

ISOLATION, PARTIAL CHARACTERIZATION,  
AND LOCALIZATION OF A RAT RENAL  
TUBULAR GLYCOPROTEIN ANTIGEN

Antibody-induced Birth Defects\*

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The appearance of birth defects after injection of heterologous antiserum directed against whole rat kidney homogenate into pregnant rats during the organogenetic period was first reported by Brent and his colleagues (1). This finding has been repeatedly confirmed and extended by other investigators (2-6). The underlying mechanism whereby teratogenic kidney antiserum induces abnormal embryonic development is not understood, although the responsible teratogenic agents are immunoglobulin G (7). It appears that the induction of congenital malformations by these immunoglobulins (Ig) does not depend on complement and other nonspecific immunologic mediators (8). The rat kidney antigens that elicit the production of these teratogenic antibodies have not been isolated since the original finding in 1961 (1), although preliminary studies using both saline-soluble (9) and saline-insoluble (10) kidney fractions indicated that the responsible antigens are glycoproteins of high molecular weights. This communication reports the isolation and partial characterization of a highly purified glycoprotein antigen that elicited the production of potent teratogenic antibodies. Immunofluorescent studies suggest that the isolated kidney antigen is a component of the proximal convoluted tubular cells. Furthermore, specific localization of the teratogenic antibodies in vivo in the visceral yolk-sac endoderm and embryonic endoderm suggest that the teratogenic antibodies may induce abnormal embryonic development by interfering with the functions of the yolk-sac placenta or by a direct effect on the embryo itself.

Materials and Methods

*Timing of Pregnancy.* A colony of random-bred Wistar rats was used. Rats were mated for 14 h overnight. Females that had been inseminated were considered to be at the beginning of the 1st d of pregnancy on the morning sperms were found. Rats were housed in stainless steel cages and given food (Purina mouse chow) and water *ad lib*.

*Extraction with Phosphate-buffered Saline (Step 1).* Purification of the glycoprotein that elicited the production of teratogenic antibodies involved six steps. Wistar rats of both sexes weighing 200-350 g were perfused with isotonic phosphate-buffered saline (PBS),<sup>1</sup> pH 7.3. Kidneys were removed, decapsulated, and immediately frozen at -60°C. Each experiment used 100 kidneys,

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<sup>1</sup> *Abbreviations used in this paper:* PBS, phosphate-buffered saline, pH 7.3; Con A, concanavalin A; gp 340K, rat renal tubular glycoprotein, 340,000 mol wt; PAGE, polyacrylamide gel electrophoresis.

and the entire isolation procedure was performed in the cold at 4°C. Kidneys were homogenized in PBS (2 ml/kidney) containing 1 mM phenylmethylsulfonylfluoride, 10 mM *N*-ethylmaleimide, 25 mM  $\epsilon$ -aminocaproic acid, and 20 mM EDTA in a Virtis model 60K homogenizer (VirTis Co., Inc., Gardiner, NY) at 10,000 rpm for 20 min. The homogenate was filtered through a stainless steel sieve (40 mesh) to remove large pieces of connective tissue and tissue debris. The filtrate was recovered and centrifuged at 105,000 *g* for 1 h. The resultant clear supernatant was recovered. The residual pellet was extracted again with PBS containing the protease inhibitors, and the homogenate was centrifuged as described in the first extraction. The resultant supernatant was pooled with the supernatant obtained from the first extraction.

*Precipitation with Ammonium Sulfate (Step 2).* The pooled supernatant was adjusted with saturated ammonium sulfate solution to make a 60% solution of  $(\text{NH}_4)_2\text{SO}_4$  and allowed to equilibrate overnight. Protein precipitates were sedimented by centrifugation at 12,000 *g*. After discarding the supernatant, the precipitates were dissolved with a minimum amount of PBS. The protein solution was then dialyzed against PBS overnight and against 20 mM sodium phosphate buffer, pH 8.0, for 2 d with multiple changes of buffer.

*Anion Exchange Chromatography (Step 3).* The nondialyzable material obtained by step 2 was concentrated to ~25 ml by an Amicon ultrafiltration unit (Amicon Corp., Scientific Sys. Div., Lexington, MA) fitted with a PM 10 membrane. Anion exchange diethylaminoethyl cellulose (DE 52) was obtained from Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England. Buffer equilibration, removal of fines, and column packing were performed according to the instructions provided by the manufacturer. The cellulose was equilibrated with 20 mM phosphate buffer, pH 8.0. A glass column with internal 25-mm Diam was packed with the equilibrated cellulose to a height of 300 mm. The concentrate from the Amicon ultrafiltration unit was clarified by centrifugation at 12,000 *g*. The resultant supernatant was applied onto the DE 52 cellulose column. A flow rate of 30 ml/h was established. The absorbance of the effluent was determined at 280 nm using a Hitachi spectrophotometer (Hitachi Ltd., Tokyo, Japan). After the pass-through peak, a crude fraction was obtained by using one-step elution method with a solution of 0.2 M NaCl without phosphate buffer. The eluted fraction thus obtained was subsequently dialyzed for 2 d against 20 mM sodium phosphate buffer, pH 7.0, containing 0.9% NaCl with several changes of buffer.

*Concanavalin A (Con A) Affinity Chromatography (Step 4).* The nondialyzable material obtained by step 3 was concentrated to ~25 ml by an Amicon ultrafiltration unit fitted with a PM 10 membrane. The concentrate was clarified by centrifugation at 12,000 *g*. The resultant supernatant was applied onto a Con A-Sepharose 4B column prepared as follows. Con A covalently bound to Sepharose 4B was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden. 1 ml of packed Sepharose 4B contained ~10 mg of Con A. 10 ml of Con A-Sepharose 4B was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.9% NaCl and packed into a glass column (10 × 100 mm). The equilibrated protein concentrate was allowed to pass through the Con A-Sepharose 4B column at a flow rate of 5 ml/h. The column was then washed with the same buffer until the absorption of the effluent at 280 nm decreased to almost the buffer level. The absorbed glycoproteins were eluted by the addition of the same buffer containing 100 mM  $\alpha$ -methyl-D-mannoside. The eluted glycoproteins were dialyzed against 10 mM phosphate buffer containing 1 M NaCl, pH 6.9, for 2 d with several changes of buffer. The nondialyzable glycoproteins were concentrated by an Amicon ultrafiltration unit fitted with a PM 10 membrane.

*Sephacryl S-300 Gel Filtration (Step 5).* A glass column was packed with Sephacryl S-300 gel (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) that had been equilibrated with 10 mM phosphate buffer containing 1 M NaCl, pH 6.9. The dimensions of the gel bed were 25 × 920 mm. The void volume of the column was determined by blue dextran 2,000. Four proteins obtained from Pharmacia Fine Chemicals were used to calibrate the column: thyroglobulin (669,000 mol wt), ferritin (440,000 mol wt), aldolase (158,000 mol wt), and chymotrypsinogen A (25,000 mol wt). After clarification by centrifugation, 5 ml of the glycoprotein concentrate obtained by step 4 was applied onto the column and allowed to pass through the column at a flow rate of 20 ml/h. The absorption of the effluent was measured at 280 nm. Tubes of individual protein peak were pooled and dialyzed against PBS for 2 d with several changes of

buffer. The nondialyzable fractions were concentrated over Amicon PM 10 membrane and clarified by centrifugation.

*Discontinuous Polyacrylamide Gel Electrophoresis (PAGE) (Step 6).* The dialyzed concentrates obtained by step 5 were analyzed and fractionated by discontinuous PAGE without sodium dodecyl sulfate. The procedure used was essentially the same as that described by Davis (11) but without the sample gel. 100–200  $\mu$ g of protein samples were subjected to electrophoresis in 5.25% separating gel. After adjustment to 15% (vol/vol) glycerol and 0.1% bromophenol blue, the samples were layered on top of a pH 6.9 spacer gel. Current of 1–2 mA per tube was used initially. After the samples had entered the spacer gel, the current was maintained at 7 mA per tube. At the completion of the electrophoretic run, the gels were removed from the tubes and rinsed with distilled water. They were frozen briefly and sliced into 2–3-mm segments. Duplicate gels were stained for proteins with 0.05% Coomassie Blue and for glycoproteins by the periodic acid-Schiff procedure (12). Proteins were extracted from the unstained gel segments with PBS by homogenizing the identical segments from several gels in a small glass homogenizer. Protein fractions thus obtained were concentrated and analyzed again by disc PAGE. An apparent highly purified glycoprotein was isolated and designated as gp 340K.

*Isolation of Antibody-Antigen Complexes with Immobilized Protein A.* Protein A, obtained from the cell wall of *Staphylococcus aureus*, is known to interact with the Fc part of the IgG molecules of several animal species, including rabbit. Rabbit IgG molecules were purified from the teratogenic antiserum directed against antigen gp 340K by precipitation at 33% ammonium sulfate saturation, pH 7.8, and subsequently by DE 52 ion-exchange cellulose equilibrated with 17.5 mM phosphate buffer, pH 6.3. The rabbit IgG molecules were obtained from the pass-through peak of the DE 52 cellulose column, and their purity was monitored by disc PAGE. Protein A covalently coupled to Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, which contained ~2 mg protein A per ml of Sepharose gel. After both the rabbit IgG molecules (20 mg) and protein A-Sepharose CL-4B (3 ml) had been equilibrated with 10 mM phosphate buffer, pH 8.0, the IgG molecules were allowed to adsorb onto the immobilized protein A column. After the column had been extensively washed with PBS, 50 mg of PBS-soluble rat kidney supernatant obtained after centrifugation at 105,000 *g* (step 1) was allowed to pass through the column at a flow rate of 5 ml/h. The column was then washed with PBS until the absorbance at 280 nm reached almost the baseline (0.08) level. The immunocomplexes were eluted from the column by the addition of 0.58% glacial acetic acid containing 0.15 M NaCl, pH 2.8, and were immediately dialyzed against several changes of PBS. The immunoprecipitates were recovered by centrifugation and stored at  $-10^{\circ}\text{C}$  to be used as immunogens.

*Production of Antisera.* New Zealand white female rabbits weighing ~2.5 kg each were used for the production of antisera by the method previously reported (13). Antisera were produced against the five protein fractions (A to E) obtained by Sephacryl S-300 gel filtration (step 5) and against proteins extracted from the unstained gels (step 6), including the gp 340K. Antiserum was also produced against the immunocomplexes obtained from the immobilized protein A column. Protein concentration of the antigens was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). A total of ~4 mg of protein each from various fractions, disc PAGE extractions, and immunocomplexes was used to immunize individual rabbits. Protein solutions or immunocomplex suspensions were emulsified with equal volume of complete Freund's adjuvant (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). Rabbits were initially immunized once in the footpads. Subsequent subcutaneous multiple-site injections were given weekly for 6–7 wk. Rabbits were bled weekly from the central artery of the ear after the fourth injection. 1 wk after the last injection, the rabbits were exsanguinated by cardiac puncture. Sera from multiple bleedings were pooled, decanted at  $56^{\circ}\text{C}$  for 30 min, and stored at  $-20^{\circ}\text{C}$ . Antibody titers of the antisera were determined by serial dilutions, and immunoprecipitation was observed by double immunodiffusion in agar gel. Only antisera of high antibody titers were used. Control sera were obtained from the rabbits before immunization.

*Immunodiffusion.* Double immunodiffusion was performed in petri dishes containing 1.0% agar gel in PBS, pH 7.3. Merthiolate or  $\text{NaN}_3$  was added as a bacteriostatic agent. Each well was of 2-mm Diam. All wells were 5 mm from each other, center to center. The gel dishes were kept in a humidified chamber at room temperature and observed for 2–3 d. After optimum

precipitin lines had developed, the gels were removed and washed in PBS for 3 d, dried, and stained with 1% amido black for proteins. The Sudan black B staining method described by Barka and Anderson (14) was used to stain dried gel diffusion slides for lipoprotein antigens. After destaining in methanol, the precipitin lines were photographed.

*Immuno-electrophoresis.* The procedure was essentially the same as that described by Garvey and his colleagues (15). After electrophoresis of the antigenic protein solution in the center well, teratogenic antiserum against gp 340K was added to the troughs. After the precipitation pattern had been developed, the slide was washed in PBS for 3 d with several changes of PBS. The slide was then dried, stained with 1% amido black, and photographed.

*Evaluation of Antisera on Embryonic Development.* The biologic effects of the antisera on embryonic development were evaluated in the following manner. On the 9th d of gestation, pregnant rats were injected intraperitoneally with various dosages of antisera. Control pregnant rats were injected similarly with preimmunization rabbit serum. All rats were anesthetized with sodium pentobarbital (30 mg/kg) before intraperitoneal injection. The mothers were killed on the 22nd d of gestation, and the fetuses were delivered by cesarean section. The fetuses were examined grossly, weighed, and fixed in Bouin's fixative for dissection. The incidence of malformations was determined by the cross-sectional technique described by Wilson (16). Three 9th d pregnant rats were injected with 0.5 ml of gp 340K antiserum per 100 g of animal weight and were killed 24 h later. Egg cylinders were subsequently dissected out from the uteri under a dissecting microscope, and individual lengths of the egg cylinders were measured. Control egg cylinders were likewise obtained from three pregnant rats injected with preimmunization rabbit serum and their measurements were recorded.

*Immunofluorescent Localization Studies.* After the intraperitoneal injection of the teratogenic antiserum directed against gp 340K into pregnant rats on the 9th d of pregnancy, the mothers were killed 24 or 48 h later. Maternal kidneys and embryonic tissues were examined for the *in vivo* localization of the injected antibodies with the direct fluorescent antibody technique as described previously (13). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. Absorption of the FITC-goat antibodies was performed twice by using 100 mg of lyophilized Wistar rat liver powder per ml of reconstituted FITC-goat antibodies for 1 h at room temperature. The absorbed FITC-goat antibodies were diluted 20-fold before use for staining. *In vitro* localization of antibodies in the rabbit antiserum directed against gp 340K was also performed on 4- $\mu$ m frozen sections of embryonic and kidney tissues obtained from untreated rats. The rabbit antiserum was twice absorbed with lyophilized rat liver powder and diluted 20-fold before use for staining the tissue sections. Examination of the stained sections were made using a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ) with an XBO ultraviolet lamp. Photographs were taken with Kodak Tri-X (ASA 400) film (Eastman Kodak Co., Rochester, NY).

## Results

*Isolation and Characterization of the Antigen.* The PBS-soluble rat kidney antigen was purified by ammonium sulfate precipitation, DE 52 cellulose chromatography, Con A affinity column, Sephacryl S-300 gel filtration, and discontinuous PAGE. Sephacryl S-300 gel filtration resolved the glycoproteins eluted from the Con A affinity column into five fractions (Fig. 1). The results of Coomassie Blue staining of disc PAGE gel of each fraction are shown in Fig. 2. All of the protein bands in each fraction were positive when stained with periodic acid-Schiff reagent. This result confirmed that all the proteins isolated by Con A affinity column were glycoproteins. The Coomassie Blue-stained gels indicated that peak A contained large aggregates of proteins that could not penetrate into the separating gel (Fig. 2). Peak B contained two distinct glycoprotein bands. Peak C contained one major broad band with some minor contamination from peak B. When antigens were extracted from regions b and c of the unstained gels B and C (Fig. 2) with PBS and reelectrophoresed by disc PAGE,

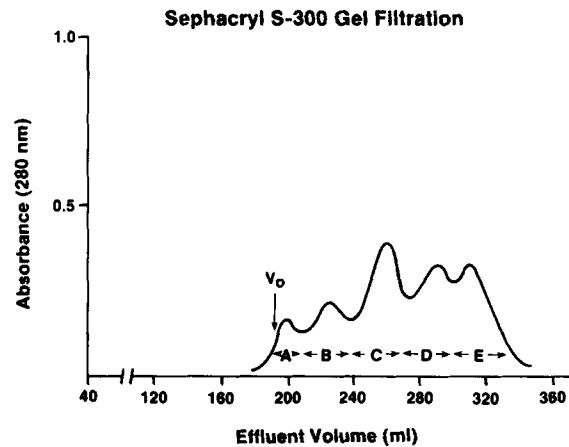


FIG. 1. A representative profile of Sephacryl S-300 gel filtration of the glycoproteins eluted from the Con A affinity column.  $V_0$  indicates the void volume as determined by blue dextran 2,000. Five fractions (A to E) were obtained.

the results indicated that region b contained two distinct bands (not shown), while one single band existed in region c (Fig. 3). The molecular weight of the major protein (gp 340K) residing in peak C of Sephacryl S-300 gel filtration was estimated to be ~340,000. This glycoprotein (gp 340K) exhibited an  $\alpha_2$ -globulin mobility in disc PAGE; it ran behind transferrin. However, the result of immunoelectrophoresis using PBS-soluble kidney supernatant (step 1) as the antigenic solution indicated that it had a mobility between  $\beta_1$ -globulin and  $\alpha_2$ -globulin. Ouchterlony diffusion analysis indicated that a single immunoprecipitin band was observed when gp 340K antiserum diffused against the crude PBS-soluble rat kidney homogenate (Fig. 4). This immunoprecipitin band failed to stain by Sudan black B, indicating the absence of lipoprotein. A single immunoprecipitin line was also observed when antiserum to the immunocomplexes diffused against PBS-soluble rat kidney homogenate, and it was in complete identity with that formed by gp 340K antiserum.

*Evaluation of Effects of Antisera on Embryonic Development.* The results of testing for the biologic effects of the antisera against various fractions are summarized in Table I. The antisera against the first three peaks (A, B, and C) of the Sephacryl S-300 gel filtration (Fig. 1) demonstrated that they were all teratogenic, whereas antisera against antigens in peak D and E seemed to have little effect on embryonic development. The antisera against proteins extracted from the unstained disc gels (region b) of peak B did not seem to have any effect on embryonic development. On the contrary, the antiserum against gp 340K extracted from the unstained disc gels (region c) from peak C was very potent embryotoxic agent. All the conceptuses were resorbed when the mothers were injected with 1 ml/100 g of animal weight. The antiserum induced birth defects in all of the surviving fetuses when injected with a dosage of 0.25 ml/100 g of animal weight. The antiserum directed against the immunocomplexes was also teratogenic: injection of 1.0 ml of antiserum per 100 g rat resulted in >80% of the surviving fetuses being malformed. All of the embryotoxic antisera tested in this study induced fetal growth retardation, congenital malformations, and embryonic death. The term fetuses whose mothers were injected with embryotoxic antisera had a much lower mean weight than those whose mothers were exposed to preimmunization sera.

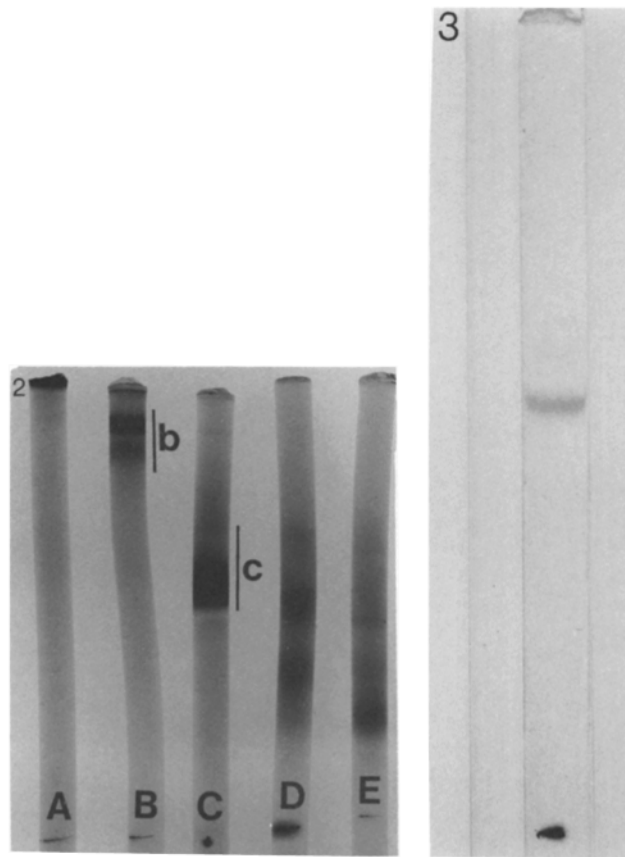


FIG. 2. Discontinuous PAGE of the five fractions (A to E) obtained by Sephacryl S-300 gel filtration. Antigens were extracted from unstained segments marked b and c from B and C, respectively.

FIG. 3. Reelectrophoresis by disc PAGE of the major glycoprotein (gp 340K) extracted from c (Fig. 2).

The biologic effects of the embryotoxic antisera appeared to be dose dependent. Defects such as anophthalmia, hydrocephaly, exencephaly, cleft palate, cleft lip, and some cardiovascular anomalies were observed. Nevertheless the most common anomaly was anophthalmia. Some of the mothers injected with lower dosage of the antiserum were allowed to deliver their abnormal offspring. Many of those with no other major abnormalities except eye defects survived and grew up to be adults of normal size and weight. The effect of the antiserum against gp 340K on the growth of the embryos at the egg cylinder stage was apparent: 24 h after the administration of the antiserum, the mean size (1.04 mm) of 36 egg cylinders was almost one-half that (1.82 mm) of the 27 controls.

*In Vivo Localization of Antibodies to Gp 340K.* Specific immunofluorescent localization of teratogenic antibodies against gp 340K was observed in the visceral yolk sac endodermal cells and embryonic endoderm of 10th d gestational age, that is, 24 h after the administration of the antiserum (Fig. 5). Similar staining was also observed in the visceral yolk sac endodermal cells 48 h after the administration of the teratogenic

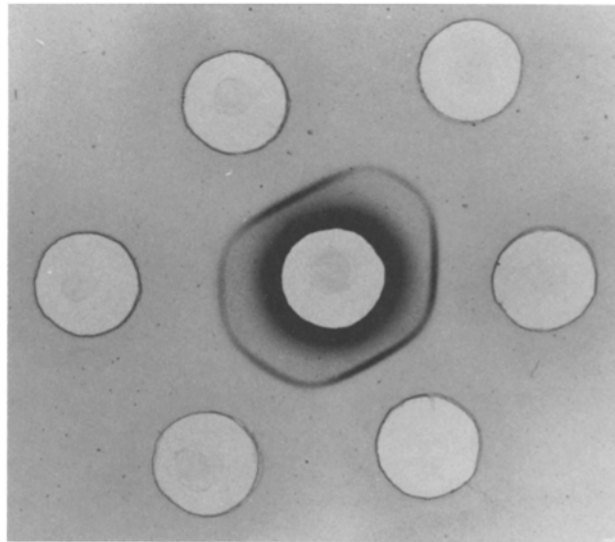


FIG. 4. Ouchterlony gel diffusion analysis. Peripheral wells contained antiserum against gp 340K. Central well contained PBS-soluble rat kidney homogenate supernatant (30 mg/ml).

TABLE I  
*Effect of Antisera on Embryonic Development*

| Rabbit antiserum               | Number of litters | ml anti-serum/<br>100 g pregnant rat | Embryonic re-sorption | Surviving fetuses |                             |                           | Egg cylinders |             |
|--------------------------------|-------------------|--------------------------------------|-----------------------|-------------------|-----------------------------|---------------------------|---------------|-------------|
|                                |                   |                                      |                       | Num-ber           | Percent with malfor-mations | Mean fetal weight at term | Num-ber       | Mean length |
|                                |                   |                                      | %                     |                   |                             | g                         |               | mm          |
| Sephacryl peak A               | S-300             | 4                                    | 1.0                   | 17                | 44                          | 100                       | 3.28          |             |
| Sephacryl peak B               | S-300             | 5                                    | 1.0                   | 15                | 51                          | 96                        | 3.75          |             |
| Sephacryl peak C               | S-300             | 5                                    | 1.0                   | 10                | 43                          | 100                       | 3.24          |             |
| Sephacryl peak D               | S-300             | 5                                    | 1.0                   | 10                | 43                          | 0                         | 4.63          |             |
| Sephacryl peak E               | S-300             | 5                                    | 1.0                   | 8                 | 59                          | 2                         | 4.70          |             |
| Disc PAGE region b             | 4                 | 1.0                                  | 12                    | 56                | 0                           | 4.65                      |               |             |
| Disc PAGE region c:<br>gp 340K | 2                 | 1.0                                  | 100                   |                   |                             |                           |               |             |
|                                | 4                 | 0.5                                  | 44                    | 31                | 90                          | 4.02                      |               |             |
|                                | 3                 | 0.5                                  |                       |                   |                             |                           | 36            | 1.04        |
|                                | 5                 | 0.25                                 | 17                    | 39                | 100                         | 3.51                      |               |             |
| Immunocomplexes                | 5                 | 1.0                                  | 15                    | 57                | 86                          | 3.29                      |               |             |
| Control preimmunization serum  | 6                 | 1.0                                  | 12                    | 60                | 2                           | 4.68                      |               |             |
|                                | 3                 | 1.0                                  |                       |                   |                             |                           | 27            | 1.82        |

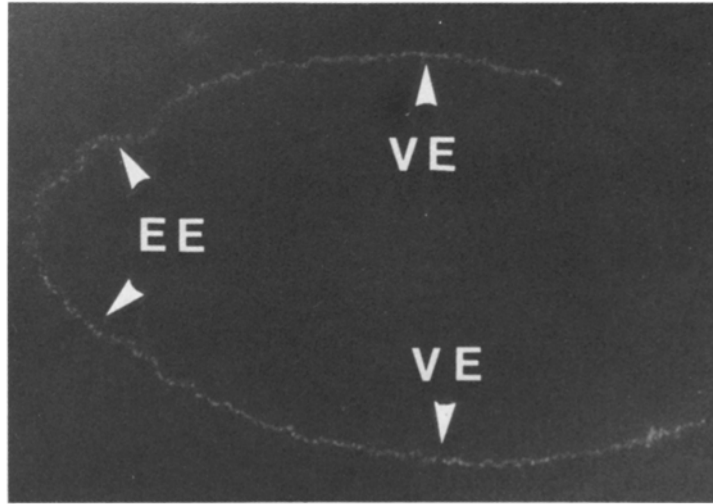


FIG. 5. Specific immunofluorescent localization of teratogenic gp 340K antibodies. The mother was injected with 0.5 ml of rabbit gp 340K antiserum on the 9th d of gestation. The embryonic sites were examined 24 h later. The visceral yolk-sac endoderm (VE) is continuous with the embryonic endoderm (EE) at this stage of embryonic development. Both structures (VE and EE) of the egg cylinder were stained. To the right of the VE was the ectoplacental cone that was not stained.  $\times 75$ .

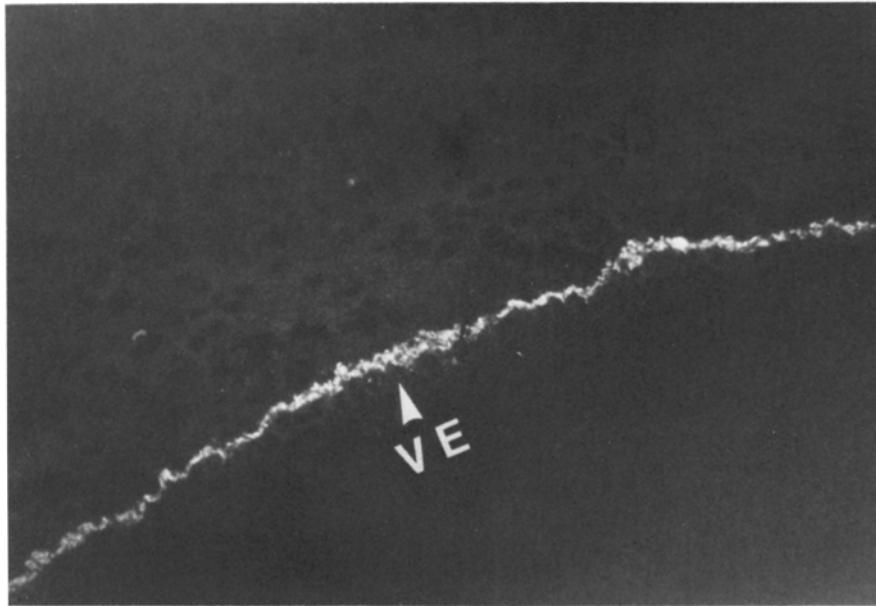


FIG. 6. Specific immunofluorescent localization of rabbit gp 340K antibodies. The mother was injected with 0.5 ml antiserum on the 9th d of gestation. Tissue was examined on the 11th d of gestation. Granular staining was observed at the apical part of the visceral yolk-sac endodermal cells (VE). At this stage of embryonic development, there was little or not staining in the embryonic endoderm. Maternal tissue is shown above the VE.  $\times 200$ .



antiserum (Fig. 6); granular staining was observed at the apical portion of the cells. Reichert's membrane (the basal lamina of the parietal yolk sac) was not stained. Furthermore, there was no antibody localization in the maternal kidney tissue. There was no immunofluorescent staining in the visceral yolk sac and embryonic endoderm obtained from control pregnant rats injected with preimmunization rabbit serum.

*In Vitro Localization of Antibodies to Gp 340K.* Specific immunofluorescent staining was observed in the visceral yolk-sac endoderm (Fig. 7) of all gestational ages examined (9th–20th d) and in the embryonic endoderm of 10th d gestational age. The staining appeared to be along the external plasma membrane of the cells. There was no staining in the basal lamina and mesenchyme of the visceral yolk sac, mesothelial cells lining the exocoelom, blood vessels, Reichert's membrane, parietal yolk-sac cells, or trophoblast cells. Specific fluorescent staining was observed in the proximal convoluted tubular cells of the rat kidney (Fig. 8). There was no staining in the renal glomeruli, blood vessels or other renal tissues.

### Discussion

Since the discovery of this interesting model in which abnormal embryonic development can be induced by antibodies, little progress has been made in the past two decades in the area of identifying the antigen(s) that elicits the production of monospecific teratogenic antibodies. This apparent slow progress was primarily caused by the inherent difficulties involved in this teratogenic model. There was no *in vitro* method to substitute the time-consuming bioassay using the incidence of malformations as the end point. To purify the responsible antigen(s), every fraction or subfraction of separated proteins had to be injected into rabbits for several weeks to produce antisera. The resulting antisera had to be tested in time-pregnant rats to determine which fraction or subfraction contained the responsible antigenic molecules. This procedure, involving the production of antisera and subsequent bioassay of the antisera, was the only method available. Further complicating the situation was the fact that some rabbits did not respond to antigenic stimulation as well as others; only antisera of proven high antibody titer could be used. It was not uncommon that an assay was not completed for 6 mo or more.

Although there might be more than one kidney antigen that is responsible for the production of teratogenic antibodies, we isolated a highly purified large molecular weight protein that elicited teratogenic antibody production. The molecule appeared to be a glycoprotein because it interacted with Con A and was PAS positive. It was neither a lipoprotein, because it was not stained by Sudan black B, nor a glycoaminoglycan (data not shown) when determined by the alcian blue method (17). The molecular weight of the glycoprotein was estimated by gel filtration to be 340,000; the actual molecular weight might be somewhat lower than this because glycoproteins exhibit abnormal behavior in gel filtration (18). The isolated molecule had an  $\alpha_2$  electrophoretic mobility in disc PAGE. However, a much broader electrophoretic pattern ( $\beta_1$ – $\alpha_2$ ) was observed when crude PBS-soluble kidney supernatant was examined by immunoelectrophoresis. It is possible that under this condition the gp 340K might be associated with other molecules with a slower mobility. It is also possible that this molecule might exhibit microheterogeneity as a result of the loss of sialic acid, or it might be an aggregate of smaller molecules. Preliminary data obtained

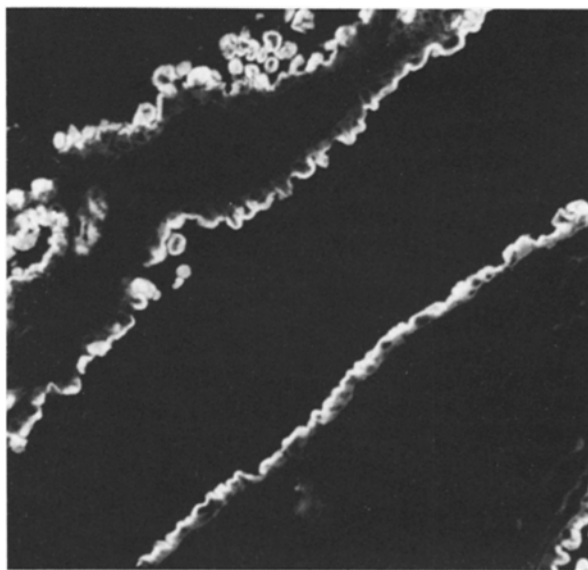


FIG. 7. In vitro immunofluorescent staining of rabbit gp 340K antibodies. Visceral yolk-sac tissue was obtained at the 14th d of gestation. Staining appeared to be along the apical plasma membrane of the visceral yolk-sac endodermal cells; some cytoplasmic staining was also observed. Similar staining was observed on the visceral yolk-sac tissue obtained at the 9th to 20th d of gestation.  $\times 150$ .

from preparative isoelectric focusing of the fraction eluted from the Con A column (step 4) suggested that the gp 340K had isoelectric points ranging from 5.6 to 6.4.

Edgington and his colleagues (19, 20) isolated three rat renal tubular epithelial antigens. RTE $\alpha_3$  and RTE $\alpha_4$  (19) were soluble only after treatment with sodium deoxycholate; RTE $\alpha_5$ , a nephritogenic lipoprotein antigen, was not associated with saline soluble fraction (20). Using pronase treatment, Naruse and his colleagues (21) isolated a rat renal tubular glycoprotein that was also nephritogenic. It is difficult to relate our gp 340K with the antigens isolated by the latter two groups of investigators because different methods were used. The gp 340K was saline soluble, and it was also present in the saline-insoluble fraction (10); it was isolated without using any detergents or enzymes. It is possible that the gp 340K and those antigens isolated by Edgington et al. and Naruse et al. might contain some common antigenic determinants.

The mechanism of action of teratogenic antibodies is not understood. Three hypotheses have been proposed (22). They are (a) secondary effect due to immunologic disease of the mother, (b) a direct effect of antibodies on the embryo proper, and (c) yolk sac placenta dysfunction.

The first hypothesis assumes that birth defects are the results of maternal immunologic disease. For example, heterologous antiserum directed against rat kidney homogenate is known to be nephrotoxic (23) and would induce "Masugi" nephritis that resembles the pathology of human glomerulonephritis. Strong evidence has been presented (13, 24) to support the view that teratogenicity and nephrotoxicity of kidney antisera are of separate biologic properties. The nephrotoxicity of the antisera did not parallel their teratogenicity (13, 24). The result of our present investigation on the in

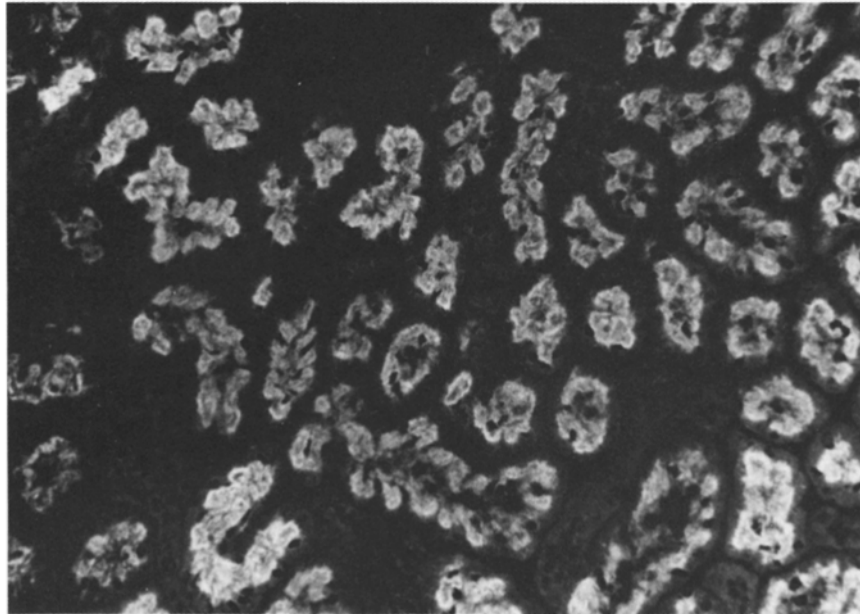


FIG. 8. In vitro immunofluorescent staining of rabbit gp 340K antibodies on the rat renal tubular cells. Cytoplasmic staining of the tubular cells was observed. The nuclei were not stained. The glomeruli and blood vessels were not stained.  $\times 150$ .

vivo immunofluorescent localization of teratogenic gp 340K antibodies indicated that the antibodies did not localize in the maternal kidney. This finding adds further support to the contention that birth defects induced by antibodies may not result from maternal kidney immunologic disease but does not exclude the possibility that malformations could result from other maternal immunologic disease.

The question whether heterologous gp 340K antibodies ever reach the embryo during the period of organogenesis is of obvious importance. Immunofluorescent localization studies demonstrated that gp 340K antibodies localized in the embryonic endoderm as well as the visceral yolk-sac endoderm at the 10th d of gestation. The importance of antibody localization in the embryonic endoderm related to embryonic development cannot be ignored, although such localization could no longer be observed in embryos of later stages. The consistent localization of gp 340K antibodies in the visceral yolk-sac endodermal cells from the 10th d to 12th d of gestation may play an important role in inducing abnormal embryonic development. The fixation of gp 340K antibodies in the visceral yolk-sac endodermal cells may interfere with normal transport or histiotrophic functions of visceral yolk-sac epithelium because the rat visceral yolk sac has been regarded as an organ for active transport of nutrient to the embryo as well as respiratory exchange between the mother and the conceptus, especially before the establishment of the chorioallantoic placenta on the 12th d of gestation.

It is conceivable that one might be able to induce rats or mice to produce teratogenic autoantibodies after injections of large amounts of gp 340K. Johnson and his colleagues (25) recently reported that rats, when injected with mouse nerve-growth factor, produced antibodies that reached the developing offspring and resulted in the

destruction of dorsal root ganglion neurons. Experiments of this nature are being considered.

### Summary

A glycoprotein with an apparent 340,000 mol wt (gp 340K) was isolated from rat kidney saline-soluble extract by ammonium sulfate precipitation, DE 52 ion-exchange cellulose chromatography, concanavalin A affinity column, Sephacryl S-300 gel filtration, and discontinuous polyacrylamide gel electrophoresis (PAGE). The relative purity of gp 340K was examined by double immunodiffusion analysis, disc PAGE, and immunoelectrophoresis. Injection of rabbit gp 340K antiserum into pregnant rats during the organogenetic period induced abnormal embryonic development, fetal growth retardation, and embryonic death. Antiserum against the immunocomplexes isolated by immobilized protein A also produced the same embryotoxic effects. The biologic effects of the antisera appeared to be dose dependent. Defects such as anophthalmia, hydrocephaly, exencephaly, cleft palate, cleft lip, and some cardiovascular anomalies were observed. The most frequently observed anomaly was anophthalmia. Immunofluorescent localization studies indicated that gp 340K antibodies localized in vivo in the visceral yolk-sac endodermal cells and the embryonic endoderm. In vitro immunofluorescent localization studies revealed that gp 340K was a component of the renal tubular cells that cross-reacted with antigen in the visceral yolk-sac endodermal cells and embryonic endoderm. The underlying mechanism whereby gp 340K antibodies induce birth defects is not known. Three hypotheses were discussed.

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### References

1. Brent, R. L., E. Averich, and V. A. Drapiewski. 1961. Production of congenital malformations using tissue antibodies. I. Kidney antisera. *Proc. Soc. Exp. Biol. Med.* **106**:523.
2. David, G., L. Mercier-Parot, and H. Tuchmann-Duplessis. 1963. Action teratogene d'heteroanticorps tissulaires. I. Production de malformations chez le rat par action d'un serum antirein. *C. R. Seances Soc. Biol. Fil.* **157**:939.
3. Mikhailov, V. M. 1976. Pathogenic action of nephrocytotoxic serum on embryonic development of albino rats. *Biul. Eksp. Biol. Med.* **63**:97.
4. Gebhardt, D. O. E., E. J. Baart de la Faille-Kuyper, and I. Nagel. 1970. The embryoletality and localization of antikidney serum in the pregnant mouse, *Mus musculus*. *Teratology*. **3**:143.
5. Barrow, M. V., and W. J. Taylor. 1971. The production of congenital defects in rats using antisera. *J. Exp. Zool.* **176**:41.
6. Vaillancourt, P., and D. J. McCallion. 1972. Inhibitory effects of nephrotoxic antisera on the growth of rat fetuses. *Am. J. Obstet. Gynecol.* **114**:255.
7. Hefton, J. M., and R. L. Brent. 1970. Characterization of 7 S molecular fractions of teratogenic antiserum. *Teratology*. **3**:202 (Abstr.)
8. Bragonier, J. R., M. M. Frank, and R. L. Brent. 1970. Production of congenital malformations using tissue antisera. VIII. Effectiveness of reduced, alkylated and digested anti-kidney antibodies. *J. Immunol.* **105**:1175.
9. Leung, C. C. K., C. H. Hung, B. G. Hudson, R. L. Brent, and R. C. Cotton. 1979.

- Congenital abnormalities induced by heterologous antisera directed against rat kidney glycoproteins isolated by concanavalin A affinity chromatography. *Pediatr. Res.* **13**:1211.
10. Leung, C. C. K., and R. L. Brent. 1980. Partial purification and characterization of trypsin-solubilized rat kidney antigens that stimulate the production of teratogenic antibodies. *J. Immunol.* **124**:1267.
  11. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404.
  12. Fairbanks, G., T. L. Stick, and D. F. H. Wallach. 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10**:2606.
  13. Leung, C. C. K., A. Urdaneta, R. P. Jensh, M. Jensen, and R. L. Brent. 1974. Evidence that different antibodies are involved in the production of immunologically induced teratogenesis and nephritis. *J. Immunol.* **113**:885.
  14. Barka, T., and P. J. Anderson. 1963. Lipids. In *Histochemistry, Theory, Practice, and Bibliography*. Harper and Row Publishers, Inc., New York. Chapter 5. 120.
  15. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Gel Electrophoresis. In *Methods in Immunology*. 3rd edition. The Benjamin Co., Inc., New York. 328.
  16. Wilson, J. G. 1964. Methods for administering agents and detecting malformations in experimental animals. In *Teratology: Principles and Techniques*. J. W. Wilson and J. Warkary, editors. University of Chicago Press, Chicago, Illinois. 251.
  17. Gold, E. W. 1979. A simple spectrophotometric method for estimating glycosaminoglycan concentrations. *Anal. Biochem.* **99**:183.
  18. Andrew, P. 1965. The gel-filtration behaviour of proteins related to the molecular weights over a wide range. *Biochem. J.* **96**:595.
  19. Edgington, T. S., R. J. Glassock, J. I. Watson, and F. J. Dixon. 1967. Characterization and isolation of specific renal tubular epithelial antigens. *J. Immunol.* **99**:1199.
  20. Edgington, T. S., R. J. Glassock, and F. J. Dixon. 1968. Autologous immune complex nephritis induced with renal tubular antigen. I. Identification and isolation of the pathogenic antigen. *J. Exp. Med.* **127**:555.
  21. Naruse, T., T. Fukasawa, N. Hirokawa, S. Oike, and Y. Miyakawa. 1976. The pathogenesis of experimental membranous glomerulonephritis induced with homologous nephritogenic tubular antigen. *J. Exp. Med.* **144**:1347.
  22. Brent, R. L. 1964. The production of congenital malformations using tissue antisera. II. The spectrum and incidence of malformations following the administration of kidney antiserum to pregnant rats. *Am. J. Anat.* **115**:525.
  23. Unanue, E. R., and F. J. Dixon. 1967. Experimental glomerulonephritis: immunological events and pathogenic mechanisms. In *Advances in Immunology*. F. J. Dixon and J. H. Humphrey, editor. Academic Press, Inc., New York. 1.
  24. Leung, C. C. K., and R. L. Brent. 1972. The production of congenital malformations using tissue antisera. X. Effectiveness of kidney antigens treated with neuraminidase or trypsin. *Pediatr. Res.* **6**:822.
  25. Johnson, E. M., P. D. Gorin, L. D. Brandeis, and J. Pearson. 1980. Dorsal root ganglion neurons are destroyed by exposure *in utero* to maternal antibody to nerve growth factor. *Science (Wash. D. C.)*. **210**:916.