

IMMUNOLOGIC STUDIES OF AUTOIMMUNE DISEASE IN
NZB/NZW F₁ MICE

I. BINDING OF FLUORESCIN-LABELED ANTINUCLEOSIDE ANTIBODIES IN LESIONS
OF LUPUS-LIKE NEPHRITIS*

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The nephritis which occurs in human systemic lupus erythematosus (SLE)¹ has a natural counterpart in the renal lesions that arise spontaneously in a strain of mice, the F₁ generation derived from crossing NZB (black) with NZW (white) inbred stock. The NZB mice were developed by Dr. Bielschowsky (1) from an outbred colony of mixed coats. These animals were found to show serologic and pathologic evidence of autoimmune hemolytic anemia, as evidenced by the appearance of a positive Coombs test, at about 6 months of age. In addition, LE cells often could be demonstrated and nuclear antibodies were present in about one-third of older mice. Some animals had severe glomerulonephritis (2).

The NZB mice were crossed with a number of other inbred lines. The F₁ hybrids could be separated into two categories: (a) one in which autoimmune hemolytic anemia was the dominant disease, and (b) another in which a severe fatal lupus-like nephritis developed. The F₁ generation, resulting from a cross of NZB with NZW mice developed renal disease within the period of 4-6 months (3). Histologic examination of kidneys of diseased mice revealed a membranous type of glomerulonephritis suggestive of lupus nephritis of man (4). Studies with fluorescein-labeled antibody to mouse globulin showed the presence of host globulin in the glomerular capillary walls (GCW) dispersed as a finely granular deposit (5-8), similar to that seen in the human disease. Fluorescein-labeled serum from human lupus, containing antibody reactive with DNA, was bound in the GCW of such animals. Antibody reactive with DNA could be eluted from the glomeruli of these animals (9, 10). The association of a lupus-like nephritis with other stigmata of SLE has established the NZB/NZW F₁ mouse as the animal of choice for the study of spontaneous glomerulonephritis resembling that seen in SLE of man.

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¹ Abbreviations: C, Cytidine-BSA conjugate; T, 5 methyluridine-BSA conjugate; BSA, bovine serum albumin; DNA, deoxyribonucleic acid; GCW, glomerular capillary walls; SLE, systemic lupus erythematosus.

It is the purpose of this paper to present further data on the localization of wholly or partially denatured DNA and host globulin in GCW of NZB/NZW F₁ mice during the course of their lupus-like nephritis. Preliminary reports of this work have been made by Andres (11) and Beiser, et al. (12). Fluorescein-labeled antibodies to mouse globulin and antibodies specific for the pyrimidine bases of DNA, thymine and cytosine, are used to search for the presence of host globulin and denatured DNA, respectively, in the glomeruli. Unlike the DNA-reactive antibodies in sera from patients with SLE, which were used by Lambert and Dixon and other workers to test for DNA in glomeruli, the antibodies specific for the pyrimidines, thymine and cytosine, do not react with native DNA (13-15).

Materials and Methods

Animals.—NZB and NZW breeding mice were purchased from the Laboratory Animal Centre, Medical Research Council Laboratories, Carshalton, Surrey, England. The two lines were maintained with sister-brother matings for the past 3 years. The F₁ generation was raised, using either NZB or NZW females, since it has been reported that transmission of autoimmune characteristics in the hybrids is not sex linked (3).

Repeated determinations of urinary protein were made in all animals, using albustix.² Selected mice were bled from the orbital sinus, at intervals, under light ether anesthesia. The blood was allowed to clot at room temperature and the serum, separated in a refrigerated centrifuge, was tested for complement, DNA, antibody to DNA, and nuclear binding antibody.

Serum Complement.—Serum complement was measured in fresh, unstored sera by a technique similar to that described by Cinader et al. (16). Sensitized sheep erythrocytes (4×10^7 cells in a volume of 0.2 ml) were added to dilutions of mouse serum in 1.0 ml of Veronal buffer, pH 7.4, containing CaCl₂, 1.5×10^{-4} M, and MgSO₄, 5×10^{-4} M. After incubation at 37°C for 60 min, unlysed cells were washed with 4 ml of cold buffer, lysed in 3 ml of water, and the optical density was determined at 412 μ m. Titers were expressed as 50% units, using the von Krogh table for calculations.

Antibodies to Denatured DNA.—Antibodies to DNA were measured in a selected group of sera by two procedures: (a) the micro complement fixation technique of Wasserman and Levine (17), and (b) gel diffusion analysis in Veronal-glycine gel at pH 7.5, employing wells 5 mm in diameter 2.5 mm apart. Heat-denatured calf thymus DNA (100°C for 10 min followed by rapid chilling) was used as antigen for C' fixation tests in concentrations of 0.1-2.08 μ g, and in gel diffusion studies at concentrations of 0.5 to 1.0 mg per ml. The results were read after 48 to 72 hr reaction at room temperature.

Free DNA.—Sera studied in gel diffusion were also tested for "free DNA" by using the serum of a patient with SLE which possessed precipitating anti-DNA activity.

Nuclear Binding Antibody.—Selected sera were tested for the presence of nuclear binding antibody at a dilution of 1:4. Human auricular appendages were quick-frozen immediately following commissuromy. Sections 4 μ thick were cut in a cryostat, incubated with the diluted mouse sera for 30 min at room temperature, washed for 10 min with phosphate-buffered saline, pH 7.2, and stained with fluorescein-labeled antibody to mouse globulin (see preparation procedures below).

Pathologic Examination.—The 107 mice used in this study were killed at monthly intervals

² Ames Co., Inc., Division of Miles Laboratories, Inc., Elkhart, Ind.

or when they were apparently moribund. An occasional mouse was autopsied shortly after death. NZB mice were killed at ages ranging from 5–19 months, and the NZW and F₁ animals at ages ranging from 2–15 months and 2–12 months, respectively. Terminal urines were obtained in most instances and selected animals were bled for serologic testing. At autopsy, kidneys and spleens were weighed and pieces fixed in Zenker's solution or buffered formalin for histologic examination by light microscopy. Other pieces were quick-frozen for immunofluorescence studies. Small fragments of kidney cortex were fixed for 1 hr in 2% osmium tetroxide, buffered at pH 7.2. After dehydration in graded acetone, the tissues were embedded in Araldite, sectioned with an LKB ultratome and studied with the aid of a Siemens Elmiskop I electron microscope.

Procedures for the Preparation of Antisera, Fluorescein Labeling, and Staining of Tissues.—Antiserum to mouse serum gamma G globulin (Cohn fraction II) was prepared by immunizing rabbits with the antigen in complete Freund's adjuvant given intracutaneously once a week for 4 wk. Immuno-electrophoretic analysis of the antiserum showed only a trace reaction with mouse serum proteins other than IgG. The preparation of the conjugates, C and T, and the schedule of immunization of rabbits with these antigens have been described (13–15).

The anti-T and anti-C sera were absorbed with BSA, when necessary, and reacted only with the homologous antigen, no cross reaction being detectable. The sera also react with denatured but not with native DNA. Controls for the fluorescein-staining experiments were prepared by absorbing samples of the fluorescein-tagged antisera with T, C, and denatured DNA. Three successive absorptions were performed with T and C, using 5 μ g N antigen per 0.5 ml serum, and with denatured DNA,³ using 40 μ g of denatured calf thymus per ml serum. After each antigen addition, the mixture was placed in a 37°C water bath for 1 hr, then refrigerated overnight. After centrifugation, the procedure was repeated. The supernatant fluids after the third addition of antigen were such as to limit the total dilution factor for the three absorptions to less than 20%. Antiserum to BSA, labeled with fluorescein, was used as an additional control.

The technique for the labeling of antibody with fluorescein, and the application of the reactant to frozen sections, have been described (18). Unlabeled antibody was applied first to some of these tissues in order to test for blocking the subsequent reaction with the labeled antibody. Before any newly conjugated fluorescent antibody was included for routine use, its optimal dilution was determined by testing on a series of sections of a tissue known to contain the specific antigen.

RESULTS

Serologic and Pathologic Studies.—All mice used in this study were weighed periodically to determine the onset of edema and were checked for urinary protein. The NZB mice reached their maximum weight of 45–55 g in about 6 months. However, this was found not to be associated with edema but was due to large fat deposits, particularly in the peritoneal cavity. During the first 7–8 months, the urine was either free of protein or contained less than 300 mg/100 ml. In later months, one-third of the NZB animals had proteinuria to the extent of 300 to 1000 mg/100 ml. The NZW mice, when fully grown, weighed from 35 to 45 g. Protein in the urine was either not detectable or present in

³ DNA was denatured by placing a solution (800 μ g/ml) containing 1% formaldehyde in a boiling water bath for ten minutes, followed by chilling quickly in an ice bath.

amounts of 30 to 300 mg/100 ml, except in 2 animals which showed values between 300 and 1000 mg/100 ml.

The findings in the NZB/NZW F₁ hybrids presented a different picture. At the end of 4 months they usually weighed between 30 and 40 g. During the 5th or 6th month, most of these mice rapidly gained weight, largely as a result of edema associated with ascites and occasionally with hydrothorax. Proteinuria in most animals reached 1000 mg/100 ml or more in the 6th to 7th month. The edema usually abated before death. Most of the F₁ mice, unless killed at prescribed intervals, were exsanguinated when approaching terminal renal failure. Only 2 females and 10 males lived 9 to 12 months.

Sera from interim or terminal bleeding in selected animals were tested for nuclear-binding antibody, for complement, and, in a few instances, for DNA and

TABLE I
Weights of Spleens and Kidneys in Adult NZB, NZW, and NZB/NZW F₁ Mice

	NZB		NZW		NZB/NZW F ₁	
	Spleen	Kidneys	Spleen	Kidneys	Spleen	Kidneys
No. animals	16	16	24	24	49	56
Mean wt, mg	530	580	115	535	163	625
Range, mg	150-3174	359-686	38-348	342-652	50-720	392-880

antibody to denatured DNA. Sera from 9 of 17 NZB mice, 10-15 months of age, were positive for nuclear binding when tested against human cardiac tissue. Using the same serum dilution, nuclear-binding activity was demonstrable in the sera of only 2 of 15 NZW mice, both 14 months of age. In contrast, 17 of 18 NZB/NZW F₁ mice, 6-9 months old, had nuclear-binding antibody.

Serum complement titrations of NZB mice showed less than five C'H50 units reflecting a congenital lack of a complement component designated MuBl (16). Complement values in NZW mice varied from 16 to 44 units, the females having the lower quantities. NZB/NZW F₁ females, even when young, showed low complement levels, rarely exceeding 15 units. Of 14 F₁ males tested for complement levels, the mean for 8 animals, 3 months or younger, was 36 units, the range being 26-43. The mean for 6 animals, 8 months or older, was 12 units with a range from less than 5 to 23.

Large spleens were usually found in NZB mice at autopsy (Table I). Four animals died from internal hemorrhages. NZW mice rarely presented gross pathology. Two 15 month old females from the imported breeding stock had spleens weighing 284 and 348 mg, respectively, which, as may be seen from Table I, are heavier than the mean. Edema was the outstanding gross pathology seen in NZB/NZW F₁ mice 6 months of age or older, at times with ascites and hydrothorax and large, occasionally finely granular kidneys. Large spleens were seen in 4 of these animals.

Lesions have not been seen in the kidneys of NZB and NZW mice at ages up to 9 months. Microscopic examination of renal tissues from F₁ animals, 6 to 12 months of age, showed cellular proliferation in the glomeruli with thickening of capillary walls. The lesions tended to vary in intensity from glomerulus to

TABLE II
Localization in Glomeruli of NZB, NZW, and NZB/NZW F₁ Mice of Mouse IgG, T, and C

Mice	No. and sex	Age	No. of mice binding fluoresceinated antibody to			Total no. F and M with T and/or C in GCW
			IgG	T	C	
		<i>mos.</i>				
NZB	9F	5-12	6/9	0/9	0/9	0/14
	5M		2/5	0/5	0/5	
	2F	14-19	2/2	2/2	1/2	2/5
	3M		3/3	0/3	0/3	
Total.....						2/19
NZW	12F	2-18	9/12	0/12	0/12	0/27
	15M		11/15	0/15	0/15	
Total.....						0/27
NZB/NZW F ₁	2F	2-5	1/2	0/2	0/2	0/5
	3M		1/3	0/3	0/3	
	2F	5-6	2/2	0/2	0/2	1/5
	3M		3/3	1/3	0/3	
	17F	6-7	17/17	7/17	11/17	15/20
	3M		3/3	2/3	2/3	
	13F	7-8	13/13	6/13	9/13	17/19
	6M		6/6	5/6	4/6	
	2F	8-12	2/2	2/2	2/2	8/12
	10M		10/10	4/10	3/10	
Total.....						41/61

glomerulus and even within different loops of a single glomerulus, but all glomeruli became involved to some extent. Figs. 1 and 2 show the hematoxylin and eosin staining of a renal section from mouse 186, a male 199 days old. This mouse was found to have circulating antibody to DNA. Fig. 1 illustrates an area in which are visible: a severe interstitial infiltration of mononuclear cells, two glomeruli with membranous changes, and an artery with a thickened wall. In Fig. 2, a glomerulus is shown at higher magnification. The GCW are thick-

ened by the presence of membranous deposits. Infiltrating mononuclear cells are localized in the interstitial tissue and around Bowman's capsule. Fig. 3 is an electron micrograph of a glomerulus from mouse 348, an F₁ female, 190 days of age. Here the typical lesions of lupus-like membranous nephritis are seen with numerous subendothelial and subepithelial deposits in the GCW.

Immunofluorescence Studies.—Summarized in Table II are the results in which frozen sections of kidneys from NZB, NZW, and NZB/NZW F₁ mice, 2–18 months of age, were tested with fluorescein-labeled antibodies to mouse IgG, T, and C. It may be seen that the majority of GCW of older mice in each group showed the presence of more globulin than normally seen. Excess host globulin was present in all F₁ mice 5 months of age or older. 2 of 19 NZB mice, both over 14 months of age, bound anti-T, and one of these also bound anti-C, in their GCW, indicating the presence of denatured DNA in this area. None of the 27 NZW mice (16 of which were over 6 months of age) were capable of binding anti-T or anti-C. In contrast, renal tissues of 40 of the 51 NZB/NZW F₁ mice, over 6 months of age, showed binding of anti-T or anti-C or both in GCW, thus indicating the presence of partially or wholly denatured DNA in the glomeruli of about 80% of these hybrids. Antiserum to BSA was bound in relatively small amount in half of all the animals. It was seen as often in the glomeruli of mice that did not bind anti-T and anti-C as in those which did. In the latter cases, antinucleoside activity was considered negative unless the staining was markedly brighter than that found with anti-BSA. Fig. 4 illustrates the binding of fluorescein-labeled antibody to mouse globulin in two glomeruli of mouse 186. The variation in distribution of the excess mouse globulin both between the two glomeruli and within the loops of the lower glomerulus on the left is apparent. In some areas, patchy distribution along the basement membrane is seen. Figs. 5 and 6 illustrate the staining with anti-T of renal sections from mouse 186 and 348, respectively. Variations in distribution similar to that seen in Fig. 4 are apparent. Fig. 7 shows another glomerulus from mouse 186 stained with anti-C.

Blocking tests were carried out by incubating tissues with specific, unlabeled antiserum prior to the application of the fluorescein-labeled antibody. The staining reaction was blocked completely.

Absorption of the fluorescein-labeled anti-T and anti-C sera with the respective homologous antigen, T and C, reduced the brightness of the binding of the fluorescent antibody by 75% or more. In order to evaluate the extent and intensity of immunofluorescent staining, serial photomicrography on 35 mm film was employed. Each section was photographed under identical conditions on the same roll of film and contact prints were made from the same strip simultaneously. Figs. 8 and 9 illustrate absorption of the specific antibody from the anti-T serum. The same glomerulus from each of two renal sections of an F₁ male mouse 338, 272 days of age, is shown: in Fig. 8, the glomerulus was brightly stained with the unabsorbed anti-T, whereas, in Fig. 9, the glomerulus,

which was treated with the absorbed anti-T, showed only slight staining. Similarly, Figs. 10 and 11 are the contact prints of two sections of the same mouse but stained with the unabsorbed and absorbed anti-C, respectively. Here again much of the binding power of the anti-C serum has been removed.

Further studies on the specificity of the anti-T and anti-C sera were carried out by cross absorption of anti-T with C and anti-C with T. The ability of denatured DNA to absorb the antibody from both anti-T and anti-C sera also was tested. It was found that staining with anti-T was not diminished by absorption with C nor was that of anti-C by absorption with T. Staining by both antisera was markedly affected by absorption with denatured DNA. Contiguous sections from a kidney of a 261 day old male F_1 mouse 491 are illustrated in Figs. 12-15. The sections have been stained with fluorescein-labeled anti-C (Fig. 12), and with the same antiserum absorbed with C (Fig. 13), absorbed with T (Fig. 14), and absorbed with denatured DNA (Fig. 15). The specificity of the reaction is readily apparent by the demonstration that absorption with C and denatured DNA removed essentially all of the staining ability, whereas the serum absorbed with T gave a reaction similar to that seen with the unabsorbed anti-C. Similar results were obtained with anti-T; T and denatured DNA absorbed the fluorescein-staining activity, while C was ineffective.

DISCUSSION

The NZB/NZW F_1 mice used in these studies represent a generation of animals which develop lupus-like nephritis spontaneously (3-8). Kessler (19) reported that these F_1 animals also have lesions of the salivary and lacrimal glands which simulate those found in Sjögren's syndrome, a disease of man often associated with some of the stigmata of SLE. Recent studies on the pathogenesis of the mouse disease have indicated that DNA (9, 10) and/or denatured DNA (11, 12) are present in the renal lesions together with mouse globulin. This is compatible with the hypothesis that DNA and/or denatured DNA complexed with specific mouse antibody is present in the GCW, and is responsible for initiating the pathology seen in the kidney. It is noteworthy that these lesions, as shown by electron microscopy and immunofluorescent studies, are similar to the lesions seen in the Dixon model of experimental membranous nephritis caused by circulating antigen-antibody complexes (20, 21).

Lambert and Dixon (9), in their studies of NZB/W F_1 mice, reported that fluorescein-labeled antibody reactive with DNA, obtained from a patient with SLE, was bound in the glomeruli of these hybrids if the tissue sections were first treated with 2 M NaCl or citrate buffer, pH 3.2, in order to partially elute the mouse globulin in the GCW. In the experiments reported here, however, satisfactory binding of the anti-T and anti-C sera was obtained without prior elution of globulin. Koffler, Schur, and Kunkel (22) found binding of fluorescein-labeled antibody from human SLE in the glomeruli of kidneys from human

lupus nephritis patients when sections of the renal tissue were first eluted with citrate buffer, pH 3.2, or 2 M NaCl, thus showing the presence of DNA in the glomeruli. As will be reported elsewhere, we have found that anti-T and anti-C sera bind to the glomeruli in lupus nephritis patients and that elution with citrate buffer or saline can increase and sharpen this staining.

The additional information reported in this paper shows that antibodies reactive with the pyrimidine bases, thymine and cytosine, which do not react with native DNA or with nuclei except at time of replication of DNA (14), are bound in large amounts in GCW of F₁ mice with lupus-like renal lesions. This indicates that the DNA localized in the glomeruli is at least partially or perhaps largely denatured. With reference to similar studies using serum from patients with SLE, it should be noted that many sera from such patients contain antibodies reactive with denatured DNA or with both native and denatured DNA. Few sera contain antibodies that react only with native DNA (23).

The question not answered, but currently under investigation, is whether antibody eluted from the glomeruli of the F₁ mice reacts with T and C. The report of Lambert and Dixon (9) states that the eluate they obtained from mouse kidneys had a high titer of nuclear-binding antibody. It is known that anti-T and anti-C will not bind to nuclei except at the time of replication of the DNA. The question of why DNA might elicit antibody to itself is, of course, basic to the understanding of the pathogenesis of those autoimmune diseases in which antibody to DNA develops. It would appear that the determination of the precise specificity of the antibody eluted from the mouse glomeruli would give valuable information in this regard, in particular with respect to the role of DNA denaturation in the initiation of the disease.

SUMMARY

For the past 3 years NZB and NZW mice have been maintained by sister-brother matings from English breeder stock. NZB/NZW F₁ hybrids developed lupus-like nephritis during the 6th to 7th month and few survived beyond the 8th month. Renal tissues of these animals were examined with fluorescein-labeled antinucleoside sera, specific for thymine and cytosine, for the presence of denatured DNA in GCW, and with labeled antibody to mouse IgG for the presence of excess host globulin in the same areas. The following results have been obtained:

(a) All 51 hybrids, over 5 months of age, had an excess of mouse globulin in GCW. 40 animals between the ages of 5 and 12 months showed, in the same areas, antigens which bound one or both of the antinucleoside antibodies.

(b) Renal tissues of 19 NZB mice, 5-19 months old, and 27 NZW mice, 2-18 months old, were examined. Excess host globulin was seen in GCW of 13 NZB and 20 NZW animals. The tissues of only two old NZB mice, 14 months of age, bound antinucleoside antibody but none of the other animals did.

The association of rapidly fatal lupus-like nephritis in NZB/NZW F₁ mice with denatured DNA and mouse globulin in GCW supports the hypothesis involving this antigen-antibody complex in the pathogenesis of the disease.

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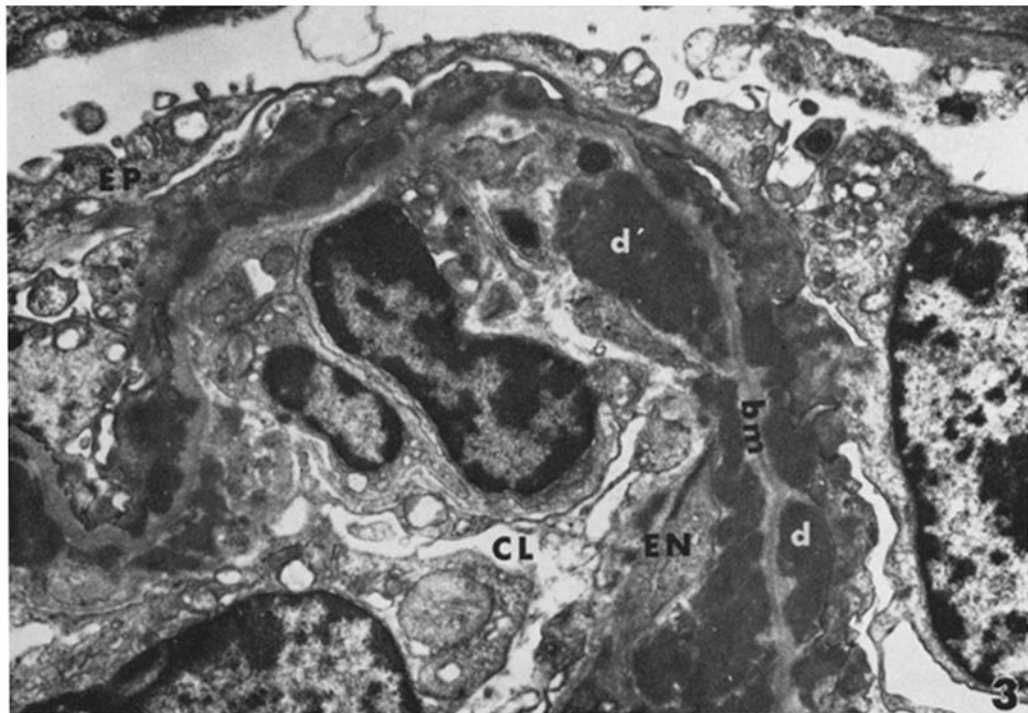
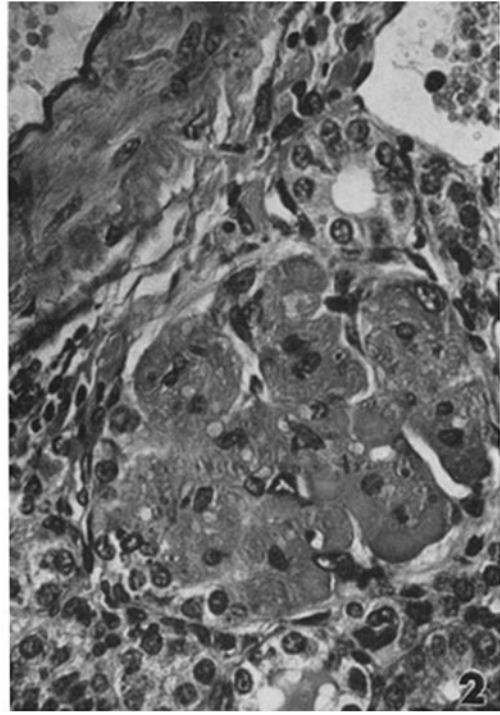
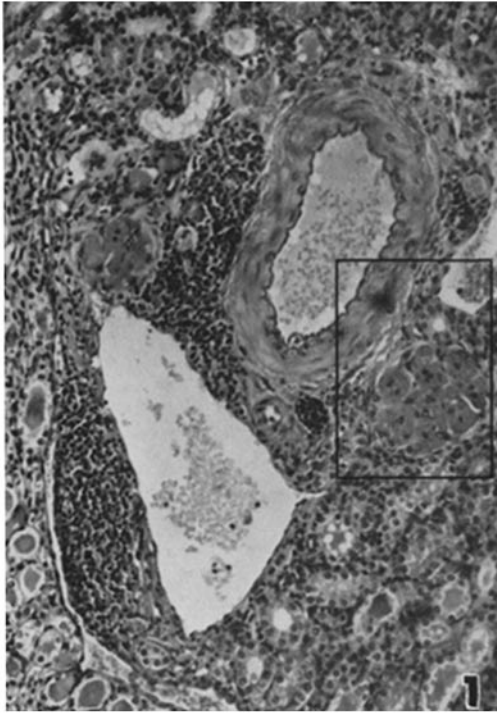
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FIG. 1. Mouse 186. The picture shows a renal section in which a severe infiltration of mononuclear cells is visible in the interstitium. Two glomeruli with membranous changes and an artery with a thickened wall are also included in this section. Hematoxylin and eosin staining. $\times 250$.

FIG. 2. Higher magnification of the boxed area in Fig. 1. The picture shows severe membranous lesions in the glomerular capillary walls. Mononuclear cells are present in the interstitium. The wall of the artery is thickened. Hematoxylin and eosin staining. $\times 600$.

FIG. 3. Electron micrograph showing part of a glomerular capillary wall of mouse 348. Electron opaque deposits are present on both sides of the basement membrane (bm) in subendothelial (d') and subepithelial (d) positions. EP, epithelium; CL, capillary lumen. Lead hydroxide staining. $\times 8000$.



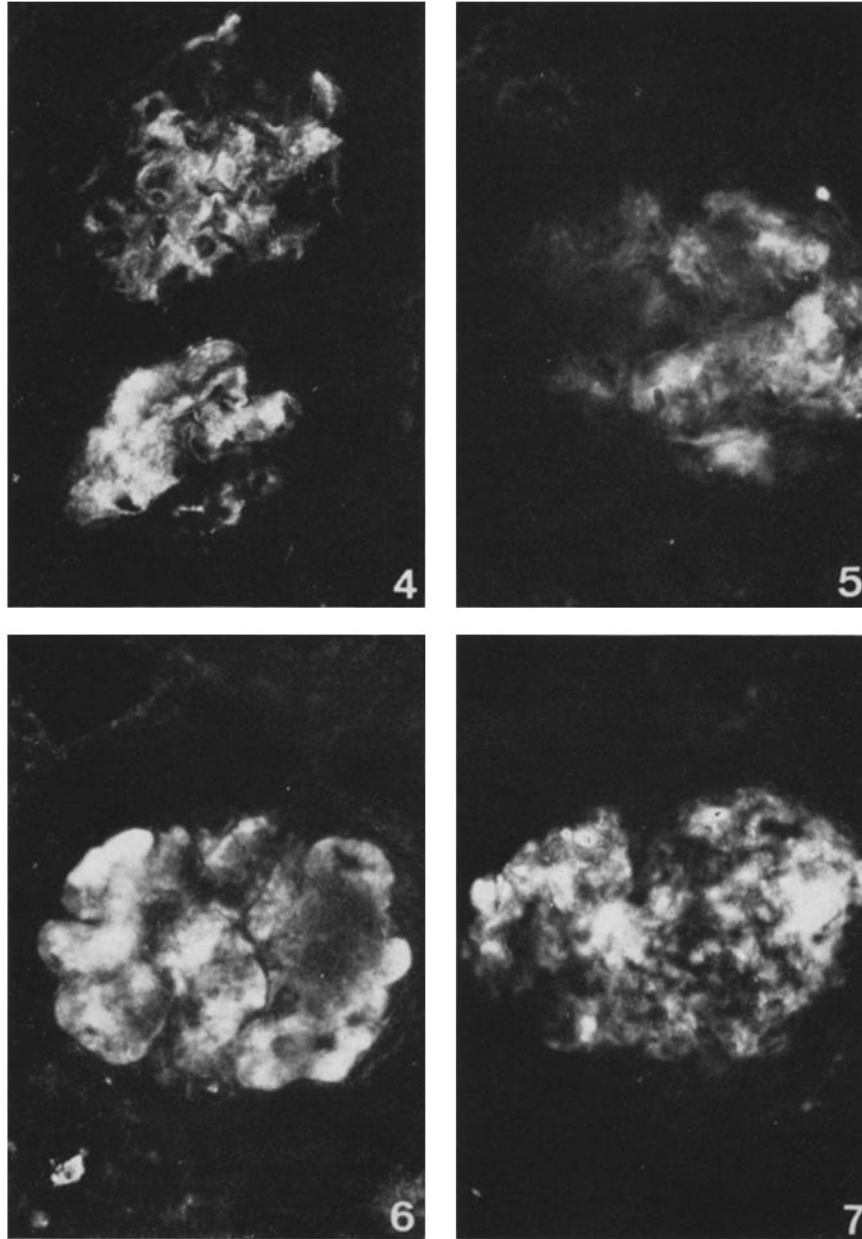
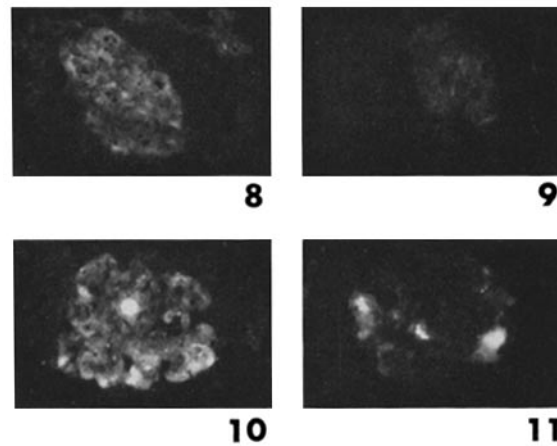


FIG. 4. Renal section of mouse 186 stained with fluorescein-labeled antibody to mouse globulin. Two glomeruli show localization in the glomerular capillary walls (GCW). $\times 300$.

FIG. 5. Renal section of mouse 186 stained with fluorescein-labeled antibody specific for thymine (A-TFL), which is localized in the glomeruli. $\times 300$.

FIG. 6. Renal section of mouse 348 stained with A-TFL. The pattern of fluorescent staining is similar to disposition of the membranous deposits seen in Fig. 2. $\times 300$.

FIG. 7. Renal section of mouse 348 stained with fluorescein-labeled antibody specific for cytosine (A-CFL). Similar localization is seen as in Fig. 5. $\times 300$.



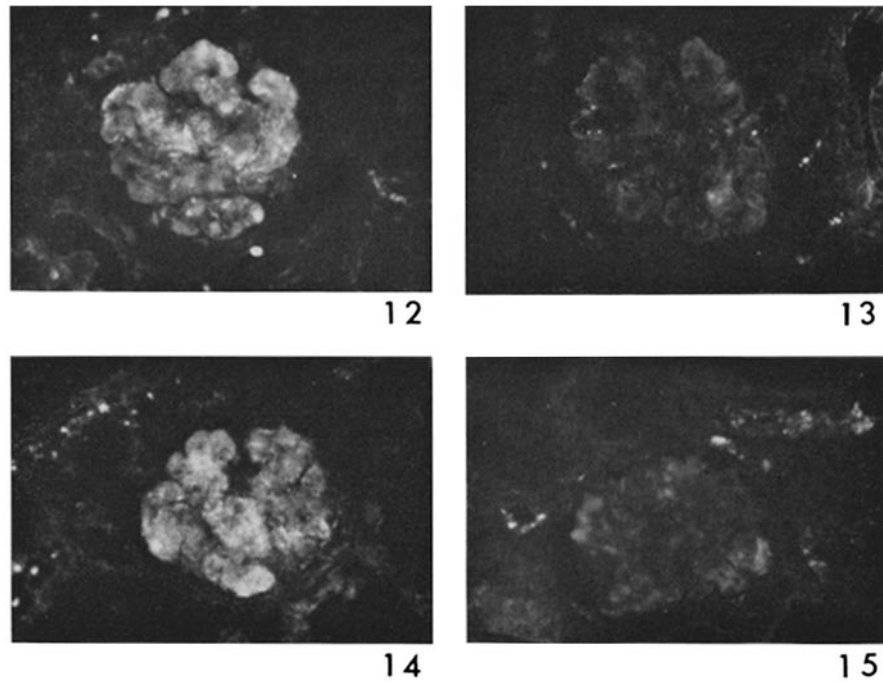
FIGS. 8-11 are contact prints made from 35 mm negative films taken with identical exposures. Intensity of fluorescence can therefore be compared directly.

FIG. 8. Contact print of a glomerulus from a renal section of mouse 338 stained with A-TFL showing glomerular localization. $\times 100$.

FIG. 9. Contact print made under identical condition as in Fig. 8 of the same glomerulus of mouse 338 "stained" with A-TFL previously absorbed with the specific antigen. The staining is barely visible. $\times 100$.

FIG. 10. Contact print of a glomerulus from another renal section of mouse 338 stained with A-CFL. Glomerular localization is seen. $\times 100$.

FIG. 11. Contact print of the same glomerulus as in Fig. 10 from a renal section of mouse 338 "stained" with A-CFL previously absorbed with the specific antigen. The contact print shows very little staining in the GCW. $\times 100$.



FIGS. 12, 13, 14, and 15. Contiguous renal sections of mouse 491, stained with fluorescein-labeled anti-C unabsorbed or absorbed with different antigens. $\times 180$. In Fig. 12, the antibody to C is bound in the GCW. Fig. 13 illustrates the result of staining with anti-C absorbed with C. The intensity of fluorescence is greatly decreased. Fig. 14 shows the results of staining with anti-C absorbed with T. The fluorescence is not diminished. In Fig. 15, the section was stained with anti-C absorbed with denatured DNA. The ability for staining the tissue by this antibody is almost completely removed.