PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 95–99 (1980)

Evidence that the Antigen of Autologous Immune Complex Glomerulonephritis of Rats is a Mannose- or Glucose-Containing Glycoprotein (40729)

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Though considerable evidence has been accumulated to show that the majority of chronic glomerulonephritis in humans is immune complex mediated (1), very little is known about the offending antigens. A rational approach to the treatment of chronic glomerulonephritis is unlikely until the source and the nature of the involved antigens are understood. Because autologous immune complex glomerulonephritis of rats (AIC) has a striking clinical histological and immunohistological similarity to the idiopathic membranous glomerulonephritis in humans (2, 3), identification of the nature of the antigen responsible for the AIC is of considerable interest. This report presents evidence that the antigen which immunohistologically has been shown to be present in the region of brush border of proximal renal tubules (BBAg) is most likely a mannose or glucose containing glycoprotein.

Material and methods. The nature of BBAg was determined immunohistochemically on fresh normal rat kidney sections by indirect immunofluorescence technique of Coons *et al.* (4).

Antibody. AIC was produced in rats by immunizing Sprague–Dawley (SD) rats with a SD rat kidney cortex fraction (FX^1A) emulsified in complete Freund's adjuvant as previously described by Glassock and Edington (2). It has been previously (2, 5) shown that when SD rats are immunized with SD FX^1A the only antibody that is deposited as part of antigen–antibody complex in the kidney of rats with AIC is the antibody which is specifically directed against a renal tubular antigen (BBAg), even though the rats are immunized with a crude renal cortex fraction which contains a number of antigens. It seems that the predominant antibody response is elicited against the BBAg, (4, 5) although it has been shown that when the Lewis rats are immunized with SD FX^1A, antibodies to tubular basement membrane can also be present in the kidney (6). This specific antibody to the BBAg was eluted from the kidneys of those rats that had severe AIC by the acid elution technique at pH 3.2 with citrate-citric acid buffer as previously described (6). The eluted antibody was subjected to immunoelectrophoresis along with normal SD rat serum, as a control. Precipitation lines were then developed with rabbit antirat whole serum in the trough. The eluted protein was found to be rat IgG. No other serum proteins were present in the eluate. The eluted antibody was then tested for its specificity for BBAg by indirect immunofluorescence, as previously described (7). These tests showed that the antibody was rat IgG and was specifically directed against the BBAg. The antibody reacted only with brush border of proximal tubules, no reaction with the glomerular components was noted. The protein concentration of the eluate was determined by Lowry's (8) method and was found to be 1.0 mg/ml.

Reagents. Fluorescein-isothiocynate (FITC)-labeled goat anti-rat IgG was obtained from Travenol Lab (Costa Mesa, Calif.). Its specificity was proved by double radial immunodiffusion in gel and by immunoelectrophoresis against fresh normal rat serum. Rabbit antirat whole serum was obtained from Cappel Laboratories (Downington, Pa.). Concanavalin A, FITC-labeled concanavalin A, Ricin II, wheat germ hemagglutinin, and α-methyl-D-mannopyranoside were obtained from Sigma Chemical Company (St. Louis, Mo.). FITC-Labeled concanavalin solution contained 1 mg protein/ml.

Immunohistochemical techniques to de-

0037-9727/80/010095-05$01.00/0

tect nature of BBAg. Fresh normal SD rat kidney was snap frozen in liquid nitrogen and 2–4-μm sections were cut in a cryostat. The sections were air-dried at room temperature for 1 hr and washed twice for 10 min each with phosphate-buffered saline, pH 7.2, (PBS). The sections were then reacted with BBAb for 1 hr at room temperature in a moist chamber. Following reaction with BBAb, the sections were again washed twice for 10 min each with PBS and then reacted with FITC-labeled goat anti-rat IgG for 1 hr at room temperature in a moist chamber. The sections were then washed again twice for 10 min each in PBS and mounted in 1:1 solution of PBS and glycerol. Some normal SD rat kidney sections were directly stained with FITC labeled concanavalin A and studied by direct immunofluorescence.

In another experiment, the sections were treated in the following sequence: first reacted with various strengths of solutions (0.1, 0.5, 1.0, 2.5, 5, 10, 20 mg/ml) of concanavalin A, Ricin II, and wheat germ hemagglutinin for 30 min, washed twice with PBS, reacted again with BBAb, washed twice with PBS, then reacted with FITC-labeled goat anti-rat IgG, washed twice in PBS, and mounted. Some sections, after reaction with concanavalin A, were washed in a solution of 1% α-methyl-D-mannopyranoside for 20 min by continuous shaking at low speed on a shaker and then reacted with FITC-labeled goat anti-rat IgG. The solutions of concanavalin A, Ricin II, wheat germ hemagglutinins, and α-methyl-D-mannopyranoside were prepared by dissolving individual reagents in PBS, pH 7.2. In addition, a solution of concanavalin A was also prepared in 1 *M* NaCl.

All sections were examined under the fluorescent microscope (Leitz, Model Dialux.)

Results. The results are summarized in Table I and Figs. 1–3. The maximum dilution of eluate at which a positive reaction for BBAb *in vitro* on normal SD kidney section was obtained was 1:4. Reaction of BBAb to BBAg on rat kidney sections was blocked by concanavalin A. A quantitative estimation of the blockade with undiluted eluate (1 mg protein/ml) was performed by incubating normal SD rat kidney sections for 30 to 60 min with various concentrations of concanavalin A, ranging from 0.1 to 20 mg/ml. Though progressively increasing blockade was obtained by increasing concentrations of concanavalin A, a complete blockade could only be achieved at a concentration of 2.5 mg/ml and an incubation period of 30 min. No difference in reactivity was noted between concanavalin A dissolved in distilled water or 1 *M* NaCl. With the 1:4 diluted eluate the blockade could be achieved at a concentration of 0.5 mg/ml. The blockade was completely reversed after two 10-min washes with 1% α-methyl-D-mannopyranoside. Ricin II and wheat germ hemagglutinin were not effec-

TABLE I. IMMUNOHISTOCHEMICAL STUDIES

Tissue	Reagents	Fluorescence for BBAg
1. NSDK[a]	+ FITC goat anti-rat IgG	Negative
2. NSDK	+ Normal rat serum + FITC goat anti-rat IgG	Negative
3. NSDK	+ FITC concanavalin A	Positive
4. NSDK	+ Rat BBAb + FITC goat anti-rat IgG	Positive
5. NSDK	+ Concanavalin A + rat BBAb + FITC goat anti-rat IgG	Negative
6. NSDK	+ Concanavalin A + methyl-*D*-mannopyranoside + rat BBAb + FITC goat anti-rat IgG	Positive
7. NSDK	+ Ricin II + rat BBAb + FITC goat anti-rat IgG	Positive
8. NSDK	+ Wheat germ hemagglutinin + rat BBAb + FITC goat anti-rat IgG	Positive

[a] NSDK, Normal Sprague–Dawley kidney section; FITC, fluorecein isothiocyanate conjugated.

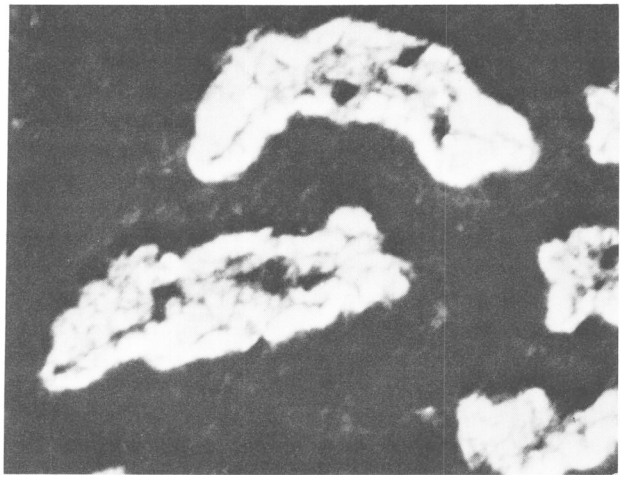

FIG. 1. Brush border antigen is shown by areas of bright fluorescence on normal SD rat kidney sections.

tive in producing blockade in any of the above concentrations with undiluted or diluted eluate. Direct immunofluorescence with FITC-labeled concanavalin A stained brush border antigen area of proximal renal tubules, tubular basement membrane area of all tubules, blood vessles, and diffusely the glomeruli.

Discussion. Purified lectins bind specifically to saccharides on the surface of cells and plasma membranes (9). They recognize specific saccharide residues, a property which can be used for the purification of carbohydrate-containing proteins and for the study of their chemical structure (9, 10). Because of the high specificities of some lectins, and the homogeneity of their saccharide-binding sites, they provide a unique opportunity for the investigation of homogenous combining sites on antigen or antibody-like molecules (9, 10). Furthermore, the binding of the lectins to the saccharide-binding sites on the surface of the cells or antigen, can be inhibited by specific sugars such as α-methyl-D-mannopyranoside in the case of concanavalin A (9, 10). We utilized these unique properties of lectins in identifying

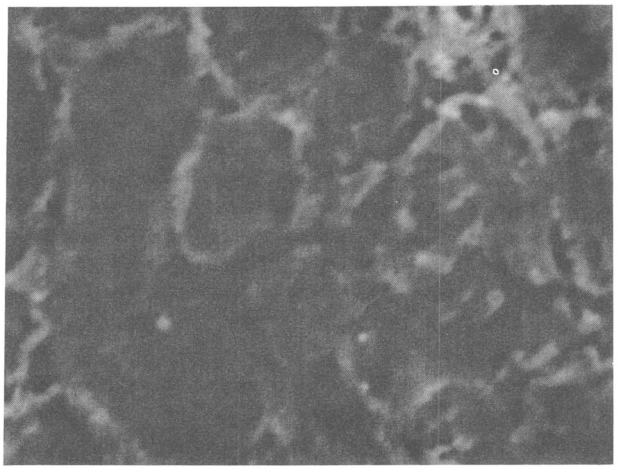

FIG. 2. The fluorescence of the brush border antigen is completely blocked by pretreatment with concanavalin A.

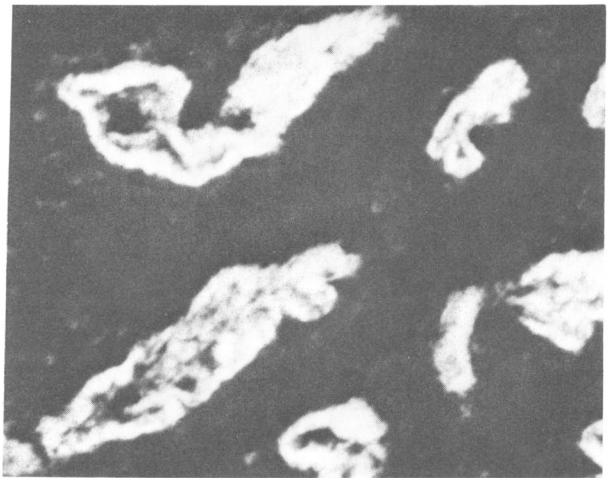

FIG. 3. The bright fluorescence reappears after treatment with α-methyl-D-mannopyranoside.

the chemical nature of the antigen of AIC of rats.

Our results show that the antigen of AIC of rats which previously has been shown to be a protein (11), is most likely a glycoprotein because its reaction with its specific antibody can be blocked by concanavalin A. Furthermore, it seems that the antigen contains either mannose or glucose, since the reaction of the antigen with concanavalin A was reversed by α-methyl-D-mannopyranoside which specifically competes with concanavalin A for the mannose- or glucose- containing receptor sites on the antigen. Since glucose is usually not found as an integral part of most tissue glycoproteins (12), it seems that at least one of the sugar moieties in the antigen of AIC is probably mannose. However, these data do not completely rule out the possibility that the concanavalin A reacted with another glycoprotein which was spatially oriented in close proximity to the BBAg molecule and this prevented the reaction of the antibody with the BBAg molecule. Experiments with Ricin II and wheat germ hemagglutinin may suggest that the antigen does not contain galactose or *N*-acetylclucosamine or that the molecular conformation prevented these sugars to react with these two lectins in this experimental setup.

Edgington and associates (11) obtained a partially purified preparation of this antigen by first homogenizing rat renal cortex and then solubilizing the antigen with desoxycholate. They found that antigen was a macromolecule of lipoprotein nature. The antigen was felt to be lipoprotein because the precipitin line in gel containing the antigen reacted with Sudan Black B. The desoxycholate detergent solubilized molecule most likely was isolated in micellar form with its membrane bound lipids and thus was thought to be a lipoprotein. Naruse and co-workers, (13) recently using a pronase digest of the renal cortex, have suggested that the proteolytic cleaved fragment of the antigen may be a glycoprotein. They based their conclusion on the staining properties of the precipitin line in polyacrylamide gel with periodic acid–Schiff reagent. The smaller protein isolated by Naruse *et al.* is most likely only a part of the whole membrane glycoprotein. Our results agree with their findings, however, it must be stressed that Naruse *et al.*, also had to digest the antigen with pronase, whereas our design of study tested the nature of the antigen on intact tissue sections, a technique in which the antigen maintains its immunochemical integrity.

The AIC of rats has a striking clinical, histological, and immunohistological similarity to the idiopathic membranous glomerulonephritis of humans (1–3). Recent observations by the Japanese investigators (14, 15) seem to suggest that even the offending antigen in both diseases may

be similar. Therefore, the identification of the chemical nature of the antigen of AIC is of considerable importance. Identification of the nature of the antigen is likely to lead to greater insight into the pathogenesis of these diseases and may also possibly provide opportunities for the treatment of these disorders.

It is interesting to point out that the antibody (BBAb) used by us did not react with any of the glomerular components with undiluted or diluted (up to 1:32) eluate. This is an important point because recently it has been claimed that heterologous antirat FX^1A when injected in isolated perfused kidney may react with a similar antigen in glomerulus (16). However, it must be pointed out that this issue is controversial and many other investigators, including the above investigators (16), have not been able to demonstrate the reaction of homologous BBAb with BBAg on rat kidney sections by indirect immunofluorescence. The disparity of these observations may be related to the differences in the design of testing and heterologous versus homologous nature of the antibody.

Summary. Chemical nature of the brush border antigen (BBAg) of the autologous immune complex glomerulonephritis (AIC) of rats was investigated by a histochemical method utilizing indirect immunofluorescence. Fresh frozen rat kidney sections, a specific antibody to BBAg (BBAb), purified lectins (concanavalin A, Ricin II, wheat germ hemagglutinin) and a specific sugar, α-methyl-D-mannopyranoside were used. The reaction of the specific antibody BBAb to its antigen BBAg on tissue sections was blocked by concanavalin A but not be Ricin II and wheat germ agglutinin. This blockade was reversed by α-methyl-D-mannopyranoside. These results suggest that the antigen of AIC of rats is a mannose or glucose containing glycoprotein.

Identification of the chemical nature of the antigen of AIC is of considerable importance because AIC has a striking clinical, histological, and immunohistological similarity to the idiopathic membranous glomerulonephritis in humans.

This work was supported by research grants from The Kidney Foundation of Ohio, Inc., and NIH Grants RR 05410-14 and AM 25782.

The author is indebted to Mrs. Sharon Monahan for her excellent technical assistance.

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Received June 5, 1979. P.S.E.B.M. 1980, Vol. 163.