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Circulatory antigens of Heymann nephritis

I. IDENTIFICATION AND PARTIAL CHARACTERIZATION

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SUMMARY

Previously, we have isolated and characterized a complex glycoprotein antigen (gp600) from the rat kidney that can induce Heymann nephritis (HN) in the rat. A monospecific antibody against the gp600 was used as a probe to document the existence of cross-reactive antigens in normal rat serum. A competitive radioimmunoassay measured the concentration in normal rat serum as being $45{\cdot}5 \pm 10{\cdot}2$ μg/ml ($n = 17$). Molecular exclusion gel chromatography of normal rat serum identified gp600 activity in three distinct peaks corresponding to the molecular weights of 150,000, 110,000 and 70,000, respectively. Soluble immune complexes of mean molecular weight $1{\cdot}1 \times 10^6$ were formed when normal rat serum was reacted with affinity-purified ^{125}I anti-gp600. Normal rat serum, electrophoresed in 8% SDS-PAGE gels, transblotted to nitrocellulose membrane and reacted with anti-gp600 by indirect immunoperoxidase technique, identified three to four bands in the molecular weight region of 66,000–80,000. Isoelectric focusing revealed these antigens to be anionic (pI of 4·5–5·5) in nature. We conclude that normal rat serum contains antigens that cross-react with gp600. Further, these antigens are anionic in nature and form soluble immune complexes with anti-gp600 *in vitro*. The relevance of these findings to the pathogenesis of HN is discussed.

INTRODUCTION

Heymann nephritis (HN) is an experimental autoimmune glomerulonephropathy described in the rat by Heymann *et al.* in 1959. The disease is usually induced by immunization of rat with the crude renal cortical proximal tubular fraction FX1A, emulsified with complete Freund's adjuvant (CFA) (Edgington, Glassock & Dixon, 1968). The glomerular pathology of HN is known to involve an autoantibody that can be detected in serum and the acid eluates of glomeruli of diseased animals. The antibody reacts avidly with an antigen in the brush border of proximal tubule as seen by indirect immunofluorescence (IIF) on normal rat kidney sections (Grupe & Kaplan, 1969). Recently, it has also been shown to react with an antigen present in the coated pits in the epithelial cell membrane at the base of the foot process by electron microscopy using indirect immunoperoxidase staining (Kerjaschki & Farquhar, 1983). It is not clear how the antigen reaches this site. One of the possibilities considered by the authors (Karjaschki & Farquhar, 1983) was the uptake of a circulatory antigen by the coated pits. Though there has been suggestive evidence in the literature to indicate that there is a circulatory Heymann antigen in the rat serum (Glassock *et al.*, 1968; Naruse *et al.*, 1976), confirmatory proof of the presence of the circulatory antigen is lacking.

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Recently, this laboratory reported the purification of a nephritogenic glycoprotein antigen of MW 600,000 (gp600) from FX1A (Makker & Singh, 1984). The antigen gp600 qualified as the antigen of HN by the following criteria: (i) it could induce active HN in a group of Lewis rats by immunization with microgram quantities of the antigen; (ii) antibody raised in rabbit against gp600 on injection into a rat produced glomerular fixation of antibodies in a granular pattern typical of passive Heymann nephritis.

In this paper we have used this antibody as a probe, and present direct evidence to suggest that antigens cross-reactive with the HN antigen circulate in the normal rat serum. These antigens have been identified and partially characterized for molecular size and charge.

MATERIALS AND METHODS

Materials

Na^{125}I was obtained from Amersham Corporation, Arlington heights, IL. Bovine serum albumin (RIA grade), thyroglobulin, ferritin, human IgG, beta-lactamase, Trizma base, glycine, amido black, Commassie brilliant blue, Tween-20, 3,3-diaminobenzidine tetrahydrochloride, *o*-dianisidine and chloramine-T were purchased from Sigma Chemical Co., St Louis, MO. Nitrocellulose sheets (pore size 0·45 μm, HAWP) were purchased from Millipore Corporation, Bedford, MA. Ultrogel A-6 beads (fractionation range $2{\cdot}5 \times 10^6$) and ampholytes (range

3·5–10·0) were from LKB, Bromma, Sweden. Sodium dodecyl sulphate, acrylamide, BIS, ammonium persulphate and TEMED were of highest grade of purity supplied by Biorad Laboratories, Richmond, CA. Biorad protein reagent was also obtained from Biorad Laboratories. Horseradish peroxidase labelled goat anti-rabbit IgG was obtained from Cappel Laboratories, West Chester, PA. Standard pI markers were supplied by FMC, Rockland, ME. Packard Tri-carb gamma scintillation spectrometer (Downer's Grove, IL) was used for gamma counting. Isoelectrofocusing was done on a flat bed using the equipment model No. 900, supplied by Hoeffer Scientific, San Francisco, CA. Ultrafiltration was done using PM-10 membrane (cut off above 10,000) in the standard Amicon ultrafiltration cell (Amicon, Lexington, MA).

Preparation of gp600 and rabbit anti-gp600 antiserum

Gp600 was isolated from the crude kidney cortical fraction FX1A by *lens culinaris* lectin affinity chromatography as previously described (Makker & Singh, 1984). The fraction isolated was physicochemically and immunologically characterized as a single glycoprotein antigen of MW 600,000. By polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), gp600 resolved into four glycoprotein subunits of MW 330,000 (gp330), 140,000 (gp140), 110,000 (gp110) and a 70,000 duplet (gp70). Antiserum to gp600 was raised in rabbits using microgram quantities of gp600 emulsified in complete Freund's adjuvant (CFA) according to the method of Vaitukaitis *et al.* (1971).

Specificity of anti-gp600 antiserum

By double immunodiffusion-in-gel, anti-gp600 antiserum gave a single precipitin line of identity against gp600 and FX1A (Makker & Singh, 1984). Indirect immunofluoresence on normal kidney sections showed intense staining of rat kidney proximal tubular brush border. The antiserum stained the brush border until a dilution of 1:1024. One mg of IgG of this antiserum given intravenously to a Lewis rat produced typical glomerular lesions of passive Heymann nephritis. The antiserum was tested for its reactivity to the subunits of gp600 by indirect immunoperoxidase technique performed on nitrocellulose blots of FX1A separated by SDS-PAGE. FX1A was prepared according to the method of Edgington *et al.* (1968) and resolved on SDS-PAGE by the method of Laemmli (1970). The separated proteins were transblotted onto a nitrocellulose membrane according to the method of Towbin, Staehelin & Gordon (1979) The membrane was blocked for reactive sites with 5% Tween-20 in PBS for 1 hr at room temperature (RT). A strip of the membrane was incubated with 1:1000 diluted anti-gp600 antibodies overnight. Another strip was similarly incubated with 1:1000 diluted normal rabbit serum (control). The strips were washed with PBS and then incubated with radioiodinated protein A. The membranes were washed with PBS and then autoradiographed on XAR-2 film at $-70°$ for 24 hr. Anti-gp600 reacted most strongly against gp70 and weakly against gp330, gp140 and gp110 subunits (data not shown).

Competitive radioimmunoassay for gp600

The method has been described by us in detail in a recent publication (Singh & Makker, 1985). Briefly, Gp600 was radioiodinated with $Na^{125}I$ to a specific activity of 4 μCi/g using the Cloramine T method (McConahey & Dixon, 1966). The label was 90% TCA precipitable, and at the working dilution of 1:3000 (final dilution in incubation mixture) the anti-gp600 bound 30% of the label. The radioimmunoassay for gp600 measurement was carried out as follows: into 12×75 mm tubes were placed 50 μl of 1:10 diluted sera or standard gp600 (0·75–24 μg), 50 μl of the label containing 20,000 counts per minute (5 ng) and 50 μl of 1:1000 diluted anti-gp600 antiserum. All dilutions were made in radioimmunoassay (RIA) buffer which consisted of 10 mM phosphate buffer, pH 7·4 , containing 130 mM NaCl, 10 mM ethylene diamine tetraacetate and 0·2% (w/v) bovine serum albumin. Controls of maximum binding (without standard gp600 or rat sera) and non-specific binding (without antibody) were included in the assay. The tubes were vortexed and incubated at 4° overnight. The next day, 20 μl of 10% (v/v) protein A-containing *Staphylococcus aureus* cells were added and the tubes vortexed and incubated for 30 min at room temperature. Protein A-containing *Staphyloccus aureus* cells were prepared according to the method described previously by us (Singh & Makker, 1983). One ml of PBS was added to all tubes after overnight incubation. The tubes were vortexed and centrifuged at 800 ***g*** for 10 min. The supernatants were decanted and bacterial pellets were counted in a gamma ray spectrometer for 1 min. A standard inhibition curve was plotted from binding data using logit-log transformation. The antigen values (gp600) of different samples were read off from the curve. Protein was determined by Biorad protein reagent.

Collection of arterial and venous blood from rats

Samples of arterial and venous blood were taken from the abdominal cavity. Arterial blood was taken with the help of a syringe and gauge 27 needle from the abdomen aorta. Venous blood was taken by similar technique from the inferior vena cava above the level of the renal veins.

Formation of immune complexes on mixing radioiodinated (^{125}I) rabbit anti-gp600 with normal rat serum in vitro

Anti-gp600 antibodies were purified by affinity chromatography on a column of gp600. The purified antibody was labelled with $Na^{125}I$ to a specific activity of 100 μCi/g by the Chloramine T method (McConahey & Dixon, 1966). A trace amount of the label was mixed with 1 ml of normal rat serum and incubated at 4° overnight. The mixture was fractionated on an Ultrogel A-6 column ($2·5 \times 100$ cm) equilibrated with PBS. The Ultrogel A-6 column was calibrated for molecular weight determination with thyroglobulin (600,000), ferritin (440,000), IgG (150,000), albumin (66,000), and β-lactamase (30,000). Five-ml fractions were collected at a pressure head of 10 cm and a flow rate of 20 ml/hr. The fractions were counted in a gamma ray spectrometer to trace the shift in the label. As a control, a similar amount of radioactive antibody was mixed with 1 ml of 2 mg/ml of BSA and chromatographed on the same column.

SDS-PAGE of normal rat serum, transblotting onto nitrocellulose and immunoperoxidase staining of transblotted proteins against anti-gp600

Normal rat serum was resolved by SDS-PAGE by the method of Laemmli (1970). Electrophoresed proteins were transblotted onto nitrocellulose by the method of Towbin *et al.* (1979). A strip of the blot was stained with amido black (0·1% in methanol:acetic acid:water 9:2:9) for 1 min and destained in the same solvent without the dye. Strips of blots were tested for

reactivity against normal rabbit serum and anti-gp600 antibodies by the method of Towbin *et al.* (1979) modified as follows: blots were blocked by immersion in 5% Tween-20 in PBS at room temperature for 1 hr. They were then washed with PBS and incubated with 1:100 diluted anti-gp600 antiserum or 1:100 diluted normal rabbit serum made in PBS containing 1% bovine serum albumin at room temperature overnight on a gentle rocker. The next day, the blots were washed with several changes of PBS over 1 hr and immersed in 1:200 diluted peroxidase-labelled goat anti-rabbit IgG conjugate made in PBS containing 1% BSA for 2 hr at room temperature. The blots were washed again with several changes of PBS over 1 hr. Peroxidase reaction was developed by dipping the blots in substrate solution containing 0·05 mg/ml of *o*-dianisidine and 0·024% H_2O_2 in 50 mM Tris-HCl buffer, pH 7·4, for 1 hr at room temperature. Reaction was stopped by washing the blots with water. The blots were stored in water and photographed by reflected light.

Isoelectric focussing of normal rat serum, transblotting to nitrocellulose and testing of reactivity of anti-gp600 antibodies on the transblot by indirect immunoperoxidase technique

Normal rat serum was electrofocused on 0·75 mm thin 5% polyacrylamide gels containing ampholine carrier ampholytes between the pH range 3·5 and 10·0. Gels were cast by photopolymerization using the gel casting kit supplied by the manufacturer. Electrofocusing was performed on a flat bed by resting the electrode assembly on top of the gel surface. The separated proteins were transblotted onto nitrocellulose by the technique of passive transfer recently described by Lanzillo *et al.* (1983). Sections of the blot containing normal rat serum and standards were stained with India ink by the method of Hancock & Tsang (1983) to confirm the transfer. Immunoperoxidase staining of the blots was carried out as follows: two sections of the blot containing normal rat serum proteins were washed three times for 10 min each with PBS containing 5% Tween-20 to saturate the remaining sites on nitrocellulose. One section was incubated with 1:500 dilution of rabbit anti-gp600 made in PBS overnight at room temperature. The other section was similarly treated with 1:500 dilution of normal rabbit serum which served as the negative control. Next day, the sections were washed three times for 20 min each with PBS. They were then incubated with 1:400 dilution of peroxidase-labelled goat anti-rabbit IgG for 90 min at room temperature. The sections were washed again with PBS and the bound peroxidase was visualized with 0·05 mg/ml of *o*-dianisidine made in 50 mM Tris-HCl buffer, pH 7·4, containing 0·024% (v/v) H_2O_2.

Molecular sizing of the HN antigens in normal rat serum by molecular exclusion gel chromatography

Five ml of normal Lewis rat serum was clarified by ultrafiltration and equilibrated with PBS. Ultrogel A-6 was packed in a 2·5 cm × 100 cm column and equilibrated with PBS at a pressure head of 10 cm. Flow rate was adjusted to 10 ml/hr. Normal rat serum was fractionated on this columm and 5-ml fractions collected. Protein was determined in the samples by A_{280} in a spectrophotometer. The gp600 cross-reacting antigens were traced in the fractions by competitive RIA for gp600 described above.

RESULTS

Concentration of antigen in normal rat sera by radioimmunoassay

In the competitive RIA for gp600, which has been described in the 'Materials and Methods', 5 ng of hot antigen were added to each tube that could be displaced completely by 2400 ng of the cold antigen, which amounts to 480 molar excess. This resulted in considerable loss of sensitivity. This point was investigated further. It was found by SDS-PAGE and autoradiography of the label that the labelled antigen degraded to smaller fragments under the oxidizing conditions of the labelling method. We believe that the small molecular weight fragments of the label bind the antibody with much higher affinity than the intact antigen. However, since the antigen was present in microgram quantities in the rat serum, the sensitivity of our RIA was adequate to measure these concentrations accurately.

Serum samples obtained randomly from 17 normal Lewis rats, of all ages and both sexes, were tested at 1:10 and 1:100 dilutions in the competitive RIA of gp600. All 17 samples gave dilution curves that paralleled the standard inhibition curve (data not shown). Antigen levels ranged between 25·4 and 65·4 with a mean ± SD of 45·5 ± 10·2 μg/ml ($n = 17$). Antigen levels were determined in arterial and venous samples from 10 normal age-matched (6–8 weeks) male Lewis rats. Arterial samples contained 30·4 ± 10·1 (Mean ± SD) μg gp600/ml and venous samples contained 27·1 ± 6·7 μg gp600/ml. The difference between the arterial and venous concentrations was not statistically significant by Student's *t*-test.

^{125}I-anti-gp600 forms an immune complex with the serum antigen *in vitro*

When radioiodinated (^{125}I) anti-gp600 was mixed with normal rat serum *in vitro* and the mixture chromatographed on a

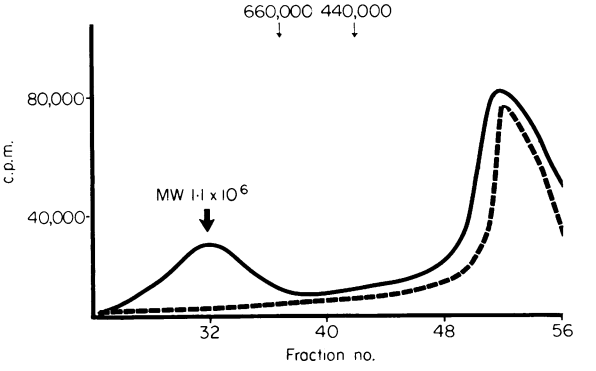

Figure 1. Formation of immune complexes by mixing rabbit anti-gp600 with normal rat serum *in vitro*. Rabbit anti-gp600 was affinity purified as described in the 'Materials and Methods' and labelled with ^{125}I to a specific activity of 100 μCi/g by the chloramine-T method. A trace amount of the label was mixed with 1 ml of normal rat serum and incubated at 4° overnight. The mixture was fractionated on an Ultrogel A-6 gel filtration column equilibrated with 10 mM phosphate buffer containing 130 mM NaCl, pH 7·4 (PBS). The Ultrogel A-6 column was calibrated for molecular weight determination with thyroglobulin (660,000), ferritin (440,000), IgG (150,000), albumin (66,000), and β-lactamase (30,000). The column eluants were counted in a gamma ray spectrometer. (——) shows the profile of the ^{125}I-anti-gp600 mixed with normal rat serum. (– – – –) shows the profile of the ^{125}I anti-gp600 alone.

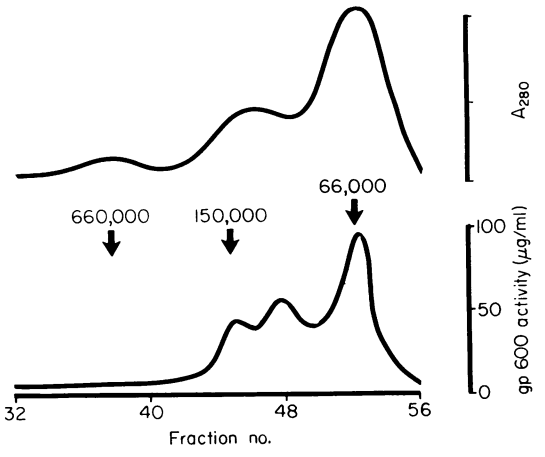

Figure 2. Fractionation of normal rat serum in the Ultrogel A-6 gel filtration column and protein concentration plotted. Gp600 concentration was measured in each fraction by RIA. Protein was estimated by A_{280}. Arrows show the positions of the standard MW markers.

molecular exclusion column (Fig. 1), it appeared that about 30% of the antibody was engaged with the serum antigen to form immune complexes of MW range of $0{\cdot}88–1{\cdot}32 \times 10^6$ with a mean MW of $1{\cdot}1 \times 10^6$. The percentage of the antibody involved in the immune complex formation was calculated from Fig. 1 by the measurement of the areas under the two peaks corresponding to the immune complex and free antibody, respectively.

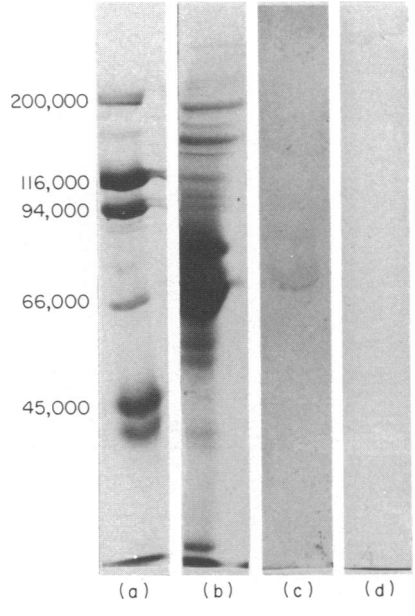

Figure 3. Reactivity of anti-gp600 antibodies to rat serum antigens. Normal rat serum proteins were resolved in 8% SDS-PAGE and transblotted onto nitrocellulose. Strips of nitrocellulose were stained by immunoperoxidase technique with 1:100 diluted anti-gp600 antibodies (c) and 1:100 diluted normal rabbit serum (d). Strip (a) is a gel strip indicating standard MW markers: ovalbumin (45,000), albumin (66,000), phosphorylase B (94,000), β-galactosidase (116,000) and myosin (200,000). Strip (b) is the gel strip showing the rat serum proteins that were transblotted.

The antigen in the serum was present in three different molecular sizes

A pool of normal Lewis rat serum was chromatographed on a molecular exclusion gel column and the eluants were tested for antigen concentration in the competitive RIA for gp600. It was observed that the antigen was present in three discreet peaks, corresponding to the molecular weight regions of 150,000, 110,000 and 70,000 respectively (Fig. 2). A large percentage (51%) of the total activity was confined to the 70,000 peak.

Direct reactivity of anti-gp600 antibodies to antigens of normal rat serum resolved by SDS-PAGE

Normal rat serum was electrophoresed on 8% SDS-PAGE gels and transblotted onto nitrocellulose according to the procedure described in the 'Materials and Methods'. The blots were tested against anti-gp600 antiserum by immunoperoxidase technique as described in the 'Materials and Methods'. Figure 3 shows that anti-gp600 reacted to three to four bands in the range of 66,000–80,000. The failure to visualize the 150,000 and 110,000 antigens by this technique but which were documented by RIA is not understood. Possible explanations considered are (i) denaturation of these antigens caused by SDS in the electrophoresis system, or (ii) the immunodominance of the 70,000 antigen species which effectively competes out the other antigens for reaction with the antibody. The non-transfer of 150,000 and 110,000 antigens from rat serum to nitrocellulose was not

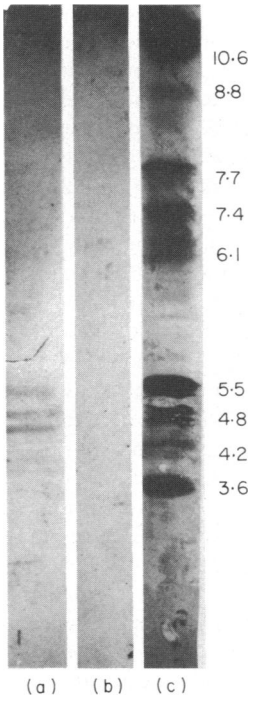

Figure 4. Reactivity of anti-gp600 antibodies to rat serum antigens resolved by isoelectricfocusing. Normal rat serum was isoelectricfocused between the pH of 3·5 and 10 and transblotted onto nitrocellulose. Strip (a) was stained with 1:500 diluted anti-gp600 antiserum by immunoperoxidase. Strip (b) was stained similarly with 1:500 diluted normal rabbit serum. Strip (c) shows the standard pI markers stained on the nitrocellulose by India ink as described in the 'Materials and Methods'.

considered as an explanation because amidoblack staining of the transfer clearly proved a faithful transfer (data not shown).

pI of the serum antigens

Normal rat serum was isoelectrifocused on a polyacrylamide gel between pH 3·5 and 10·0 and transblotted onto nitrocellulose as described in the 'Materials and Methods'. The focused serum proteins on the nitrocellulose were tested against anti-gp600 antiserum by indirect immunoperoxidase staining of the blot. Figure 4 shows that the three to four protein bands that reacted with anti-gp600 antibodies were all anionic proteins with pI in the range of 4·5–5·5.

DISCUSSION

Using a monospecific antiserum, anti-gp600, as a probe, we have documented that there are antigens present in normal rat serum which cross-react with gp600—the nephritogenic kidney antigen of Heymann nephritis. Multiple antigens were present in rat serum that cross-reacted with gp600, though a 70,000 antigen seemed to be the immunodominant species. The antigens were found to be anionic in nature.

When radioiodinated antibody anti-gp600 was mixed with normal rat serum, the immune complexes that resulted had a mean molecular weight of $1{\cdot}1 \times 10^6$. The large size indicates the complexity of these immune complexes. Since the complexes were not isolated for further characterization, it is difficult to say whether they contained a single antigen or multiple antigens. If we assume that the antibody engaged the immunodominant 70,000 antigen, then highly latticed structures such as $Ab_4\,Ag_4$ to $Ab_5\,Ag_5$ can be considered to be present in the immune complex material. From this experiment, we prefer to conclude only that reactive antigens are present in norml rat serum without going into the size of the immune complexes, because in the *in vivo* situation of both active and passive Heymann nephritis the size of the complexes will be dictated by the concentrations of the antigen and antibody which are probably constantly changing and of which we have little knowledge.

Edgington, Glassock & Dixon (1968) and Glassock *et al.* (1968) were the first investigators to report the isolation of the antigen of HN from the crude kidney fraction FX1A. Using physicochemical methods of separation, they isolated a fraction called RTE-α5 (renal tubular epithelial antigen) which was found to be highly nephritogenic to the rat. Attempts to detect circulating RTE-α5 in normal animals using micro-Ouchterlony and immunofluorescent inhibition techniques were not successful. However, in normal Lewis rats it was found that passively administered hyperimmune rabbit anti RTE-α had an accelerated removal from the circulation when compared to non-immune rabbit gamma globulin. This rapid elimination of the antibody led the investigators to believe that the injected antibody formed circulatory immune complexes with a circulating antigen which resulted in rapid clearance of the antibody from blood.

Later, Naruse *et al.* (1976) provided more direct evidence to indicate that there is a circulatory antigen present in normal rat serum. These investigators obtained the antigen of HN in a soluble form by enzymatic (pronase) digestion of renal epithelial segments. Further separation of the antigen was carried out by physicochemical methods. They called their antigen Tub-Ag, and radioimmunoassay for Tub-Ag was developed. Using this RIA, they documented the presence of the antigen in normal rat serum. Tub-Ag activity appeared as a single peak ahead of the position of gamma globulin on gel filtration fractions of rat serum. When serum was electrophoresed and the fractions tested for Tub-Ag activity, it was observed that the activity was present in three different peaks distributed in regions of alpha and beta globulins. The antigen level in the rat serum diminished as the antibody titres rose in the active disease, suggesting that circulatory antigen was being sequestered as immune complexes. Interestingly, these investigators observed three antigens in the serum with widely differing electrophoretic mobilities but with identical molecular weights. On the other hand we have observed the antigens in serum to be present in three differing molecular sizes, namely 70,000, 110,000 and 150,000 respectively. The discrepancy in the two reports may be in the differing specificities of the anti-Tub-Ag and anti-gp600 antibodies used by the two investigators. Because the Tub-Ag was not resolved on SDS-PAGE for its subunits and not tested for reactivity to its subunits, it is difficult to compare the specificities of the two antibodies. Due to the non-availability of SDS-PAGE and transblotting techniques at that time, the direct binding of the anti-Tub-Ag to serum proteins could not be demonstrated. The pI of the antigen was also not reported. Neither Glassock *et al.* (1968) nor Naruse *et al.* (1976) attempted to isolate the circulatory antigens and study them further.

A hypothesis that is usually forwarded to explain the Heymann lesion is the *in situ* formation of complexes attracted by the HN antigen purported to be present in the glomerular capillary wall. Recently, Kerjaschki & Farquhar (1983) demonstrated the presence of the Heymann antigen in the glomerular epithelial cell at the base of the foot process, providing support to this hypothesis. In light of these observations, the rapid elimination of anti-RTE-α5 antibodies from circulation in the experiments of Glassock *et al.* (1968) could partly be due to the binding of the antibody directly to the glomerular antigen, and the diminution in the antigen levels in serum in the Naruse experiment could be due to the interaction of the serum antigen with the *in situ* formed complexes.

The role circulating Heymann antigens play in the pathogenesis of this disease can only be speculated at this time. First, it is possible that the circulatory antigens may bind to the capillary wall by virtue of non-immune mechanisms and give rise to foci for the binding of the antibody *in situ*. Several recent experimental studies have appeared in the literature that have shown that circulating cationic proteins are attracted to the fixed negative charges located in the glomerular capillary wall (Gauthier, Mannick & Striker, 1982; Gallo *et al.*, 1983; Caulin-Glazer, Gallo & Lamm, 1983). The fact that the antigens we have isolated are anionic in nature makes that possibility unlikely, though not improbable because anionic circular DNA has been shown to bind to the capillary wall (Izui, Lambert & Miescher, 1976). Second, it is also possible that the antigens may interact with the glomerular bound antibody, which was primarily bound by *in situ* mechanism and contribute in the cross-linking and enlargement of these immune complexes. Suggestion that this may be occuring has come from the studies of Edgington *et al.* (1968), who demonstrated by immunofluorescence technique that there was increase of FX1A antigens contained in the glomerular immune deposits of HN rats with progression of disease. Studies to investigate this question using the antigens

we have isolated are in progress in our laboratory. Third, the antigens can potentially form circulatory immune complexes with the autoantibody and contribute to the pathogenesis of the disease over and above the *in situ* immunopathogenesis. In order to prove that circulatory immune complexes play part in the pathogenesis of HN, it is imperative to demonstrate that the Heymann antibody forms immune complexes with the serum antigen *in vivo*. In this study we have shown that the heterologous anti-gp600 definitely formed immune complexes *in vitro* with normal rat serum. Though this result in no way proves that Heymann autoantibody forms circulatory immune complexes in the active Heymann nephritis situation, it does, however, raise that possibility.

The fact that the heterologous anti-gp600 formed definite immune complexes with normal rat serum antigens *in vitro* suggests that the circulatory antigen may have a role in the pathogenesis of passive Heymann nephritis, which is usually produced 4–5 days after passive administration of a heterologous antibody to the tubular brush border complex (like anti-FX1A). The characterization of immune complexes formed *in vivo* in the passive HN model therefore needs to be studied further.

At this time, the source of the circulatory antigen can again only be speculated. Could the antigen be of renal origin? Data on the antigen levels in arterial and venous blood samples was indecisive. Recently, we have shown the presence of Heymann cross-reactive antigens in membranes of many rat tissues by radioimmunoassay and immunoprecipition (Singh & Makker, 1985). It is possible that the circulating antigen may be present as a catabolite from the normal turnover of membrane proteins. Some of the questions raised above on the exact role of the circulatory antigen in pathogenesis of Heymann nephritis can be attempted after the isolation and purification of these antigens. These studies are in progress.

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