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The Role of Circulating Antigen in the Formation of Immune Deposits in Experimental Membranous Nephropathy (42430)

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Abstract. To investigate the role of circulating antigen in the formation of subepithelial immune deposits in the Heymann rat model of membranous nephropathy, the renal uptake and site of renal deposition of intravenously injected renal tubular antigen (F×1A) was studied. F×1A, (15, 30, 60, and 600 μg) radiolabeled with ^{125}I, and bovine serum albumin (BSA; 15 μg) labeled with ^{131}I were intravenously injected into naive rats. Plasma clearance and organ uptake of brush border antigens were determined. Of the injected F×1A, 75% was cleared from the circulation by 1 hr as compared to 10% of the BSA. Uptake of F×1A by heart, lung, and spleen was less than 1% at all doses studied. Renal uptake of F×1A (29.8 μg/g tissue) was greater than that for liver (4.75 μg/g), spleen, heart, and lung (each less than 1 μg/g tissue). Evaluation of washed renal homogenate and isolated glomeruli confirmed specific tissue-associated F×1A antigen. Direct immunofluorescence demonstrated deposits of F×1A antigen along the glomerular capillary wall in animals injected with F×1A. Small scattered electron dense deposits were demonstrated in the subepithelial space. Similar binding could be reproduced *in vitro* by incubating cryostat sections of normal rat kidney or isolated glomeruli with solubilized F×1A antigens. Direct binding of a tubular antigen to a constituent of the glomerulus could initiate *in situ* immune complex formation, and may explain the variably demonstrable "cross-reactivity" of the Heymann antibody with the glomerular capillary wall.

Autologous immune complex nephritis in rats (Heymann nephritis) is a well-characterized animal model of membranous nephropathy (1). Animals given a single injection of crude kidney homogenate or more purified preparations of the brush border of the proximal renal tubule develop antibody to a tubular antigen (2–4). This is followed by subepithelial immune deposit formation in the glomerulus. Approximately 8 weeks following immunization, the animals develop proteinuria and clinical nephrotic syndrome. Initial studies of the pathogenic mechanism indicated that brush border antigen combined with autoantibody to form circulating immune complexes that deposited in the glomerulus and caused disease (5). Using heterologous antiserum to F×1A (2), a crude preparation of the brush border antigen, Van Damme *et al.* (6), and Couser and his colleagues (7), demonstrated direct binding of antiserum to a proposed intrinsic component of the glomerular capillary wall. These observations led to the hypothesis that immune deposits in Heymann nephritis resulted only from *in situ* formation, namely, cross-reactivity of antibody to the renal tubule with a glomerular antigen. Recent studies have provided data which support both circulating immune complex (8–10) and *in situ* (9, 11, 12) mechanisms in the formation of subepithelial deposits. The precise role of each mechanism in the initiation and perpetuation of the disease remains controversial. In addition, it has recently been suggested that more than one antigen may be involved in the disease (9, 13, 14). Thus, further study is necessary to define all of the factors that contribute to subepithelial deposit formation.

In a previous study from this laboratory (10), alterations in mononuclear phagocyte activity were shown to correspond with the amount of glomerular immune complex deposition in active Heymann nephritis. Organ uptake of immune complexes of brush border antigen and its antibody was determined as a measure of the efficiency of removal of these complexes by phagocytes. As controls, these experiments included normal animals and measurements of uptake of complexes and free antigen by organs other than the liver and spleen. Unexpectedly, it was noted that renal uptake of a brush border antigen was greater

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than that previously reported for other injected materials (15–17). Further evaluation of the renal uptake of injected brush border antigens is the subject of this report.

Materials and Methods. *Reagents.* The following materials were purchased from the manufacturer: male Lewis rats weighing 250 g (M. A. Bioproducts, Bethesda, Md.), Enzymobeads (Bio-Rad, Richmond, Calif.), ^{125}I, ^{131}I (New England Nuclear, Boston, Mass.), bovine serum albumin (Miles Laboratories, Elkhart, Ind.), ceruloplasmin (Sigma Chemical Co., St. Louis, Mo.), fluorescein-conjugated rabbit anti-rat IgG (Cappell Laboratories, Cochranville, Pa.). Sheep anti-rat F×1A was prepared, absorbed, and fluorescein conjugated as previously described (9). This antiserum has been extensively absorbed with glomeruli and anti-idiotypic antibody to anti-F×1A has been removed. It does not bind to normal rat glomeruli as detected by indirect immunofluorescence using cryostat sections of rat kidney or isolated glomeruli or by binding of radiolabeled antibody to isolated glomeruli. This is in contrast to the unabsorbed antiserum which also binds to gp330, the tubular antigen that is shared with the glomerulus.

Proximal renal tubular antigens were prepared as F×1A by the standard method (2). F×1A was then solubilized in 1% sodium deoxycholate–Tris-buffered saline. Following centrifugation at 105,000*g*, the supernatant was precipitated with 25% saturated ammonium sulfate, dialyzed against water, and lyophilized (2). This material was weighed and dissolved in phosphate-buffered saline (PBS), and clarified by centrifugation prior to radioiodination with ^{125}I using Enzymobeads by directions provided on the package insert. Free iodine was removed using a Bio-Rad P-6DG column. This preparation contains only those components of the particulate F×1A that are soluble in PBS. The composition of this material was assessed by polyacrylamide gel electrophoresis as described below and by isolectric focusing by published methods (18). Prior to injection, the radiolabeled F×1A was mixed with the appropriate amount of ^{131}I-labeled BSA that had been similarly prepared. In addition, these results were compared to organ uptake of radiolabeled ceruloplasmin and aggregated rat gamma globulin (8). Ceruloplasmin was selected for comparison to F×1A as it is a circulating glycoprotein of 160,000 Da with a pI of 4.4 (19) which is in the same range as the PBS-soluble components of F×1A (4). Dosages are given below.

Experimental procedures. Male Lewis rats weighing 250 g were divided into four groups of five each. All animals received 15 μg ^{131}I-labeled BSA. Simultaneously, animals received one of the following doses of ^{125}I-labeled F×1A: Group I, 15 μg; Group II, 30 μg; Group III, 60 μg: Group IV, 600 μg. In a separate experiment, organ uptake of F×1A was compared to the uptake of ceruloplasmin and heat-aggregated rat gamma globulin. Each rat (five rats per group) received 15 μg of the appropriate protein. In both experiments, 20 μl of blood was collected from the tip of the tail at 1, 3, 5, 8, 10, 20, 30, and 60 min following intravenous injection of the antigens. One hour after injection of material the animals were exsanguinated under ether anesthesia. Heart, lung, liver, spleen, and kidneys were removed, washed free of blood with cold isotonic saline, blotted dry, and weighed. The two kidneys were divided into four halves prior to weighing. One piece was prepared for histology as described below. The second piece was counted for total radioactivity. The third piece was homogenized in a Brinkman homogenizer for 10 sec at high speed, and washed four times with cold PBS. The homogenate was then counted for radioactivity. The fourth piece was minced in cold PBS, sedimented by centrifugation, and washed three times with cold PBS. Glomeruli were isolated by seiving as previously described (20). Isolated glomeruli were washed with PBS, the number of glomeruli was counted using a hemocytometer, and the associated radioactivity was determined. Blood and organ radioactivity were determined using a Packard multichannel gamma counter.

Organ bound counts were calculated using the paired-label formula previously described (21). Specific organ bound counts were expressed as a percentage of the injected dose, nanograms per gram of tissue, and micrograms per organ. The rate of plasma clearance of antigen was calculated as a percentage of the amount present at 1 min which was assigned the 100% value. Total counts were corrected for trichloroacetic acid precipitable counts.

Elution of glomerular-bound antigen. Following counting of radioactivity of the isolated

glomeruli, the glomeruli were eluted with 0.5 *M* NaCl, 0.2 *M* glycine buffer, pH 2.8, by stirring at room temperature (22). The eluate was collected after 1 hr and again after 5 hr. Of the bound radioactivity, 60% was recovered in the supernate after 1 hr of elution. Another 10% was recovered after 4 additional hr of elution. The eluates were restored to neutral pH and concentrated to 100-μl volumes. Samples were suspended 1:1 in 0.1 *M* Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 2% glycerol, and 1 *M* β-mercaptoethanol and heated to 100°C for 3 min. Gels were prepared with 5.5–10% polyacrylamide (2.67% crosslinking) containing 0.1% SDS and 0.5 *M* urea. The samples were electrophoresed at 30 mA constant current per gel for 4 hr using a standard Laemmli system of discontinuous buffers (23). Molecular weight standards (Bio-Rad) were simultaneously analyzed. The polypeptides were stained with silver stain (Bio-Rad). Serial 5-mm sections of each electrophoretic lane were counted for radioactivity. The glomerular eluate was compared to similarly treated starting material.

Histology. Portions of kidney were snap frozen in precooled isopentane. Cryostat sections (2 μm) were fixed with acetone, washed with PBS, and stained with fluorescein-conjugated sheep anti-rat F×1A. Stained sections were studied using a Leitz Dialux microscope equipped for epi-illumination with a 100-W Osram bulb. Tissue samples for electron microscopy were fixed in 2.5% glutaraldehyde, postfixed in Osmium tetroxide, and embedded in Epon.

In vitro binding of antigen. F×1A (1 mg/ml) was suspended in PBS. Twenty-four hours following suspension, the insoluble material was removed by centrifugation. The supernate of this preparation was incubated with cryostat sections of normal rat kidney and isolated glomeruli (20) (room temperature, 30 min), washed three times with cold PBS, and stained with fluorescein-conjugated sheep anti-rat F×1A.

Results. *Plasma clearance.* The results of the plasma clearance are shown in Fig. 1. There were no differences in the rates of clearance of either BSA or F×1A for the various dosage groups; thus, results for all animals were grouped in the figure. BSA was minimally cleared from plasma following an initial distribution in plasma. In contrast, approximately 75% of the injected F×1A was rapidly removed from the circulation. There was no evidence of saturation of clearance mechanisms, as there was no change in the clearance curve at the highest dose studied.

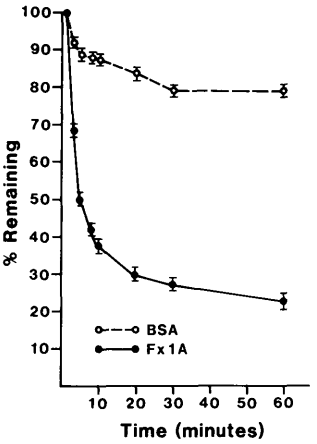

FIG. 1. The plasma clearance of the injected material is plotted as the percentage remaining versus time. (○) BSA, (●) F×1A. As there were no differences between the groups that received different doses, all the data were grouped for this figure.

Organ uptake. The results of the organ uptake of F×1A after correction for nonspecific protein uptake are shown in Table I. As noted by both the rate of plasma clearance and organ uptake, clearance and uptake of F×1A were not saturated at any dose studied. Isolated glomeruli showed no evidence of BSA binding. Total glomerular binding for the 600-μg dose of F×1A was 210 ng per 76,000 glomeruli or 2.76 pg/glomerulus. Renal uptake of F×1A was greater than ceruloplasmin or aggregated rat gamma globulin (Table II).

Histology. Immunofluorescence microscopy of renal tissue demonstrated fine granular deposits of F×1A antigen along the glomerular capillary wall which increased in intensity with increasing dose of F×1A administered. In addition, protein reabsorption droplets in the cytoplasm of the proximal tubule stained for F×1A antigens. Large reabsorption droplets were also seen by electron microscopy. These suggest that some of the F×1A antigens are filtered by the glomerulus and reabsorbed by the proximal tubule. Both glomerular and tu-

TABLE I. ORGAN DEPOSITION OF Fx1A

Dose of Fx1A (μg)		Heart	Liver	Lung	Spleen	Kidney	Homog	Glom
15	$\bar{X}$	0.001	0.123	0.071	0.072	0.748	0.187	0.007
	SD	0.001	0.022	0.011	0.012	0.127	0.039	0.001
30	$\bar{X}$	0.002	0.199	0.140	0.164	1.428	0.330	0.009
	SD	0.004	0.040	0.005	0.024	0.126	0.086	0.003
60	$\bar{X}$	0.006	0.450	0.273	0.288	2.457	0.719	0.024
	SD	0.005	0.103	0.051	0.046	0.424	0.170	0.008
600	$\bar{X}$	0.012	4.340	4.875	3.060	28.696	8.029	0.210
	SD	0.005	0.645	1.079	1.015	3.716	0.793	0.060

Note. Kidney, whole kidney data; Homog, washed kidney homogenate; Glom, data for isolated glomeruli. All results are expressed as μg of Fx1A deposited/g tissue (after correction for nonspecific uptake), except glomerular data which represents μg deposited/76,000 glomeruli.

bular staining for FX1A antigens are shown in Fig. 2. Electron microscopic examination of glomeruli demonstrated small, scattered, subepithelial electron dense deposits (Fig. 3).

Glomerular elution. Following reduction and polyacrylamide gel electrophoresis of the radiolabeled FX1A used for injection, multiple polypeptide bands are detected (Fig. 4). Molecular mass estimates of these proteins vary from 330 to less than 30 kDa. Only five of these bands are detected by counting for radioactivity. The radiolabeled bands correspond to proteins of 330, 96, 68, 60, and 50 kDa. All of the counts associated with the glomerular eluates corresponded to the 68-kDa band. Isoelectric focusing of PBS-soluble components of FX1A showed isoelectric points ranging from 4.4 to 5.6.

In vitro studies. Immunofluorescent staining of cryostat sections of normal rat kidney preincubated with PBS-soluble components of FX1A (shown in Fig. 5A) demonstrated binding of a FX1A antigen along the glomerular capillary wall in a pattern identical to that shown in Fig. 2B. Incubation of isolated glomeruli with absorbed, fluorescein-conjugated sheep anti-rat FX1A shows no evidence of binding to a FX1A antigen (Fig. 5B). The incubation of isolated glomeruli with FX1A resulted in diffuse, granular staining over the surface of the spherical, decapsulated glomerulus (Fig. 5C). In some areas the FX1A antigen appears aggregated where photographic confluence of adjacent granules in different focal planes occurs. Some of the confluent granules may represent aggregation or rearrangement of antigen on the surface of the glomerular epithelial cell during the hour of incubation

TABLE II. ORGAN DEPOSITION OF Fx1A, CERULOPLASMIN, AND AGGREGATED GAMMA GLOBULIN

	n		Liver	Lung	Spleen	Kidney
Fx1A	5	$\bar{X}$	0.138	0.051	0.155	0.644
		SD	0.024	0.008	0.012	0.029
Ceruloplasmin	5	$\bar{X}$	0.343	0.028	0.034	0.186
		SD	0.031	0.005	0.004	0.018
ARG	5	$\bar{X}$	0.147	0.038	0.335	0.052
		SD	0.018	0.020	0.046	0.012

Note. The results represent the group means ± 1 SD for organ uptake of Fx1A, ceruloplasmin, and aggregated rat gamma globulin (ARG) expressed as μg/g tissue. Each rat was given 15 μg of the designated protein and organ uptake was measured 60 min after injection.

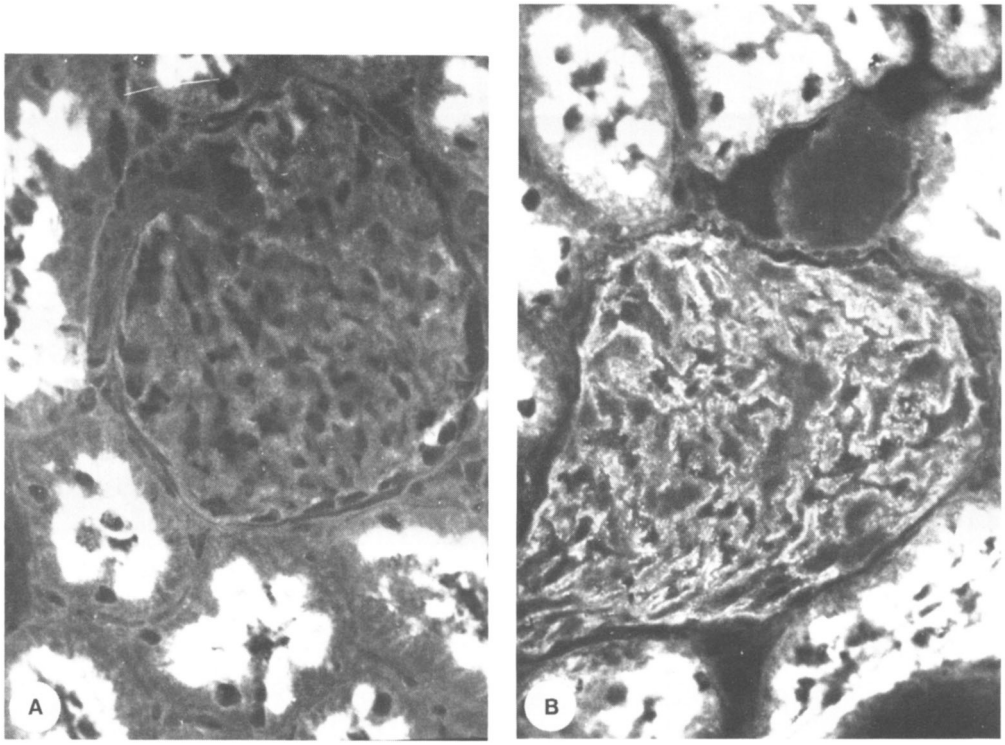

FIG. 2. Direct immunofluorescence of kidney sections stained with fluorescein-conjugated sheep anti-rat F×1A. (A) Normal rat kidney showing staining of the tubular brush border and no glomerular staining; (B) kidney from a rat injected with 600 μg of F×1A showing staining of the tubular brush border and fine granular deposits of F×1A along the glomerular capillary wall.

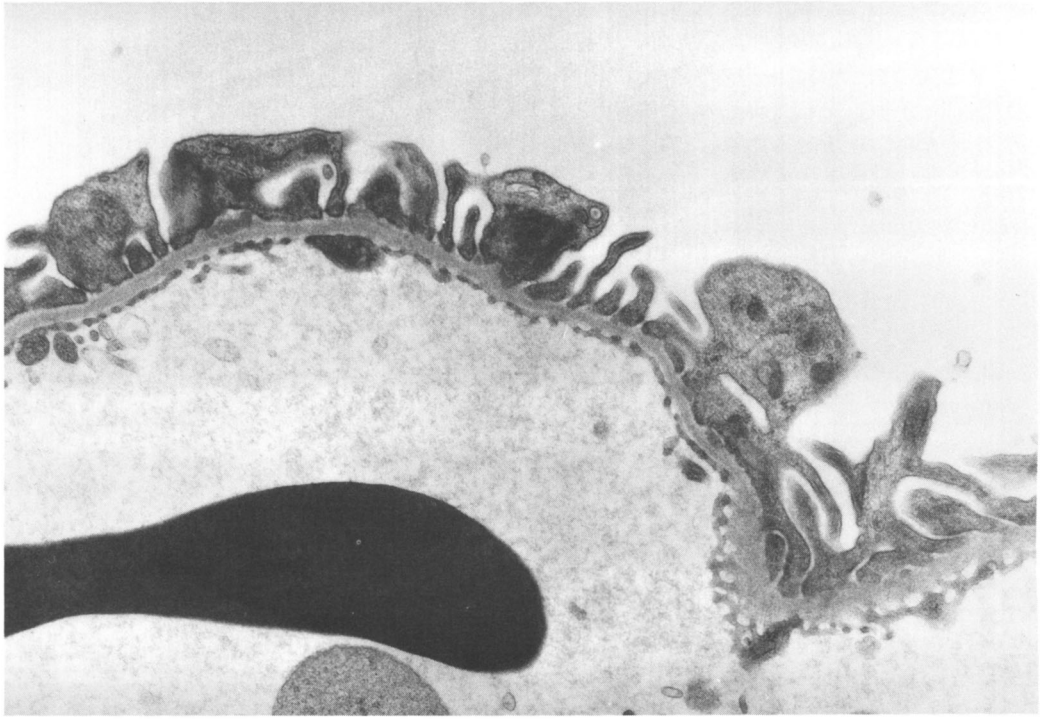

FIG. 3. Electron micrograph from a rat injected with 15 μg of F×1A showing scattered electron dense deposits in the subepithelial space of the glomerulus. (13,650×)

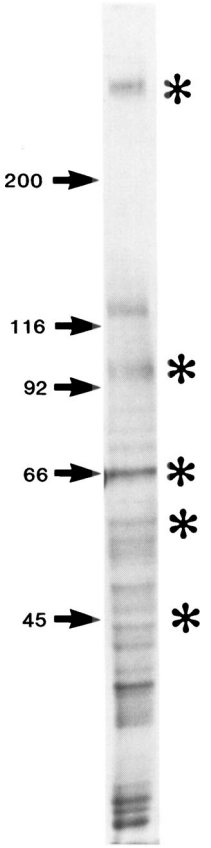

FIG. 4. Electrophoresis of F×1A used for injection. Silver staining of the gel shows the major polypeptide bands. The molecular weight markers are shown on the left. The starred (*) bands represent those that are radiolabeled. Material eluted from the glomeruli of F×1A injected rats corresponds to the 68-kDa band.

time. Occasional glomeruli in the same preparation remained covered with Bowmans capsule. These glomeruli showed no staining for F×1A antigen on their surface, suggesting that the antigen does not bind nonspecifically to glomerular tissue.

Discussion. The subepithelial immune deposits in the Heymann model of membranous nephropathy contain renal tubular antigen and its antibody (5, 10, 12). Both antigen and antibody (1, 3), as well as circulating immune complexes (8), are present in the circulation of these animals. Subepithelial immune deposits have been postulated to form by passive deposition of circulating immune complexes and by *in situ* binding of antibody to the tubule with an antigen that is shared by the glomerulus. The results of the present experiment demonstrate that a component of F×1A binds directly to the renal glomerulus. The glomerular binding of tubular antigen has also been suggested by previous studies. Cornish *et al.* (24) reported increased glomerular accumulation of IgG deposits in rats with heterologous passive Heymann nephritis given prior intraperitoneal injections of a tubular antigen preparation. However, they did not study renal tissues for antigen deposition prior to administration of antibody. In similar studies, rabbits given intraperitoneal injections of PBS-solubilized tubular antigens developed granular capillary wall deposits of tubular antigen detectable by immunofluorescence microscopy (25). Kidneys from rabbits not injected with antigen lacked evidence of capillary wall deposits of tubular antigen. The demonstration that a component of F×1A is normally present in the circulation (26) suggests that glomerular binding of this antigen may occur naturally and thereby participate in immune deposit formation in rats with circulating antibody. In addition, circulating immune complexes containing this antigen might bind to the glomerulus by the same mechanism.

The presence of proximal tubular reabsorption droplets containing F×1A antigen as detected by immunofluorescence suggests that some of the administered antigen is filtered and reabsorbed. However, the studies of renal homogenates and isolated glomeruli confirm that the F×1A taken up by the kidney is associated with renal tissue and not simply accounted for by peptide fragments in urine. The glomerular binding of a component(s) of F×1A accounts for a small percentage of the total renal-associated material. Evaluation of the renal eluates demonstrate that only one of the proteins present in F×1A binds to the glomerulus. Estimates from radioactive counting of the starting material suggest that the protein that binds to the glomerulus accounts for less than 20% of the injected protein. Considerable controversy exists regarding the nature of the Heymann antigen(s) and the relationship of individual components of F×1A to other components. None of the components have been purified to homogeneity, although various preparations have been enriched for certain components. Further evaluation of the

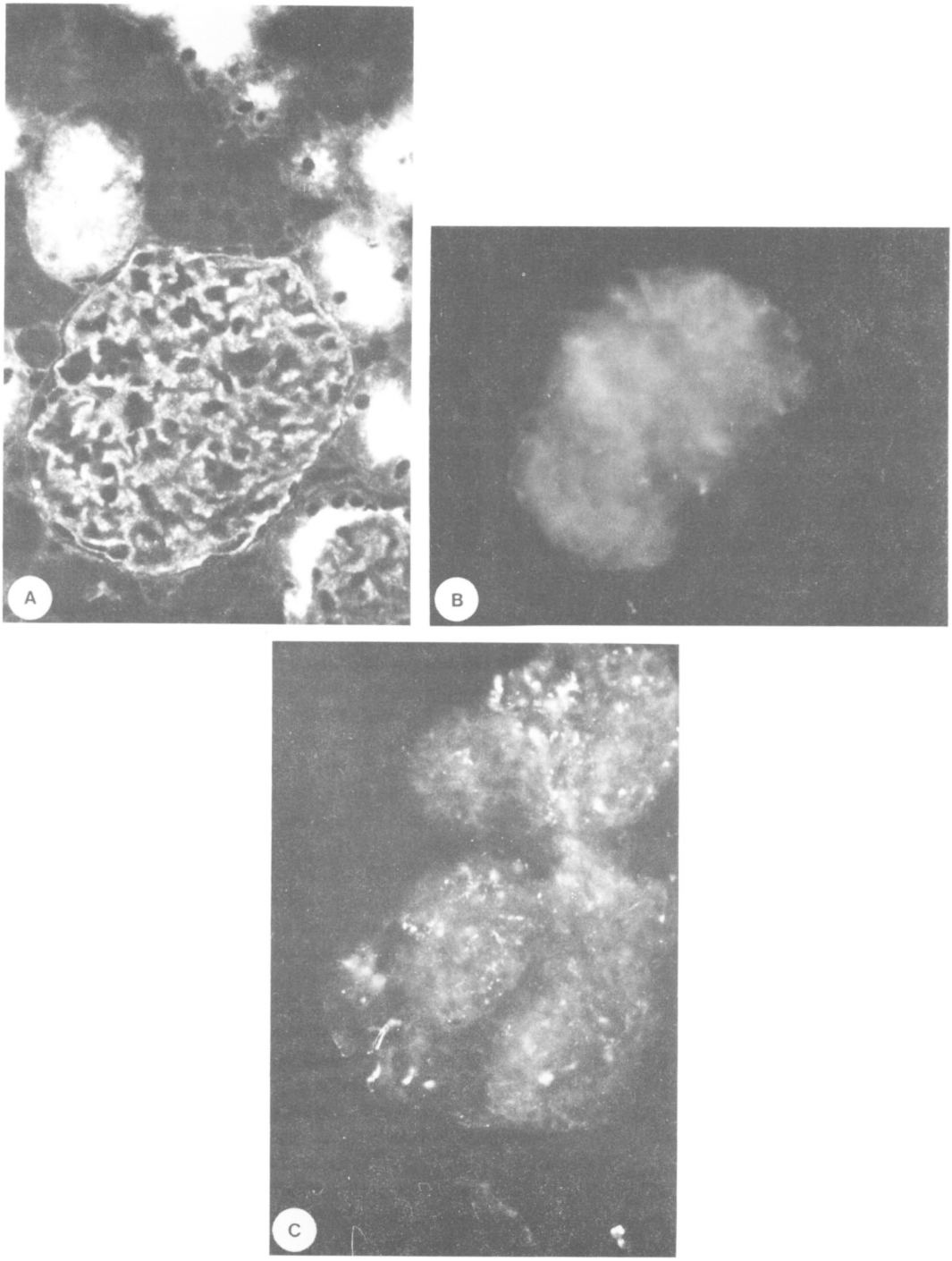

FIG. 5. *In vitro* binding of F×1A. Normal rat kidney sections or isolated glomeruli were stained with fluorescein-conjugated sheep anti-rat F×1A (1:256 dilution) after incubation with PBS alone or PBS-solubilized F×1A. Normal rat kidney incubated with PBS showed staining of the tubular brush border and no staining of the glomerulus identical to that shown in Fig. 2A. (A) Normal rat kidney incubated with F×1A showing fine granular deposits of F×1A along the glomerular capillary wall, as well as staining of the brush border; (B) an isolated glomerulus showing no evidence of antibody binding above background staining. To enhance photographic visualization, these glomeruli were incubated with fluorescein-conjugated sheep anti-rat F×1A at a 1:4 dilution. Exposure time for this micrograph was 4 min in contrast to 8 sec for all other immunofluorescence micrographs. (C) An isolated glomerulus that was incubated with F×1A showing diffuse fine granular binding of F×1A antigen to the surface of the glomerular epithelial cells with areas of aggregated antigen. (320×)

kinetics of glomerular binding must await purification of the subcomponent of F×1A that accounts for glomerular binding.

The relationship of this antigen to the "nephritogenic" antigen responsible for induction of Heymann nephritis remains to be defined. Kerjaschki and Farquhar (4) reported disease induction with a glycoprotein component of F×1A with an apparent molecular mass of 330,000 Da. Since that time, Natori and Shibata (27) have reported that the antigen is 170,000 Da in size when isolated using pronase digests of tubular fractions. Makker and Singh (28) reported that the Heymann antigen is 600,000 Da and only expresses smaller fragments when subjected to denaturing conditions. However, they (26) identified the Heymann antigen in rat plasma. The circulating antigen is approximately 60–70 kDa which corresponds to that of the F×1A antigen that bound to the glomerulus in the present study. Singh and Schwartz (29) have also reported the induction of active Heymann nephritis by immunization with this component of F×1A. The tubular antigen that has been isolated from glomerular immune deposits from rats with active Heymann nephritis is of similar molecular mass (30). No other tubular antigen was detected in these glomerular eluates. The variable estimates of the size of the Heymann antigen may result from the variety of procedures that have been employed during isolation. Proteolytic enzymes in plasma may account for the smaller size of the antigen in the circulation. Further study is needed to clarify the relationships between the molecules described by the various investigators.

The site of glomerular binding appears to be the subepithelial space as demonstrated by electron microscopy. The mechanism responsible for the subepithelial binding is unknown. The anionic charge of the glomerular capillary wall has been shown to influence binding of cationic antigens from the circulation. Estimates of isolelectric points of the Heymann antigens vary, depending upon the detergent used to solubilize F×1A, but all estimates are anionic (4). In the present study, the isoelectric points of the PBS-soluble components of F×1A range from 4.4 to 5.6. Thus, it is unlikely that the binding reported here is on a charge basis. It is unclear how deposits of molecules with molecular weights of under 200,000 Da could lead to electron dense deposits. We postulate that the F×1A antigen binds to the surface of the glomerular epithelial cell. Following binding, patching may lead to aggregation of the protein, making it electron dense. However, this remains to be substantiated.

The mechanism responsible for subepithelial deposit formation in the Heymann model of membranous nephropathy remains controversial. Immune complexes of F×1A and antibody are present in the circulation of rats with Heymann nephritis (8) and their accumulation in the kidney is altered by modifications of mononuclear phagocyte system function (10). Despite the support for an immune complex pathogenesis, cross-reactivity of binding of antibody to the tubular brush border with an intrinsic glomerular antigen (31) has been demonstrated (11–13). These observations support the role of *in situ* binding of antibody in the glomerulus. Additional data (9, 13, 14, 30, 32) suggest that at least two antigen–antibody systems may contribute to the development of immune deposits in this model. Binding of a tubular antigen from the circulation to the glomerular epithelial cell surface could explain some of the data which heretofore have been difficult to reconcile.

Binding of F×1A antigen to the epithelial cell surface, could serve as a "planted" antigen for subsequent antibody binding and immune deposit formation. In addition, it could account for the variable reports of cross-reactivity of heterologous, polyclonal antibodies made to the brush border with intrinsic glomerular antigens. Although the presence of gp330 on the tubular brush border and glomerular epithelial cell is well documented (31), additional antigens appear to play a role in glomerular immune deposit formation in both active (14, 30) and pasive (32) Heymann nephritis. These additional antigens and their respective antibodies may not necessarily localize in the glomerulus by the same mechanism as occurs for the gp330 system. Clarification of the complex factors that result in subepithelial immune deposit formation must await further study. However, we propose that a circulating brush border antigen binds to the surface of the glomerular epithelial cell. Circulating antibody to this "planted" antigen binds *in situ,* followed by repeated deposition

of circulating antigen. The progressive accretion of antigen and antibody then perpetuates immune deposit formation. This does not preclude the additional deposition of circulating immune complexes that contain the brush border antigen and its antibody. Unlike gp330, antibody to the 68-kDa molecule does not appear to recognize an intrinsic glomerular antigen (30). Definition of the role of antibodies that bind to intrinsic glomerular antigens, and to common and restricted antigens of the tubule and glomerulus, will be accomplished only after each of the components of F×1A has been separated and monoclonal reagents to them are available.

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