A Specific Soluble Substance Involved in Nephrotoxic Nephritis.* (18827)

LEON R. COLE, WILLIAM J. CROMARTIE, AND DENNIS W. WATSON.
From the Departments of Medicine and Bacteriology, University of Minnesota, Minneapolis.

The experimental nephritis induced in rats by the injection of anti-rat kidney serum has been studied intensively since it was first described by Masugi(1). Smadel(2) obtained anti-rat kidney serum by repeated intraperitoneal injections into rabbits of suspensions of perfused rat kidney. The anti-rat kidney serum when injected intravenously into rats, produced either fulminating or chronic nephritis, depending upon the dosage. This nephrotoxic nephritis was well characterized by Smadel(2,4) and Smadel and Farr(3). Little information has been obtained, however, as to the exact nature of the kidney antigen which gives rise to nephrotoxic antibodies. Swift and Smadel(5) found that when a suspension of ground-up kidney was placed in the refrigerator for 2 days and centrifuged for one-half hour at 2,500 r.p.m. some antigen occurred in a layer of suspended particulate matter overlying a bottom layer of the more solid material. Two investigators have presented evidence that the antigen is located in the glomeruli and not in other portions of the kidney: Solomon *et al.*(6), by separating kidney into glomerular and tubular fractions, demonstrated that only the glomeruli could absorb the nephrotoxic rabbit serum globulin; Pressman *et al.*(7), in experiments that employed radioactive anti-mouse kidney serum, demonstrated localization of radioactivity in the glomeruli and showed that the anti-kidney serum is largely organ-specific. Eisen *et al.*(8) found that the kidney antigen could not be dissolved by a variety of solvents and noted its resistance to heat. Loeb *et al.*(9) produced nephritis in the rat by the injection of anti-rat placenta serum which had been developed in rabbits.

It is the purpose of this communication to report that a substance has been obtained in solution from rat kidney which will combine with the nephrotoxic antibodies and render anti-kidney serum harmless to the rat. This material has not previously been obtained in solution. Some of the chemical properties of this material have been characterized. The substance was assayed by absorption in anti-kidney serum which was then injected into rats. When no nephritis ensued, it was believed that the nephrotoxic antibody in the serum had combined with a specific soluble substance. The fact that it was obtained in a soluble form greatly increases the possibility of further characterization of the substance which is responsible for the phenomenon of nephrotoxic nephritis.

Methods and materials. I. Preparation of anti-rat kidney serum. Wistar rats weighing 200 to 300 g were sacrificed and perfused with 2 liters of 0.85% sodium chloride solution. The perfusate entered through a cannula inserted in the thoracic aorta and escaped by way of an incision in the vena cava. The kidneys were frozen and stored. In preparation for injection, kidneys were ground in a mortar under aseptic conditions and injected intraperitoneally into rabbits following the schedule of Smadel(2). Pooled rabbit serum was distributed to 2 ml ampules, frozen

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3. Smadel, J. E., and Farr, L. E., *J. Exper. Med.*, 1937, v65, 527.
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6. Solomon, D. H., Gardella, J. W., Fanger, H., Dethier, F. M., and Ferrebee, J. W., *J. Exper. Med.*, 1949, v90, 267.
7. Pressman, D., Hill, R. F., and Foote, F. W., *Science*, 1949, v109, 65.
8. Eisen, H. N., and Pressman, D., *J. Immunol.*, 1950, v64, 487.
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in a dry ice-cello-solve solution and stored at −70°C. Before use, the serum complement was destroyed by heating one half hour at 56°C and the serum was absorbed 4 times with rat red blood cells and plasma. It was found that the injection intravenously of 2 ml into a 150 g rat would cause death through renal failure in about 2 weeks. This dose was given on one day or on 2 consecutive days. The onset of nephritis was almost always within 24 hours although infrequently it was delayed until 96 hours.

II. *Methods of absorption.* 2 ml amounts of serum were used in assaying the ability of various fractions of the kidney to absorb the nephrotoxic antibody. 0.4 ml of material to be tested for this capacity was added to the serum and allowed to stand in the refrigerator from 4 to 9 hours. The serum was then centrifuged at 2,500 r.p.m. for 15 minutes and the precipitate discarded. Four or five such absorptions were done, making the total amount of material added about 1.6 ml. Almost all kidney material tested gave precipitation, regardless of the extent of removal of nephrotoxic antibody. Hence it would appear that many antibodies were present which were not harmful to the rat. When solid material was assayed for antigenic activity, 4 absorptions were also done.

III. *Examination of rats.* Wistar rats of weight 150 g to 250 g were used. The absorbed sera were injected intravenously by employing the veins of the tail or feet. Nephritis was tested for by quantitative 24 hour urine protein determinations using the Shevky-Stafford method(10), and by histological examination of the kidneys. These two methods corroborated each other consistently. Twenty-four hour urine specimens were collected in a urine-feces separator and one drop of formalin was added as a preservative. A series of normal controls established that the 24-hour output of protein ranged from 0 to 6 mg. Since an occasional larger rat excreted as much as 10 mg, a 24-hour excretion of 10 mg of protein per 24 hours was taken as the upper limit of normal. In contrast to this finding, rats with nephritis usually excreted over 100 mg of protein daily. Animals were fasted during urine collections. The rats were killed by ether anesthesia. The kidneys were fixed in 10% formalin and were stained with hematoxylin and eosin and with the Hotchkiss connective tissue stain. The nephritis produced in our rats was essentially the same as that described by Smadel(2-3). Early histological changes consisted essentially of enlarged glomerular tufts with thickened basement membranes and few erythrocytes in the glomerular capillaries. There was a slight increase in the cellularity of the tufts.

Experimental. I. *Experiments with whole kidney.* Suspension of whole kidney readily absorbed out the nephrotoxic antibodies. A suspension of half of a kidney was found to absorb the antibodies present in 2 ml of serum. Lyophilized kidney was equally as effective. Lyophilized kidney was placed in a Soxhlet apparatus and extracted with a mixture of ether-alcohol, 1 part to 3 parts, for 8 hours. The material still retained its ability to absorb out the nephrotoxic antibody from serum. Because of the results described by Loeb *et al.*(9), the capacity of rat placenta to absorb sera was tested. Two ml of serum was absorbed with a total of 4 ground-up placentas which had been obtained from rats almost at parturition. The absorbed serum failed to produce nephritis. Nine rats were protected in this manner. In contrast to these findings, 2 placentas failed to absorb the nephrotoxic antibodies from 2 ml of serum. The antigen responsible for the nephrotoxic effect thus appears to be present in the placenta, though possibly at a lesser concentration than in kidney tissue.

II. *Experiments with solvents.* The extraction of kidney with water, saline, alkaline and acid solutions, alcohol or diethylene glycol failed to bring the antigen into solution. Animals subject to test with sera which had been absorbed with these materials invariably developed nephritis. An extract of an autolysate of kidney prepared by incubation at 37°C for 36 hours did not contain the antigen.

10. Kolmer, J. A., and Boerner, F., *Approved Laboratory Technique,* Appleton-Century-Crofts, Inc., N. Y., Fourth Edition, 1945, p. 133.

TABLE I. Effect of Absorption of Nephrotoxic Serum with Specific Rat Kidney Soluble Substance upon Excretion of Urinary Protein.

	Rat No.	Source of material used for absorption	24 hr urinary protein output Days after injection				
			5	6	10	11	12
			mg	mg	mg	mg	mg
Test rats	1	Kidney		6	6	6	
	2			4		9	
	3			4	6	8	
	4			1	0	0	
	5			2		8	
	6			1	0	0	
Control rats	7	Muscle	147			Slight ascites	311
	8		61			Anasarca	151
	9		147				144
	10		116			,,	151
	11		130				173
	12		130			Ascites	151

III. *Digestion of kidney with trypsin.* These experiments led to the production of the specific soluble substance in solution and hence will be described in detail. Twelve kidneys, totaling about 12 g in weight, were ground in a mortar, made into a thick paste with 0.85% sodium chloride and buffered with 0.1 M sodium borate to pH 8. To this was added 100 mg of crystalline trypsin. The suspension was placed in a 37°C incubator and stirred frequently. Digestion by the trypsin caused the pH to drop rapidly. Hence a pH determination was performed every half hour and borate added to restore the pH to 8. Incubation was continued for 3 hours, at the end of which time little drop in pH was being obtained. The suspension was then heated in a water bath at 60°C for 30 minutes to inactivate the trypsin. Since the volume of the suspension was about 30 ml another 10 ml of 0.85% sodium chloride were added so as to fill the large cup of a Spinco Preparative Model "L" ultracentrifuge. The suspension was centrifuged at 27,000 r.p.m. for 35 minutes. This made available a mucoid, homogeneous, clear tea-colored supernatant fluid which was pipetted from the precipitate. It was found that a total of 0.75 ml of this clear supernatant would precipitate with 2 ml of serum to remove the nephrotoxic antibodies. This supernatant in 5 portions, was added at intervals of from 4 to 8 hours. At the time this report was written a total of 24 rats had been protected from nephritis by precipitating antibodies from the sera with this material before injection. Five different batches of the material were prepared by the above method and each was found to be effective.

Three types of control experiments were carried out to exclude trypsin activity as the protective agent: (1) Twenty-five mg of trypsin were dissolved in 10 ml of rat plasma and a pH 8 was maintained by the addition of a small amount of 0.1 M sodium borate. Three samples of serum were absorbed with this solution and injected into rats, all of whom developed nephritis. (2) To 2 ml of serum was added 10 mg trypsin and the serum was then absorbed 4 times of 4 hours each with rat red blood cells. This serum was nephrotoxic to a rat. (3) In the last control experiment the procedure for obtaining specific soluble substance as described above was followed except that 12 g of muscle from the thigh and abdomen were substituted for 12 g of kidney. A preparation of kidney material was made at the same time. Six sera were absorbed with the supernatant from muscle at the same time that 6 sera were absorbed with supernatant from the kidney preparation. The twelve sera were injected into rats of 250 g on the same day. The results are given in Table I. It can be seen that the trypsin-digested kidney preparation gave uniform protection to 6 rats. On the

other hand the trypsin-digested muscle preparation failed to protect; all 6 animals developed nephritis.

Some protected rats have been kept for periods of up to 3 weeks without developing proteinuria or kidney lesions by histological examination.

IV. *Further purification of the supernatant of trypsin-digested kidney.* The results of further attempts to isolate the specific soluble substance now in progress will be reported in a later communication. Suffice it to say at present that the substance is stable for several weeks at alkaline pH in the refrigerator, and that it is not sedimented at 35,000 r.p.m. for 120 minutes in the Spinco 40 preparative centrifuge.

Discussion. The studies described above suggest that the antigen involved in the production of nephrotoxic nephritis is a substance bound within the tissues of the kidney by protein molecules, and hence cannot be obtained in solution by simple extraction with solvents. Digestion with trypsin probably destroys the protein which binds the specific soluble substance herein described and permits it to escape into solution. Possibly the antigen concerned is a protein in combination with a polysaccharide or lipid and a portion of this complex molecule is resistant to trypsin because of the non-protein components. We are at present attempting to produce nephrotoxic antibody by injecting this soluble material into rabbits, and our preliminary results indicate that the material is not antigenic. If no such antibody is produced, we may assume that trypsin digestion splits off a hapten group from a larger antigenic molecule. If antibodies can be produced to this specific soluble substance the entire antigen molecule must exist in solution.

Nephrotoxic nephritis is an unusual experimental phenomenon in that one insult to the kidney can produce a chronic and progressive disease. Isolation of the specific antigen or hapten in purified form will permit more precise studies of this experimental nephritis. Certainly it must have some unique properties because only antibodies to it will produce this type of nephritis. Antibodies to the many other antigens in the rat kidney do not produce this picture. The nature of this specific substance should be of interest in the study of certain diseases such as rheumatic fever, glomerulonephritis, and periarteritis nodosa, which are thought likely to be on an allergic basis. If these human diseases are due to the action of an "autoantibody" upon tissue antigen or hapten, the antigenic substance may be of similar nature to that which produces nephrotoxic antibody when injected into rabbits. At least, it does not seem surprising that attempts in the past to find antibodies to saline extracts of the heart muscle in rheumatic fever patients and to saline extracts of kidney tissue in glomerulonephritis patients, have been fruitless.

Summary. A specific soluble substance which will combine with the nephrotoxic antibody of anti-rat kidney serum was obtained in solution. The material was liberated into solution by digesting rat kidney with trypsin at pH 8. This substance was assayed by its ability to absorb antibodies from nephrotoxic serum. It protected a total of 24 rats without failure. Control experiments excluded trypsin activity as being the protective substance. Further characterization should be possible now that this specific material has been obtained in solution.

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