

AUTOLOGOUS IMMUNE COMPLEX NEPHRITIS INDUCED WITH RENAL TUBULAR ANTIGEN

I. IDENTIFICATION AND ISOLATION OF THE PATHOGENETIC ANTIGEN*

BY THOMAS S. EDGINGTON,† M.D., RICHARD J. GLASSOCK,‡, § M.D., AND
FRANK J. DIXON, M.D.

*(From the Department of Experimental Pathology, Scripps Clinic and
Research Foundation, La Jolla, California 92037)*

PLATES 70-73

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A form of experimental allergic glomerulonephritis induced with renal tubular antigen has recently been advanced as a model of an autologous immune complex disease (1). This form of immunologically mediated renal disease, first described by Heymann (2) as an autoimmune nephrosis (3, 4), is a chronic progressive membranous glomerulonephritis which exhibits, in common with certain forms of human glomerulonephritis (5, 6), the hallmarks of antigen-antibody complex-induced nephritis, namely: (a) the granular deposition of γ - and β_{1C} -globulins along the glomerular capillary walls (7-9); and (b) the appearance of electron-opaque deposits along the subepithelial aspects of the glomerular basement membranes (10). In addition it has been demonstrated that the nephritogenic antigen(s) is not of glomerular origin (1, 11, 12), but is a renal tubular epithelial (RTE) antigen; and recent studies have suggested its derivation from the brush border cells of the proximal convoluted tubule of the kidney (1). This report is concerned with the identification, isolation, and partial characterization of the nephritogenic antigen which is responsible for the induction and perpetuation of this form of autologous immune complex (AIC) nephritis. The nephritogenic antigen is distinct from the previously described RTE-specific antigens (13) and has been designated RTE- α_6 . Typical AIC nephritis has been induced with microgram doses of purified RTE- α_6 .

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§ Present address: Department of Medicine, Harbor General Hospital, Torrance, Calif., and the University of California, Los Angeles. Work performed during a leave of absence from the Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

and the antigenic identity of the glomerular-deposited nephritogenetic antigen has been confirmed. The accompanying paper (14) is concerned with the pathogenetic mechanisms involved in AIC nephritis and exposition of the general pathogenetic concept.

Materials and Methods

Preparation of Crude Kidney Fractions.—Sprague-Dawley rat kidneys were fractionated, much as previously described (13), into a predominantly renal tubular epithelial suspension, fraction 1 (Fx1), which in turn was subfractionated into the saline insoluble ultracentrifugally sedimentable fraction 1A (Fx1A) and the ultracentrifuge supernatant, fraction 1B (Fx1B). In addition, whole glomeruli were isolated from the initial 400 g sediments by washing; and glomerular basement membranes were isolated from washed glomeruli as described by Krakower and Greenspon (15). All fractions were dialyzed against or washed with distilled water at 4°C, lyophilized, and stored at -35°C.

Lyophilized Whole Rat Tissues.—Rats were anesthetized, and their vascular system was perfused with cold phosphate-buffered saline (PBS). Tissues were removed, minced in 10 volumes of PBS, and homogenized for 2 min at 0-2°C in a Waring Blendor. The suspension was dialyzed at 4°C against distilled water, lyophilized, and stored at -35°C.

Preparation of Rabbit Antisera to Rat RTE Antigens and Urine.—Antisera to Fx1A and two of the previously isolated RTE-specific antigens, RTE- α_3 and RTE- α_4 , were prepared by repeated immunization of male New Zealand white rabbits with the appropriate antigen in incomplete adjuvant as previously described (13). Antisera specific for renal tubular epithelium, anti-RTE, was prepared by four absorptions of anti-Fx1A with 1 mg lyophilized rat serum and 2 mg Fx1B per ml of antiserum. Antiserum to whole urinary protein was prepared by immunization of rabbits with 10 mg of normal rat urine which had first been dialyzed and then lyophilized. The antigen was administered first in the foot-pads and then subcutaneously at two successive weekly intervals. Antiserum was subsequently drawn 7 days following an additional immunization. γ_2 -globulin fractions of all antisera were then prepared by 50% ammonium sulfate precipitation which was followed by elution from DEAE-cellulose at 0.0175 M sodium phosphate, pH 6.5 (16).

Fluoresceinated Antibodies.—Immunoelectrophoretically specific antisera to rabbit γ -globulin (anti-RGG) were prepared in sheep by repeated immunization with rabbit γ_2 -globulin (16). Antisera to rat γ -globulin (anti-RrGG) were prepared by immunization of rabbits with rat γ_2 -globulin isolated in the same fashion. Rabbit antisera to rat β_{1C} -globulin (anti- β_{1C}) were prepared according to the method described by Mardiney and Müller-Eberhard (17). The γ_2 -globulin fraction of each antiserum was fluoresceinated according to the method of Clark and Shephard (18); and nonspecific staining was removed by absorption with acetone-precipitated sheep liver powder or alternatively the fluoresceinated antibody (fluorescein: protein ratio of 1-2) was reisolated chromatographically as described by Wood et. al. (19).

Immunofluorescent Staining Techniques.—The techniques employed for conventional immunofluorescent studies were much as originally described by Coons and Kaplan (20). Demonstration of RTE- α_5 , deposited in glomeruli from rats with AIC nephritis has been described in a brief report (1). Blocks of rat kidney were snap-frozen in a liquid nitrogen and stored at -35°C. Sections were cut in a cryostat at 4 μ and allowed to adhere to the glass microslides overnight at 4°C in a humid box. Glomerular-deposited γ -globulin was then partially eluted in 2.5 M potassium thiocyanate (KSCN) at 37°C for 2 hr followed by 15 min at 56°C. The sections were washed three times for 8 min in PBS and were then fixed first for 10 min in ether-ethanol (1:1) followed by 20 min in 95% ethanol. Following three 5 min washes in PBS, the sections were incubated for 45 min with rabbit antiserum (usually the γ_2 -globu-

lin fractions). The sections were washed three times for 5 min in PBS and then stained with fluoresceinated anti-RGG, which had been absorbed with rat γ -globulin, for 45 min, then washed and examined. A single pool of anti-RTE (γ_2 -globulin at a concentration of 0.5 mg protein/ml) was routinely used for the first antibody layer. Negative controls included saline and anti-RTE, which had been absorbed three times with 5 mg Fx1A/mg γ_2 -globulin.

Fluorescent Antibody Inhibition Test (FIT).—The presence and concentration of RTE- α_5 or a cross-reactive material in various tissue fractions or extracts was evaluated by the capacity of such test material to inhibit the staining by anti-RTE of glomerular deposited RTE- α_5 in kidneys from rats with AIC nephritis. A single pool of anti-RTE was used to stain the glomerular deposited RTE- α_5 as described above. The kidneys from three rats with advanced disease were employed throughout the investigation. 50 μ l of anti-RTE γ_2 -globulin at 1.0 mg protein/ml was mixed and incubated with the same volume of test solution or suspension overnight at 4°C in 3 ml conical precipitation tubes. Following resuspension and centrifugation for 30 min at 1000 g, the supernate was used as the first antibody layer as described above for the immunofluorescent demonstration of glomerular deposited RTE- α_5 . The minimum concentration of test material required to abolish the immunofluorescent reaction with glomerular deposited antigen was expressed as the inhibiting concentration (μ g nitrogen/mg antibody). Test preparations were assayed at twofold serial dilutions, and the results were reproducible to within one dilution. Chromatographic and electrophoretic fractions were qualitatively assayed at full strength.

Crude Solubilized RTE Antigen Preparation.—The RTE-specific antigens were isolated in a crude, but soluble, form by slight modification of previously described techniques (13). 1 g of Fx1A was homogenized in 50 ml of 1% sodium desoxycholate in 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 8.0. The suspension was stirred at 4°C for 2–4 hr, and then centrifuged at 105,000 g for 45 min at 4–8°C. The supernate containing the RTE-specific antigens was carefully decanted and retained. The precipitate was dialyzed against distilled water and lyophilized. Saturated ammonium sulfate was added by slow drip during constant stirring at 4°C to bring the sodium desoxycholate soluble extract to 25% ammonium sulfate saturation. The resultant suspension was centrifuged at 78,640 g for 45 min and the precipitate was dialyzed against distilled water and lyophilized. The supernatant was extensively dialyzed against saline at 4°C, then concentrated to 5 ml by pressure dialysis. This latter fraction contained the previously described RTE-specific antigens (13) as well as RTE- α_5 in a soluble form as determined by immunoelectrophoretic and Ouchterlony techniques, and the fluorescent antibody inhibition test.

Preparatory Electrophoresis.—The crude solubilized RTE-specific antigen fraction, recovered as the 25% ammonium sulfate supernatant, was electrophoretically fractionated on a Pevikon block in 0.05 M Veronal pH 8.2 as described by Müller-Eberhard (21). The origin was placed 4 inches from the cathodal margin of a 5 × 18 inch block and electrophoresis was carried out at 4°C with a potential gradient of 8.9 v/inch for 18 hr. The block was cut at 1 inch intervals and a sample of the initial eluate was taken for the fluorescent antibody inhibition assay. The segments were subsequently eluted twice following the addition each time of 5 ml saline. The protein recovered from each segment was determined by the Folin technique. Fractions which contained RTE- α_5 were pooled, dialyzed against saline, and concentrated to approximately 20 ml. This product was assayed semiquantitatively for RTE- α_5 by the fluorescent antibody inhibition test.

Molecular Sieving on Sephadex G-200.—The bead form of Sephadex G-200 was dry sieved and the 270–325 fraction (45–53 μ bead size) was hydrated in 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 8.0 and packed to 95 cm in a 3 cm column under constant flow at 3.5 ml/cm² cross-sectional area/hour. The sample, recovered from Pevikon block electrophoresis, was concentrated to 2 ml and gently applied to the column, and 5 ml fractions of the effluent

were collected automatically and assayed for RTE- α_5 as well as other RTE-specific antigens. Fractions containing RTE- α_5 were pooled and concentrated by negative pressure dialysis.

DEAE Sephadex Chromatography.—Sephadex A-50, equilibrated with 0.0175 M sodium phosphate, pH 6.5 was packed to 25 cm in a 1.5 cm diameter column. The sample, previously dialyzed against this same starting buffer was applied and the column was washed with 200 ml of starting buffer. A salt gradient was formed for elution of the RTE-specific antigens, and employed a 150 ml beaker with starting buffer and magnetic stirrer as the mixing flask and a 125 ml Erlenmeyer flask containing 1.4 M NaCl in starting buffer as the reservoir. Effluent fractions of 3 ml were collected and assayed for RTE- α_5 as well as other antigens.

Macromolecular Exclusion Chromatography.—4% granulated agarose (SAG-4) and 6% granulated agarose (SAG-6)¹ were packed to 85 cm in a 1.5 cm diameter column following equilibration with 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 8.0. The sample, concentrated by negative pressure dialysis to approximately 0.5 ml, was applied and effluent fractions of 3 ml each were collected. Fractions containing RTE- α_5 as determined by the fluorescent antibody inhibition test were pooled, then concentrated by negative pressure dialysis.

Ultracentrifugation.—Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge at 52, 640 rpm and 20°C. Phosphate buffer $\mu = 0.1$, pH 7.0 was used as solvent. Observed sedimentation velocities were corrected for the viscosity and density of the buffer ($S_{20,w}$), but were not extrapolated to zero sample concentration.

Anti-RTE- α_5 .—Antibodies to RTE- α_5 were elicited by immunizing rabbits with an initial foot-pad injection of 3.8 μ g N of RTE- α_5 in incomplete adjuvant and giving two boosters of the same composition subcutaneously at weekly intervals. 7 days following the last injection the animals were bled.

Immunodiffusion Techniques.—Immunoelectrophoresis was carried out in 1% agarose in 0.025 M Veronal, 0.0025 M EDTA, pH 8.6 on glass microslides. Ouchterlony plates were identical except for the use of 0.1 M Veronal, 0.01 M EDTA, pH 8.6 buffer. Following introduction of reagents the slides were incubated at 4–5°C for 1–4 days to develop the immune precipitin lines.

Subcellular Fractions of Renal Tubular Epithelium.—Subcellular fractions (SC) were prepared from rat kidneys as previously described and lyophilized (13). SCI was the 105,000 g supernatant phase or "cell sap". SCII was the microsome fraction and the ribosomes were designated SCII-A. The microsomal fraction was further resolved into reassociated microsomal membranes (SCII-B), and a nonreassociable fraction of (SCII-C). SCIII was a composite of subcellular particulate constituents ranging in density from that of the lysosome to organelles as large as nuclei.

Nephritogenic Bioassay.—Lewis strain rats,² weighing 100–180 g, were used to assay the capacity of various tissue fractions and isolated antigens to induce typical AIC nephritis. The test antigens, suspended in saline, were vigorously mixed with an equal volume of complete adjuvant (1 part Aracel, 9 parts Bayloll F, 4 mg *Mycobacterium tuberculosis* H.37Ra³/ml.) Each rat was injected with 0.125 ml of emulsion in each rear foot-pad. Usually only one immunization was employed; however, under certain circumstances a single additional subcutaneous injection of similar composition was given at a later date. 24-hr urines were collected at biweekly or weekly intervals for 12 wk or longer. Quantitative urinary protein was determined following precipitation with 3% sulfosalicylic acid (22) by determination of the optical density at 550 m μ . Normal mean urinary protein of nonimmunized rats weighing 200–280 g was 1.45 \pm 1.07mg/24 hr. Mean proteinuria of Lewis rats 90 days following injection of saline in complete Freund's adjuvant was 2.96 \pm 1.14 mg/24 hr. Proteinuria of 10 mg or

¹ Gallard-Schlesinger Chemical Corp., Carle Place, L. I., N. Y.

² Microbiological Associates, Walkersville, Md.

³ Difco Laboratories, Detroit, Mich.

greater per day was considered abnormal, and was accepted as presumptive evidence of disease. Further support for the presence of AIC nephritis depended on the immunofluorescent demonstration of granular deposits of host γ - and β_{1C} -globulins along glomerular capillary walls and the presence of RTE α_5 in the glomerular deposits. Renal tissue was also fixed in 10% buffered formalin and stained according to the periodic acid-Schiff technique for histologic evaluation.

RESULTS

In a previous study it was shown that the nephritogenic antigen was one of several antigens specific for renal tubular epithelium and further that it was normally localized in the brush border of cells of the proximal segment of the nephron. In AIC nephritis this antigen was deposited in a granular fashion, apparently as part of antigen-antibody complexes, along glomerular capillary walls (1). A subsequent study led to the identification and isolation of two RTE-specific antigens, RTE- α_3 and RTE- α_4 , neither of which proved to be nephritogenic (13). It thus appeared that one or more additional RTE-specific antigens must be present in renal tubular epithelium and this latter antigen(s), designated RTE- α_5 , was by definition the antigen found in glomeruli of rats with AIC nephritis.

Two methods of assay for RTE- α_5 were employed. The first was the nephritogenic bioassay. The appearance of proteinuria and/or the granular deposits of γ -globulin in the affected glomeruli, usually within 90 days, was accepted as evidence of AIC nephritis and indicated the presence of RTE- α_5 in the immunizing material. The second technique was the fluorescent antibody inhibition test in which it was observed that absorption of the specific antibody, anti-RTE, with a tissue fraction known to be nephritogenic by bioassay would abolish the specific immunofluorescent staining of this antigen in glomeruli of rats with AIC nephritis. In Fig. 1 the deposits of RTE- α_5 present in the glomeruli of a rat with well-established AIC nephritis are demonstrated immunohistochemically. The fine granular or beaded character of the deposits can be readily appreciated. Many granules are discrete while others have become confluent along the glomerular capillary walls. Little if any of the antigen appears in the mesangial regions. When the anti-RTE was absorbed twice with Fx1A at 5 mg dry weight/mg Ab, or purified RTE- α_5 at 2 μ g N/mg Ab the immunochemical specificity was demonstrated by abolition of the reaction as shown on the right. Some selected observations are presented in Table I to illustrate the relationship between these two assay methods. RTE- α_5 was associated only with the saline insoluble subcellular constituents of renal tubular epithelial cells that were sedimentable in the ultracentrifuge (Fx1A), and was not found in the saline soluble supernatant fraction (Fx1B). The antigen was similarly not detectable in glomerular-rich sediment, normal urine, or in liver by either assay method.

As shown in Table II, RTE- α_5 appeared in significant concentration in only

one of the major subcellular fractions of renal tubular epithelium. This fraction, SCIII contained subcellular fragments and organelles denser than microsomes. Although whole microsomes (SCII) contained only trace quantities of RTE- α_5 , this antigen was present in significant concentration in a subfraction

TABLE I
Distribution of RTE- α_5

Tissue fraction	Nephritogenic bioassay		Immunofluorescent inhibition test	
	Result	Dose	Result	Concentration
Antigen		<i>mg</i>		<i>mg/mg Ab</i>
Saline-control	0	—	0	—
Fx1A	+*	0.5-10	+	5
Fx1B	0	10-20	0	20
Glomerular sediment	0	10	0	20
Glomerular basement membrane	0	10	0	20
Urine†	0	5	0	20
Liver	0	10-50	0	20

* 80-100% of rats showed proteinuria and granular deposits of γ -globulin along glomerular capillary walls by day 90.

† Dialyzed against distilled water and lyophilized.

TABLE II
Distribution of RTE- α_5 in Subcellular Fractions of Renal Tubular Epithelium

Subcellular fraction		Fluorescent antibody inhibition test* Inhibition
SCI	Cell sap	0
SCII	Microsomes	Slight
SCII-A	Ribosomes	Slight
SCII-B	Reassociated membranes	0
SCII-C	Nonreassociable fraction of microsomal membranes	Complete
SCIII	Lysomes → nuclei	Complete

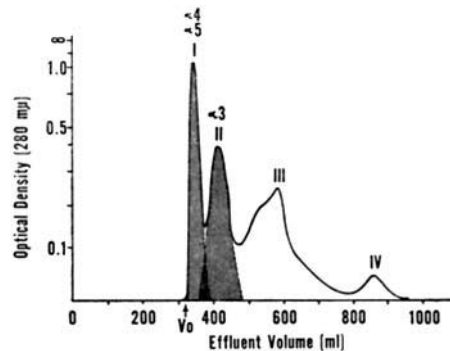
* All antigens used at 5 mg/mg antibody γ_2 -globulin

(SCIIC) of microsomal membranes which was not reassociated by 0.01 M $MgCl_2$ following dissociation with sodium desoxycholate.

The tissue distribution of RTE- α_5 was further evaluated by the fluorescent antibody inhibition test employing whole lyophilized tissues at 50 mg/mg Ab. No inhibition was observed with rat brain, heart, lung, spleen, stomach, bowel, skeletal muscle, prostate, seminal vesicles, testis, plasma, serum, or γ -globu-

lin. If present in extrarenal tissues the concentration was insufficient for detection by this assay method. Evidence for the excretion of RTE- α_5 in minute quantities was provided by the observation that anti-rat urine would specifically stain the glomerular deposited RTE- α_5 in kidneys from rats with AIC nephritis.

Dissociation of RTE- α_5 from Fx1A and recovery in the soluble phase was observed with 1 mg sodium desoxycholate/10 mg Fx1A; however maximal solubilization required approximately 10 mg sodium desoxycholate/10 mg Fx1A. Under the latter conditions approximately 70% of the Fx1A was rendered soluble. Utilizing the desoxycholate-soluble fraction it was found that a considerable degree of purification could be achieved by fractional ammonium sulfate precipitation. The supernatant from 25% ammonium sulfate fraction



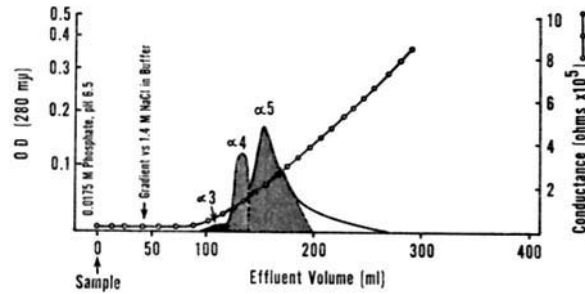
TEXT-FIG. 1. Molecular exclusion chromatography (G-200) of the RTE- α_5 -active fraction from Pevikon block electrophoresis. RTE- α_5 activity, assayed by FIT, was recovered in association with RTE- α_4 in the exclusion peak. RTE- α_3 as well as other uncharacterized material was retarded.

contained approximately 5% of the starting material and was active in the immunofluorescent inhibition test at 100 μ g N/mg Ab.

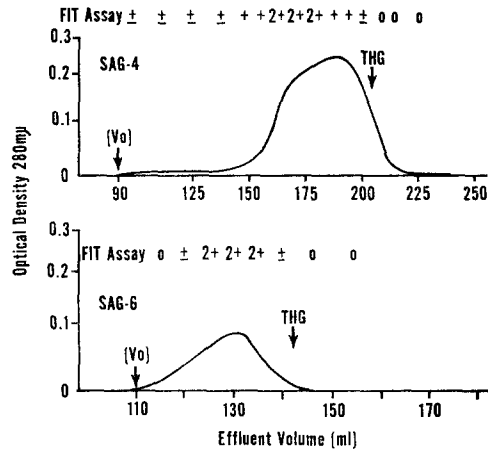
Preparative block electrophoresis was next employed to separate RTE- α_5 activity, along with both RTE- α_3 and RTE- α_4 , from much of the nonorgan-specific antigenic material. As previously described (13), these kidney-specific antigens were recovered in a broad zone corresponding to the migration of serum α -globulins located 9 $\frac{1}{2}$ –13 $\frac{1}{2}$ inches from the cathodal end of the block. Approximately 54% of the recovered protein was present in this RTE- α_5 -enriched zone.

Significant purification of RTE- α_5 was achieved by repeated molecular exclusion chromatography on Sephadex G-200 (Text-fig. 1). RTE- α_5 activity was recovered in the exclusion peak with RTE- α_4 ; and other antigenic material was not detectable in this fraction. This material was active in the immunofluorescent inhibition test at 25 μ g N/mg Ab and was nephritogenic in the

bioassay at 10 μg N per rat, although only two of five rats showed proteinuria at this dose. At 40 μg N the uniform immunohistochemical evidence of disease was supported by the presence of proteinuria in four of five rats (157 mg mean protein/24 hr). No evidence of disease was observed at the 2.5 μg dose level.



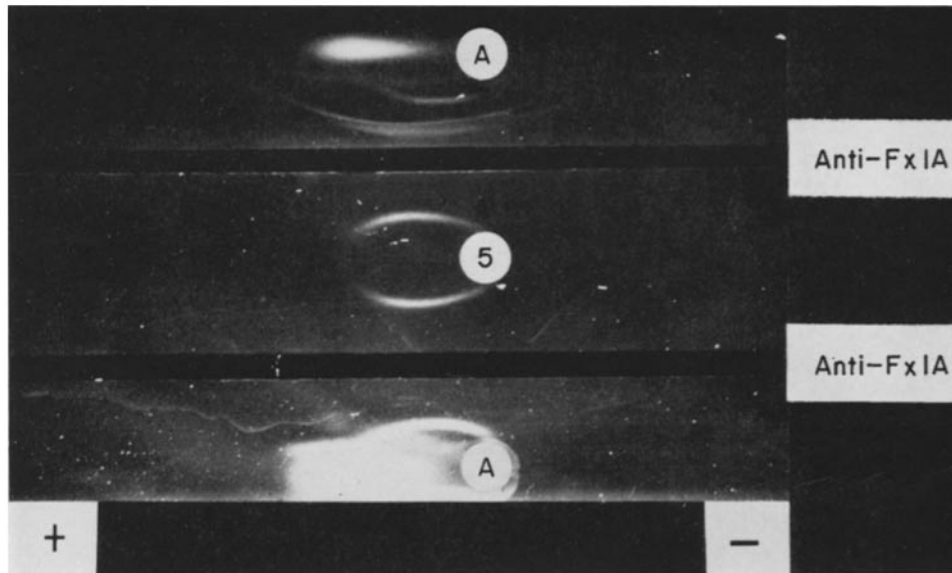
TEXT-FIG. 2. Ion exchange chromatography on DEAE-Sephadex (A-50) of the first peak recovered from Sephadex G-200 molecular exclusion chromatography. Four regions of the elution can be discriminated. Residual RTE- α_3 was recovered first and was closely followed by RTE- α_4 . The major peak contained all RTE- α_3 activity as indicated by FIT. The tail of eluted material was devoid of antigenic activity.



TEXT-FIG. 3. Final purification by repeated molecular exclusion chromatography on agarose. The partially purified RTE- α_5 from DEAE-Sephadex was first passed through 4% agarose (SAG-4, above) and RTE- α_5 activity was recovered predominantly in the heavier segment of the major retarded elution peak. The elution volume of thyroglobulin (THG) is indicated for reference. Peak RTE- α_5 activity was recovered at approximately $1.7 \times V_0$. Following repeated isolation of the RTE- α_5 fraction on the same column the sample was transferred to 6% agarose (SAG-6) and the RTE- α_5 reisolated. A final repeat isolation of RTE- α_5 on SAG-6 is illustrated. The asymmetry of the eluted peak may reflect slight aggregation or protein: protein interaction.

Partial separation of RTE- α_5 and RTE- α_4 was observed following ion exchange chromatography on DEAE Sephadex (Text-fig. 2). Using Ouchterlony slides the antigenic expression of RTE- α_4 was found equally in the RTE- α_4 and RTE- α_5 regions; however, RTE- α_5 activity was present only in the latter.

Columns of 4 and 6% granulated agarose were employed for final purification of RTE- α_5 . The RTE- α_5 fraction from DEAE-Sephadex was first passed through 4% agarose and RTE- α_5 activity was identified in greatest concen-



TEXT-FIG. 4. Immunoelectrophoretic characterization of purified RTE- α_5 (1.5 v/cm \times 60 min.). Only a single immune precipitin line was developed with both anti-Fx1A and anti-RTE reacted against RTE- α_5 in the center well (5) at 90 μ g N/ml. Fx1A-DOC was utilized in the outer wells (A) at 20 mg/ml.

tration in the first half of the major elution peak, but it was also found in low concentration in a spread of material extending from the void volume through a broad zone to the major elution peak (Text-fig. 3). It would appear likely that the elution of RTE- α_5 activity through a broad range of molecular sizes larger than the prime recovery of this antigen is a reflection of aggregation. The region of greatest RTE- α_5 activity was concentrated and repassed through the same column and activity was again recovered, but only at approximately $1.7 \times V_0$ which corresponds to the major RTE- α_5 activity on the first passage through this gel. The RTE- α_5 preparation was then passed twice through a 6% agarose column and the area of greatest activity was recovered on each occasion. The antigen was eluted rather broadly; however, the area of greatest activity corresponded to approximately $1.7 \times V_0$ on 4% agarose which is

slightly smaller volume than that found with thyroglobulin. The elution volume for RTE- α_5 corresponds to a Stoke's radius in the order of 100 A.

The purified RTE- α_5 had an $S_{20,w}$ of approximately 28.6 when examined at a concentration of 90 $\mu\text{g N/ml}$. Due to the paucity of purified antigen further ultracentrifugal characterization was not feasible. Immunoelectrophoretic analysis confirmed that the isolated RTE- α_5 was reasonably purified (Text-fig. 4); and the migration corresponded roughly to that previously described for RTE- α_4 (13). Reasonable purity was further suggested by the formation of only a single precipitin line in gel when developed with anti-RTE or anti-Fx1A. Additional antigens were not detectable by gel diffusion against rabbit anti-whole serum or anti-RTE- α_3 . The presence of lipid in this antigen was

TABLE III
Serological Relationships of the RTE-Specific Antigens

Antiserum	Glomerular-deposited RTE- α_5 *	Isolated RTE- α_5 †	RTE- α_4 †	RTE- α_3 †
Anti-RTE	+	+	+	+
Anti- α_3	0	0	0	+
Anti- α_4	0	+	+	0
Anti- α_5	+	+	+	0
Anti α_5 (absorbed with RTE- α_4)	+	+	0	0

* Reactivity with the antigen determined by the immunofluorescent method.

† Biochemically isolated antigens tested in Ouchterlony slides against the indicated antiserum.

indicated by the subsequent staining of the RTE- α_5 precipitin line with Sudan Black B in a fashion similar to RTE- α_4 (13).

The immunochemical interrelationships of the three RTE-specific antigens were briefly explored (Table III). The identity of RTE- α_5 as distinct from RTE- α_3 and RTE- α_4 , which were the only two RTE-specific antigens previously recognized (13), was verified by the observation that the RTE- α_5 found in glomeruli from rats with AIC nephritis did not react with either anti- α_3 or anti- α_4 . Anti- α_2 did not form an immunoprecipitin line with RTE- α_5 in Ouchterlony slides and these two antigens are clearly unrelated; however, anti- α_3 and anti- α_4 appeared to form lines of identity with each other when either RTE- α_5 or RTE- α_4 were employed as the antigen. Following repeated absorption of anti- α_5 with excess RTE- α_4 this antiserum did not react with RTE- α_4 but still reacted with glomerular deposited RTE- α_5 by immunofluorescence as well as isolated RTE- α_5 in an Ouchterlony slide.

Anti- α_5 , absorbed with RTE- α_4 , and anti- α_4 reacted in Ouchterlony slides with RTE- α_5 to give a line of identity. It would appear that RTE- α_5 is present

on a common large molecule that also contains some RTE- α_4 . However, RTE- α_4 has also been recovered on a similar size but less highly charged molecule that does not possess the RTE- α_5 antigenic determinants. In that the glomerular deposited RTE- α_5 is not associated with RTE- α_4 it would appear that a segment, or perhaps only a small fragment, of the isolated molecule containing the RTE- α_5 -antigenic determinants is involved in the pathogenesis of AIC nephritis.

These present isolates of RTE- α_5 were active in the immunofluorescent inhibition test at 2-5 $\mu\text{g N/mg Ab}$ which represented a 100- to 200-fold greater

TABLE IV
Nephritogenicity Following a Single Immunization with Renal Tubular Epithelial Fractions of Rat Kidney

Antigen	Dose	Proteinuria*	No. with Disease†
	$\mu\text{g N}$	$\text{mg}/24 \text{ hr}$	
Fx1A	380	87.5	5/5
	38	63.3	3/4
	3.8	4.9	0/5
Fx1B	380	3.3	0/9
RTE- α_5	3	73§	1/3
	6	22.8	5/5

* Determined 12 wk following immunization.

† Indicates the number which developed abnormal proteinuria and the number of rats in the test group. Deposition of γ - and β_{1C} -globulins in a granular fashion along glomerular capillary walls was confirmed by immunofluorescent methods.

§ One rat had proteinuria of 216 mg/24 hr. The other two had normal urinary protein. All three rats had granular deposits of γ -globulin in their glomeruli, but β_{1C} -globulin was found only in the rat with abnormal proteinuria.

|| Two injections of 3 μg given 8 wk apart.

activity than found for Fx1A. Of three rats who received a single immunization with 3 $\mu\text{g N}$ of RTE- α_5 in complete adjuvant, one developed proteinuria of 216 mg/day with well-established AIC nephritis by immunofluorescent criteria, and the other two rats had a definite but mild granular deposition of γ -globulin along their glomerular capillary walls but no abnormal proteinuria (Table IV). Neither of the nonproteinuric rats had β_{1C} -globulin deposited in conjunction with the γ -globulin. Five additional rats were immunized initially with 3 $\mu\text{g N}$ of RTE- α_5 and were given an additional booster of the same composition subcutaneously 8 wk later. All five had disease by immunohistological criteria with deposit of both γ - and β_{1C} -globulins. The mean proteinuria was 22.8 mg/24 hr 4 wk after the booster and the level of proteinuria was rising at

12 wk. The pathologic features of AIC nephritis after immunization with 3 μ g N of RTE- α_5 were similar to those found after immunization once with Fx1A; and consisted of the progressive appearance of fine PAS-positive deposits along the subepithelial aspect of the glomerular basement membranes in the absence of significant endothelial or epithelial proliferation. The effect of the deposits was to impart a distinctly thickened appearance to the glomerular basement membranes. In Fig. 2 a glomerulus from a rat immunized 12 wk previously with 3 μ g N of RTE- α_5 and exhibiting over 200 mg proteinuria per day is shown. Fine granular deposits were present along the subepithelial aspects of glomerular basement membranes. The glomerular basement membranes of a control rat of the same age were quite smooth and regular (Fig. 3). γ -Globulin was found as a confluent granular deposit along the glomerular basement membranes (Fig. 4), and β_{1C} -globulin was found in a similar distribution. Following partial elution of sections with potassium thiocyanate, RTE- α_5 was demonstrated as a fine beading along the glomerular capillary walls (Fig. 5). All rats given a secondary immunization with purified RTE- α_5 in adjuvant showed well-developed γ -globulin, β_{1C} -globulin, as well as RTE- α_5 along glomerular capillary walls. Multiple intraperitoneal injections of Fx1A in complete adjuvant caused heavy glomerular deposits of RTE- α_5 as shown in Fig. 6. The granular deposition of RTE- α_5 along glomerular capillary walls is clearly demonstrated with anti-RTE- α_5 . For comparison a normal rat kidney, reacted in parallel with the same antiserum, is shown (Fig. 7). No trace of antigen is found in the normal glomerulus irregardless of whether or not it is eluted with potassium thiocyanate. Glomeruli from rats with nephrotoxic nephritis and aminonucleoside nephrosis are similarly negative.

Immunofluorescent studies of a variety of normal rat tissues using antisera to purified RTE- α_5 showed that this antigen was distributed in a selective fashion identical to RTE- α_3 and RTE- α_4 (13); namely only in the brush border and apex of epithelial cells of the proximal convoluted tubule of the kidney and small amounts in the brush border of mucosal cells of the small bowel.

DISCUSSION

Considerable evidence has been presented to support an immune complex pathogenesis for AIC nephritis. Not only the morphological and immunohistochemical observations which indicate that nonglomerular antigen, antibody, and complement are deposited in a discrete or particulate fashion in the glomeruli of these rats (1, 7-10, 12) but also the unique and nonglomerular derivation of the nephritogenic antigen strongly suggest such pathogenetic mechanism. This normal renal tubular epithelial antigen, designated RTE- α_5 , has been found primarily in the proximal convoluted tubules with small amounts detectable in the brush border of the mucosal of the small bowel. Other tissues did not contain detectable amounts.

RTE- α_5 has been recovered as a large lipoprotein molecule with an $S_{20,w}$ of 28.6 and an electrophoretic mobility comparable with the α -globulins of serum. This molecule would appear to be a cell membrane subunit probably derived from the plasma membrane of the brush border or intracellular membranes of certain renal tubular epithelial cells. The nephritogenic activity has been rather diffusely recovered through various microparticulate subcellular fractions, most abundantly in the mitochondrial fraction (9, 11, 12). This is in agreement with the known variable sedimentation behavior of plasma membranes (23, 24).

The basis for suggesting RTE- α_5 as a plasma membrane component is morphologic as well as physicochemical. This antigen has been localized immunohistochemically to the brush border of the epithelial cells of the proximal convoluted tubules. Ultrastructurally the brush border appears to be a prodigiously redundant system of plasma membranes containing a small quantity of cytoplasmic matrix (25). In view of the membrane-bound character of the antigen it would appear to be a derivation of these membranes rather than a soluble or loosely associated molecule in this region of the cell. The normal functional role of RTE- α_5 in the cell is not known; however, the cytological and tissue distribution is remarkably similar to certain ATPases (26) and alkaline phosphatases (27), and a similar role in active transport is an attractive hypothesis.

The character of the isolated RTE- α_5 is not unlike some of the isolated histocompatibility antigen preparations which are also membrane-bound lipoproteins and are associated with additional antigenic factors of diverse character (24, 28). The RTE- α_5 antigen is isolated as a very large molecule which may possess other unrelated antigenic sites. Some evidence suggests that RTE- α_4 , a previously described RTE specific, nonnephritogenic antigen, may also be expressed in the isolated RTE- α_5 lipoprotein. In their considerations of the structural and functional integration of cellular membranes, Green and Tzagoloff have proposed that specific cellular membranes are composed of structurally similar protein: phospholipid subunits. The specific functional protein may vary considerably in individual subunits, however, when associated into membranes a highly sophisticated and enzymatically integrated functional system is achieved (29). The large RTE- α_5 lipoprotein molecule which has been isolated may represent one of these subunits.

Physicochemical isolation of the antigen has been achieved by dissociation of a crude cellular membrane fraction of kidney with sodium desoxycholate and gradual purification by salt precipitation, preparative electrophoresis, molecular exclusion chromatography, and ion-exchange chromatography. The final product appears reasonably purified, and it is active in the fluorescent antibody inhibition test at concentrations as low as 2 μ g N. By contrast, the crude cellular membrane fraction, representing 4.3% of wet weight of renal

cortex (13), showed inhibition at a concentration no less than 380 $\mu\text{g N}$. It would appear that a concentration of RTE- α_5 in the order of 200-fold has been achieved.

We have observed moderately heavy γ -globulin deposits with little or no complement, in the glomeruli of some rats which did not develop abnormal proteinuria greater than 10 mg/24 hr following a single immunization with a solubilized nephritogenic fraction of kidney. Following a second immunization with the same material β_{1C} usually appeared in the lesions and proteinuria was observed. These observations suggest that the soluble forms of RTE- α_5 may not be as effective in eliciting complement-fixing antibody. The complement-dependent character of glomerular basement membrane injury has been well recognized in the nephrotoxic nephritis model and presumably is also involved as a major mediator in AIC nephritis (30, 31). If the immunohistochemical observations are accepted as evidence of disease, then all rats immunized once with 3 $\mu\text{g N}$ of purified RTE- α_5 develop disease.

In the course of this investigation techniques have been developed for the identification of the antigenic constituent of glomerular deposited immune complexes. This technique involves partial elution from cryostat sections of AIC-nephritic kidneys of the glomerular deposited γ -globulin and complement with the subsequent immunohistochemical demonstration of the antigen by specific antisera. Without elution, the glomerular deposited RTE- α_5 , in AIC nephritis, will usually not react by the immunofluorescent technique, although occasionally in advanced disease a weak reaction may be observed. Apparently the deposited immune complexes become saturated with antibody so that all available antigenic sites are covered or sterically hindered. Partial elution of antibody from the immune complexes presumably exposes sufficient antigenic sites on the RTE- α_5 to permit reaction with specific antisera. Elution of some, but not all, γ -globulin from the glomeruli of NZB hybrid mice has also been a necessary prerequisite to the immunohistochemical demonstration of nuclear antigens (32); and similar techniques appear necessary for the demonstration of DNA in the glomerular deposits in systemic lupus erythematosus (33). This technique may find additional application in the study of a variety of forms of human glomerulonephritis which are characterized by granular or lumpy deposits of γ -globulin and complement along the glomerular capillary walls.

It has been found possible to utilize the immunofluorescent techniques for the demonstration of glomerular deposited antigen not only in a qualitative fashion, but also to adapt it so as to provide a semiquantitative assay for the same antigen in various preparations. Utilizing standard blocks of tissue and antibody to RTE antigens at a concentration twice that necessary to demonstrate the glomerular deposited RTE- α_5 , we found that the capacity of a given tissue fraction to inhibit the reaction when mixed with the antibody was im-

munologically specific, reproducible, and sensitive to approximately 2 μg N of isolated RTE- α_5 . Such an assay system may find application in isolation of the pathogenetic antigens in other forms of glomerulonephritis mediated by circulating immune complexes.

SUMMARY

The nephritogenic antigen, responsible for the immunogenic stimulus in experimental allergic glomerulonephritis induced with tubular antigen, has been identified as a renal tubular epithelial (RTE)-specific antigen and has been isolated in a relatively purified form. This antigen, RTE- α_5 , is a distinct and antigenically specific lipoprotein of high density which is derived primarily from the brush border of proximal convoluted tubular epithelium of the rat kidney. It has been suggested that this molecule may be a plasma membrane subunit. Immunization of rats with as little as 3 μg N of RTE- α_5 in complete Freund's adjuvant has effectively induced this form of membranous glomerulonephritis. RTE- α_5 is not a constituent of normal rat glomeruli; however, with the onset of autologous immune complex nephritis it is deposited in a granular fashion along glomerular capillary walls indistinguishable from the deposits of γ -globulin and complement. The antigenic specificity of this antigen and its tissue derivation has been explored, and the observations support the autologous immune complex pathogenesis of the glomerulonephritis induced in rats by immunization with renal tubular antigen.

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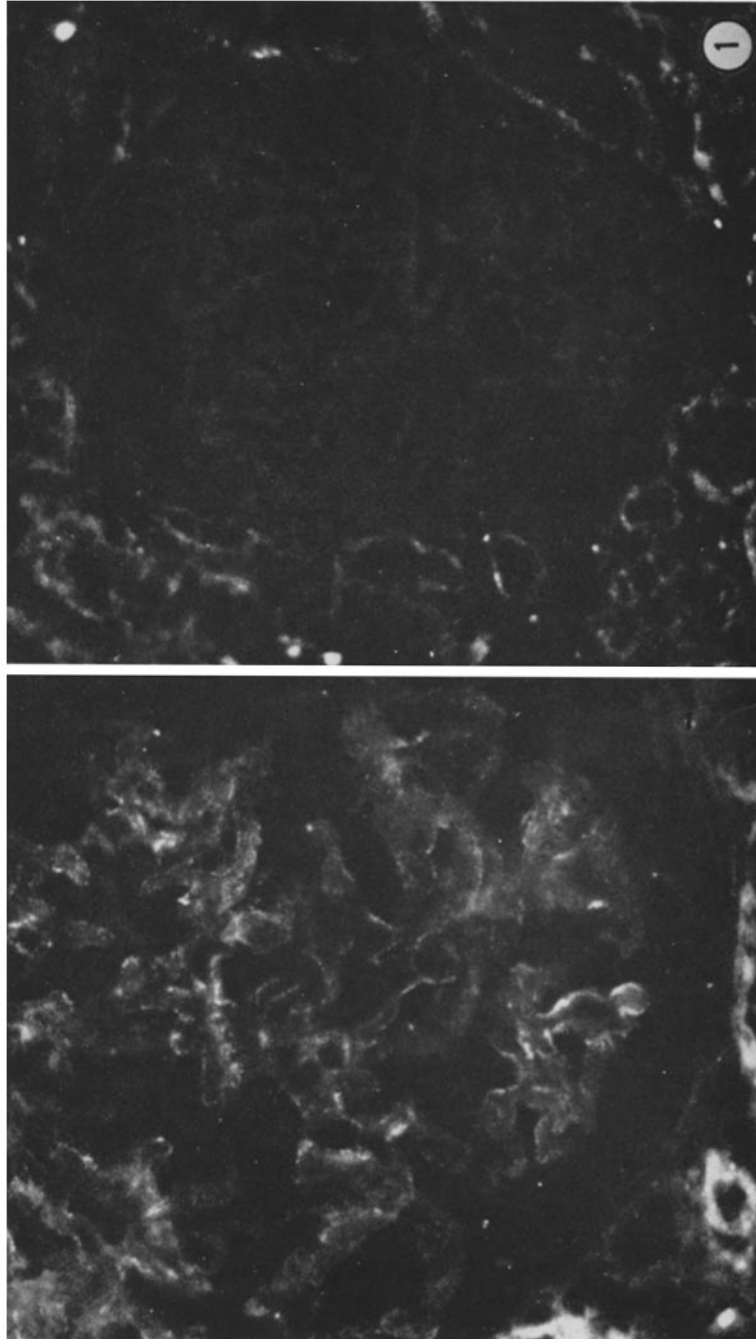
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EXPLANATION OF PLATES

PLATE 70

FIG. 1. Fluorescent antibody inhibition test (FIT). Glomerular deposited RTE-specific antigen is demonstrated on the left in one of the standard sections from a rat with AIC nephritis. Following absorption of the anti-RTE with 5 mg Fx1A/mg γ -globulin (right), the immunofluorescent reaction with glomerular deposited nephritogenic antigen was abolished. \times 300.

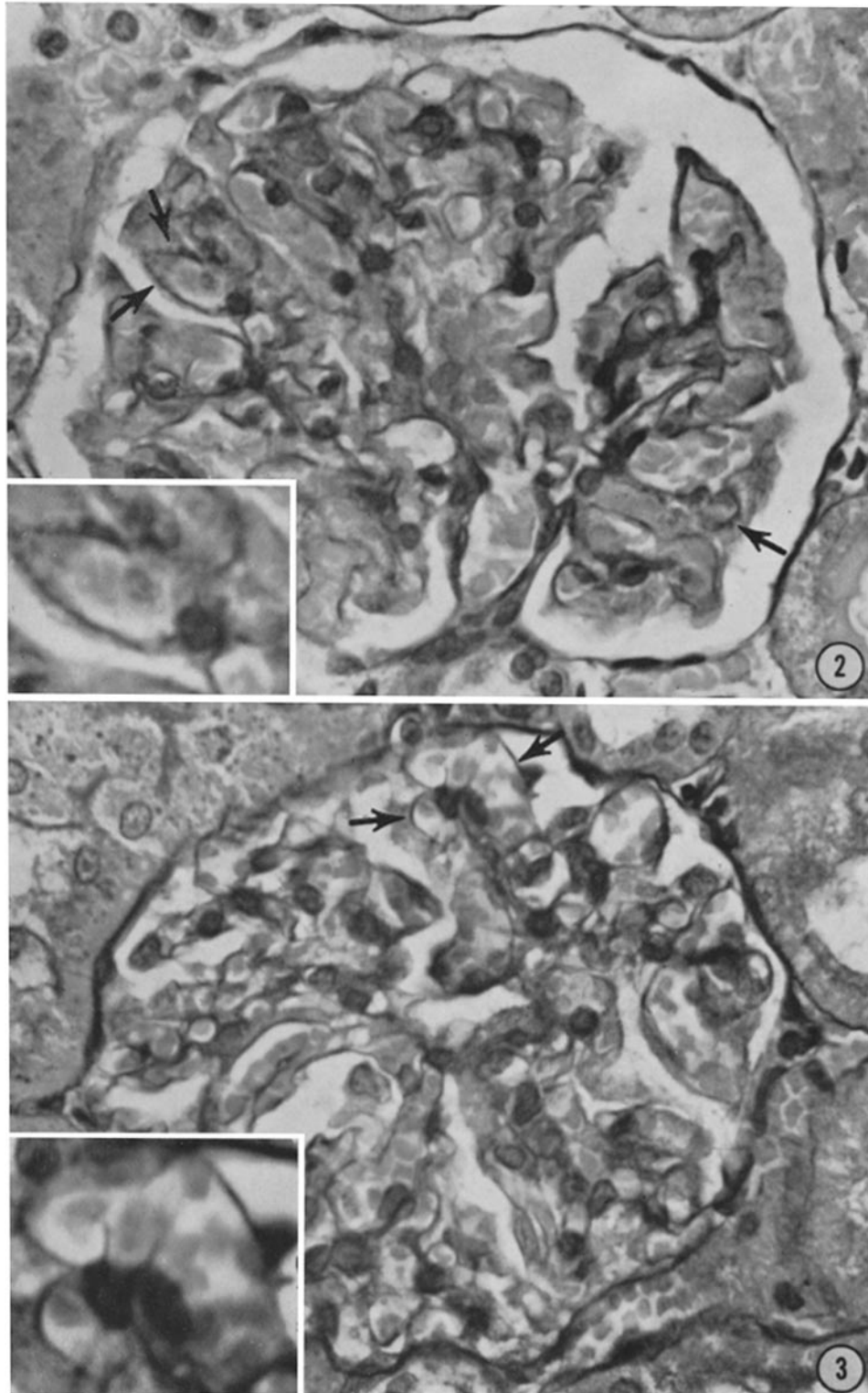


(Edgington et al.: Autologous immune complex nephritis. I)

PLATE 71

FIG. 2. Photomicrograph of a typical glomerulus from a rat in which AIC nephritis was induced with 3 μ g N of purified RTE- α_5 . 12 wk following immunization proteinuria was 216 mg/24 hr and histopathologic changes indicative of an early membranous glomerulonephritis were observed in all glomeruli. Fine PAS-positive granular deposits could be recognized along the subepithelial aspects of many glomerular basement membranes (arrows and inset). The glomerular basement membrane per se was not significantly thickened. Polymorphonuclear leukocytes were not present in increased number, and there was no proliferative response on the part of either endothelial or epithelial cells. PAS stain. \times 450. (Inset, \times 975).

FIG. 3. A glomerulus from a control rat of the same assay group that received saline in adjuvant. Subepithelial aspects of the glomerular basement membranes are devoid of the granular deposits demonstrated in Fig. 4 (arrows and inset). PAS stain. \times 450. (Inset, \times 975).

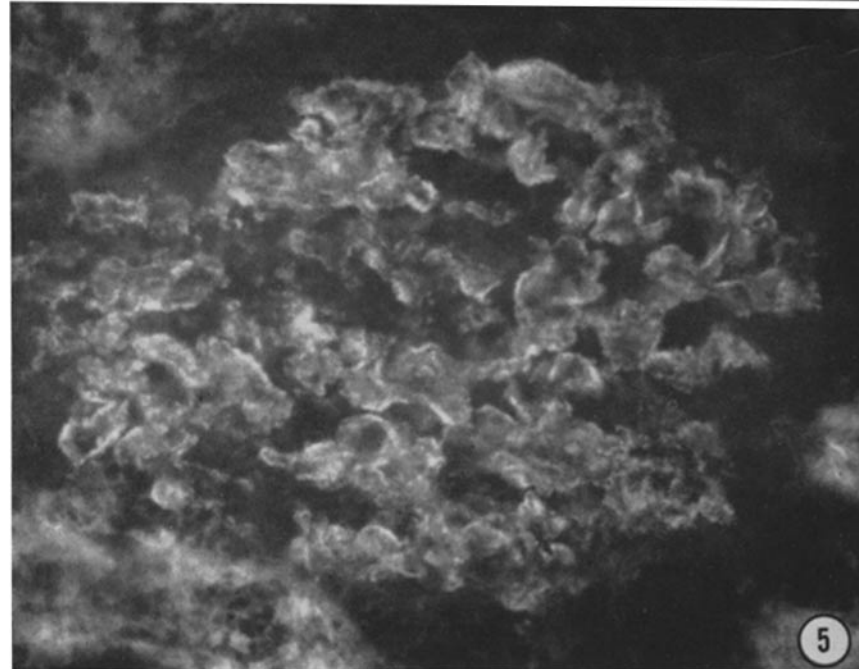
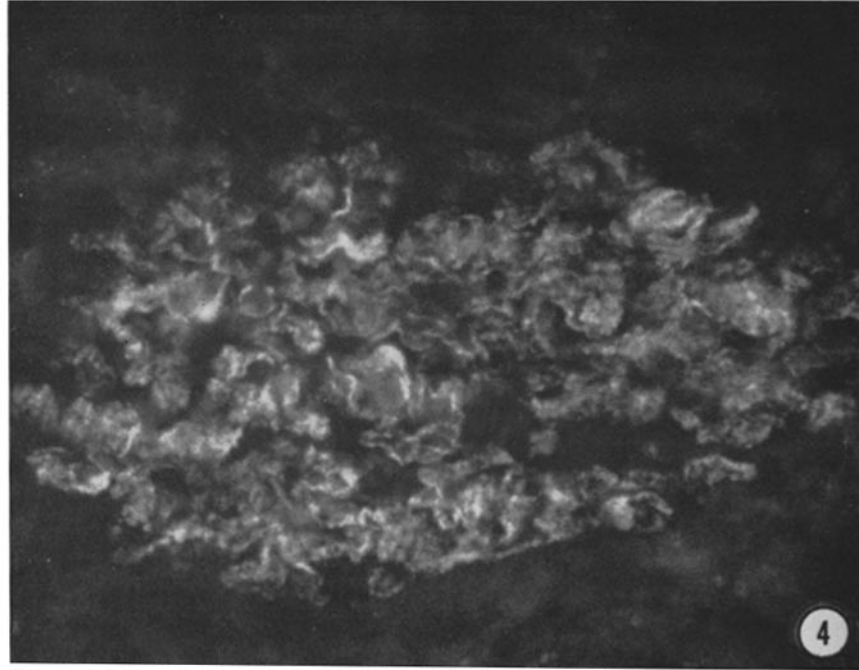


(Edgington et al.: Autologous immune complex nephritis. I)

PLATE 72

FIG. 4. Immunofluorescent demonstration of fine granular deposits of γ -globulin along the glomerular capillary walls in a rat with AIC nephritis induced with purified RTE- α_5 (same as Fig. 2. The γ -globulin is more extensive than might be suggested by the histopathological examination. \times 540.

FIG. 5. Immunofluorescent demonstration of RTE- α_5 along glomerular capillary walls in a fashion analogous to the γ -globulin deposits. The RTE- α_5 is present as fine granules which frequently appear confluent. This section is from the same rat illustrated in Figs. 4 and 6. \times 540.

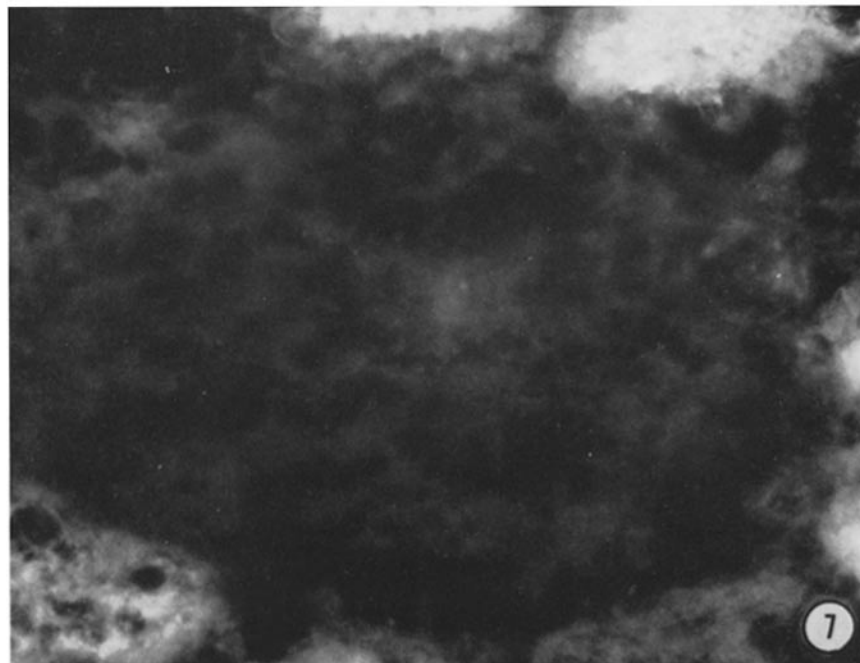
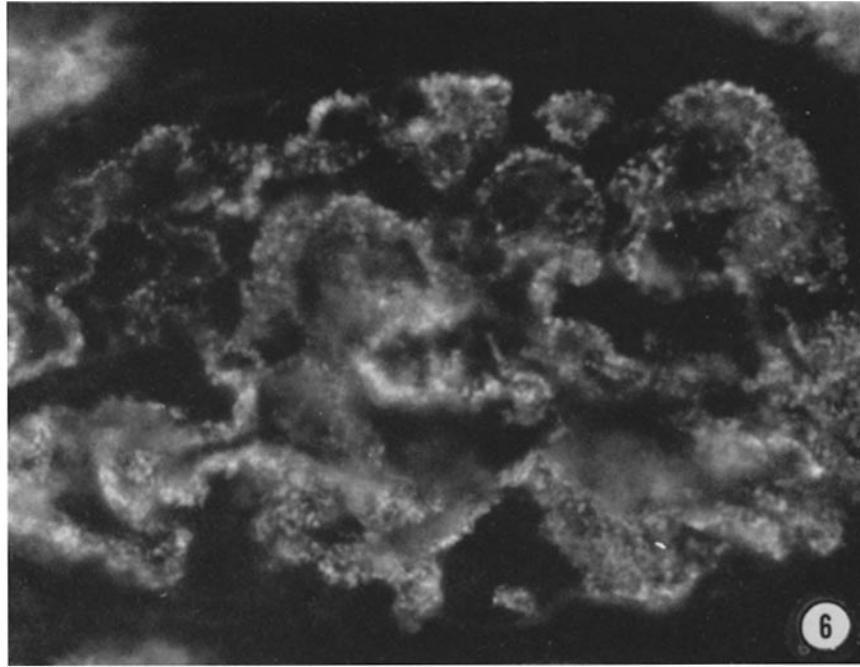


(Edgington et al.: Autologous immune complex nephritis. I)

PLATE 73

FIG. 6. Immunohistochemical demonstration of the beadlike character of the deposits of RTE- α_5 along the glomerular capillary walls of a rat with AIC nephritis for 1 yr. Antiserum to purified RTE- α_5 was employed in order to confirm the antigenic identity of the isolated antigen and the glomerular-deposited antigen. \times 865.

FIG. 7. RTE- α_5 is not found in the glomerulus of a normal Lewis rat. Prepared in parallel with the section shown in Fig. 6. \times 865.



(Edgington et al.: Autologous immune complex nephritis. I)