Nephritogenic Antigenicity of Chromatographic Fractions of Trypsin and Pronase Glomerular Digests* (34067)

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Experimental glomerulonephritis induced in various animals by the injection of anti-kidney (AK) serum, has been studied intensively since it was first described by Masugi (5). It is well established that the nephritogenic antigen is located in the glomerulus (4). A soluble material, capable of neutralizing the anti-kidney antibodies, was isolated from tryptic digests of the renal cortex by numerous investigators (2, 3, 9). We have found evidence that the major nephritogenic antigen is sialoglycoprotein (10). The specific reactivity of sialic acid with colloidal iron provided a method of localizing the nephritogenic antigens in the cell membranes of the glomerular cells.

The present study provides additional proof that the nephritogenic antigen is sialoprotein and that degradation of the protein component leads to a loss of its neutralizing capacity. Chromatographic fractions of trypsin-digested glomerular membrane preparations (GMP) are compared with those of pronase digests to determine their relative protein, total sugar, and sialic acid content. In addition, their ability to neutralize the nephrotoxic anti-kidney serum is tested and compared by *in vivo* assay.

Experimental Procedures. Preparation of glomeruli and basement membrane fractions. The glomeruli used in this study were prepared from either bovine renal cortices or mouse kidneys. The isolation of these glomeruli was based on the method of Krakower and Greenspon (4) using stainless-steel sieves and repeated sedimentation in 0.15 *M* NaCl. Basement membrane fractions were obtained from the isolated beef glomeruli by ultrasonic disruption in 1.5 *M* NaCl. A Branson Sonifier model S125 with a microprobe was used at the No. 6 power setting. The fractions were sonicated for 5 min, with several minutes of cooling time allowed after each 1-min burst. Repeated trials showed that such sonication was sufficient for total disruption of the glomeruli. The sonicated material was centrifuged for 10 min at 1500 rpm in an International centrifuge. The sediment which contained cell membranes and basement membranes was designated as the "glomerular membrane preparation" (GMP).

Proteolytic digestion of kidney preparations. Trypsin digestion. Basement membrane and whole glomerular preparations were suspended in 0.1 *M* Tris HCl buffer (pH 8.0) and trypsin was added to equal a 1/100 enzyme-substrate ratio. The mixture was stirred at 37° for 24 hr. After digestion, the soluble material was separated by centrifugation at 15,000 rpm and dialyzed exhaustively against distilled water. It was then lyophilized and stored in the cold.

Pronase digestion. This enzyme was added to equal 1% of the weight of the glomerular fractions in 0.1 *M* Tris-acetate buffer (pH 7.8) containing 5×10^{-4} *M* $CaCl_2$ and stirred at 37° for 24 hr. At the end of the incubation, the soluble material was separated by centrifugation, dialyzed, lyophilized, and stored in the cold.

Chromatography. Columns of Sephadex G-200 (2.5 × 40 cm) were used for the fractionation of both the trypsin and pronase digests. The 10-ml fractions were collected continually and monitored at 280 mμ on an LKB Uvicord II for 16 hr. The approximate molecular weight of the fractions was deter-

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mined by chromatographing blue dextran, gamma globulin, and albumin on the same G-200 column. Aliquotes of the enzyme fractions were routinely analyzed for their total neutral sugar and sialic acid content. Total neutral sugars were determined by the anthrone procedure of Morris (8) using glucose as the standard. Sialic acid was determined by the thiobarbituric acid assay of Warren (11) after it was liberated from the protein by hydrolysis in 0.1 *N* H_2SO_4 at 80° for 60 min, or after it was treated with vibrio cholerae neuraminidase using *N*-acetyl neuraminic acid as the standard. This sugar was also determined directly on column fractions by the resorcinol method of Svennerholm as modified by Cassidy *et al* (1). The fractions in each elution peak marked A, B, and C were pooled, excessively dialyzed against water, lyophilized, and used in the neutralization tests.

In vivo *assay of GMP enzyme digests and chromatographic fractions for anti-kidney serum neutralizing capacity.* Anti-kidney serum was produced in rabbits according to the method reported elsewhere (6). Nephritogenicity of the glomerular fractions was tested by neutralization of the AK serum. Absorption was carried out by mixing glomeruli or their equivalent fractions with anti-kidney serum and placing the mixture in the refrigerator overnight. This was followed by centrifugation at 15,000 rpm for 30 min to remove any precipitate which might have formed.

Groups of eight female mice were injected (ip) with either unabsorbed or absorbed anti-kidney serum and the ensuing proteinuria was measured by precipitation with 3% sulphosalicylic acid (7).

Results. Kinetics of trypsin digestion in relation to sialoprotein release. It has been observed that the rate at which sialoproteins are removed from kidney tissues varies with the concentration of the enzyme. At a 1/100 enzyme-substrate ratio, 24-hr digestion liberated almost all of the sialic acid–containing glycoproteins. Figure 1 illustrates the time curve of the trypsin digestion of the beef glomerular preparation. Digestion was initially rapid. The sialic acid content in this incubation mixture was 0.5 mg after the first hour and obtained a maximum of ≅ 1.5 mg after 24 hr. For the complete removal of sialoprotein, replacement of the soluble reaction product with fresh trypsin and buffer was necessary. The trypsin probably became inhibited by the soluble polyanion reaction product. The soluble trypsin fraction contained two to three times more sialic acid and carbohydrate than the GMP.

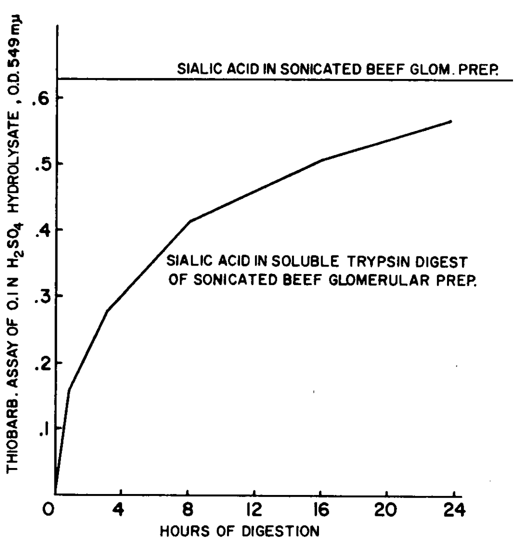

FIG. 1. Time curve of trypsin digestion of sonicated beef glomerular preparation measured by the release of sialoprotein. (Trypsin 23 mg/2.3 g substrate in 15 ml of 0.1 *M* Tris HCl buffer (pH 8.5) at 37° was used for the digestion. Sialic acid was released from both the starting material (horizontal line) and the soluble trypsin digest (curve) by hydrolysis with 0.1 *N* H_2SO_4 at 80° for 60 min. The concentration of sialic acid was determined by the thiobarbituric acid assay.

Chromatography of the soluble trypsin digest. After trypsin digestion of the GMP, the soluble digest was fractionated by gel filtration on Sephadex G-200. Figure 2 (top) shows the resolution of the digest into three fractions A, B, and C, with C predominating. They all contain sialic acid and hexoses. Fraction A moved with blue dextran in the void volume. Since its descending limb coincided with the gamma globulin peak, its molecular weight must have been in the range of 100,000–200,000. Fraction B overlapped the albumin peak, therefore, its molecular weight

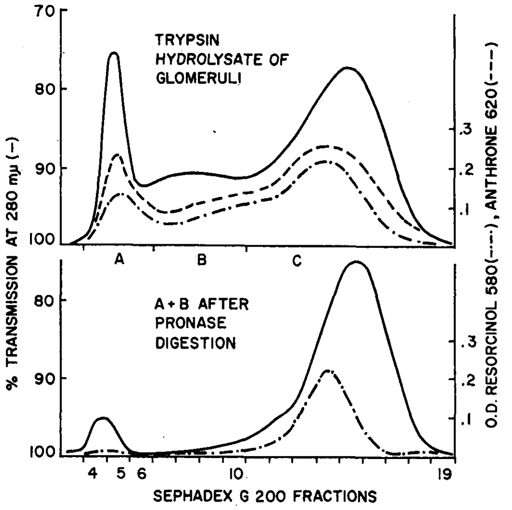

FIG. 2. Chromatogram of soluble trypsin digest of GMP (top). Note the three peak areas A, B, and C, and the presence of sialic acid in all three. A and B rechromatographed after pronase digestion (bottom). Note the emergence of a prominent C peak. Most of the sialic acid is present in this peak.

must have been in the 20,000–100,000 range. Peak C corresponded to a molecular weight of approximately 10,000.

Chromatography of the pronase digest. To determine whether the decreasing molecular weight of chromatographic fractions results from progressive protein breakdown, another experiment was performed with a less specific protease. The GMP and the pooled A and B fractions of the trypsin digest were digested with pronase and fractionated on Sephadex G-200. The result of the separation is plotted in Fig. 2 (bottom). Very small quantities of peaks A and B could be detected after pronase digestion. Almost all of the sialic acid and more than 90% of the hexoses were found in a single peak. This peak appeared at the same location as peak C produced by trypsin digestion of the GMP.

Bioassay of enzyme digests and their chromatographic fractions for neutralizing capacity. To measure the neutralizing activity present in the three glycoprotein fractions of the trypsin digest, the samples within each peak were pooled and studied separately. Figure 3 shows the result of a typical neutralization experiment in which the sum of the average daily urinary protein output per mouse from each experimental group is compared with the proteinuria of mice injected with unabsorbed anti-kidney serum. The bar diagram clearly indicates that fraction C is completely inactive while fractions A and B are both able to absorb the nephrotoxic antibody.

In contrast, pronase digests of the glomeruli and of the pool of A and B trypsin frac-

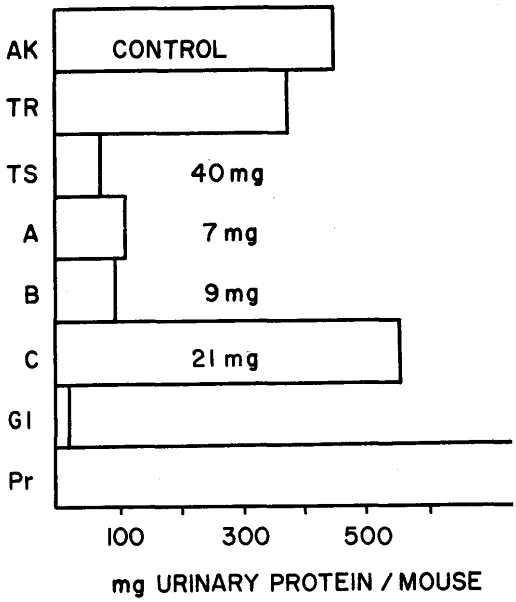

FIG. 3. Bar diagram of proteinuria in mice injected with unabsorbed and absorbed AK serum. A kidney pool of 40 mice yielded 150 mg of lyophilized glomeruli. This amount, routinely used in a single absorption, was sufficient to quantitatively remove all of the nephrotoxic antibody present in 10 ml of antikidney serum. The soluble trypsin digest obtained from 150 mg of glomeruli weighed 40 mg, while its residue weighed 37 mg. The chromatographic peaks A, B, and C were obtained from another 150-mg glomerular batch and weighed 7, 9, and 21 mg respectively. Note the physiological quantity of proteinuria in mice injected with AK serum absorbed with pure glomeruli (G1) and the marked reduction of proteinuria in mice injected with AK serum absorbed with the soluble trypsin digest (TS) or with either the A or B chromatographic fractions. Note the absence of protection in mice injected with AK serum absorbed with the residue of the trypsin digest (TR), with peak C or with the pronase (Pr) digest. Observe the actual enhancement with the latter two.

tions were not capable of neutralizing the nephrotoxic antibody.

Discussion. Our postulate that a portion of the nephritogenic antigen consists of sialic acid residues is based on several observations. Earlier reports (10) have shown that:

1. Neuraminidase treatment of kidney sections prevented the localization of colloidal iron in the glomeruli. Treatment of kidney sections with anti-kidney antibodies also prevented the deposition of colloidal iron. These findings suggest that the colloidal iron reaction is associated with sialic acid and that antibodies directed at the sialyl end group of the glomeruli are present in the anti-kidney serum.
2. Neuraminidase digestion of whole glomeruli decreased their capacity to neutralize the nephrotoxic antibodies. This finding provides evidence that the nephrotoxic antibodies act directly on the morphological elements of the glomeruli containing the sialic acid residue. Furthermore, Cole *et al.* have shown that the nephrotoxic activity of anti-kidney antibodies can be neutralized by the soluble material obtained from the trypsin digestion of homogenized rat kidneys. We have found that this same treatment released almost all of the sialic acid in the form of glycoproteins.

In the present study, the chromatographic distribution of the sialoglycoproteins of trypsin digests were found to be heterogeneous. A pool of the tubes constituting the C peak revealed no neutralizing activity. On the other hand, the high molecular weight fractions (pools of the A and B peaks) were able to neutralize the nephrotoxic antibodies. Although the conditions of the isolation were not severe—the peptide chain splitting being minimal—some loss of the activity of the neutralizing substances occurred. This was indicated by the incomplete absorption of nephrotoxic antibodies.

The behavior of the inactive trypsin peak C was identical with that of the single peak produced by pronase digestion of the glomeruli. This peak may represent the basic heteropolysaccharide unit of the intact nephritogenic antigen. Conversely, the heterogeneity of this sialoglycoprotein may indicate that these carbohydrates are the degradation products of a more complex protein core. This view seems to be supported by the fact that excessive degradation of the protein core of the antigen by pronase digestion resulted in its inability to neutralize the nephrotoxic antibodies.

No conclusion can be made about the original form of the neutralizing substance in the kidney because of the enzymic digestion used for extraction. Cole *et al.* (2) suggested that the material was a protein, possibly associated with a polysaccharide or lipid because their antigen was trypsin-resistant. Shibata, on the other hand, suggested that the nephritogenicity of the trypsin-solubilized antigens was closely related to the high level of polysaccharide content (9).

Glycopeptides accounting for the carbohydrate present in the insoluble glomerular trypsin residue have not been studied in detail because the residue apparently lost all of its ability to neutralize the nephrotoxic antibodies.

Summary. Because previous studies indicated that the nephritogenic antigen is sialoprotein, glomeruli were subjected to enzymic digestion and chromatographed to compare their sialic acid content and their ability to neutralize the anti-kidney antiserum.

The soluble fractions of glomerular trypsin digests contained all the sialic acid and the entire capacity to neutralize the anti-kidney serum. Sephadex G-200 chromatography was capable of separating the soluble digests into two high (A and B) and one low molecular weight compenent (C). They all contained sialic acid, but only the higher molecular weight fragments possessed the neutralizing capacity.

Pronase digests also contained most of the sialic acid, but these were completely inactive. Their chromatogram consisted almost entirely of the low molecular weight C peak, indicating that this less discriminating proteolytic enzyme completely degraded the protein portion of the antigen.

These findings seem to provide additional proof that the nephritogenic antigen is sialoprotein, but that degradation of the pro-

tein component leads to a loss of the neutralizing capacity.

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