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Basement Membrane Specific Antisera Produced to Solubilized Tissue Fractions

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Summary. Attempts were made to produce antisera to solubilized tissue fractions rich in basement membranes and reticulin. Murine tissue fractions solubilized with sodium hydroxide elicited precipitating antibodies upon injection into rabbits. Although no nephrotoxic effect was observed upon injecting the rabbit antisera into mice, the antisera were fixed to the glomerular basement membrane, and not elsewhere, within 5 minutes of injection and remained fixed for at least 3 weeks.

Specificity studies suggested that in addition to unique antigens, reticulin and epithelial basement membranes share a common antigen which is responsible for the similar *in vitro* immunofluorescence produced by antisera to tissue fractions rich in one or the other of these components.

INTRODUCTION

Since Lindemann in 1900 first demonstrated that heterologous antiserum to kidney could elicit glomerulonephritis upon injection into an animal of the species from which the kidney was obtained, 'nephrotoxic nephritis' has been used as an experimental model by investigators concerned with human glomerulonephritis. The major source of the nephrotoxic antigens was shortly localized to the cortex of the kidney (Pearce, 1903) then to the glomerulus (Solomon, Gardella, Fanger, Dethier and Ferrebee, 1949; Greenspon and Krakower, 1950) and finally to the glomerular capillary basement membranes (Krakower and Greenspon, 1951). Other investigators have shown that the kidney is not the sole source of nephrotoxic antigens since antisera to liver (Pearce, 1903) lung (Chikemitsu, 1940) and placenta (Seegal and Loeb, 1946) have also produced nephrotoxic nephritis.

The nephrotoxic antigens are insoluble in saline and in a variety of other solvents (Eisen and Pressman, 1950). The insolubility of the antigenic preparations in neutral media has discouraged immunological analyses of nephrotoxic antigens by many of the common immunological techniques such as gel diffusion and hemagglutination. Thus the only effective criterion by which the early investigators could study these antigens was their ability to elicit the production of nephrotoxic nephritis or inhibit the pathological consequences of the injection of nephrotoxic antisera.

Many attempts have been made to circumvent the difficulties inherent in working with insoluble antigens. Steblay and Lepper (1961) were able to show a correlation between nephrotoxicity and complement fixation titres of their nephrotoxic antisera. Although complement fixation is one of the few *in vitro* methods applicable to the study of insoluble

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antigens, it has not been extensively used in the study of nephrotoxic antigens. Others have attempted to render the antigenic material soluble by enzymatic digestion. Goodman and Baxter (1956) were able to isolate a soluble factor from rat kidney by tryptic digestion that would neutralize antisera although the tryptic digests were incapable of inducing the formation of nephrotoxic antibodies upon injection into rabbits. Milazzo (1957) also found nephrotoxin-neutralizing activity in both the soluble and insoluble fractions of tryptic digests of kidney. Further treatment of the insoluble fraction with pepsin or papain destroyed the activity.

Although much of the original interest in this field was confined to nephrotoxicity, the development of immunofluorescent techniques made it possible for investigators to study the reactions between nephrotoxic antisera and antigens in any tissue and it became apparent that nephrotoxic antisera would react with both basement membranes and reticulin in all tissues if tested *in vitro*. Cruickshank and Hill (1953) used nephrotoxic antisera prepared against whole rat kidney, isolated rat glomeruli and whole rat lung. They found that fluorescent conjugates of all of these antisera produced specific fluorescence of basement membranes and reticulin when applied to frozen sections of a large number of rat tissues. Shortly after, Mellors and his co-workers (Mellors, Siegal and Pressman, 1955; Ortega and Mellors, 1956) showed by immunofluorescence that although nephrotoxic antisera localize on basement membranes and reticulin in any tissue by *in vitro* techniques, the same antisera localize almost exclusively on glomerular basement membranes if given *in vivo* to rats.

A method for obtaining soluble antigenic material capable of inducing antisera with strong affinity for basement membranes was suggested to us by the demonstration of Pierce and associates that the saline-insoluble material prepared from a neoplastic basement membrane of mice and rendered soluble by treatment with sodium hydroxide gave a precipitin reaction with antiserum to the non-solubilized antigenic material (Pierce, Midgley, Sri Ram and Feldman, 1962).

We felt that antisera to soluble antigens would be valuable for several reasons provided that the antisera retained specificity for basement membranes. They would enable us to study the antigens of basement membranes and reticulin by techniques other than immunofluorescence which in the past had not been utilized due to their requirement of soluble antigens. Secondly, since the availability of soluble nephrotoxic antigens would facilitate the study of nephrotoxicity, we attempted to produce nephrotoxic antisera with basement membrane-rich tissue extracts which had been rendered soluble by treatment with sodium hydroxide.

MATERIALS AND METHODS

Antigens

Solubilized neoplastic basement membrane (*SNBM*). The neoplastic basement membrane used in these studies has been shown by Pierce and co-workers (Pierce, Midgley and Sri Ram, 1963; Pierce, Beals, Sri Ram and Midgley, 1964) to be identical to normal epithelial basement membranes histochemically, immunohistochemically and electron microscopically. It was chosen because it can easily be obtained in large amounts. The method used for preparing the antigenic material was adapted from the method of Pierce and his coworkers. Cream and white mice of the 129 J strain (Roscoe B. Jackson Laboratories, Bar Harbor, Maine) bearing parietal yolk sac carcinomas in the intraperitoneal form were obtained through the kindness of Dr G. B. Pierce (University of Michigan, Ann

Arbor). The tumours were transplanted into the abdominal cavity of forty-six male mice. Tumour was collected from the mice by weekly paracentesis until the mice died, at which time the remaining tumour was expressed by paracentesis. All specimens were stored at −20°. A suspension of the tumour in an equal volume of 8 per cent NaCl was homogenized in a Virtis high speed homogenizer and the homogenate centrifuged at 1400 ***g*** for 30 minutes. The insoluble fraction was washed five times in 8 per cent NaCl and finally in distilled water by resuspension and recentrifugation. The insoluble residue rich in neoplastic basement membrane (NBM) was dialysed against distilled water overnight and lyophilized. A small amount of 1 N NaOH was added to 50 mg of NBM. After stirring at room temperature for 5 minutes, the suspension was centrifuged. The supernatant was dialysed against 0·1 M Veronal buffer, pH 8·6 at 4° for 2 days with several changes of buffer. NaOH was added to the undissolved residue and the process of dissolution, centrifugation and dialysis repeated until all the material was in solution or until a small residue resistant to NaOH remained. The contents of the dialysis sacs were pooled and the volume was adjusted to 5 ml by the addition of Veronal buffer.

Solubilized spleen residue (*SSR*). Mouse spleens were homogenized in 8 per cent NaCl, washed, dialysed and lyophilized in the same manner as NBM to obtain the 8 per cent saline-insoluble fraction. Solubilized spleen residue (SSR) was made from the lyophilized spleen residue by treatment with 1 N NaOH, centrifugation and dialysis using the same method as described for the preparation of SNMB.

Normal mouse serum (*NMS*). Normal adult Swiss mice were bled and the serum separated by centrifugation. After dialysis against distilled water, the serum was lyophilized.

Antisera

Rabbit antiserum to solubilized neoplastic basement membrane (*anti-SNBM*). Monthly intramuscular injections of 5 mg of SNBM emulsified in 0·5 ml of complete Freund's adjuvant were given to two rabbits. The rabbits were bled from the ear vein 1 week after each injection and the sera were tested against SNBM by Ouchterlony gel-diffusion. After the fourth injection precipitating antibodies were demonstrable and the sera were pooled and used in subsequent experiments.

Rabbit antiserum to solubilized spleen residue (*anti-SSR*). The procedure followed for the preparation of anti-SSR was identical to that stated above for anti-SNBM. One of the rabbits died and the other produced precipitating antibodies after the fourth injection.

Fluorescein-conjugated goat antiserum to rabbit γ-globulin (*anti-RGG-FITC*). The γ-globulin fraction of goat antiserum to rabbit γ-globulin, labelled with fluorescein isothiocyanate, was obtained from Hyland Laboratories, Los Angeles, and the specificity confirmed by immunoelectrophoresis.

In vitro *localization of antisera*

The reaction of anti-SNBM and anti-SSR with mouse tissues was tested by the indirect fluorescent antibody technique, using the tissues of uninjected mice (see below). The test antisera were absorbed with lyophilized whole mouse serum (8 mg/ml of antiserum) to remove antibodies to mouse serum proteins. Anti-RGG-FITC was used as the labelled component in the test. Our method of performing the test has been described previously (Myers, Sargent and Cohen, 1965). Control sections for this test were prepared by: (1) substituting normal rabbit serum for the antisera, and (2) omitting the specific antisera and applying anti-RGG-FITC directly to the tissues.

In vivo *localization of antisera*

Adult Swiss mice were injected with experimental and control sera according to the following schedule. Ten mice received 0·1 ml of anti-SNBM by intravenous injection into the tail vein. Another ten mice received similar injection of anti-SSR. Two mice from each of these groups were killed after 5 minutes, 1 hour, 6 hours, 24 hours and 3 weeks. Controls consisted of four mice injected in the same manner with normal rabbit serum and two mice that were not injected. Two of the mice receiving normal rabbit serum were killed after 5 minutes, and the other two after 24 hours. The liver, kidney, spleen, lung, heart and intestines were removed for further study. A portion of each tissue was fixed in formalin and processed routinely for histologic studies. Another portion of each tissue was fixed in 95 per cent ethanol at 4° overnight, processed, embedded, sectioned and prepared for fluorescent staining according to the method of Sainte-Marie (1962). The tissues were studied for the distribution of rabbit γ-globulin by the direct fluorescent antibody technique following the procedure of Nairn (1962). The anti-RGG-FITC employed for the detection of rabbit γ-globulin was absorbed with guinea-pig liver homogenates just prior to use. In addition to the control tissues, the specificity of the reaction was checked by substituting fluorescein-conjugated goat anti-human 7S γ-globulin antiserum for the anti RGG-FITC.

Specificity studies

Gel-diffusion tests were performed by the method of Ouchterlony (Ouchterlony, 1958) in 5-cm Petri dishes containing 0·75 per cent agar in 0·1 M phosphate buffer, pH 7·4, containing 1 : 10,000 Merthiolate as a preservative. Three holes were cut in the agar plates equidistant from one another. In each plate one of the antisera (anti-SNBM, anti-SSR) was allowed to diffuse against the two antigens (SNBM and SSR).

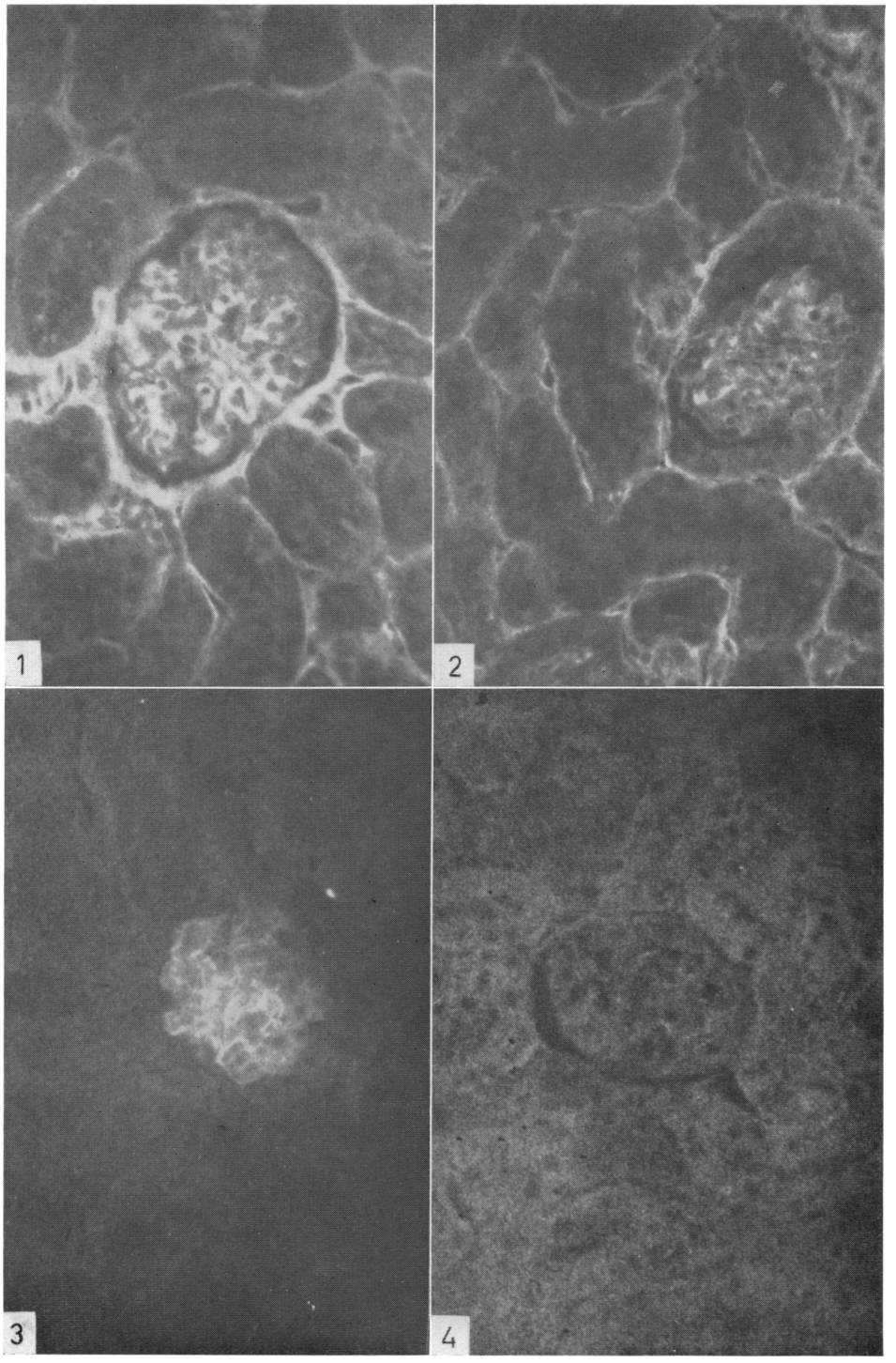
Haemagglutination–inhibition tests were used to estimate the degree of cross-reactivity between the two solubilized tissue antigen preparations. Aliquots of each antiserum were incubated with SSR, SNBM and normal mouse serum (NMS) for 30 minutes at 37° and centrifuged. The ratio of test inhibitor to antiserum was 12 mg test inhibitor per ml of undiluted antiserum in all cases. This ratio was chosen because it gave significant but not total inhibition in preliminary experiments. NMS was tested for inhibition to exclude false positive results from species specific rather than 'organ' specific antigens. The unabsorbed and the absorbed antisera were tested by the tanned cell haemagglutination technique of Boyden (1951) for their haemagglutination titres with cells sensitized with SNBM and SSR. The optimal sensitizing concentrations for the antigens (SNBM, 0·3 mg/ml; SSR, 0·05 mg/ml) were determined by trial and error. The inhibitory activity of each antigen was evaluated by its capacity to decrease the titre of the antiserum after absorption.

FIG. 1. Normal mouse kidney. Localization of anti-SNBM by indirect immunofluorescence. Specific fluorescence is present in the glomerular basement membrane, Bowman's capsule and tubular basement membranes. × 170.

FIG. 2. Normal mouse kidney. Localization of anti-SSR by indirect immunofluorescence. Pattern of staining is similar to that with anti-SNBM. × 170.

FIG. 3. Section of kidney from mouse injected 5 minutes prior to killing with anti-SSR. Specific fluorescence is confined to the glomerular basement membrane. This pattern was observed in kidney sections from all of the experimental animals. × 170.

FIG. 4. Section of kidney from mouse injected 5 minutes prior to killing with normal rabbit serum. Photograph is overexposed to show outline of glomerulus (centre) and tubules by autofluorescence. No specific fluorescence is seen. × 170.



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RESULTS

In vitro LOCALIZATION OF ANTISERA

By the indirect fluorescent antibody test anti-SNBM and anti-SSR stained basement membranes and reticulin in all mouse tissues studied (Figs. 1 and 2). This pattern appeared virtually identical to that obtained by others using nephrotoxic antisera in rats (Cruickshank and Hill, 1953) and by Midgley and Pierce (1963), using antiserum prepared against insoluble neoplastic basement membranes.

In vivo LOCALIZATION OF ANTISERA

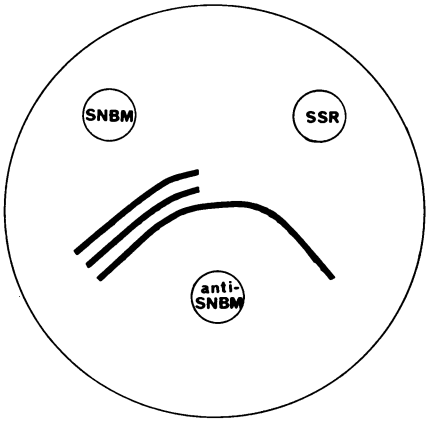
All mice receiving injections of anti-SNBM and anti-SSR showed rabbit γ-globulin localized on glomerular basement membranes (Fig. 3) and not elsewhere. There was no observable difference in the intensity or distribution of fluorescence related to the time of killing or the antiserum injected. No specific fluorescence was observed in sections stained with the antiserum to human γ-globulin and there was no fluorescence in the control tissues stained with anti-RGG-FITC (Fig. 4).

Sections of the kidneys stained with haematoxylin and eosin and with the periodic acid–Schiff stain failed to reveal evidence of nephrotoxicity. Although occasional diseased glomeruli were observed, the number of affected glomeruli and the extent of glomerular damage did not differ significantly between the experimental and control groups.

SPECIFICITY STUDIES

By gel-diffusion, three precipitin arcs developed between anti-SNBM and SNBM. A single line developed between anti-SNBM and SSR which showed a reaction of identity with one of the three lines between anti-SNBM and SNBM (Fig. 5).

A single band of identity developed between anti-SSR and both antigens. The segment of the arc between anti-SSR and SSR eventually split into two components which never separated completely (Fig. 6). The results of haemagglutination–inhibition (Table 1) also suggest partial immunologic identity between the antigens since both SSR and SNBM were capable of some cross-inhibition. In all systems, there was no significant inhibition by normal mouse serum.



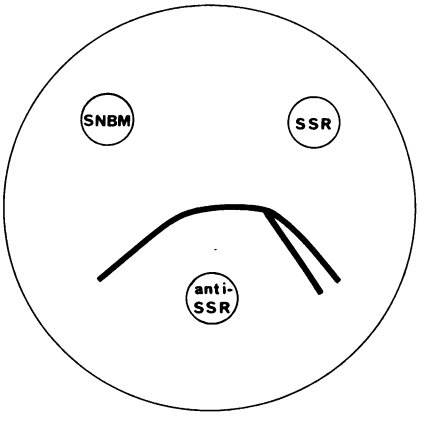


FIG. 5 (left). Gel diffusion, Ouchterlony technique, in Petri dish. Antigen preparations SSR and SNBM were reacted with anti-SNBM.

FIG. 6 (right). Gel diffusion, Ouchterlony technique, in Petri dish. Antigen preparations SSR and SNBM were reacted with anti-SSR.

TABLE 1

HAEMAGGLUTINATION–INHIBITION STUDIES

Antiserum	Material used for absorption (12 mg/ml)	Titre* of antiserum with cells sensitized with:	
		SNBM	SSR
Anti-SNEM	—	8 tubes	7 tubes
	SNBM	3 tubes	6 tubes
	SSR	6 tubes	0 tubes
	NMS	8 tubes	7 tubes
Anti-SSR	—	7 tubes	9 tubes
	SSR	1 tube	2 tubes
	SNBM	1 tube	7 tubes
	NMS	6 tubes	9 tubes

* The titre is expressed as the last of a series of two-fold dilutions of antiserum showing complete agglutination.

DISCUSSION

The *in vitro* and *in vivo* fluorescent antibody studies indicate that treating basement membrane-rich tissue fractions with sodium hydroxide in order to render them soluble does not destroy the ability of the fraction to elicit basement membrane reactive antibodies. Although it is possible that there are other basement membrane antigens which have been denatured or that a quantitative loss of antigen resulted from the treatment, antigens remaining after treatment retained the major property described by others for non-solubilized basement membrane-rich preparations—the ability to elicit antiserum which reacts with basement membrane (and reticulin) generally *in vitro* and which localizes on glomerular basement membranes *in vivo*. The antisera appear to have antibodies for common antigens in basement membranes and reticulin by the three tests employed: (1) fluorescent antibody, (2) gel-diffusion, and (3) haemagglutination–inhibition. The latter two tests gave results which suggest that reticulin-rich tissues and basement membrane-rich epithelial tissues also have unique antigens. Whether the non cross-reacting antigens are involved in the staining by fluorescent antibody cannot be determined on the basis of these experiments. However, fluorescent antibody studies which we have recently completed (unpublished) gave results similar to those of Pierce *et al.* (1964) who found incomplete inhibition of immunofluorescence when antiserum to a non-solubilized reticulin-rich tissue residue was absorbed with a homogenate of epithelial basement membrane and vice versa. The failure to obtain any significant inhibition of any of the four combinations of antiserum and sensitized red cells by normal mouse serum indicates that the reactions are 'organ' specific and do not result from common species antigens.

The failure to induce glomerulonephritis upon the injection of antiserum which localized preferentially on the glomerular basement membrane is not too surprising since others have reported similar results. Rothbard and Watson (1959) prepared rabbit antiserum to rat tail collagen which localized on glomerular basement membranes. The antiserum alone had no nephrotoxic effect when injected into rats but when Freund's adjuvant was administered with the antiserum, renal lesions developed (Rothbard and Watson, 1959, 1961). Rothbard and Watson theorized that the rabbit anti-collagen antiserum localized on the rat glomerular basement membrane and, while not toxic in itself, elicited the formation of antibodies by the rat to at least the γ-globulin portion of the

anti-collagen antiserum under the increased antigenic stimulus of the adjuvant; these antibodies then combined with the rabbit globulin on the basement membrane, causing renal disease.

Markowitz (1960) compared glomerular basement membranes, lung basement membranes and tendon fibrils for antigenicity and concluded that these tissue components shared identical or related antigens. He showed that nephrotoxic antisera, after absorption with tendon preparations, were no longer nephrotoxic, which would indicate that tendon collagen contains the nephrotoxic antigens. This finding does not support Rothbard and Watson (1959, 1961) who concluded that antibodies to collagen localized on glomerular basement membranes but were not in themselves nephrotoxic. The findings of Hassòn, Bevans and Seegal (1957) may explain this discrepancy. They found that nephritis in the rat caused by the injection of duck anti-rat kidney antiserum could be immediate or delayed in onset, depending on the potency of the antiserum. The less potent antisera may have contained insufficient antibodies to cause nephritis but induced formation of antibodies by the rat to the injected duck globulins which then complexed with the duck antibodies fixed on the basement membrane. The presence of host γ-globulin on the glomerular basement membrane, which has been postulated as a possible cause of nephrotoxicity (Rothbard and Watson, 1959, 1961; Hasson *et al.*, 1957), has been demonstrated in the lesions of nephrotoxic nephritis (Hasson *et al.*, 1957). It has also been shown that antigen–antibody complexes on the glomerular basement membrane can cause nephritis even without antibodies specific for the basement membrane (Dixon, Feldman and Vasquez, 1961).

These findings suggest that any antiserum which fixes on the glomerular basement membrane can be nephrotoxic under the proper conditions. Our mice may not have produced antibodies to the injected rabbit antiserum in sufficient titre to develop disease as a result of antigen–antibody complexes on the glomerular basement membrane; perhaps, like the rats studied by Rothbard and Watson (1959, 1961), a strong immune reaction to rabbit γ-globulin requires the additive effect of an adjuvant. A second possibility is that renal disease was evoked by the antisera but that the criterion of morphologic damage was not sensitive enough to permit recognition of the disease. Unfortunately, urine was not obtained from our mice for studies of functional damage.

We believe that antisera to the saline-insoluble basement membrane-rich organ fractions have potential applications to the study of nephrotoxicity, and that these antisera and the solubilized antigens to which they are directed will prove useful in the analysis of basement membrane antigens, essential to our understanding of glomerulonephritis and other diseases with basement membrane abnormalities.

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