{50}, standard hemolytic complement activity; EA cells, sheep red blood cells sensitized with rabbit antiserum; PT, proximal tubules.

reversal, was done in the same buffer for 12 hr at 120 V. The gel was washed several times in PBS and stained with Coomassie Blue.

Generation and purification of C5a. A component with anaphylatoxic activity, generated by incubating ε-aminocaproic acid- (Sigma) treated HS with 20 mg/ml of Fx1A for 75 min at 37°C, was purified according to the technique of Vallota and Mueller-Eberhard (6) involving a three-step chromatographic procedure. For reference, C5a was generated by incubating ε-aminocaproic acid-treated HS with baker's yeast spores (6, 15). Polyacrylamide gel electrophoresis of either anaphylatoxin preparation gave a single band (15). The minimal effective concentration of reference C5a, as tested on guinea pig ileum (16), was 2.5×10^{-10} M. The m.w. of the Fx1A-generated anaphylatoxin as well as that of the reference C5a were calculated according to the method of Andrews (17). A Sephadex G-100 column (3.5 x 200 cm) equilibrated with acetic acid sodium acetate, pH 3.7, ionic strength 0.1 was employed. ^{125}I-bovine serum albumin (66,000 daltons) (crystallized BSA, Behringwerke, AG), hemoglobin (64,500 daltons) (Behringwerke, AG), peroxidase (40,000 daltons) (Boehringer, Mannheim, West Germany), cytochrome *c* (12,400 daltons) (Boehringer), and ^{125}I glucagon (3600 daltons) (Behringwerke, AG) were used as marker substances. Labeling of the C5a preparations with ^{125}I was performed by the lactoperoxidase-glucose procedure (18). Iodination was followed by extensive dialysis against Rexyn 201 anion-exchange resin (Fisher Scientific Co., Fairlawn, NY) containing PBS, centrifugation, and affinity chromatography (19). The m.w. of the reference C5a was ~17,500.

Reference C3a was generated by incubation of ε-aminocaproic acid-treated HS with inulin. Isolation was performed by the method of Hugli *et al.* (19). Polyacrylamide gel electrophoresis of the C3a preparation gave a single band (15). The m.w. of C3a estimated by the method of Andrews (17) was ~9000. The minimal effective concentration of C3a to induce guinea pig ileum contraction was 5×10^{-8} M.

RESULTS

Fixation of C by cryostat sections of rat kidney. After incubation of sections of rat kidney with HS or RS, deposits of human or rat immunoglobulins were never observed in renal structures. The results of the study of fixation of C to sections of rat kidney are summarized in Table I. Incubation of acetone-fixed kidney sections with HS or RS resulted in fixation of C3 along the luminal border of proximal tubules (PT) (Fig. 1). Omission of HS or RS abolished the immunofluorescence reaction. Properdin, but not C1q or C4, could be shown to be localized at the same site. Except for the walls of the vessels, other kidney structures did not appear to fix C. Binding of C was unaffected by Mg EGTA or the enzyme inhibitors Aprotin or PMSF. In contrast, C fixation was completely abolished by heat-inactivation of HS or RS (Fig. 2), by treating HS or RS with EDTA or zymosan, by incubation of HS or RS with Fx1A, or by HS pretreated with the IgG fraction of an antiserum against human C3 or properdin. HS pretreated with the IgG fraction of anti-human C1q serum partially blocked C fixation. Liver or small intestine cryostat sections did not bind C. In unfixed sections, the C activation by PT was weak and not well localized. Sections fixed in absolute ethanol (5 min), methanol (5 min), or 3.5% paraformaldehyde (6 min) were able to fix C weakly. C fixation, however, was abolished when these fixations were prolonged for 15 min.

TABLE I

In vitro fixation of various human complement components along the luminal side of proximal tubules in the rat kidney

Treatment of Normal Fresh Human Serum	C3	Properdin	C1q	C4
None	+++[a]	+++	0	0
56°C, 30 min	0[a]	0	0	0
EDTA	0[a]	0	0	0
Mg EGTA	+++[a]	+++	0	0
Aprotin^R	+++[a]	+++	0	0
PMSF	+++[a]	ND	0	0
Fx1A	0[a]	0	0	0
Zymosan	0[a]	0	0	0
Anti-C3 IgG	0	0	0	0
Anti-properdin IgG	0	0	0	0
Anti-C1q IgG	+	++	0	0

[a] Similar results were obtained for rat C3 by using rats as source for fresh serum. ND, not done. For further details, see *Materials and Methods.*

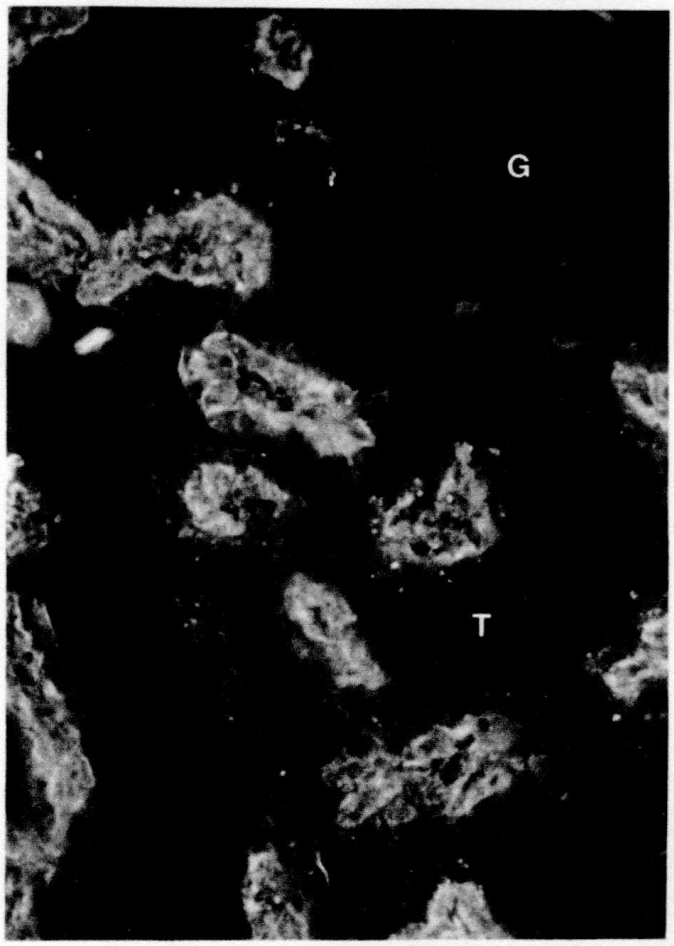

Figure 1. Frozen section of cortical rat kidney fixed in acetone and incubated first with fresh normal human serum (HS) and then stained for human C3. Binding of C to the brush border of proximal tubules is observed. A glomerulus (G) and distal tubules (*T*) do not show C fixation. × 300.

C activation by Fx1A. Incubation of HS or RS with Fx1A caused a significant decrease in the CH_{50} value. Incubation with the glomerular preparation did not lead to C consumption (Fig. 3). C consumption was not inhibited by treatment of fresh serum with Mg EGTA. Treatment with EDTA, however, blocked C consumption. In HS incubated with Fx1A, a reduction in native C3 and the appearance of C3c and C3d could be demonstrated (Fig. 4). C3 activation was blocked by EDTA but not by Mg EGTA.

Generation of anaphylatoxic activity by incubation of human serum with Fx1A. One hundred microliters of serum, first incubated with Fx1A and dialyzed to remove C3a, induced contractions of guinea pig ileum. The Fx1A-generated active serum component exhibited cross-tachyphylaxis with purified human C5a used as reference, but not with C3a. The purified Fx1A-generated active serum component appeared to have the same m.w. (17,500) as the reference C5a. In addition, the minimal effective concentration of the component (5×10^{-10} M), as tested on guinea pig ileum, was comparable to that of the reference C5a.

DISCUSSION

The results of the present experiments, utilizing the immunofluorescence technique, indicate that the brush border of PT in acetone-fixed, and less in unfixed, cryostat sections of kidney is capable of directly activating the C system. After incubation of rat kidney sections with HS, deposits of human IgG, IgA, or IgM along the luminal side of PT were never observed. This finding excludes the possibility that HS used in these experiments contained antibodies reacting with the brush

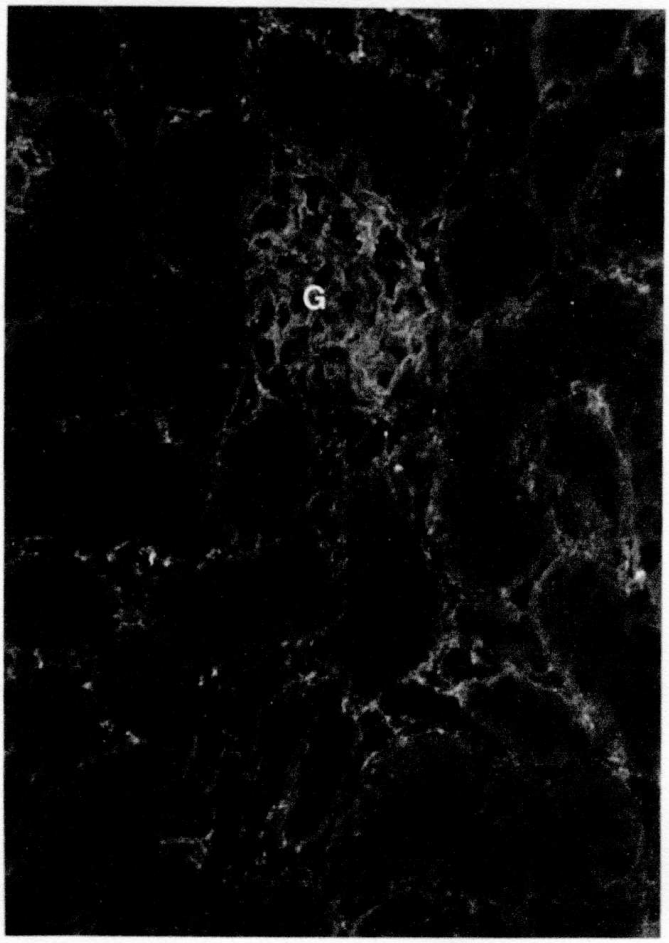

Figure 2. Frozen section of cortical rat kidney fixed in acetone and incubated first with heat-inactivated HS and then stained for human C3. No binding of C to the brush border of proximal tubules is observed. G, glomerulus. × 300.

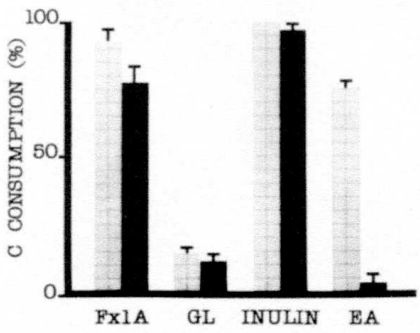

Figure 3. C consumption in untreated (*grey bars*) or Mg EGTA-treated (*black bars*) HS incubated with Fx1A, a glomerular preparation (*GL*), inulin, or sensitized sheep red blood cells (*EA*). Each bar represents 5 experiments ± 1 SE. For details see *Materials and Methods* and *Results*.

border of convoluted PT (20). The occurrence in human sera of antibodies directed against brush border antigens of PT seems a very rare event (21) and has never been observed in our laboratory. Likewise, after incubation of rat kidney sections with RS, tubular deposits of rat immunoglobulins or IgG were never observed. Acetone fixation of the cryostat sections was helpful for precise identification of the site of C fixation. C fixation was also detected in the walls of the vessels, as described by Linder (22). Activation of C by other tubular segments or by glomeruli was not observed. Furthermore, liver tissue or the brush border of the villi of the small intestine did not fix C. In agreement with our observations is a report by Beeson and Rowley (23) describing the strong anticomplementary effect of kidney tissue, and an incidental reference by Bartolotti and Peters (24), who while investigating C fixation *in vitro* by immune complexes lodged in glomeruli, noted localization of variable amount of C3 in tubules of normal as well as nephritic rabbits when the kidney tissue was incubated with C4-deficient guinea pig serum.[4]

The following findings favor the interpretation that the C system was mainly activated via the alternative pathway: First, after incubation of kidney sections with fresh serum, C3, and properdin, but no C1q or C4, could be demonstrated along the brush border of PT. Second, addition to fresh serum of Mg EGTA, which blocks the classical but not the alternative pathway, did not influence the binding of C to the tubules. Failure of two enzyme inhibitors to prevent tubular fixation of C3 makes it unlikely that the C system was activated by enzymes present in PT.

The contention, based on the results obtained from the studies by immunofluorescence techniques, that brush border of PT may directly activate the C system was strengthened by experiments involving incubation of fresh serum with partially purified brush border present in a kidney fraction called Fx1A (8). The addition of Fx1A to fresh serum abrogated the ability of C to bind to cryostat sections of kidney, promoted the consumption of total C hemolytic activity, and generated C3 breakdown products and an anaphylatoxin with the characteristics of C5a. Mg EGTA added to fresh serum induced only a small decrease in C consumption. Thus, C activation mainly took place via the alternative pathway. At the same time, the latter observation suggests that a small amount of C was consumed via the classical pathway. This interpretation would explain the partial reduction in C binding to brush border in kidney sections when the sections were incubated with fresh human serum preabsorbed with an anti-human C1q antiserum (Table I).

In contrast to the tubular fraction, the glomerular preparation failed to activate the C system. A similar result was described by Lambert *et al.* (25). These authors showed that although intact glomerular basement membrane was ineffective, alternative pathway C consumption could be induced by soluble products obtained from pronase-digested glomerular basement membrane. These findings suggest that if direct activation of the C system in gomeruli occurs, as hypothesized in human basement membrane dense deposit disease (25) or in rabbits with hyperlipemia (26), the glomerular basement membrane must have been altered by an as yet unknown mechanism.

Although in normal condition C components are not filtered by glomeruli, their presence has been demonstrated in the urine of patients with nephrotic syndrome due to a variety of causes (27, 28). In these patients the C system may be activated by the brush border of PT. Such activation could lead to damage of PT. This possibility is supported by results of micropuncture studies demonstrating functional as well as morphologic lesions induced by intraluminal perfusion of PT with fresh serum (29). Because inactivation of the C system prevented the development of tubular lesions, they seem to be caused by C-mediated cell injury (30). These results might be interpreted as indicating that the activator of the C system is localized on the cell surface. Interestingly, incubation of fresh serum with a brush border fraction abolished the damaging effect of serum on PT (30). It is hypothesized, therefore, that in patients with aselective proteinuria, damage of PT (31–33) may be sustained from direct activation of the C system by the plasma membrane of the brush border.

[4] After this paper was submitted for publication, P. J. Baker, B. P. Croker, and S. G. Osofsky published evidence (Kidney Int. 20:437, 1981) that dead cultured kidney cells initiate C activation that results in binding of C3 to the cell surface.

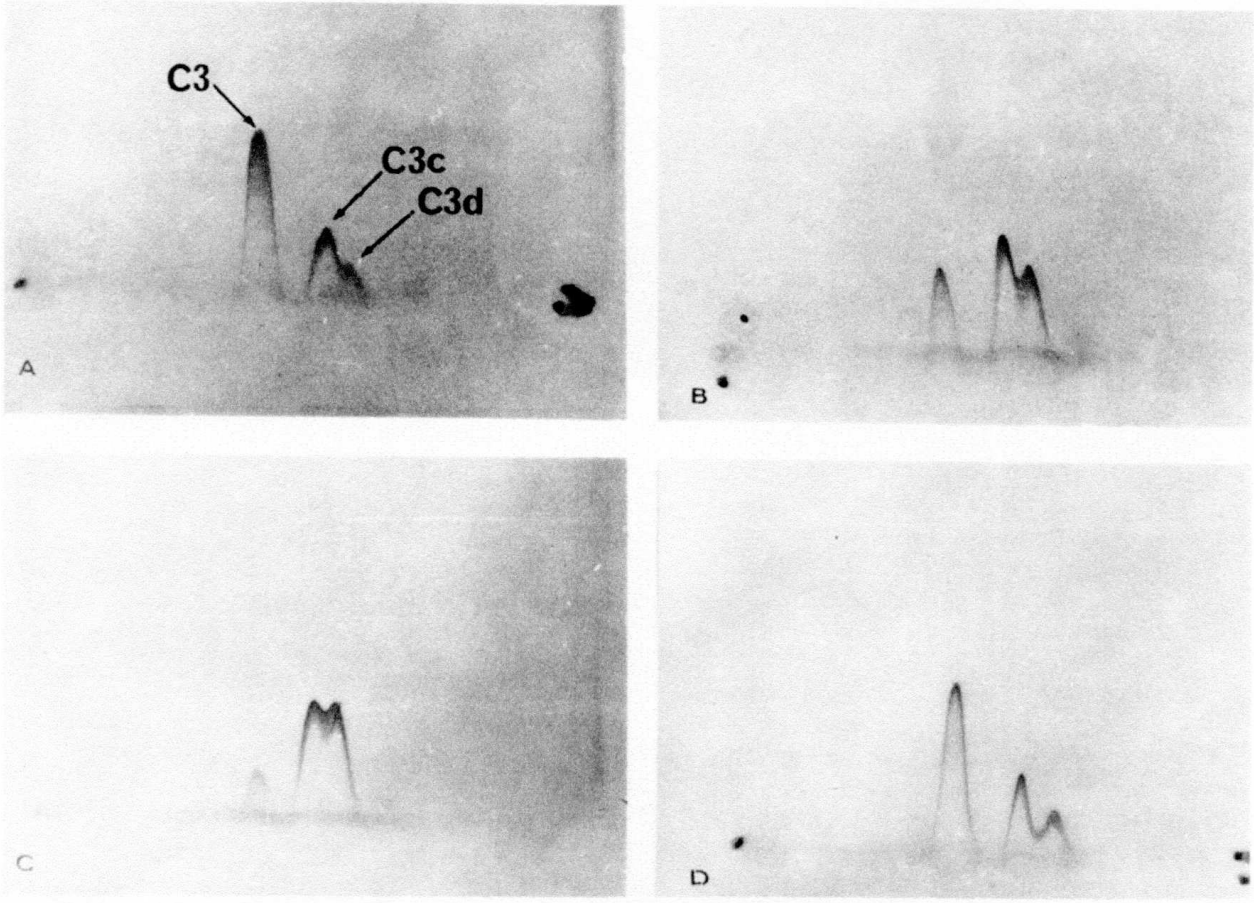

Figure 4. Results of a representative experiment performed by bidirectional immuno-electrophoresis showing the breakdown of native C3 into C3c and C3d upon incubation of HS with Fx1A. *A*, Mg EGTA-treated HS. *B*, MG EGTA-treated HS after incubation for 1 hr with Fx1A. *C*, Mg EGTA-treated HS after incubation for 3 hr with Fx1A. *D*, HS similarly treated as the serum shown in C, except for the addition of Fx1A. For details, see *Materials and Methods*.

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