AUTOIMMUNE DISEASE IN NZB/BL MICE

I. Pathology and Pathogenesis of a Model System of Spontaneous Glomerulonephritis*

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Plates 4 to 9

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During the last year we have studied autoimmune manifestations and pathological changes in mice of the inbred strain NZB/Bl, confirming and extending observations of others at the University of Otago Medical School, Dunedin, New Zealand, (1, 7, 8, 18–21), where the strain was developed by Dr. Marianne Bielschowsky, and at the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia (2, 9, 24–26, 32, 42). The present report deals mainly with the pathology and the pathogenesis of glomerulonephritis in NZB/Bl mice, a model system of membranous glomerulonephritis with spontaneous and insidious onset, progressing through chronic stages, and almost certainly induced by immunological, and autoimmune, mechanisms.

Methods and Materials

Animals.—Dr. Marianne Bielschowsky kindly sent us a breeding nucleus of 4 males and 9 females from the sixtieth generation of her inbred strain of NZB/Bl mice. A colony of many hundred has been derived from this stock by brother-sister matings in first and second litters, now in the sixty-third generation. The diet has consisted of purina laboratory chow supplemented with lettuce and carrots and free access to water. Nursing does also received bread soaked in cows' milk, with 2 drops of cod liver oil added. A silica gel insecticide (permadust, Laboratory Essentials, Inc., West Islip, New York) was dusted on the wood-shaving beddings of mice over 3 weeks of age. Daily inspections, biweekly or monthly weighings, and laboratory observations were made, and are continuing to be made, on non-breeding males and females of this colony beginning at about 2 to 3 months of age and on retired breeders from 4 to 12 months of age. A few retired NZB/Bl breeders were also received for study from Dr. Harry J. Robinson of Merck Institute for Therapeutic Research, Rahway, New Jersey. The breeding nucleus of this colony came from Dr. Derrick Rowley of the University of Adelaide, Australia. The present report is based upon 528 laboratory examinations of 38 NZB/Bl mice of both

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sexes from 2 to 12 months of age and the complete autopsy and histopathological examination of 16 NZB/Bl mice from 1 day to 12 months of age.

Thirty Swiss-Webster male albino mice were used as normal controls; on these 92 laboratory examinations and 10 complete autopsies were performed. In correlative studies, rabbit antiserum to mouse kidney was injected into 11 Swiss-Webster mice. 6 animals had proteinuria and 1 generalized ascites. The kidneys were studied at 2 days, 1, 2, and 4 weeks; nephrotoxic nephritis occurred in 10 mice. Homologous (mouse) and heterologous (rabbit) immune complexes (with ovalbumin or bovine serum albumin as antigen, in slight excess) were given to 50 Swiss-Webster mice in single or multiple intravenous injections, with cumulative doses up to 7 mg of antibody; kidneys studied at 1 to 18 days showed little more than transient acute glomerulitis.

Hematology.—Bleedings from the periorbital sinus provided one or two capillary tubes of blood (heparinized or clotted) sufficient for hematological, as well as chemical and serological, study. Standard methods were used: microhematocrit, hemoglobin determination (20 μl sample) by the cyan-hematin method (using a Bausch and Lomb spectrometer, spectronic 20), total and differential leukocyte and reticulocyte counts.

Blood Chemistry.—Ultramicromethods (Beckman system, model 150) were used for the determination of total serum protein (biuret method, 5 μl sample) and serum urea nitrogen (method of Fearon and Friedman, 10 μl sample). Serum proteins were also analyzed by paper electrophoresis (LKB system, model 3276B).

Urinanalysis.—A drop of urine was collected in a test tube. Proteinuria was detected by reagent strips (albustix, Ames Company, Inc. Elkhart, Indiana) with color calibrations at 30 mg (+), 100 mg (++), 300 mg (+++), and over 1000 mg (++++) protein per 100 ml verified by mouse serum albumin solutions, and was also estimated by volume of precipitate produced by a standard amount of rabbit antiserum to mouse serum proteins. The test for sugar also used reagent strips (clinistix, Ames Co.). Normal findings (20 examinations on 20 Swiss-Webster mice) were (−) or at most (+) proteinuria and (−) sugar. The urine was also always examined microscopically without centrifugation.

Antiglobulin (Coombs) Test.—The antiglobulin (Coombs) reagents were rabbit antiserums to mouse serum proteins, to mouse globulins, and to mouse immunoglobulins absorbed with many times washed, normal mouse red cells to remove natural and acquired hemagglutinins. The first antiserum mentioned was obtained from rabbits hyperimmunized with lyophilized mouse serum emulsified in Freund's complete adjuvant, the second and most used was a commercial preparation (sylvana), and the immunizing antigens for the third were purified mouse serum γ-globulins prepared by Dr. Leonhard Korngold by zone electrophoresis on starch. The direct and the indirect Coombs tests were conventional procedures carried out in test tubes (10 × 75 mm) with macroscopic agglutination as positive end-points. Known strong reactors from the NZB/Bl mouse strain served as positive controls. Normal Swiss-Webster mice were always negative. In the direct Coombs procedure, mouse red cells were first washed four times in isotonic saline to remove serum proteins not adsorbed by the cells, diluted to a 2 per cent suspension, mixed with an equal volume of Coombs reagent, and centrifuged at 3400 RPM for ½ minute. In the indirect Coombs procedure, the test antigens were normal Swiss-Webster mouse red cells which had been washed three times in pH 7.4-buffered saline, incubated with a standard papain solution at 37°C for 10 minutes, washed again, and diluted to a 4 per cent suspension. The test serums (and similarly red cell eluates or tissue extracts) were mixed with an equal volume of the papain-treated red cell suspension, centrifuged for ½ minute, observed, resuspended, and incubated at 37°C for 60 minutes, centrifuged for ½ minute, observed ("warm saline" agglutination), washed five times in isotonic saline, resuspended, and finally mixed with an equal volume of Coombs reagent and centrifuged once more.

Eluates of NZB/Bl Mouse Red Cells.—These were prepared by the method of Weiner (52); three times washed, tightly packed red cells (0.2 to 0.5 ml) were frozen and lysed at −70°C, thawed, and mixed with 50 per cent ethanol at −6°C to precipitate the antibodies which were then separated by centrifugation, resuspended, and dissolved in isotonic saline at 37°C for 60 minutes. The final volume of eluate was two times the volume of lysed cells; antibody activity was determined by the indirect Coombs test.

Extracts of NZB/Bl Mouse Spleens and Kidneys.—Two to three dozen frozen sections of spleen or kidney, (mounted on slides as for immunofluorescence study, but not fixed) were extracted at 4°C for 2 to 16 hours in vertical staining jars containing pH 3.4 citrate-buffered isotonic saline. Thereafter the tissue extracts were brought to pH 7.4 by dialysis against phosphate-buffered isotonic saline and concentrated to a volume of about 1 ml by pervaporation. Aliquots were assayed by the indirect Coombs test and by immunofluorescence.

Latex Fixation Tests.—These were of three types identical with those sometimes used in the clinical serology of human rheumatic diseases and utilizing latex carrier particles coated with test antigens: a slide test (Hyland, Los Angeles) for lupus factor with calf thymus DNA-histone as antigen; a slide test (Hyland) and a tube dilution test for rheumatoid factor with human γ-globulin (Cohn fraction II) as antigen. These procedures were always controlled with positive serums from patients with systemic lupus erythematosus and rheumatoid arthritis, as well as negative controls. In the test for lupus-like factor, mouse serums were diluted 1:2; for rheumatoid-like factors, serum dilutions began at 1:20.

Histopathology.—Complete autopsies were performed on 16 NZB/Bl mice of both sexes including 10 that were 4 to 12 months of age with average body weight of 37 gm and range of 30 to 43 gm, and 10 normal Swiss-Webster mice with average body weight of 33 gm and range of 24 to 43 gm. Animals were killed by cervical dislocation, and tissue blocks were immediately processed for study by light microscopy, immunofluorescence, and electron microscopy. The fixatives of choice for paraffin-embedding procedures were as follows: Vandegrift's for hematoxylin-eosin and periodic acid-Schiff stains; Carnoy's for periodic acid-methenamine silver (28) and Feulgen stains; and 4 per cent neutral formaldehyde for Congo red, crystal violet, iron, and bacteria stains. Paraffin sections were cut at 1 μ thickness for the periodic acid-methenamine silver stain, and at 4 μ for the remaining procedures.

Immunofluorescence.—Mouse tissue blocks were rapidly frozen at −70°C, sectioned at 4 μ with a rotary microtome in a cryostat at about −25°C, air-dried, and fixed with absolute acetone at room temperature for 10 minutes. Immediately before staining, sections were washed three times in pH 7.6 phosphate-buffered isotonic saline. The immunizing antigens used to raise antiserums in hyperimmunized rabbits were mouse γ-globulins purified by starch zone electrophoresis, mouse serum albumin (Pentex, Inc., Kankakee, Illinois), and normal Swiss-Webster mouse kidney powder, a many times washed saline-insoluble sediment. The antibody-active fractions were prepared from the antiserum by cold ethanol precipitation or by column chromatography on DEAE-cellulose and labeled with fluorescein isothiocyanate (Baltimore Biological Laboratories, Baltimore). The fluorescein:protein ratios were 1:67 by weight in the reaction mixtures and approximately 4:1 (molecular) in the final dialyzed and absorbed products. The fluorescent antibodies to mouse γ-globulins were monospecific, reacting only with the γ-globulins (immunoglobulins) of mouse serum as shown by immunodiffusion and immunoelectrophoresis. The fluorescent antibodies to mouse serum albumin were likewise shown to be monospecific. The fluorescent antibodies to mouse kidney did not react with mouse serum proteins, did localize *in vivo* on mouse glomerular capillary basement membrane and *in vitro* on glomerular and tubular basement membranes; and the parent antiserum was nephrotoxic. The specificity of immunofluorescent staining was demonstrated by blocking reactions, absorption of fluorescent antibodies with homologous antigen, and prior treatment of sections with unlabeled homologous antibodies or antiserum. The fluorescent

antibodies were absorbed with tissue powders (50 mg each of rabbit bone marrow and mouse liver powder per ml) and diluted to 1:8 to 1:16 (equivalent to 1 to 2 mg protein per ml) prior to use; incubation times were 45 minutes at room temperature. The indirect procedure was used in the test for antinuclear factors; mouse serum, diluted 1:2, and followed, after washing, by fluorescent antibodies to mouse γ-globulins. Tissue extracts were studied similarly by the indirect immunofluorescence procedure; here the period of incubation was 2 hours at 4°C.

Other Methods,—Studies to be reported more extensively at a later date were done in collaboration with Dr. Chen Ya Huang, in electron microscopy, and in collaboration with Dr. Leonhard Korngold, immunoelectrophoresis and ultracentrifugal analysis.

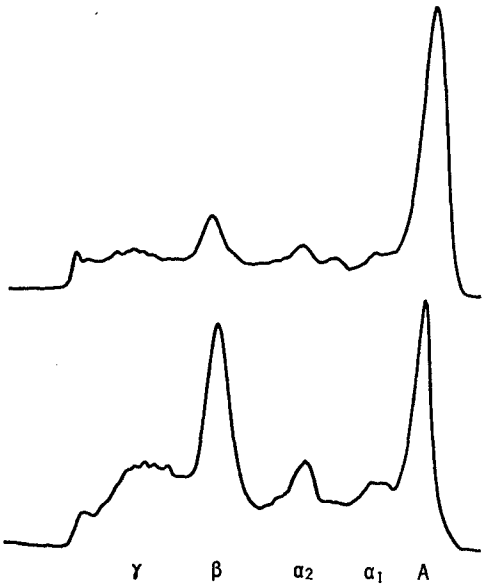
RESULTS

Hypergammaglobulinemia.—The normal total serum protein concentration and the protein partition (per cent of total) as determined by paper electrophoresis were as follows (99 per cent confidence intervals for measurements on 11 to 20 normal Swiss-Webster mice): total protein, 5.5 to 5.9 gm/100 ml; albumin, 41 to 53 per cent; α_1-globulins, 6 to 12 per cent; α_2-globulins, 13 to 17 per cent; β-globulins, 13 to 21 per cent, and γ-globulins, 10 to 14 per cent. Studies involving 87 determinations on 29 NZB/Bl mice at monthly intervals, beginning at 2 months, indicated that at 6 months, 9 of 12 mice had elevations of total proteins (the range was 6.1 to 7.1 gm/100 ml), at 7 months 4 of 6 mice had relative increases of γ-globulins (19 to 30 per cent of total proteins), and 2 of these had γ-globulin values of 1.8 and 2.1 gm/100 ml, that is, more than twice the normal concentration. Hypergammaglobulinemia once established usually persisted; in addition elevations of β-globulins, shown by immunoelectrophoretic analysis to be "fast" γ-globulins or γ_M-globulins, were observed in 2 of 5 mice at 10 months of age. An example of changing electrophoretic patterns is shown in Text-fig. 1.

Autoantibodies to Red Cells.—The direct antiglobulin (Coombs) test for incomplete antibodies localized on red cells was negative in 21 NZB/Bl mice at 2 and 3 months of age, the first positive reactions occurred at 4 months, about 50 per cent of mice were positive at 7 months, and thereafter the number of positives increased with age. Rabbit antiserums to mouse globulins, to mouse γ-globulins, and to mouse serum proteins gave comparable results as Coombs reagents although the first mentioned was the most used. These data based upon 102 direct tests on 38 NZB/Bl mice from 2 to 12 months of age and mainly in the sixty-first generation, were comparable to those first reported by Bielschowsky, Helyer, and Howie (8) at about the forty-second generation. Normal control mice were consistently Coombs negative.

Eluates prepared in three separate instances from saline-washed, Coombs-positive NZB/Bl red cells contained incomplete antibodies with affinity for papain–treated red cells of young NZB/Bl mice or Swiss-Webster mice. Serums from Coombs-positive NZB/Bl mice contained warm saline agglutinins (in low titers, up to 1:20) and warm incomplete antibodies (with titers of 1:320

or more in the indirect Coombs test). These were confirmatory observations (8, 21, 24, 32). When one serum was fractionated by density gradient ultracentrifugation, the antibodies were not detectable in the lighter fractions, amongst which were the 7S γ-globulins, but were present in the heavier fractions containing 19S γ_M-globulins. Furthermore the antibodies in serum or in eluates of washed Coombs-positive NZB/Bl red cells were inactivated by treatment with 0.1 M 2-mercaptoethanol as are other γ_M-globulins (11).



TEXT-FIG. 1. Paper electrophoresis patterns (migration to the right) of serum proteins for an NZB/Bl mouse: above, at 8 months of age; below, at 10 months of age.

Lupus-Like Factors.—Antinuclear factors were detected in the sera of only 1 (weakly positive) of 15 NZB/Bl mice (from 4 to 12 months of age) using the immunofluorescence procedure and frozen sections of autologous, isologous, or homologous mouse tissues (kidney, liver, thyroid) as test antigens. Latex fixation tests (Hyland) with calf thymus DNA-histone were negative in all 70 examinations performed on 26 NZB/Bl mice over 4 months of age. Our findings were not inconsistent with the studies of Helyer and Howie (21) in which only 2 of 43 NZB/Bl mice had positive lupus erythematosus cell tests. However a high (45 per cent) incidence of serum antinuclear factors was reported by Norins and Holmes (42) in NZB/Bl mice over 2 months of age using the immunofluorescence procedure with a commercial antiglobulin reagent and alcohol-fixed smears of human blood leukocytes as nucleoprotein test antigens. Aside from differences in immunofluorescence technique, en-

vironmental factors may contribute to the varying frequency of antinuclear factors reported for two identical strains of mice (47).

Rheumatoid-Like Factors.—The serums of 2 of 28 NZB/Bl mice subjected to monthly examinations became positive at about 7 months of age in latex fixation tests with human γ-globulin (Cohn fraction II), using slide (Hyland) and tube methods (titer 1:80). The rheumatoid-like factors were not detectable in lighter fractions obtained by density gradient ultracentrifugation of a positive serum but were present in γ_M-globulin–containing fractions (titers up to 1:80), and were inactivated by treatment with 0.1 M 2-mercaptoethanol. The rheumatoid-like factors, γ_M-globulins, were different from the autoantibodies to red cells; the latter, but not the former, were absorbed with papain-treated mouse red cells. Their significance is not known; conceivably they are anti-antibodies (39) although we have not yet devised a satisfactory test to demonstrate this.

Lymphatic Tissues.—The spleens of 10 adult NZB/Bl mice, one to three times the normal weight of 0.2 gm and the site of extramedullary hematopoiesis, contained numerous plasma cells in red pulp (21, 24) and germinal centers (up to 10 per section) forming immunoglobulins as shown by immunofluorescence. These immunoglobulins were readily extracted from frozen sections of spleen in three separate experiments by overnight incubation at 4°C in pH 3.4 citrate-buffered isotonic saline. This extract, brought to pH 7.4 by dialysis and concentrated by pervaporation, reacted positively (in the indirect Coombs test) with papain-treated mouse red cells. The extracted sections, thereafter brought to pH 7.4, had lost cell-localized immunoglobulins as shown by immunofluorescence. It was clear, in comparison with other lymphatic tissues (lymph nodes, thymus) similarly studied, that the bulk of autoantibodies to red cells were formed in the spleen, which was also the main site of red cell destruction as shown by depositions of hemosiderin in, or outside of, macrophages in the spleen of mice with high reticulocyte counts (values greater than 5 per cent occurred at 6 months, rising thereafter) and low hematocrits (less than 40 per cent by the 8th month, falling thereafter). More extensive hematological data have been published by Helyer and Howie (21), Long, Holmes, and Burnet (32), and Holmes and Burnet (24).

The thymus glands of 10 NZB/Bl mice, 4 to 12 months of age and showing about the same size (0.01 to 0.07 gm) and degree of thymic atrophy as found in Swiss-Webster mice of comparable ages, usually contained numerous lymphocytes, some with proliferative activity, and sometimes an abundance of plasma cells (2, 9), including immature, mature, and Russell-body types, and mast cells (9) and in one instance germinal centers (9, 24). A few plasma cells of all types and the germinal centers also contained immunoglobulins.

Pathology of Glomerulonephritis.—Thorough and correlative histologic, immunofluorescence, and electron microscopic (27) studies were made of both

kidneys of 10 NZB/Bl mice (6 males and 4 females) sacrificed (to avoid postmortem artefacts) at 4 to 12 months of age. Two of these mice, males at 5 and 6 months of age, had essentially normal kidneys; the remainder had glomerulonephritis. The magnitudes of glomerular structural alterations, a measure of the severity of glomerulonephritis, were designated as follows: minimal by the criteria of light microscopy (confirmed by electron microscopy); maximal (as judged from comparative studies of human glomerulonephritis); and intermediate between these extremes. The severity of glomerulonephritis was then: minimal, 1 animal at 4 months; intermediate, 3 animals at 7, 8, and 10 months; and maximal, 4 animals at 8, 10, 11, and 12 months.

The glomerular disease was spontaneous, insidious, chronic, and progressive. The structural alterations (membranous and lobular glomerulonephritis) were more serious than was apparent from ordinary laboratory examinations: only 3 of 8 animals had significant proteinuria (++ to +++), the urinary sediment contained no red cells and but a few granular casts, and the blood (serum) urea nitrogen was, with one exception, within normal limits (for which the 99 per cent confidence interval is 21 to 29 mg/100 ml) as were also 95 determinations of blood urea nitrogen on 33 NZB/Bl mice from 2 to 11 months of age. The blood urea nitrogen rose to 41 mg/100 ml in 1 animal with severe glomerulonephritis. It is to be noted however that none of the mice were followed to the final terminal course of their disease.

The kidneys were grossly pale to dark brown, a color possibly imparted in part by tubular depositions of hemosiderin, (21), smooth surfaced, and approximately normal in weight (average 0.48, range 0.33 to 0.60 gm per pair). The earliest and lasting histological change was hyaline thickening (18, 19, 24) of the capillary walls and adjacent intercapillary regions of the glomerular tufts, corresponding mainly to capillary basement membrane and mesangium when studied by electron microscopy (27). The hyaline materials were stained "soft" pink with eosin, Fig. 1; bright magenta (carbohydrate-rich) and homogeneous or occasionally granular with the periodic acid-Schiff stain, Figs. 2 to 4; and black (carbohydrate-rich) with subepithelial membranous club-like protrusions in the peripheral capillary loops and a fibrillar structure (spongy fibers) in axial (mesangial) regions when stained with the periodic acid-methenamine silver technique of Jones (28), Figs. 5–7. Distributed focally and diffusely in the glomerular tuft and eventually sparing no glomerulus, the hyaline, granular, and fibrillar materials produced narrowing of capillary lumens by concentric or eccentric encroachment in peripheral or axial regions, very much in the manner of human glomerulonephritis (3, 5, 28, 49, 50, 41, 6). Dilatations of capillaries were sometimes seen. In the later stages hyaline lobulation, Fig. 3, and sclerosis, Figs. 4, 6, and 7, of the glomerular tufts occurred. Thus the lesions corresponded to those seen in human focal and diffuse membranous, chronic lobular, and lastly, sclerosing glomerulonephritis. The focal membran-

ous change, Fig. 2, resembled the "wire-loop" lesion of lupus nephritis (19, 1), and the diffuse lesion was similar to that of amyloid in glomerular amyloidosis, as has been noted (24) but excluded by special studies (Congo red and crystal violet stains and birefringence were negative) and homogeneous ultrastructure (27).

Hyaline thrombi, containing delicate layers of indistinctly granular PAS-positive materials, Fig. 8, which were PTAH-negative, not fibrin, and very likely platelet thrombi, occurred in the lumens and along the walls of dilated capillaries of some glomeruli in one example of severe membranous glomerulonephritis. Protein casts (PAS-positive) were present in tubular lumens of glomerulonephritic kidneys; plasma cells and lymphocytes sometimes infiltrated the medullary peritubular spaces and the pelvic fat tissue. There were no additional microscopic changes of note.

At the level of electron optical resolution, the glomerular membranous lesion consisted mainly of homogenous swelling of the capillary basement membrane, with effacement of the laminae rarae and protrusions into subendothelial and subepithelial spaces, Fig. 19, and accumulation of basement membrane-like materials in the mesangium. These findings will be described in detail elsewhere (27).

Pathogenesis of Glomerulonephritis.—In all instances of glomerulonephritis in NZB/Bl mice, the glomerular tufts contained mouse immunoglobulins, Figs. 9 to 13, identified by fluorescent rabbit antibodies and at sites corresponding to the distribution of eosinophilic and PAS-positive materials in the focal and diffuse membranous and lobular lesions and in amounts increasing with the severity of glomerular disease. No more than trace amounts of mouse serum albumin were detected in the glomerular tufts by immunofluorescence, Fig. 14, although albumin was present in tubular protein casts, Fig. 15. The glomeruli of normal mouse kidneys (young NZB/Bl or adult Swiss-Webster) contained at most traces of mouse immunoglobulins.

The mouse immunoglobulins were extracted from frozen sections (24 slides in a jar at a time) of glomerulonephritic NZB/Bl kidneys in three separate animal experiments by 3-hour and overnight incubations at 4°C in pH 3.4 citrate-buffered isotonic saline (13). Traces of mouse immunoglobulins, as shown by immunofluorescence, remained in the glomerular tufts after the shorter period of extraction, Fig. 18; these sections, still intact and always equilibrated to pH 7.6 for further study, retained mouse glomerular and tubular basement antigens (23) identifiable by fluorescent rabbit antibodies to normal Swiss-Webster mouse kidney (saline insoluble components). Overnight extraction of glomerulonephritic kidneys at low pH removed virtually all of the immunoglobulins but also decreased the intensity of staining of basement membrane antigens with fluorescent rabbit antibodies to mouse kidney (at pH 7.6). The mouse immunoglobulins in overnight extracts of glomerulone-

phritic kidneys, after restoration to pH 7.6 by dialysis and concentration by pervaporation, recombined with the glomerular tufts in (pH 7.6) sections of autologous or isologous glomerulonephritic NZB/Bl kidneys from which *in vivo* localized immunoglobulins had been extracted by 3 hours' incubation at pH 3.4 but not those extracted overnight. The pattern of recombination with glomerular tufts, Fig. 17, was similar to that of the *in vivo* localized immunoglobulins. The extracted immunoglobulins in each instance did not show affinity for papain-treated mouse red cells in the indirect Coombs test, nor for autologous or isologous cell nuclei in the immunofluorescence test, nor for extracted (or unextracted) kidney sections of normal adult Swiss-Webster mice or healthy young NZB/Bl mice. Eluates prepared from Coombs-positive NZB/Bl red cells and shown by the indirect Coombs test to have affinity for mouse red cells did not combine with the 3 hours' extracted glomerular tufts as shown by immunofluorescence. Further work is in progress.

The glomerular tufts in glomerulonephritic NZB/Bl kidneys appeared, in comparison with the delicate pattern in the normal mouse, to contain thicker or more massive distributions of basement membrane-related antigens detectable with fluorescent rabbit antibodies to normal mouse kidney, Fig. 16. Nevertheless absorption of glomerulonephritic NZB/Bl kidney extracts with normal Swiss-Webster mouse kidney powder, the saline-insoluble immunizing antigens used in raising the rabbit antiserum and capable of absorbing all nephrotoxic and kidney-localizing properties from it, did not abolish the *in vitro* affinities shown by the mouse immunoglobulins for autologous or isologous glomerular tufts. Thus the rabbit and the mouse antibodies apparently were not directed against identical antigenic determinants.

The serum (diluted 1:8) of 3 NZB/Bl mice with severe glomerulonephritis contained immunoglobulins with *in vitro* affinity for 3 hours' extracted autologous or isologous glomerulonephritic tufts as shown by immunofluorescence. Thus circulating as well as localized antibodies were demonstrated. The immunogenic materials in glomerulonephritic kidneys may have been formed in the glomerular tufts or accumulated in them from some other source, such as the circulating plasma; they corresponded in location to polysaccharide-rich capillary basement membrane and mesangial materials.

DISCUSSION

The immunological aspects of human and experimental glomerulonephritis have been under investigation for many decades and reviewed elsewhere (14, 33, 34, 44, 51, 37). Glomerulonephritis has been experimentally induced by hypersensitivity (36, 46, 17, 40, 16, 12) and autoimmunity (22, 48) and occurs also as a sequela of a viral disease (31). Our studies, extending those of Helyer and Howie (18, 19), Holmes and Burnet (24), and Aarons (1) have shown that focal and diffuse membranous and chronic lobular glomerulonephritis,

with remarkable similarity to a spectrum of human nephrotic renal diseases, occur spontaneously in NZB/Bl mice and that autoantibodies with anti-kidney specificities are localized in the glomerular lesions and demonstrable in the serum. The underlying pathogenic mechanism is almost certainly autoimmunity; induction or acceleration of the disease by transfer of antibodies or immunocompetent cells and its amelioration by contrary measures should provide further proof.

The mechanisms of autoimmunity are, broadly speaking, separable into two categories (33, 10, 39); altered (loss of) immunological tolerance, and altered antigen. When several autoantibodies are detected in an individual or an inbred strain such as the NZB/Bl mice and these antibodies show reactivity not only with self components but with presumably unaltered antigens of normal individuals, then a loss of immunological tolerance to self seems to be the simpler hypothesis. Presumably, heritable or acquired departures from the normal have occurred in one or more sublines of the family of immunocompetent cells. The NZB/Bl inbred strain of mice, developed by Marianne Bielschowsky (8) is a unique and model system of spontaneous autoimmune hemolytic anemia and apparently of autoimmune glomerulonephritis, both of which show many similarities to the corresponding human disease. The outbred F_1 hybrids uniformly develop lupus factors and lupus nephritis (18, 19). The thymus gland has an influence, possibly a major one, on these autoimmune manifestations (20, 24).

The immunogenic materials in the NZB/Bl glomerulonephritic kidneys may have been formed in the glomerular tufts during the course of the disease or may have accumulated in them from some other source, such as circulating immune complexes in plasma (16, 12). They corresponded in location to polysaccharide-rich (protein) materials of glomerular capillary basement membrane and mesangium but were not identical with the basement membrane antigens which raise nephrotoxic antibodies (29, 4, 30, 23, 38) on injection in foreign species of animals. While it has been suggested in other work that the human red cell and glomerulus share a common antigen (34, 35), there was in the present study no evidence that red cells and glomerular tufts of NZB/Bl mice shared common (cross-reacting) autoantigens. Rask-Nielsen (45) observed Coombs positive hemolytic anemia, hypergammaglobulinemia, and splenic, renal, and hepatic amyloidosis in mice receiving inoculations of subcellular leukemic material, and possibly infected with mycoplasma (PPLO). Oliner, Schwartz, and Dameshek (43) studied runt disease (graft against host reaction) in mice; Coombs positive hemolytic anemia, abnormalities of serum electrophoretic patterns, and other manifestations of autoimmunity occurred in these mice.

The present study is the first recorded in which the spleen has been identified directly at the cellular level as the main site of formation of autoantibodies to

red cells; the spleen was also the main site of red cell destruction. One serum was fractionated by density gradient ultracentrifugation; the "warm" autoantibodies to red cells were present in heavier fractions containing γ_M-globulins. More studies are in progress. The usual finding in human autoimmune hemolytic disease is that warm autoantibodies are γ_G-globulins and "cold" autoantibodies are γ_M-globulins (15).

SUMMARY AND CONCLUSIONS

This study, based upon 528 laboratory examinations and 16 complete autopsies of NZB/Bl mice, deals with autoimmune manifestations (as shown by hypergammaglobulinemia, Coombs positive hemolytic anemia, and the occasional presence of lupus- and rheumatoid-like factors) and mainly with the pathology and the pathogenesis of glomerulonephritis in these mice, a model system of membranous glomerulonephritis with spontaneous and insidious onset, progression through chronic stages, and almost certainly induced by immunological, and autoimmune, mechanisms. The earliest and lasting histological change was hyaline thickening of the capillary walls and adjacent intercapillary regions of the glomerular tufts, corresponding in location to polysaccharide-rich capillary basement membrane and mesangial materials. Distributed focally and diffusely in the glomerular tuft and eventually sparing no glomerulus, hyaline, granular, and fibrillar ("spongy fiber") materials produced narrowing of capillary lumens by concentric or eccentric encroachment upon them. In the later stages hyaline lobulation and sclerosis of the glomerular tufts occurred. Thus the lesions corresponded to those seen in human focal and diffuse membranous, chronic lobular, and lastly (intracapillary) sclerosing glomerulonephritis.

In all instances of glomerulonephritis the glomerular tufts contained selective localizations of mouse immunoglobulins corresponding in distribution to that of the hyaline and (PAS-positive) polysaccharide-rich materials in the focal and diffuse membranous and lobular lesions and in amounts increasing with the severity of glomerular disease. The mouse immunoglobulins were extracted from frozen sections of glomerulonephritic kidneys and were then capable of recombination with glomerular tufts in sections of autologous or isologous glomerulonephritic kidneys from which *in vivo* localized immunoglobulins had been extracted. The pattern of recombination with glomerular tufts was similar to that of *in vivo* localized immunoglobulins. The extracted immunoglobulins did not show affinity for mouse red cells (in the indirect Coombs test) nor for autologous or isologous cell nuclei (in the immunofluorescence test). The serum of mice with severe glomerulonephritis contained immunoglobulins with *in vitro* affinity for extracted autologous or isologous glomerular tufts. Thus circulating as well as localized antibodies were demonstrated. The immunogenic materials (autoantigens) may have been formed

in the glomerular tufts or accumulated in them from some other source, such as the circulating plasma; however they corresponded in location to polysaccharide-rich capillary basement membrane and mesangial materials.

The spleen was identified at the cellular level as the main site of formation of autoantibodies to red cells, as well as the main site of red cell destruction. Some evidence was brought forth suggesting that these autoantibodies were "heavy" or γ_M-globulins. More studies are in progress.

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BIBLIOGRAPHY

1. Aarons, I., Renal immunofluorescence in NZB/NZW mice, *Nature*, 1964, **203,** 1080.
2. Abbot, A., and Burnet, F. M., Electron microscopy of the thymus in NZB mice, *J. Path. and Bact.*, 1964, **88,** 243.
3. Allen, A. C., The Kidney. Medical and Surgical Diseases, New York, Grune and Stratton, 2nd edition, 1962, 151–323.
4. Baxter, J. H., and Goodman, H. C., Nephrotoxic serum nephritis in rats. I. Distribution and specificity of the antigen responsible for the production of nephrotoxic antibodies, *J. Exp. Med.*, 1956, **104,** 467.
5. Bell, E. T., Renal Diseases, Philadelphia, Lea and Febiger, 1946, 141–253.
6. Bencosme, S. A., and Bergman, B. J., The ultrastructure of human and experimental glomerular lesions, *Internat. Rev. Exp. Path.*, 1962, **1,** 139.
7. Bielschowsky, Marianne, and Bielschowsky, F., Reaction of the reticular tissue of mice with autoimmune haemolytic anaemia to 2-aminofluorene, *Nature,* 1962, **194,** 692.
8. Bielschowsky, Marianne, Helyer, B. J., and Howie, J. B., Spontaneous haemolytic anaemia in mice of the NZB/Bl strain, *Proc. Univ. Otago Med. School*, 1959, **37,** 9.
9. Burnet, F. M., and Holmes, Margaret C., Thymic changes in the mouse strain NZB in relation to the autoimmune state, *J. Path. and Bact.*, 1964, **88,** 229.
10. Dameshek, W., Schwartz, R., and Oliner, H., Current concepts of autoimmunization: an interpretive review, *Blood*, 1961, **17,** 775.
11. Deutsch, H. F., and Morton, J. I., Dissociation of human serum macroglobulin, *Science*, 1957, **125,** 600.
12. Dixon, F. J., Feldman, J. D., and Vazquez, J. J., Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis, *J. Exp. Med.*, 1961, **113,** 899.

13. Freedman, P., and Markowitz, A. S., Isolation of antibody-like gamma-globulin from lupus glomeruli, *Brit. Med. J.*, 1962, **1,** 1175.
14. Freedman, P., and Peters, J. H., Immunologic aspects of renal disease, *New England J. Med.*, 1959, **261,** 1275.
15. Fudenberg, H. H., and Kunkel, H. G., Physical properties of the red cell agglutinins in acquired hemolytic anemia, *J. Exp. Med.*, 1957, **106,** 689.
16. Germuth, Jr., F. G., A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type, *J. Exp. Med.*, 1953, **92,** 257.
17. Hawn, C. V. Z., and Janeway, C. A., Histological and serological sequences in experimental hypersensitivity, *J. Exp. Med.*, 1947, **85,** 571.
18. Helyer, B. J., and Howie, J. B., Positive lupus erythematosus tests in a cross-bred strain of mice NZB/Bl-NZY/Bl, *Proc. Univ. Otago Med. School*, 1961, **39,** 17.
19. Helyer, B. J., and Howie, J. B., Renal disease associated with positive lupus erythematosus tests in a cross-bred strain of mice, *Nature*, 1963, **197,** 197.
20. Helyer, B. J., and Howie, J. B., The thymus and autoimmune disease, *Lancet*, 1963, **2,** 1026.
21. Helyer, B. J., and Howie, J. B., Spontaneous autoimmune disease in NZB/Bl mice, *Brit. J. Haematol.*, 1963, **9,** 119.
22. Heymann, W., Jackel, D. B., Harwood, S., Wilson, S. G. F., and Hunter, J. L. P., Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 660.
23. Hill, A. G. S., and Cruickshank, B., A study of antigenic components of kidney tissue, *Brit. J. Exp. Path.*, 1953, **34,** 27.
24. Holmes, Margaret C., and Burnet, F. M., The natural history of autoimmune disease in NZB mice. A comparison with the pattern of human autoimmune manifestations, *Ann. Int. Med.*, 1963, **59,** 265.
25. Holmes, Margaret C., and Burnet, F. M., The influence of splenectomy in NZB mice, *Australian J. Exp. Biol. and Med. Sc.*, 1963, **41,** 449.
26. Holmes, Margaret C., Gorrie, J., and Burnet, F. M., Transmission by splenic cells of an autoimmune disease occurring spontaneously in mice, *Lancet*, 1961, **2,** 638.
27. Huang, C. Y., and Mellors, R. C., to be published.
28. Jones, D. B., Nephrotic glomerulonephritis, *Am. J. Path.*, 1957, **33,** 313.
29. Krakower, C. A., and Greenspon, S. A., Localization of nephrotoxic antigens within the isolated renal glomerulus, *Arch. Path.*, 1951, **51,** 629.
30. Krakower, C. A., and Greenspon, S. A., The localization of the "nephrotoxic" antigen(s) in extraglomerular tissues. Observations including a measure of its concentration in certain locales, *Arch. Path.*, 1958, **66,** 364.
31. Leader, R. W., Lower animals, spontaneous disease, and man, *Arch. Path.*, 1964, **78,** 390.
32. Long, G., Holmes, Margaret C., and Burnet, F. M., Autoantibodies produced against mouse erythrocytes in NZB mice, *Australian J. Exp. Biol. and Med. Sc.*, 1963, **41,** 315.

33. Mackay, I. R., and Burnet, F. M., Autoimmune Diseases, Springfield, Illinois, Charles C. Thomas, 1963, 182–196.
34. Markowitz, A. S., Interactions of anti-glomerular basement membrane antisera, *Immunology*, 1960, **3,** 117.
35. Markowitz, A. S., Common antigen between human red cell and glomerulus, *Nature*, 1962, **196,** 487.
36. Masugi, M., Über das Wesen der spezifischen Veränderungen der Niere und der Leber durch das Nephrotoxin bzw. das Hepatotoxin. Zugleich ein Beitrag zur Pathogenese der Glomerulonephritis und der elkamptoschen Lebererkrankung, *Beitr. path. Anat. u. allg. Path.*, 1933, **91,** 82.
37. Mellors, R. C., Glomerulonephritis in man and experimental animals. Immunologic mechanisms of renal injury, in press.
38. Mellors, R. C., Siegel, M., and Pressman, D., Analytical pathology. Histochemical demonstration of antibody localization in tissues with special reference to the antigenic components of kidney and lung, *Lab. Inv.*, 1955, **4,** 69.
39. Milgrom, F., and Witebsky, E., Autoantibodies and autoimmune disease, *J. Am. Med. Assn.*, 1962, **181,** 706.
40. More, R. H., and Waugh, D., Diffuse glomerulonephritis produced in rabbits by massive injections of bovine serum gamma globulin, *J. Exp. Med.*, 1949, **89,** 541.
41. Movat, H. Z., Steiner, J. W., and Huhn, D., The fine structure of the glomerulus in acute glomerulonephritis, *Lab. Inv.*, 1962, **11,** 117.
42. Norins, L. C., and Holmes, Margaret C., Antinuclear factor in mice, *J. Immunol.*, 1964, **93,** 148.
43. Oliner, H., Schwartz, R., and Dameshek, W., Studies in experimental autoimmune disorders. I. Clinical and laboratory features of autoimmunization (runt disease) in the mouse, *Blood*, 1961, **17,** 20.
44. Peters, J. H., and Freedman, P., Immunologic aspects of renal disease, *New England J. Med.*, 1959, **261,** 1166, 1225.
45. Rask-Nielsen, R., Coombs-positive hemolytic anemia and generalized amyloidosis in mice following transmission of subcellular leukemic material, *Proc. Soc. Exp. Biol. and Med.*, 1964, **116,** 1154.
46. Rich, A. R., and Gregory, J. E., The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity, *Bull. Johns Hopkins Hosp.*, 1943, **72,** 65.
47. Shulman, L. E., Gumpel, J. M., D'Angelo, W. A., Souhami, R. L., Stevens, M. B., Townes, A. S., and Masi, A. T., Antinuclear factor in inbred strains of mice: the possible role of environmental influence. 11th Interim Scientific Session of the American Rheumatism Association, Washington, D.C., 1964.
48. Steblay, R. W., Glomerulonephritis induced in sheep by injections of heterologous glomerular basement membrane and Freund's complete adjuvant, *J. Exp. Med.*, 1962, **116,** 253.
49. Steiner, J. W., Movat, H. Z., Yendt, E. R., and Slater, R. J., Studies on chronic glomerulonephritis. II. A concept of the morphogenesis of the disease based on light and electron microscopic observations, *Canad. Med. Assn. J.*, 1962, **86,** 195.

50. Steiner, J. W., Slater, R. J., and Movat, H. Z., Studies on lipoid nephrosis in children and adolescents. I. The fine structural changes in "pure" nephrosis. *Lab. Inv.*, 1961, **10,** 763.
51. Vorlaender, K. O., Klinische immunologie der nierewerkrankungen, *in* Immunopathologie in Klinik und Forschung, (P. Miescher, and K. O. Vorlaender, editors), Stuttgart, Georg Thieme 1961, 379–421.
52. Weiner, W., Eluting red-cell antibodies: a method and its application, *Brit. J. Haematol.*, 1957, **3,** 276.

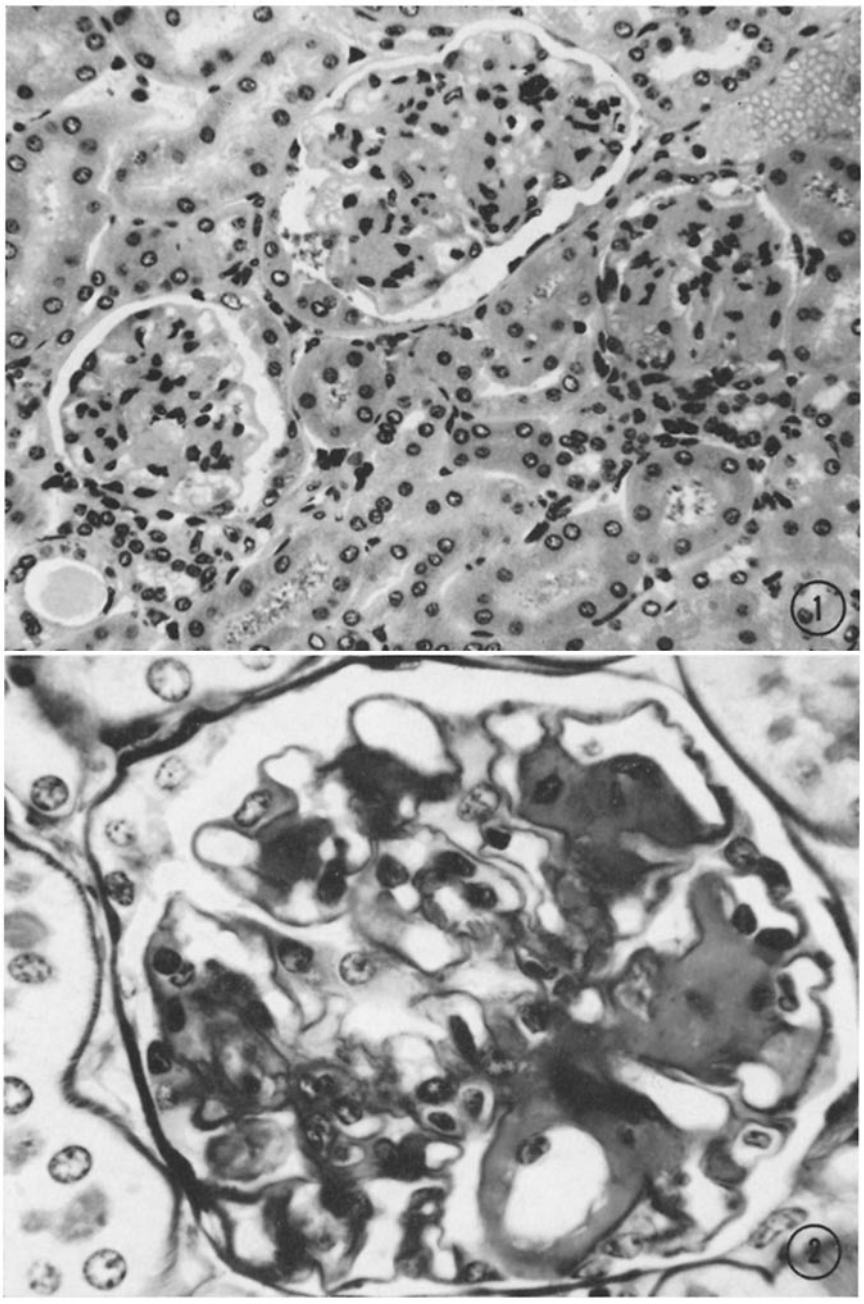
EXPLANATION OF PLATES

FIGS. 1 to 19. Figs. 1 to 8 are photomicrographs of stained paraffin sections; Figs. 9 to 18 are immunofluorescence photomicrographs of frozen sections; Fig. 19 is an electron micrograph. All figures illustrate NZB/Bl glomerulonephritic kidneys from 2 males and 2 females at 7 to 12 months of age; each animal was Coombs positive, antinuclear factor negative.

PLATE 4

FIG. 1. Diffuse membranous glomerulonephritis; a tubular protein cast is present at lower left although proteinuria was only one plus. Hematoxylin-eosin. × 384.

FIG. 2. Focal membranous glomerular lesion (at 6 o'clock) resembling "wire-loop" lesion of lupus nephritis. Periodic acid-Schiff reaction. × 960.

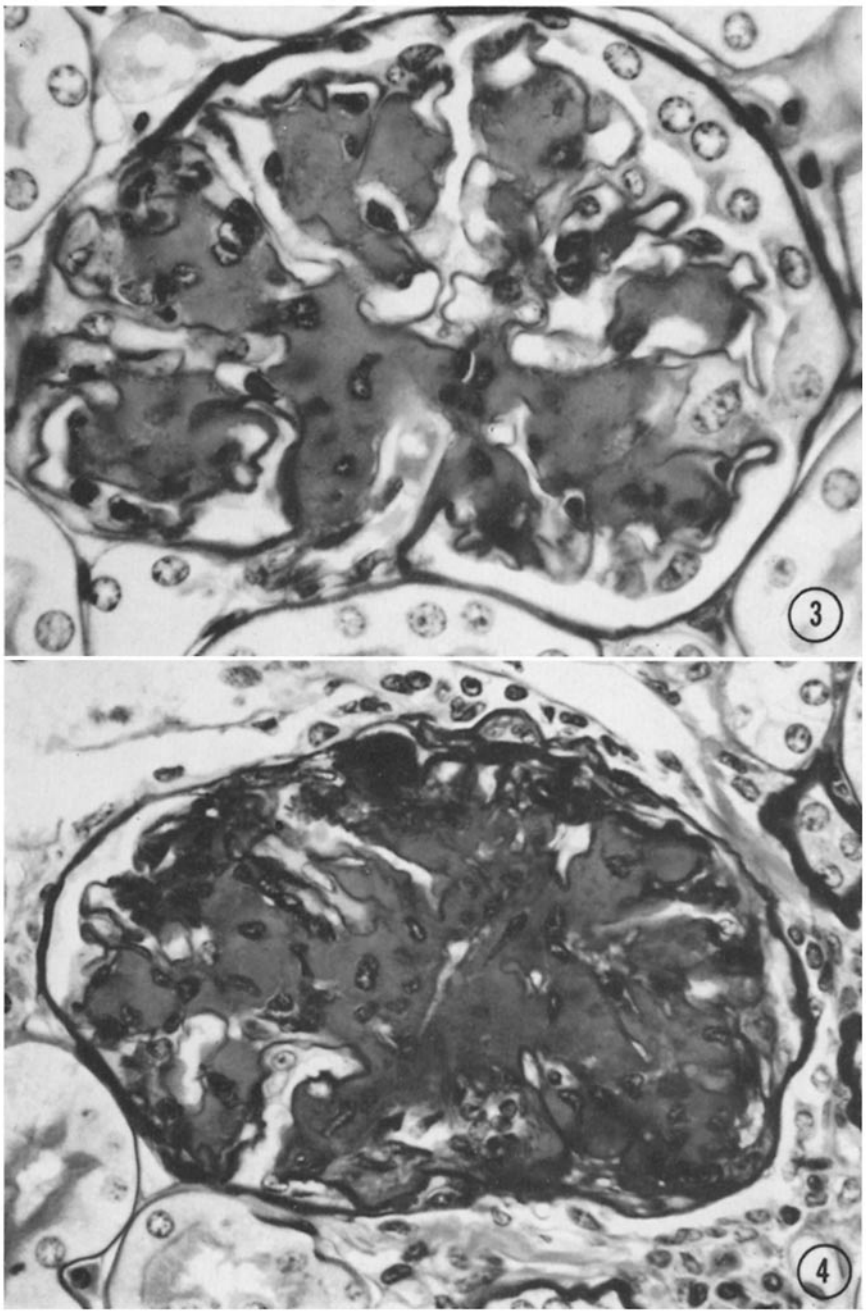


(Mellors: Autoimmune disease in NZB/Bl mice. I)

PLATE 5

FIG. 3. Membranous and lobular glomerular lesion, with thickening of wall of afferent arteriole (at 6 o'clock). Periodic acid-Schiff reaction. × 960.

FIG. 4. Hyaline sclerosis of glomerular tuft; a few capillaries with narrow lumens remain. Periodic acid-Schiff reaction. × 672.



(Mellors: Autoimmune disease in NZB/Bl mice. I)

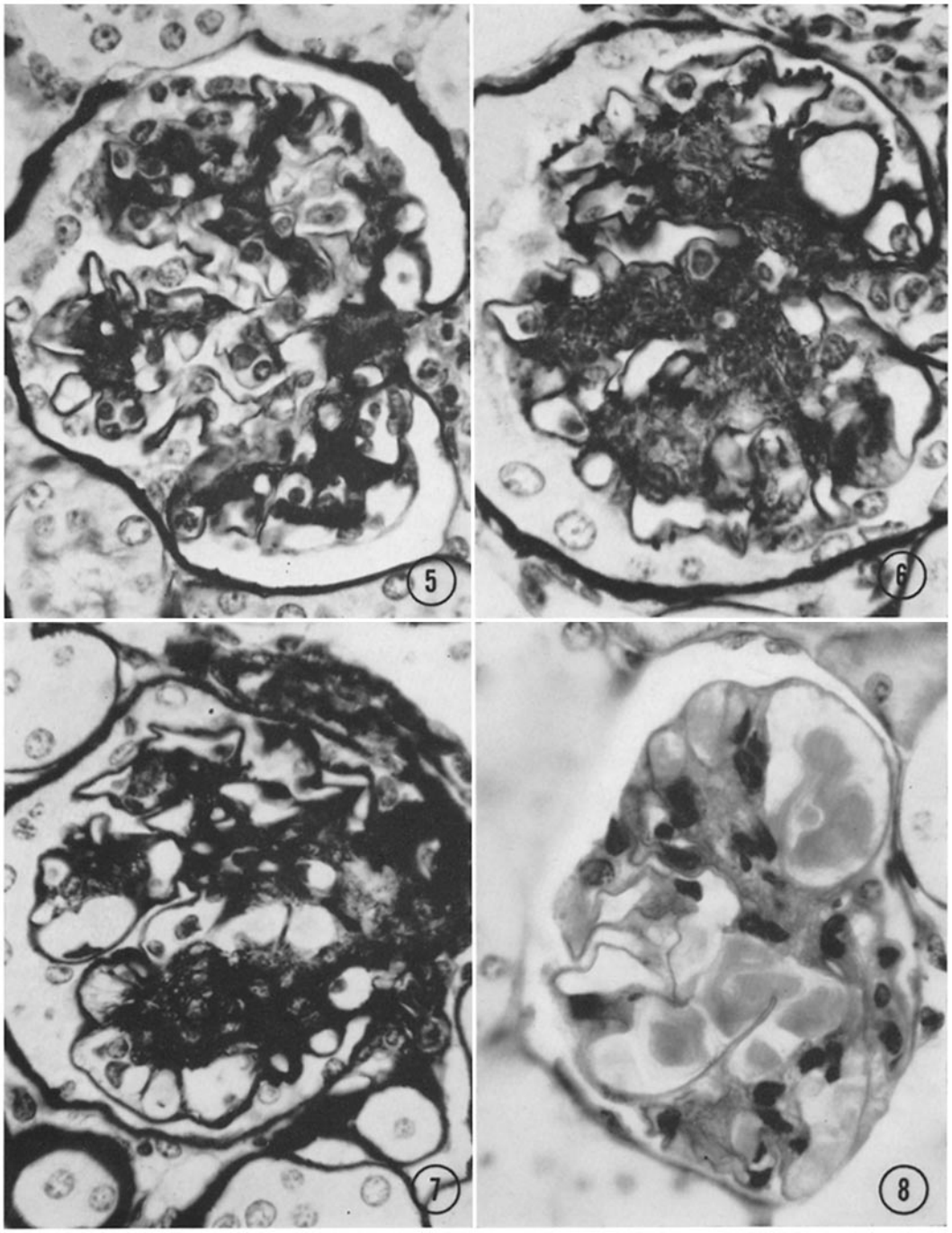
PLATE 6

FIG. 5. Early membranous and mesangial (intercapillary) lesion. Periodic acid-methenamine sliver. × 800.

FIG. 6. Membranous thickening of capillary walls with prominent subepithelial protrusions (at 1 and 2 o'clock) and accumulation of "spongy fibers" in mesangial (axial) region of tuft. Periodic acid-methenamine silver. × 1000.

FIG. 7. Membranous and sclerosing mesangial lesion. Periodic acid-methenamine silver. × 800.

FIG. 8. Laminated, PAS-positive, hyaline thrombi, probably platelet thrombi, in lumens of dilated capillaries of glomerular tuft, in glomerulonephritic animal but resembling renal lesion of thrombotic thrombocytopenic purpura. Periodic acid-Schiff reaction. × 1000.



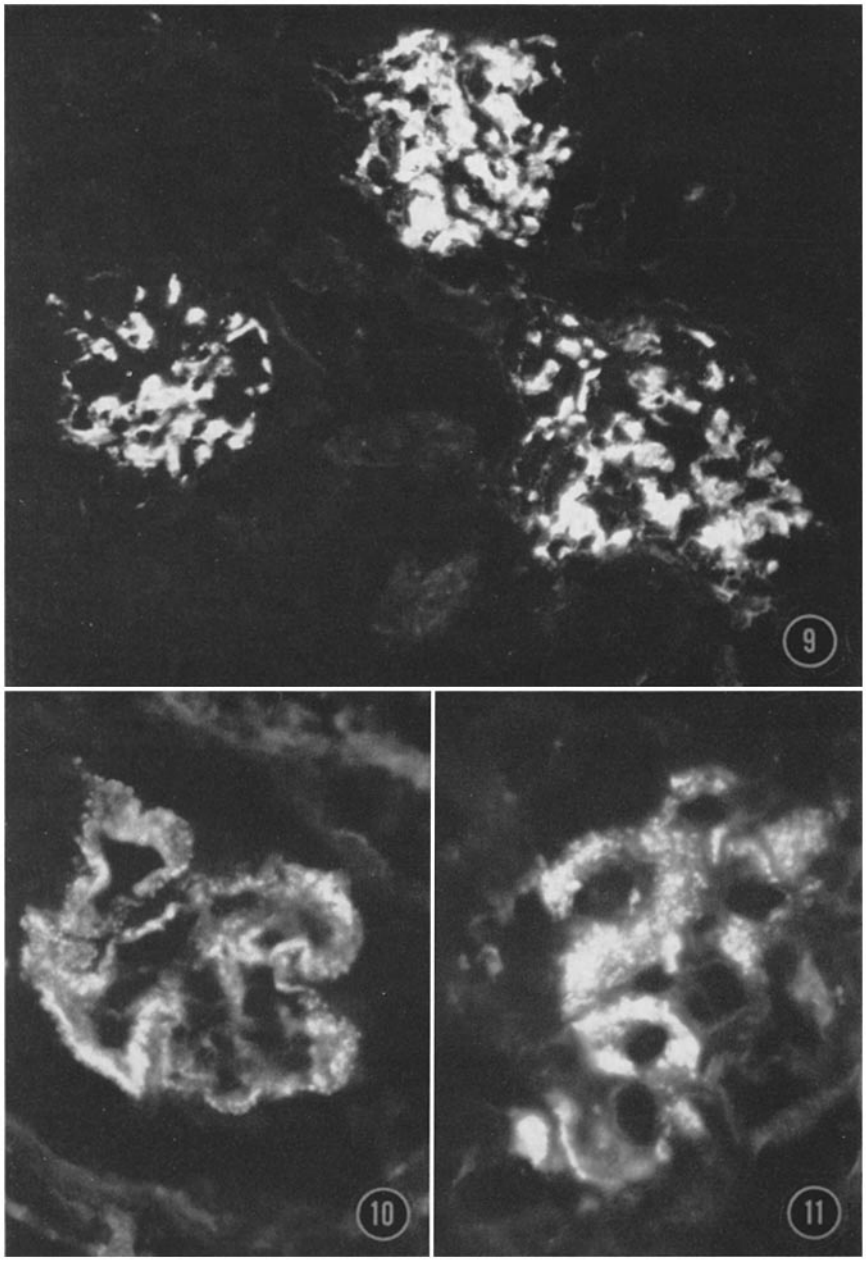
(Mellors: Autoimmune disease in NZB/Bl mice. I)

PLATE 7

FIG. 9. Localization of mouse immunoglobulins in 3 glomerular tufts, representative of all, in glomerulonephritis of NZB/B1 mice. Fluorescent rabbit antibodies to mouse immunoglobulins (immunofluorescence). × 318.

FIG. 10. Membranous localization of mouse immunoglobulins in capillary walls of lobule of glomerular tuft. Immunofluorescence. × 850.

FIG. 11. Focal localization of mouse immunoglobulins in intercapillary (mesangial) region of glomerular tuft. Immunofluorescence. × 1300.



(Mellors: Autoimmune disease in NZB/Bl mice. I)

PLATE 8

FIG. 12. Diffuse localization of mouse immunoglobulins with mesangial or lobular distribution in glomerular tuft. Immunofluorescence. × 630.

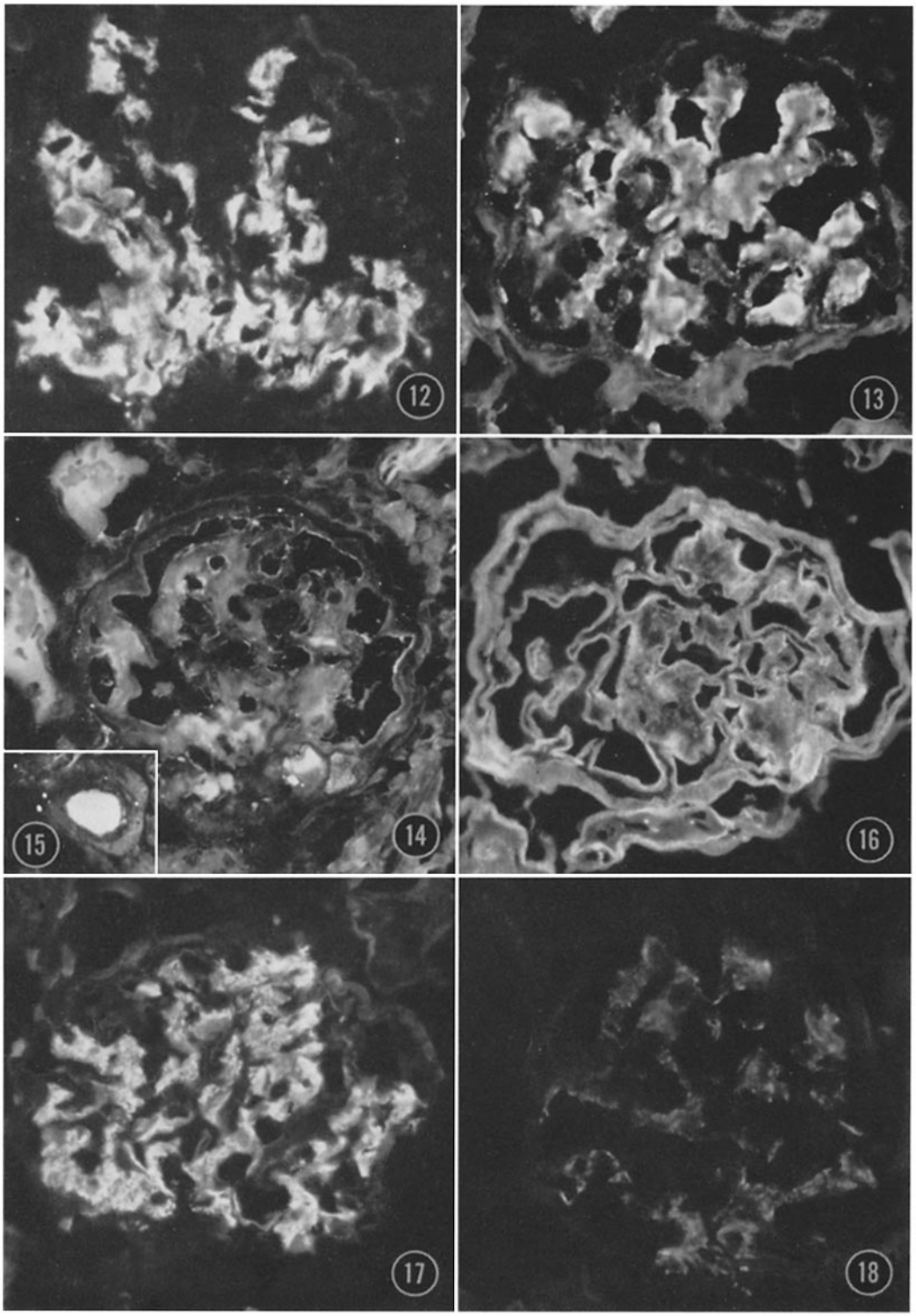
FIG. 13. Diffuse localization of mouse immunoglobulins with continuous membranous and lobular distribution in glomerular tuft. Immunofluorescence. × 400.

FIGS. 14 and 15. Mouse serum albumin is localized in the glomerulonephritic tuft in no more than trace amounts, Fig. 14, but is present in a tubular protein cast, Fig. 15. Fluorescent rabbit antibodies to mouse serum albumin. × 400.

FIG. 16. Glomerular tuft, capsular, and tubular basement antigens. Fluorescent rabbit antibodies to mouse kidney. × 500.

FIG. 17. Recombination with glomerular tufts of autologous immunoglobulins extracted at pH 3.4 from glomerulonephritic kidneys. Fluorescent rabbit antibodies to mouse immunoglobulins. × 600.

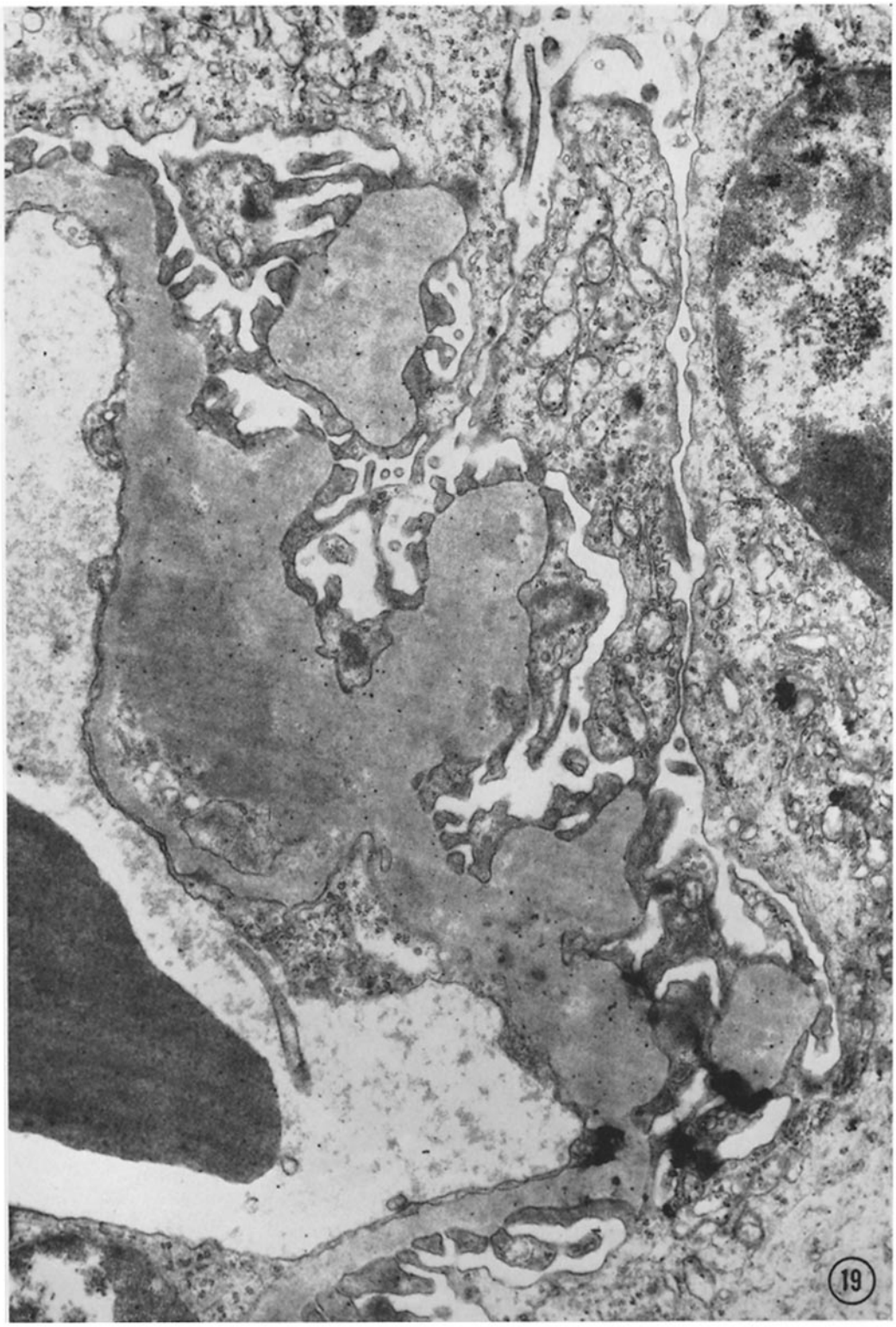
FIG. 18. Only traces of mouse immunoglobulins remain in glomerular tuft after 3 hours' extraction at pH 3.4. A control for Fig. 17, identical in all respects except not exposed to autologous immunoglobulins extracted from glomerulonephritic kidney. Fluorescent antibodies to mouse immunoglobulins. × 600.



(Mellors: Autoimmune disease in NZB/Bl mice. I)

PLATE 9

FIG. 19. The glomerular membranous lesion consists of homogeneous swelling of the capillary basement membrane (along the diagonal from upper left to lower right) with obliteration of laminae rarae and accumulations in subendothelial (towards the left) and subepithelial (towards the right) spaces. The foot processes of the epithelial cell (toward the right) and the urinary spaces appear relatively normal. Electron micrograph. × 15,000.



(Mellors: Autoimmune disease in NZB/Bl mice. I)