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Immunological Relation of Basement Membrane and a Serum Beta Globulin in the Mouse

DEMONSTRATION OF RENAL BASEMENT MEMBRANE ALTERATION IN MICE INJECTED WITH STREPTOLYSIN S

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Summary. Antiserum to a fraction of mouse β globulin isolated by electrophoresis and column chromatography was found reactive by immunofluorescence with renal basement membrane of glomeruli and tubules, and with basement membrane and connective tissue in other organs. This method for staining renal basement membrane was applied to the study of renal lesions produced in mice by streptolysin S. In lesions characterized by early changes of acute tubular necrosis a marked decrease in ability to stain tubular basement membrane was observed. Reappearance of staining property in basement membrane was linked to regeneration of diseased tubules. Repeated injections of streptolysin S produced alterations in staining also of glomerular basement membrane

INTRODUCTION

Several groups of investigators (Kelly and Winn, 1958; Hinkle, Partin and West, 1960; Tan, Hackel and Kaplan, 1961) have described production of renal lesions in mice by intraperitoneal implantation of diffusion chambers containing group A streptococci. These renal lesions were characterized primarily by tubular necrosis. In studies from this laboratory of the streptococcal factor responsible for the renal lesions it was shown that intraperitoneal injections of streptolysin S, a non-antigenic extracellular toxin of group A streptococci, produced identical lesions (Tan and Kaplan, 1962a). By immunohistochemical methods rabbit antiserum to mouse globulins was shown to react intensely with renal glomerular and tubular basement membrane of normal mice but to give a different or negative reaction with basement membrane of mice exposed to diffusion chambers or to streptolysin S (Tan and Kaplan, 1962b). This reaction with basement membrane was found related to antibody to a β-globulin component of normal mouse serum.

The immunological relation of serum β-globulin component to basement membrane and other connective tissue structures, the method of isolation of this component from mouse serum, and its physico-chemical properties are described in this report. Antiserum to the isolated β-globulin component was used to show an alteration of basement membrane in mice treated with streptolysin S.

* This work was performed during the tenure of an Established Investigatorship of the American Heart Association.

MATERIALS AND METHODS

Fractionation of Mouse Serum Proteins

Male albino mice of the Harvard Webster or CFW strains, between 2 and 4 months, were used for all experiments. Serum was obtained from mice by cutting the axillary vessels and collecting the blood that pooled in the axillary pouch. Blood from several animals was pooled and serum separated after allowing the clot to retract at 37° for 1 hour and overnight in the refrigerator.

Serum proteins were fractionated by preparative zone electrophoresis on potato starch or GEON 427 (Goodrich Chemical Co., Cleveland, Ohio), a polyvinyl chloride resin, as supporting media (Kunkel, 1954) with barbital buffer of pH 8·6, ionic strength 0·1. Four to 8 ml. of serum were applied to a block 45 × 21 × 1·2 cm., and electrophoresis carried out at 4° for 45 hours under a constant voltage of 600 V. and a current of 50–60 mA. The block was cut into 1 cm. segments and the protein recovered by stirring the supporting medium in 5 ml. saline and filtering by suction through a sintered glass filter. Protein content was determined by a modified Folin-Ciocalteu procedure (Kunkel and Tiselius, 1951).

Separation of serum proteins was also carried out by column chromatography on diethylaminoethyl (DEAE) cellulose. For whole serum the method of Fahey, McCoy and Goulian (1958), using gradient elution, was adopted for developing the chromatogram. Aliquots of 4 ml. of effluent eluate were collected automatically. Chromatography was also employed for further fractionation of the β globulins obtained from zone electrophoresis. For this procedure, stepwise elution with phosphate buffer was used as is described in detail in the text.

Concentration of protein fractions obtained by zone electrophoresis or column chromatography was performed by a combination of ultrafiltration and pervaporation. This method was developed because it was found necessary to concentrate relatively small volumes of protein solution, and other methods of concentration such as freeze-drying altered the reactive properties of certain of the protein fractions under study.The apparatus consisted of a large-sized desiccator which was fitted with a two-hole rubber stopper. A short glass tube in one of these holes was connected to the outside with thick-walled rubber tubing fitted with an adjustable screw clamp; the inside end of the glass tubing was connected to a rubber tube that extended to the bottom of the desiccator. Glass tubing in the other hole of the rubber stopper was connected to a Y tube, one arm leading to a vacuum line via a manostat and the other to a mercury manometer. The solutions to be concentrated were placed in single-bulb 100 mm. drying tubes. The bottom end of each drying tube was fitted with a short length of ¼ in. cellulose dialysis tubing. The latter was tied on itself in two knots at its free end and secured firmly to the drying tube by a tightly stretched rubber band around the glass constriction of the drying tube. After the vessel was filled with protein solution, air bubbles were displaced from the dialysis sac and the tube fitted tightly with a rubber stopper. The vessels were mounted on a plastic rack and the ultrafiltrate collected if desired in test-tube caps placed beneath the dialysis sacs. The rack was placed in the desiccator, the cover sealed with petroleum jelly and concentration carried out in the cold at 6° with a partial vacuum of 35–45 mm. Hg. By adjusting the screw clamp it was possible to permit a steady flow of air into the desiccator at the desired negative pressure and thus combine pervaporation with ultrafiltration under partial vacuum. Over a 24 hour period, approximately 5 ml. of liquid passed through each dialysis sac.

Immunization Schedules

The reactive fraction of β globulin obtained from column chromatography was concentrated by the above procedure and used for immunization of New Zealand white rabbits. An emulsion of equal volumes of a 2 mg./ml. protein solution and Difco complete Freund's adjuvant was used. The initial injection of 1 ml. emulsion was given in the footpads followed by four weekly intramuscular injections of 1 ml. of the same emulsion. After a 4 week rest period, a second course of immunization was given. Rabbits were bled by cardiac puncture 7 days after the last injection.

Fluorescent Conjugates

Antibody to mouse serum fractions was precipitated from rabbit antiserum at half-saturation with ammonium sulphate and the globulins conjugated with fluorescein isothiocyanate in the absence of organic solvents.

Two batches of fluorescent conjugate were used in the study. The initial observations were made with a conjugate of rabbit antibody to a mixture of mouse globulins precipitated from mouse serum at half-saturation with ammonium sulphate.* The second batch was made from rabbit antibody to the specific reactive fraction of β globulin obtained by electrophoretic and chromatographic separation. Both these conjugates were diluted in saline to determine the highest dilutions at which they retained optimum staining properties. For the former preparation this was at 1 : 4 dilution and for the latter at 1 : 16 dilution. At these dilutions, 'non-specific' staining was found to be insignificant and the conjugates were employed for staining without absorption with organ powders.

Direct Staining of Frozen Sections

Unfixed frozen sections of mouse tissues were cut at a thickness of 4 μ in a cryostat. After air drying, the sections were washed in buffered saline (phosphate 0·01 M, pH 7·2) for two 10 minute periods on a shaking machine to remove serum proteins and soluble tissue antigens. The washed sections were stained directly with fluorescent antibody for 1 hour in a moist chamber at room temperature. The sections were then rinsed in saline, washed in two changes of saline for 10 minutes and mounted in glycerol buffer. The fluorescence microscope used has been described previously (Kushner and Kaplan, 1961).

Absorption and Inhibition Tests with Conjugate

Immunofluorescence absorption tests permitted identification of the reactive serum fraction responsible for staining of basement membrane. For this procedure, 0·05 ml. of conjugate was mixed with 0·05 ml. of electrophoretic or chromatographic fraction in a small test tube, incubated in a 37° water bath for 60 minutes and left overnight in the refrigerator. The absorbed conjugate was then spun at 2000 rev./min. for 30 minutes and the supernatant used for direct staining as described. Inhibition of staining indicated presence of basement membrane-related antigen in the test fraction. Mixtures of conjugates and buffer solutions of similar ionic strength and pH as the test protein solutions were used as controls.

Inhibition of staining by prior application of unlabelled antiserum to the section was demonstrated as follows. The antiserum was applied to the washed section and allowed to react at room temperature for 30 minutes. The section was then rinsed in buffered saline and washed for 20 minutes with two changes of saline. Conjugate was then applied to the

* Rabbit anti-mouse globulin serum, lot No. 629606, supplied by the Sylvana Chemical Co., Orange, New Jersey.

section, allowed to react for 15 minutes and the section washed and mounted as previously described.

Streptolysin S

The group A C203S strain was obtained through the courtesy of Dr. A. W. Bernheimer, New York University College of Medicine, New York, and was used for the production of streptolysin S according to the procedure of Bernheimer (1949). Such preparations were reported by Bernheimer to show no activity for streptokinase, proteinase or hyaluronidase, but to contain desoxyribonuclease. The unit of activity of streptolysin S preparations was determined by the amount required to produce 50 per cent haemolysis of 1 ml. of a 0·7 per cent suspension of washed human red cells (Bernheimer, 1944). The streptococcal toxin prepared in this laboratory was found to produce the same histological changes in the kidney as that of preparations kindly supplied by Dr. Bernheimer.

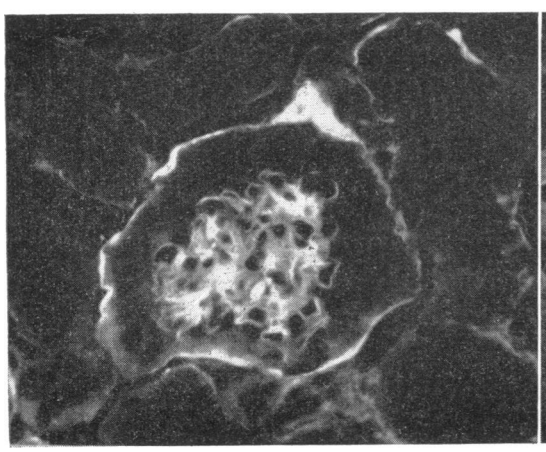

FIG. 1

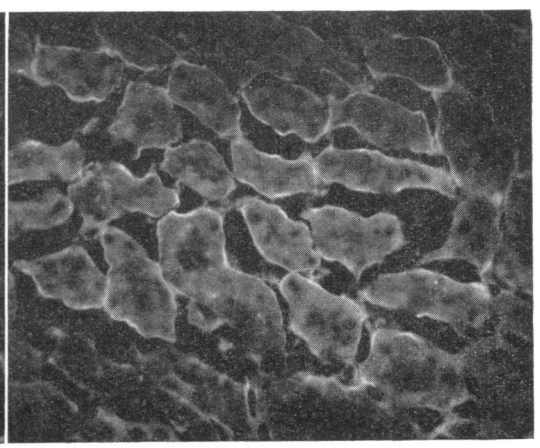

FIG. 2

FIG. 1. Fluorescent staining of normal mouse kidney with conjugate of antiserum to mouse globulin fraction. There is discrete linear staining of peripheral glomerular capillary loops and staining of structures in the central axial stalks of the glomerulus. The basement membranes of Bowman's capsule and of adjacent tubular segments are also stained. × 360.

FIG. 2. Fluorescent staining of the cortical convoluted tubules of normal mouse kidney. There is discrete linear staining of basement membrane around each tubular segment. × 281.

Injection of Streptolysin S

Male albino mice of the Harvard Webster or CFW strains between 2 and 4 months old were used. Lyophilized streptolysin S was dissolved in buffered saline pH 7·0 containing M/12·9 sodium chloride and M/15 potassium phosphate and injected intraperitoneally. Previous studies had indicated that 3000 haemolytic units of streptolysin S dissolved in 1 ml. saline and administered in a single injection was sufficient to produce the type of renal lesion reported (Tan and Kaplan, 1962a). In the present study some animals were also injected with 5000 units of streptolysin S in order to demonstrate the more severe pathological changes observed by immunohistochemical staining. Animals were killed by exsanguination under light ether anaesthesia. One kidney was quick-frozen in dry ice–alcohol for immunofluorescent studies and the other fixed in 10 per cent buffered formalin for histochemical studies. The method of staining frozen sections with fluorescent conjugate was as described above. Tissue sections were always washed in saline to remove

unbound tissue antigens or serum proteins before staining with fluorescent conjugate. The fluorescent conjugate employed in these experiments was prepared from antiserum to reactive β-globulin fraction as described.

RESULTS

STAINING OF NORMAL KIDNEY

Sections of washed normal mouse kidney exposed to dilute conjugate prepared from rabbit antibody to mouse globulin fraction showed the following staining reactions:

(1) In the glomerulus, the staining was most intense in the region of the axial stalks and in this area individual structures were not always differentiated due to the intense staining. In the region of peripheral glomerular tufts, staining of individual capillary loops could be clearly differentiated as single curved lines. Basement membrane of Bowman's capsule showed bright staining (Fig. 1).

(2) In the region of the convoluted tubules of the cortex, the basement membrane of tubules reacted with moderate intensity and were discrete, clearly outlined structures (Fig. 2). The staining of tubular basement membrane was weaker and less uniform at the cortico-medullary junction and was very weak or absent in the collecting tubules of the medulla.

ISOLATION OF MOUSE SERUM FRACTION RELATED TO BASEMENT MEMBRANE STAINING

The immunofluorescent staining of renal basement membrane by this antiglobulin conjugate suggested an immunological relationship between basement membrane and a component or components of serum globulin. Reaction with kidney was tested after absorption of conjugate with isolated fractions of mouse serum separated by starch-block electrophoresis. As shown in Fig. 3, anti-mouse globulin conjugate absorbed with

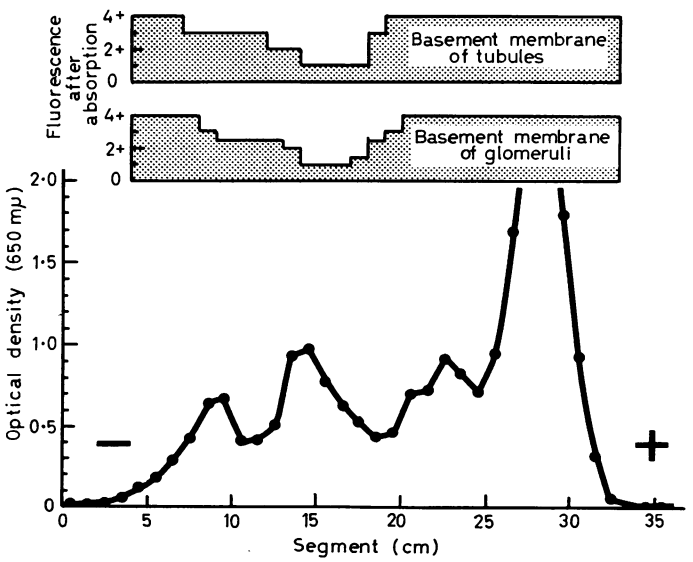

FIG. 3. Zone electrophoresis of normal mouse serum. The fluorescent conjugate of antiserum to mouse globulin was absorbed with aliquots of protein fractions and the supernatants were tested for staining of kidney. The serum fractions inhibiting immunofluorescent staining were most concentrated in the β-globulin zone.

protein fractions from the β-globulin zone failed to stain renal basement membrane, while absorption with fractions from albumin, α and γ zones was without effect. The component of mouse serum related to basement-membrane staining thus migrated as a β globulin on zone electrophoresis in starch or polyvinyl chloride, and appeared to be most highly concentrated in the fast-moving portions of β globulin and in the fractions between the α and β peaks.

Mouse serum was next fractionated by DEAE cellulose column chromatography using elution with a buffer gradient varying from 0·01 M phosphate, pH 8·0 to 0·30 M phosphate, pH 4·3. Four protein peaks were obtained and absorption of conjugate with eluate fractions demonstrated that the absorbing serum fraction was associated with the last broad peak eluted from the column (Fig. 4). Other investigators using the same gradient

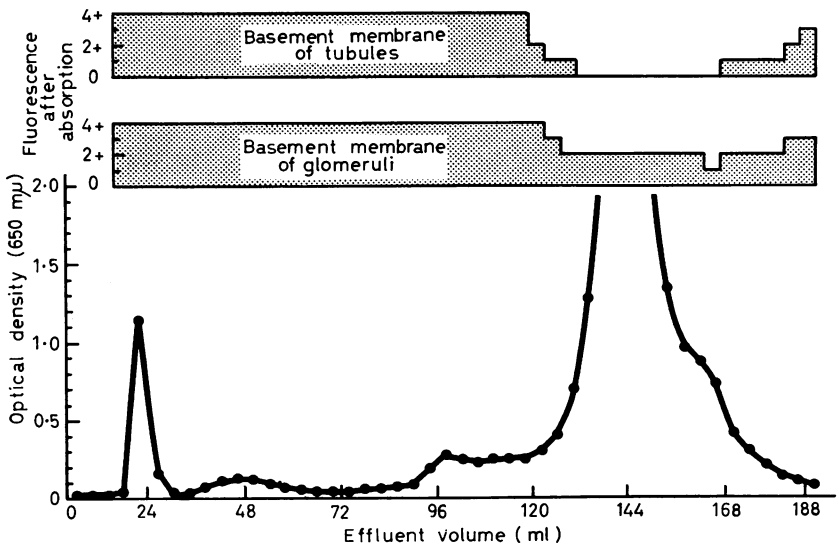

FIG. 4. DEAE column chromatography of normal mouse serum employing gradient elution. The same absorption procedure was used as in Fig. 3. The reactive fractions were eluted from the column in the final broad peak.

elution procedure and subjecting the chromatographic fractions to paper electrophoresis have shown that β globulin, albumin and other serum proteins are present in this chromatographic fraction (Fahey *et al.*, 1958).

In the final method of separation adopted, β globulin was first separated from other protein fractions by zone electrophoresis and the reactive fractions in the β-globulin zone identified by immunofluorescent-absorption tests. These fractions were pooled, dialysed against 0·01 M sodium phosphate, pH 8·0, and passed through a DEAE cellulose column equilibrated with the same buffer. The adsorbed proteins were eluted with a stepwise change of buffers. The column was first treated with the equilibrating buffer to remove proteins not adsorbed to the column. The second buffer, 0·07 M sodium phosphate, pH 6·0, had been determined by previous experiments to elute most of the remaining non-reactive fractions of β globulin; the final buffer, 0·3 M sodium phosphate, pH 4·3, was used to recover the final active fraction (Fig. 5). As shown in Fig. 6, conjugate absorbed with this last fraction adjusted to neutrality showed complete absence of staining either of glomerulus or of basement membrane of tubules.

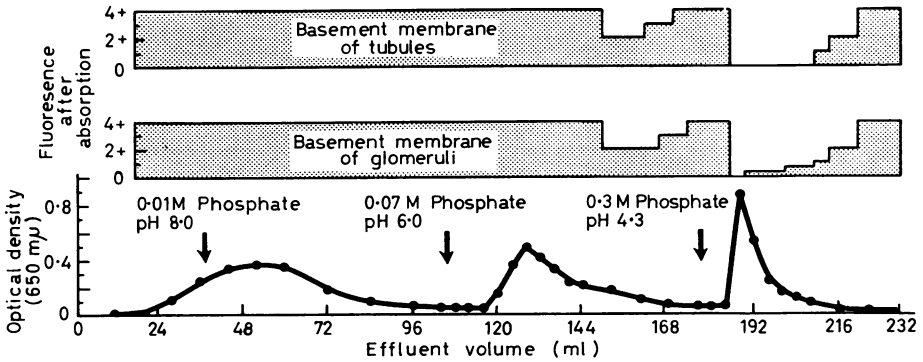

FIG. 5. DEAE column chromatography of mouse β-globulins obtained by zone electrophoresis. Stepwise elution permitted separation of reactive fraction in eluates obtained at 0·3 M phosphate, pH 4·3.

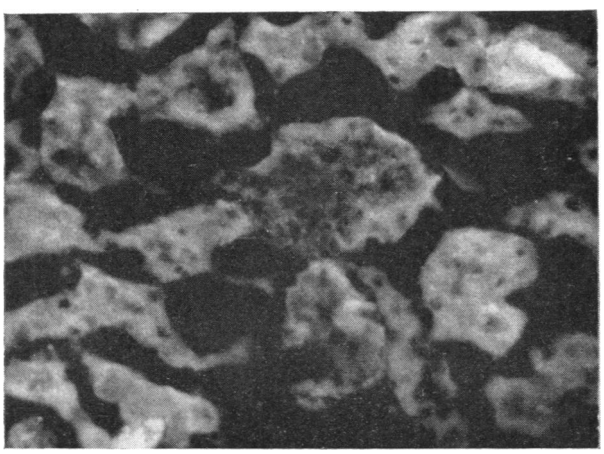

FIG. 6. Reaction of anti-mouse globulin conjugate absorbed with active chromatographic fraction of β-globulin. Little or no fluorescent staining is seen in glomerulus (centre) or in basement membranes of tubules. Greyness of tubular cytoplasm is due to over-exposure in photography to bring out structural details. × 312.

PREPARATION OF SPECIFIC ANTISERUM

Immunization of rabbits was undertaken with this last chromatographic fraction of β globulin. The active eluates were pooled and concentrated by ultrafiltration and pervaporation to a protein content of 2 mg./ml., emulsified in an equal volume of Freund's complete adjuvant, and injected weekly as described. The resulting antisera, which gave strong precipitin reactions with the active serum fractions, were tested for reaction with basement membrane. Application of unlabelled antisera to kidney sections prior to staining with anti-mouse globulin conjugate resulted in complete inhibition of staining in tubular basement membrane, while only traces of staining were left in glomeruli. Normal rabbit serum, which was collected before the immunization, did not inhibit the staining reaction of conjugate. A fluorescent conjugate, prepared from this antiserum, and a conjugate of antibody to mouse globulin (see below) were found to react identically with mouse kidney.

PROPERTIES OF REACTIVE SERUM FRACTION

Preliminary observations have been made on certain physico-chemical properties of the fraction of β globulin immunologically related to renal basement membrane. Ultracentrifugation, which was kindly done for us by Dr. Lena Lewis, of the Cleveland Clinic Research Foundation, was performed at solution density of 1·063 g./ml. according to the method of de Lalla and Gofman (1954) and showed that the reactive component in either whole serum or isolated chromatographic fraction did not belong to the low-density class lipoproteins. In starch-gel electrophoresis (Smithies, 1955) the reactive chromatographic fraction of β globulin was shown to have an electrophoretic mobility of albumin or pre-albumin when stained with amido-black 10B. No lipid material was detected in these starch-gel strips stained with Oil Red O. In agar-diffusion studies employing the Ouchterlony double-diffusion method, at least three closely grouped precipitin lines were observed between the chromatographic fraction and its specific rabbit antiserum.

IMMUNOFLUORESCENT REACTION WITH OTHER ORGANS

Several mouse organs were examined to determine whether other connective-tissue structures were specifically related to β-globulin component comparable to renal basement membrane. Washed sections of mouse organs were directly stained with fluorescent conjugate in the same way as kidney sections. Absorption tests with the reactive chromatographic serum fraction were carried out as controls of specific staining. It was observed that conjugates prepared from antiserum to mouse globulin fraction or to the reactive chromatographic fraction of β globulin showed the same specific pattern of staining in any one organ examined. The organs examined included cardiac muscle, skeletal muscle, liver, spleen and testis. In cardiac and skeletal muscle there was staining of the perimysium around large muscle bundles and of the endomysium surrounding individual muscle fibres (Figs. 7 and 8). In the liver a delicate linear staining surrounding hepatic cell cords

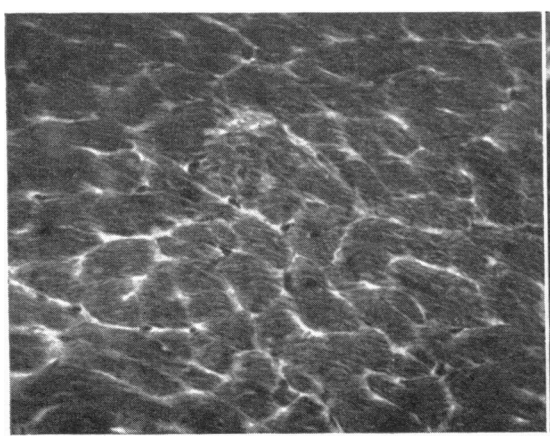

FIG. 7

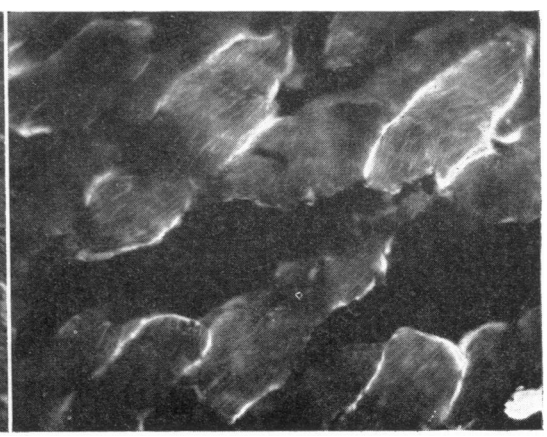

FIG. 8

FIG. 7. Staining of endomysium of cardiac muscle by fluorescent antibody to reactive β-globulin fraction. × 225.

FIG. 8. Staining of endomysium of skeletal muscle with same conjugate. × 225.

was observed which corresponded with the distribution of reticulin (Fig. 9). In the spleen there was staining of trabeculae and of the fine reticulin network in lymph follicles and red pulp (Fig. 10). In the testis staining involved the connective tissue and basement membrane surrounding the seminiferous tubules (Fig. 11).

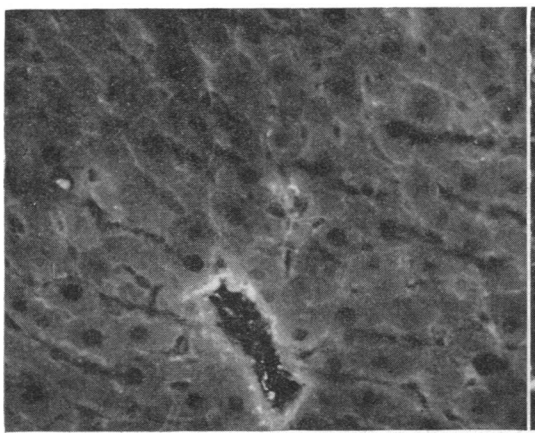

FIG. 9

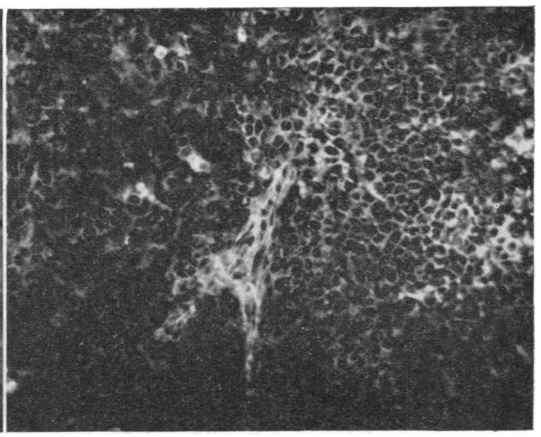

FIG. 10

FIG. 9. Normal mouse liver. Staining of reticulin in sinusoidal walls and around hepatic cells and of material around portal vein. × 225.

FIG. 10. Normal mouse spleen. Specific immunofluorescent reaction with material applied to collagen fibres of trabecula and with reticulin network of lymphoid follicles and red pulp. × 225.

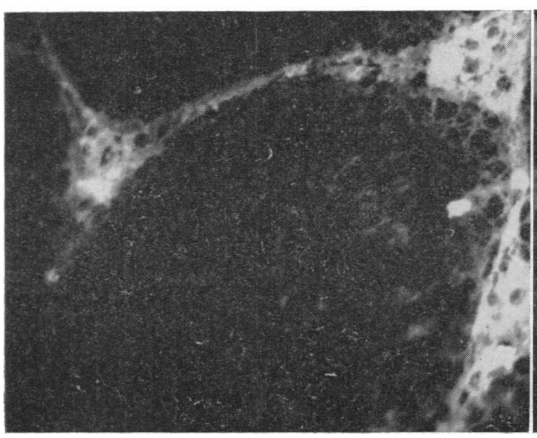

FIG. 11

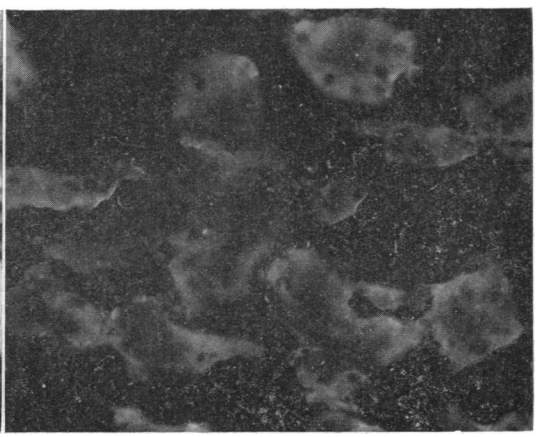

FIG. 12

FIG. 11. Testis. Reaction with basement membrane and peritubular connective tissue. × 225.

FIG. 12. Kidney of mouse given 5000 haemolytic units of streptolysin S and killed at 12 hours. Loss of staining reaction of tubular basement membrane except in a few segments. Stained with fluorescent antibody to isolated β-globulin component. × 225.

ALTERED STAINING OF TUBULAR BASEMENT MEMBRANE RESULTING FROM STREPTOLYSIN S

In mice that were given 3000 or 5000 haemolytic units of streptolysin S intraperitoneally in a single injection and killed 12–24 hours later, diminution of tubular basement-membrane staining was seen (Fig. 12), in contrast with reaction of normal control kidneys

(Fig. 13). In most tubular segments there was absence of staining, and in others only slight staining of basement membrane remained. Staining of glomerular basement membrane was not noticeably diminished or altered at this dose of toxin. Formalin-fixed

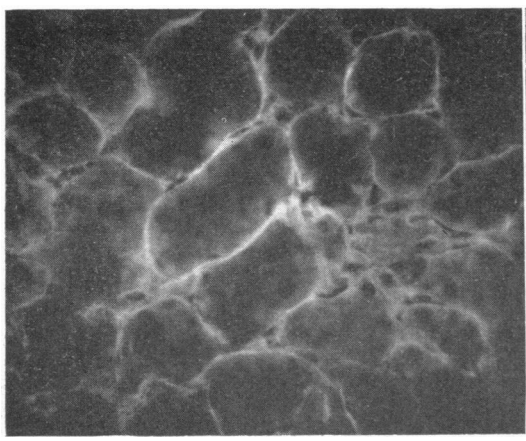

FIG. 13

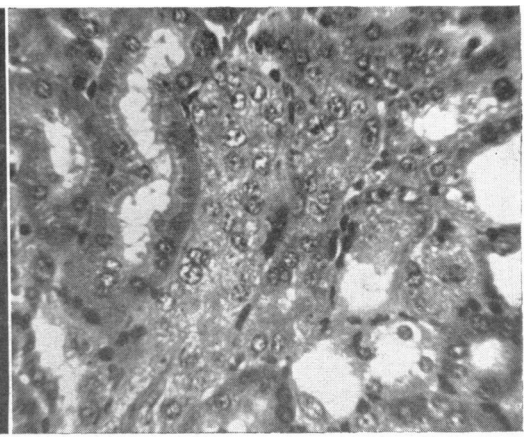

FIG. 14

FIG. 13. Normal mouse kidney. Reaction of tubular basement membrane with fluorescent antibody to isolated β-globulin fraction. × 275.

FIG. 14. Tubular regeneration in mouse given 3000 haemolytic units of streptolysin S and killed 7 days later. Haematoxylin and eosin. × 225.

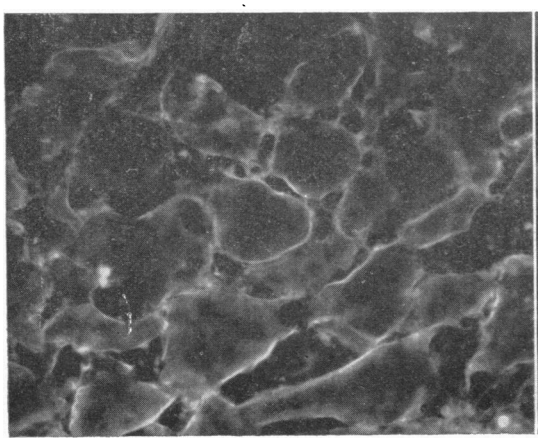

FIG. 15

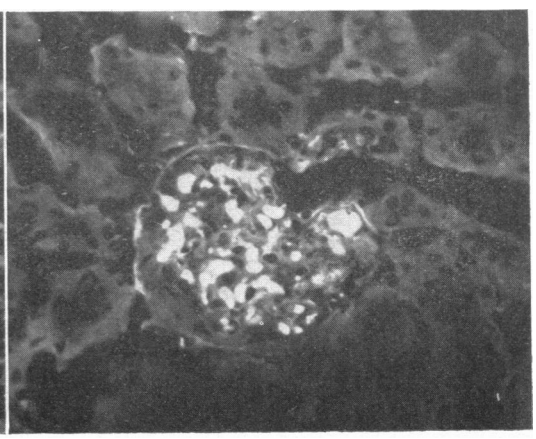

FIG. 16

FIG. 15. Reappearance of tubular basement-membrane reaction in regenerating tubules. This kidney was from same mouse as in Fig. 14. × 225.

FIG. 16. Kidney section from mouse given two injections of 5000 haemolytic units of streptolysin S. Eosinophilic masses in glomerulus are stained brilliantly with fluorescent conjugate. × 225.

kidney stained with the periodic acid–Schiff (PAS) method showed smudging and fragmentation of tubular basement membrane compared to the reaction of normal mouse kidney. PAS-stained sections showed no alteration of glomerular basement membrane. Animals given similar doses of streptolysin S were killed at various intervals after injection.

It was observed that alteration of basement-membrane staining was not present at 6 hours, became apparent at 9 hours, and persisted for 36–48 hours after injection. After this period, staining of tubular basement membrane was observed again. The return of basement-membrane staining was seen in association with regeneration of damaged tubular segments. Fig. 14 illustrates the tubular regeneration in an animal that was sacrificed 7 days after injection of streptolysin S. The majority of tubular basement membranes reacted with fluorescent antibody in this kidney (Fig. 15).

A similar course of events was observed in mice with implanted intraperitoneal diffusion chambers containing group A streptococci. Tubular basement-membrane staining was observed to disappear during the period of acute tubular necrosis and to return after tubular regeneration.

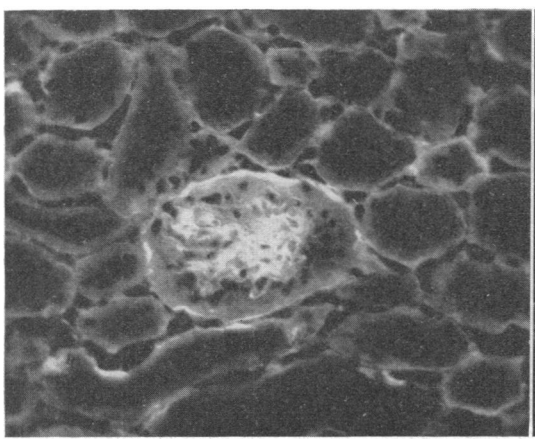

FIG. 17

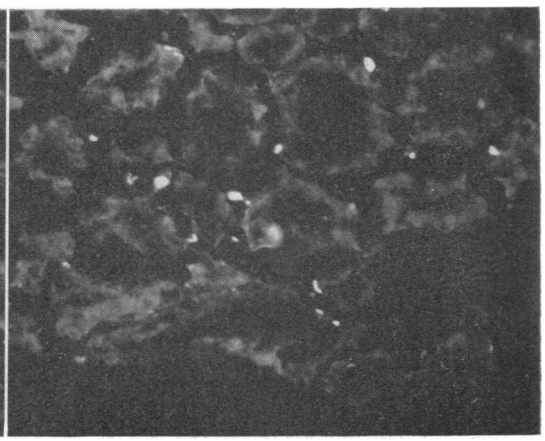

FIG. 18

FIG. 17. Reaction of normal mouse glomerulus with fluorescent anti-β-globulin component. × 225.

FIG. 18. Loss of tubular basement-membrane reaction in mouse given two injections of streptolysin S. In place of the tubular basement-membrane staining, focal accumulations of fluorescent material are present in the intertubular spaces and at the periphery of some tubular segments. × 225.

Although it was shown that a single injection of streptolysin S resulted in altered staining reaction of tubular basement membrane with fluorescent conjugate, no alteration of staining in glomerular basement membrane was observed. The effect of a second injection was examined. Intraperitoneal injection of 5000 haemolytic units of streptolysin S was followed in 3 hours by a similar injection of toxin, and the kidneys examined 5 hours after this last injection.

In sections of formalin-fixed kidney stained with haematoxylin and eosin, the glomerular capillaries were seen to be filled with homogeneous eosinophilic material which was PAS-positive. When sections of frozen kidney were stained with fluorescent conjugate, large accumulations of fluorescent material were present in the glomerulus together with a marked decrease in staining of glomerular capillary loops. This is illustrated in Fig. 16 and is contrasted with staining of glomerulus in a normal control kidney (Fig. 17). Tubular basement-membrane staining was absent and in certain areas focal aggregates of staining were present in the intertubular spaces (Fig. 18). The latter finding has been

seen in some animals receiving single injections of streptolysin S but the glomerular changes have not been observed except after a second injection.

DISCUSSION

The evidence for the presence in normal mouse serum of components immunologically related to renal basement membrane and other connective-tissue structures was demonstrated by immunofluorescent methods. The criteria for specificity of immunofluorescent reactions have been outlined (Coons and Kaplan, 1950), and in this study they have been fulfilled by the following conditions.

(1) Staining reaction of conjugate was specifically removed by absorption with an isolated chromatographic fraction of β globulin. Other serum protein fractions were not effective.

(2) Staining reaction was inhibited by unconjugated homologous antiserum.

(3) Heterologous rabbit antibody conjugates, e.g. to γ globulins from various species, did not show a similar staining reaction.

The reactive mouse β-globulin fraction has been shown to be immunologically related to glomerular capillary loops, tubular basement membrane, reticulin of liver and spleen, material applied to collagen fibres in splenic trabeculae, endomysial and perimysial connective tissue of heart and skeletal muscle, and basement membrane and connective-tissue elements of testis. It is not certain whether these immunofluorescent reactions involve a single common antigen in these structures, or whether they reflect participation of multiple antigen–antibody systems. Previous studies relating to the antigenic properties of glomeruli and tubular basement-membrane preparations are pertinent to this question. Cruickshank and Hill (1953) used the fluorescent antibody technique to demonstrate that rabbit antiserum to isolated renal glomeruli of the rat contained antibodies reactive with basement membranes and reticulin in many organs. These observations were subsequently extended by Scott (1957, 1959), who studied the comparative immunofluorescent properties of antisera to human glomeruli and synovia in cross-inhibition and 'mixed-staining' reactions, and described three groups of reactive connective-tissue components: the first, related to glomerular basement membrane, media of arteries and arterioles and possibly also renal tubular basement membrane; the second, related to reticulin in liver and spleen, sarcolemma of muscle, and media of large arteries; the third, related to fibrillar elements in vascular adventitia, interstitial connective tissue, and collagenous trabeculae. Reaction with collagen bundles was not described. However, Rothbard and Watson (1961) prepared rabbit antiserum to collagen extracted from rat-tail tendon and presented evidence of *in vivo* localization of such antibody in basement membranes of glomeruli and tubules of appropriately pretreated rats. In other serological studies, employing flocculation reactions and cross-absorption tests, Goodman, Greenspon and Krakower (1955) reported that preparations of canine glomerular and tubular basement membrane contained common as well as unrelated antigenic components, and that both elements were related immunologically with collagen fibres from tendon and cornea.

These combined studies have suggested that the serological reaction of anti-glomerulus serum with glomerular, tubular and other basement membranes, and with reticulin and collagenous fibrils may involve, in part, an immunological reaction with antigenically similar constituents of these elements.

The immunological properties of antiserum to isolated mouse β-globulin component

might similarly pertain to an antigenic relationship of glomeruli, tubular basement membrane, reticulin and collagenous connective tissue. However, the alternative possibility that multiple antigens may be present in the isolated β-globulin fraction which are individually related to one or more of these structures, has not been excluded. Agar-diffusion studies revealed three lines between isolated β-globulin component and antiserum, and the relation of these components to the immunofluorescent properties of the antiserum remains to be clarified.

The possibility that a similar serum fraction possessing immunological relationship to connective tissue is present in the sera of other species of animals has been suggested by the studies of some investigators. In the rabbit, Heller, Gakulis and Zimmerman (1959) reported that antiserum to rabbit tendon showed a precipitin reaction with rabbit serum by agar diffusion. Milazzo (1957) demonstrated that antiserum to human globulin reacted with human glomerular antigen by capillary precipitin tests. It appears likely that the presence in serum of components related to connective tissue might not be peculiar to the mouse.

The present report has described an alteration of basement membrane in mice given intraperitoneal injections of streptolysin S, or implanted with diffusion chambers containing group A streptococci. This alteration was shown by loss of ability of tubular basement membrane to react with fluorescein-conjugated antibody to the related β-globulin fraction. This effect of streptolysin S could be observed as early as 9 hours after intraperitoneal injection. At this stage, histopathological changes in kidney studied by light microscopy consisted only of hyaline granulation in renal tubular cells and decreased PAS staining of tubular basement membrane. The rapidity with which alteration of basement-membrane staining was observed by immunofluorescence after injection of streptolysin S and the relative paucity of concomitant pathological change in other renal structures suggested that the initial noxious effect of the toxin on the kidney might perhaps be directed towards this connective-tissue structure. Of possible relevance to this latter consideration is the report of Markowitz (1960) of a common antigenic determinant in rat basement membrane and red cell. The question should be considered whether the haemolytic activity of streptolysin S on erythrocytes and its effect on basement membrane may be related by virtue of a common or related substrate on these structures. Alternatively the *in vivo* effect of streptolysin S on basement membranes may have resulted from an action on tubular cells.

The present work has further indicated that only tubular basement membrane was altered by a single intraperitoneal injection of 3000–5000 units of streptolysin S and that alterations in glomeruli could not be detected under these conditions. If damage to tubular basement membrane was the precursor of subsequent damage to tubular cells, it would be in agreement with earlier observations that renal tubular necrosis was the main feature of streptolysin S-induced renal disease in mice. How alteration or damage of basement membrane would lead to tubular cell necrosis is a question that cannot be adequately answered at the present.

Although altered immunofluorescent staining of glomeruli could not be observed with a single injection of streptolysin S, the study with two injections of this toxin showed that glomerular basement membrane was also susceptible to the effect of the toxin. The masses of eosinophilic material filling glomerular capillary loops also stained with fluorescent conjugate. This suggested that the material might be derived from glomerular basement membrane since there was concomitant loss in staining of the capillary loops themselves.

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