Antigen Size and Charge in Immune Complex Glomerulonephritis

II. Passive Induction of Immune Deposits With Dextran–Anti-dextran Immune Complexes

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Utilizing dextrans of restricted sizes (10,000, 70,000, 500,000 daltons), modified with regard to charge (neutral, polycationic, polyanionic) and an anti-dextran murine IgA myeloma, W3129, the authors have examined a model that may be used in the study of the combined effect of size and charge on renal deposition of immune complexes. Polycationic DEAE dextran complexes, using the 10,000 dalton antigen, showed a mesangiocapillary pattern of deposition. The other antigens showed focal to diffuse mesangial localization of varying degree. This indicates the potential usefulness of this system in examining the factors important in glomerular immune injury. The relevance to other observations, importance of polysaccharide antigens, and role in circulating versus *in situ* or "planted" immune complex models are considered. (Am J Pathol 1983, 111:298–306)

IT IS CURRENTLY believed that the bulk of glomerulonephritis is due to immunologically mediated injury. There are two mechanisms by which immune complexes could accumulate in the glomerulus: by deposition of circulating soluble complexes or by *in situ* formation of immune aggregates due to the interaction of antibody with antigen already in the basement membrane. Such antigen may be a component of the basement membrane or may accumulate there as a consequence of its physical or chemical properties.[1–7] There is evidence that subendothelial and/or mesangial localization are due to the first,[8–10] while membranous disease follows the second.[3–5,11]

Whereas many properties of immune complexes have been examined to account for these observations—ie, size, antibody affinity, immunoglobulin class—none have proved entirely satisfactory. The presence of fixed negative charges on the filtration barrier[12–15] has suggested that antigen and/or immune complex localization might be charge-dependent.[17–23]

To investigate this proposition, we have employed modified dextrans of graded molecular weight, which can be precisely described. This system allows a multiparametric analysis of the several factors believed to play a role in this mechanism of renal injury. The use of myelomas and hybridomas permits complete definition of the antibody component. In addition, this model allows comparison with physiologic probes, examines the role of polysaccharides as antigens in immune complex injury, and serves as an animal counterpart to human IgA nephropathy.[22]

In previous studies we have explored the ability of active immunization with dextrans to produce glomerulonephritis. Such nephritis was characterized by IgA, IgM, and C3 deposition, largely in the mesangium, with a pattern of glomerular reaction that appeared to vary with antigenic charge.[23] To examine more definitively the relation of size and charge of the antigen to deposition and response, we have pro-

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posed a passive model in which the antibody component is held constant through the use of an IgA myeloma, W3129, specific for 1,6-linked dextran. If such a model leads to immune deposition and glomerular change, then other myelomas and/or hybridomas (which are already available) of varying classes and affinities can be employed for precise determination of the relative contributions each factor makes to glomerular localization and injury.

Materials and Methods

Preparation of Antigens

Neutral dextran (ND) prepared from native dextran produced by *L mesenteroides* strain B512 was purchased (Pharmacia, Upsala, Sweden) in three different size ranges (10,000, 70,000, and 500,000 daltons). These were refractionated by gel chromatography to further restrict the size range. Two grams (per run) of 10,000-dalton ND were eluted against gravity from a 1500-ml (75 × 5 cm, internal diameter [ID]) Sephacryl S-200 (Pharmacia) column at a rate of 25 ml/hr with 0.05 M ammonium bicarbonate (pH 8.2). Fractions (15-ml) were collected and analyzed for hexose content by the anthrone reaction.[24] Fractions within 200 ml from the peak were pooled and lyophilized. NDs of 70,000 and 500,000 daltons were refractionated on 500 ml (96 × 2.5 cm, ID) AcA 22 (LKB, Bromma, Sweden) and Sepharose 6B (Pharmacia) columns, respectively. Fractions (7 ml each) were collected and analyzed as above, and those within 60 ml of the peak were pooled and lyophilized. Rechromatography of material fractionated in this manner confirmed the size range restriction of the original purchased dextran.

We used portions of the material obtained from the gel filtration of the NDs to synthesize dextran sulfate (DS), using a copper-catalyzed reaction of 8-quinolyl sulfate with ND. Both the 8-quinolyl sulfate and the DS were synthesized according to a modification of the procedure of Nagasawa and Yoshidome.[25] Similarly, we used portions of ND to synthesize polycationic DEAE dextran (DD) through a basic reaction of 2-chlorotriethylamine hydrochloride (Sigma Chemical Co., St. Louis, Mo) and ND, using the procedure of McKernan and Ricketts.[26]

Following synthesis of DS and DD in each of the molecular size ranges, samples were assessed for possible size change induced by the synthetic process. This was done by rechromatography on the same columns that had initially been used to restrict the size range.[19]

The isoelectric points and mole/mole functional group substitutions of the synthesized antigens were determined by isoelectric focusing and titration, respectively.[23,25] Isoelectric focusing was carried out on a 100-ml LKB isoelectric focusing column in a 0–50% sucrose density gradient using pH 3–10 ampholines (Ampholytes, Pharmacia). The pH was measured on successive fractions from the column, which were then dialyzed against distilled water and analyzed for hexose content by the anthrone reaction.[24] The titrations were carried out as follows: 1% solutions of DD and DS were passed through an anion exchange column (Amberlite) and a cation exchange column (Dowex) respectively. The columns were washed until the effluents were neutral. The combined washings were titrated with 0.1 M standardized HCl (for DD) and 0.1 M standardized NaOH (for DS). The pH of the solutions and the volume of standardized solutions added were recorded, and the titration curves were plotted.[27]

Preparation of Antibody

The W3129 myeloma protein (anti-1,6-linked dextran, IgA) was used as the antibody component of the immune complex.[28] This was obtained as a tumor (gift of Dr. Sherie Morrison, Columbia University) and passed into untreated BALB/c mice. Ascitic fluid from tumor-carrying mice was processed by 50% ammonium sulfate precipitation, followed by dialysis in phosphate-buffered saline (PBS, pH 7.4). The IgA content of the ammonium sulfate precipitate was determined by radial immunodiffusion.[29] Purified IgA myeloma protein RPC22 was used for standardization.[30] Anti-dextran precipitin analysis of the W3129 by radioimmunoassay employed a modification of the procedure of Matsuuchi and Morrison,[31] as follows.

Radioimmunoassay

Radioimmunoassay[32] was used to determine the amount of IgA anti-dextran bound to antigen at equivalence. Tritiated 69,000-dalton dextran[25] (174 mCi/g, Amersham, Arlington Heights, Ill) suspended in 10 ml of distilled water was used as a stock solution; 10 μl of ascitic fluid (10–15 mg/ml protein, not more than 5 mg specific antibody) in 90 μl of distilled water were incubated at 4 C overnight with 5–100 μl of tritiated dextran stock solution diluted 1:10. The resulting anti-dextran complexes were precipitated by a sandwich technique. Rabbit anti-mouse IgA (10 μl, prepared in this laboratory) was added to the immune complex mixture and incubated at 4 C for 1 hour. This was followed by a 1-hour incubation at 4 C with 10 μl of goat anti-

rabbit IgG (prepared in this laboratory). The resulting precipitate was removed by centrifugation, and an aliquot of the supernatant was counted in 5 ml of Scintiverse Universal Scintillation Cocktail (Fisher) on a Searle Mark III liquid scintillation counter. A precipitin curve (Figure 1) was plotted with the use of the percentage of total counts in the precipitate as the *y*-axis and the amount of tritiated dextran added to the antibody mixture on the *x*-axis.[33] It was found from this analysis that 0.006 mg of 69,000-dalton dextran bound to 1 mg of antibody at equivalence. This corresponded to 78.9 moles of antibody per mole of antigen.

Immune Complex Preparation and Characterization

Soluble immune complexes were prepared from the 9 dextran antigens and the W3129 myeloma protein in 40 × antigen excess as calculated from the precipitin curve of 69,000-dalton dextran. Molecular weight adjustments were made for the antigen sizes used. The total amount of antigen was added to the requisite antibody at room temperature and incubated with agitation for 60 minutes (20 C). Sizing of the immune complex mixtures was performed by sucrose density gradient ultracentrifugation in a 10–40% linear gradient in PBS, pH 7.4. Solutions containing 0.2 mg of antibody protein were applied to 10.5-ml gradients and centrifuged at 140,000*g* for 18 hours in an IEC B-60 ultracentrifuge using a Type SB-283 swinging bucket rotor (International Equipment Company, Needham Heights, Mass). Mouse IgA, human fibrinogen, and human IgM were used as reference markers. Thirty 0.35-ml fractions were collected from the top of the gradient and were analyzed by immunodiffusion. ^{125}I-labeled dextran antigens, synthesized in this laboratory as per the procedure of Keck[34] without antibody, were also subjected to sucrose ultracentrifugation.

Complex Charge

The relative electrophoretic mobilities of all immune complex mixtures were examined in 0.75% agarose gels in pH 7.4 Michaelis buffer. Complexes were prepared as described above. Free and bound antibody was detected by the use of appropriate antiserums. Comparisons were made between the mobility of the IgA component of each immune complex mixture and the IgA component run without antigen. These two samples were run in parallel for each size and charge of dextran.

Injection Protocol

Male Swiss Webster mice (Taconic Farms, Germantown, New York) were divided into 10 groups of 4 mice each for use in these studies. Immune complexes prepared with the 9 dextran antigens were injected into the mice such that 4 mg of specific antibody protein was administered in 2 divided doses over a 16-hour period. Group I received only the antibody component of the immune complex. Control groups received antigen alone and saline. Animals were sacrificed by exsanguination under ether anesthesia 18 hours after the final injection. Kidneys were removed and examined.

Preparation and Analysis of Kidney Sections

Sections of renal tissue were prepared for light, immunofluorescence, and electron microscopy as previously described.[23] Immunofluoresence examination included assessment of sections for mouse IgG_1, IgM, IgA, C3, and fibrinogen by the use of the indirect technique.[35] Dextran deposition was determined with the use of a triple sandwich technique employing MOPC 104E myeloma protein (anti-1,3-linked dextran, IgM) as the primary antiserum. For this study sections were overlayered and incubated with purified MOPC 104E protein,[23] washed, exposed to specific rabbit anti-mouse IgM,[23] washed, and finally overlayered and incubated with FITC-goat anti-rabbit IgG (prepared in this laboratory). After a final washing (each time with two rinses of PBS for 5 minutes), sections were examined. Controls employing substitution (normal mouse serum for MOPC 104E or rabbit anti-mouse IgG_1) and blocking (dextran or mouse IgM) were used to confirm specificity. The kidney sections were randomly coded and examined for the presence of immune complexes (immunofluorescence and electron microscopy) and tissue injury (light and electron microscopy) in a blind fashion by two independent observers.

Results

The properties of the antigens used in this study are shown in Table 1. Discrete differences in charge density were not considered in this study. The major

Table 1—Antigen Characterization

Antigen		pI	Substitution mole/mole
Neutral dextran	10,000	7	–
Neutral dextran	70,000	7	–
Neutral dextran	500,000	7	–
Dextran sulfate	10,000	3	41.8
Dextran sulfate	70,000	<3	201.6
Dextran sulfate	500,000	<3	50.0
DEAE-Dextran	10,000	>10	2.0
DEAE-Dextran	70,000	>10	42.0
DEAE-Dextran	500,000	9.1–9.5	625.0

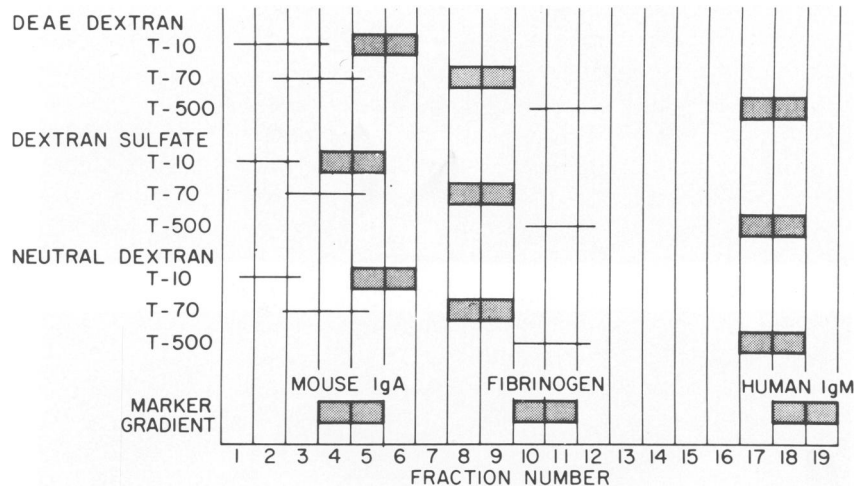

Figure 1—Ultracentrifugation of performed W3129-dextran immune complexes at 140,000*g* for 18 hours at 4 C. Fractions assayed for mouse IgA, human fibrinogen, and/or human IgM by immunodiffusion. The *horizontal lines* indicate the location of antigens alone. The *hatched rectangles* represent the immune complexes.

goal with respect to antigen charge was to synthesize antigens that were polyanionic and polycationic. The isoelectric points of the dextran sulfates were all clearly acidic, and those of the diethylaminoethyl dextrans were basic. Molar substitution ratios indicate the degree of derivatization. Of much greater importance were the properties of the complexes presented to the animals. In Figure 1, the sizes of the complexes are graphically portrayed, as determined by sedimentation analysis. Two points are of note: the complex size is largely dependent on the size of the antigen and is not influenced by the charge of the dextran, and the ratio of antigen to antibody on a molar basis is low (compared with many protein antigen systems). This low antigen-antibody ratio is due to high density of identical determinants on the linear polysaccharides. The equivalence point for the system studied was in accord with this fact; ie, one mole of 69,000-dalton neutral dextran bound 78.9 moles W3129 IgA anti-dextran myeloma. This is an order of magnitude more than is usually encountered in a bovine serum albumin (BSA)-anti BSA system.[32] The precipitin curve (Figure 2), as one would predict, had an extensive plateau region. In great antigen excess (40×) it is likely that an average ratio of two antigen molecules per IgA molecule was achieved to account for the sedimentation data.

To verify that the modified dextrans did lead to anionic and cationic *complexes*, immunoelectrophoresis in the presence of antigen (40× equivalence) was carried out (Figure 3). Studies were conducted at pH 7.4 with the use of a Michaelis buffer to approximate the physiologic milieu. The anodic and cathodic displacements were as expected. The greater migration of the DEAE-dextran complex, compared with the dextran sulfate complex is probably due to the heavier derivatization of the former.

In Table 2 the immunofluorescence data are summarized. Of greatest significance, perhaps, was that only 10,000-dalton DEAE-dextran, the smallest cationic antigen, showed a subendothelial site of deposition. The amount of deposition was least in the 10,000 dextran sulfate and 500,000 DEAE-dextran groups, where half of the animals were clearly negative. The most usual pattern was that of mesangial deposition, which ranged from a scant focal pattern to one resembling dense deposits in the animals given complexes containing high-molecular-weight dextran sulfate. These extremes—ie, (mesangocapillary) subendothelial versus heavy mesangial—are illustrated in Figure 4. The patterns were highly reproducible from animal to animal, and observer agreement was consistently present. In view of the high degree of internal consistency and the lack of intragroup variability, statistical analysis (2 × 2 test of independence using the G statistic) indicated that the reliability could be achieved despite the small number of

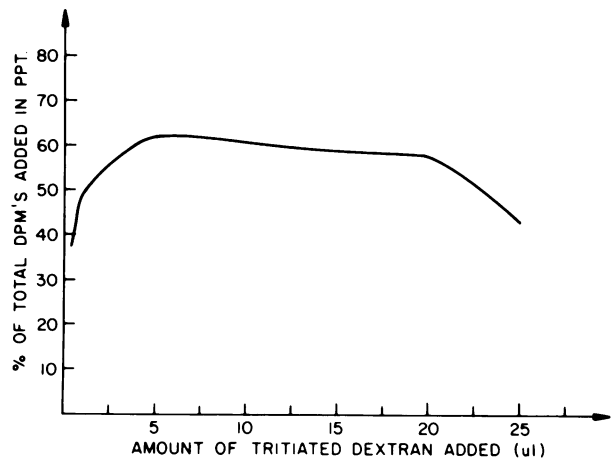

Figure 2—Precipitin curve for the reaction of 69,000-dalton neutral dextran with W3129 (IgA, anti-1,6) myeloma protein. Radioimmunoassay with ^{3}H-dextran.

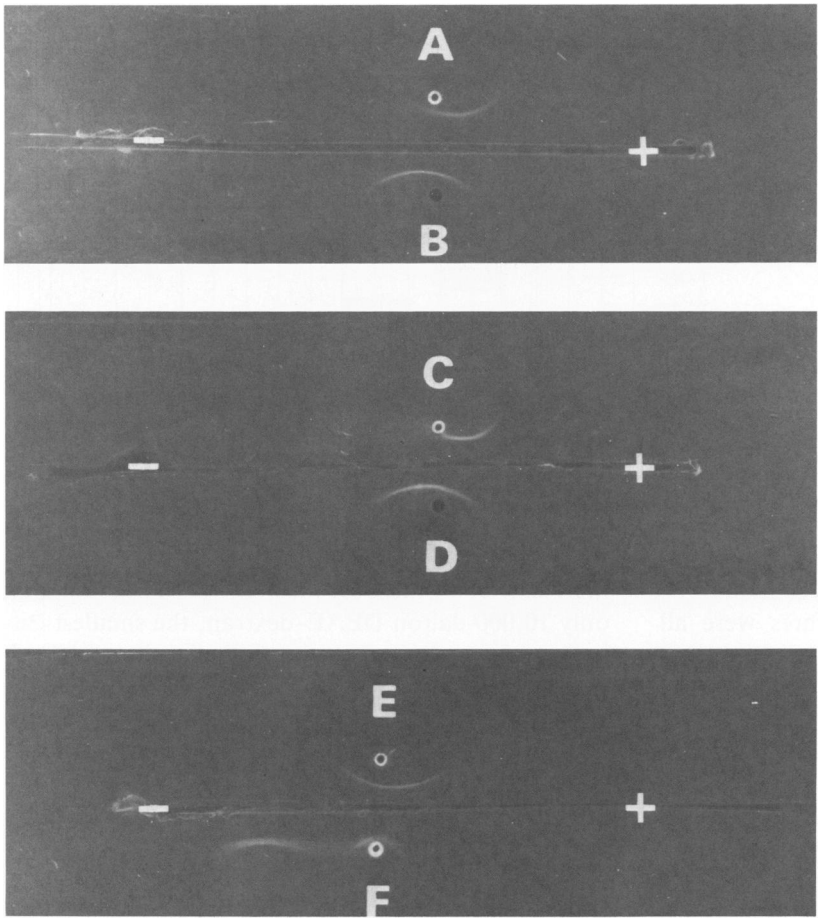

Figure 3—Immunoelectrophoresis of preformed W3129-dextran immune complexes. Wells A, D, and E contain W3129 myeloma protein alone. Wells B, C, and F contain preformed neutral dextran-W3129, dextran sulfate-W3129 and DEAE-dextran-W3129 immune complexes, respectively. The trough in all plates contained rabbit antimouse IgA; 500,000-dalton antigens were used to prepare the immune complexes shown in this photograph. This analysis was also performed for the other complexes with similar results (not shown).

animals used, ie, 4 per group. All controls were clearly negative. No IgG_1, IgM, or fibrinogen were seen in any animal save for trace amounts of mesangial IgM noted randomly in about 5% of experimental and control mice. No lesions were seen or dextran detected in any animal that received antigen alone. Studies of dextran clearance in the literature,[36,37,38] confirmed using ^{125}I-dextran antigens,[31] indicate that small amounts of antigen would be free in the circulation as well as in the kidney at the time of the second injection as well as at sacrifice. They would, however, be below the level of detection possible with the system employed here.

Table 2—Immunofluorescent Localization of Complexes*

Antigen charge	Antigen size (daltons)		
	10×10^3	70×10^3	500×10^3
+ (DEAE-dextran)	+ +, B, D	+ + +, S, D	+, S, V
0 (Neutral dextran)	+ +, S, D	+ +, S, F	+ +, S, F
− (Dextran sulfate)	+, S, F	+ +, S, V	+ + +, S, V
Antigen alone (all modifications)	−	−	−
Antibody alone	−	−	−

* In all cases localization of IgA, dextran, and C3 were similar: +, minimal; + +, moderate; + + +, heavy; B, subendothelial; S, mesangial; D, diffuse; F, focal; V, diffuse with segmental accentuation. Values are the mean scores for the group.

These observations were confirmed by electron microscopy. All experimental animals except those treated with 10,000-dalton DEAE-dextran complexes showed mesangial deposits (Figure 5). In the low-molecular-weight cationic complex groups, there was extensive deposition of flocculent material in a mesangiocapillary (subendothelial) location (Figure 6).

A final note should be made of the animals' responses to the IgA anti-dextran complexes. We had initially planned to give three injections over a 24-hour period (as has typically been done in anti-protein-protein passive immune complex studies[8]. The animals, however, became exceedingly ill, as shown by profound sluggishness and ruffling of fur. They continued in this state or deteriorated, and we terminated the study prematurely. The animals did not appear to have anaphylactic reactions, and control animals given antigen or antibody alone in identical doses showed no ill effects.

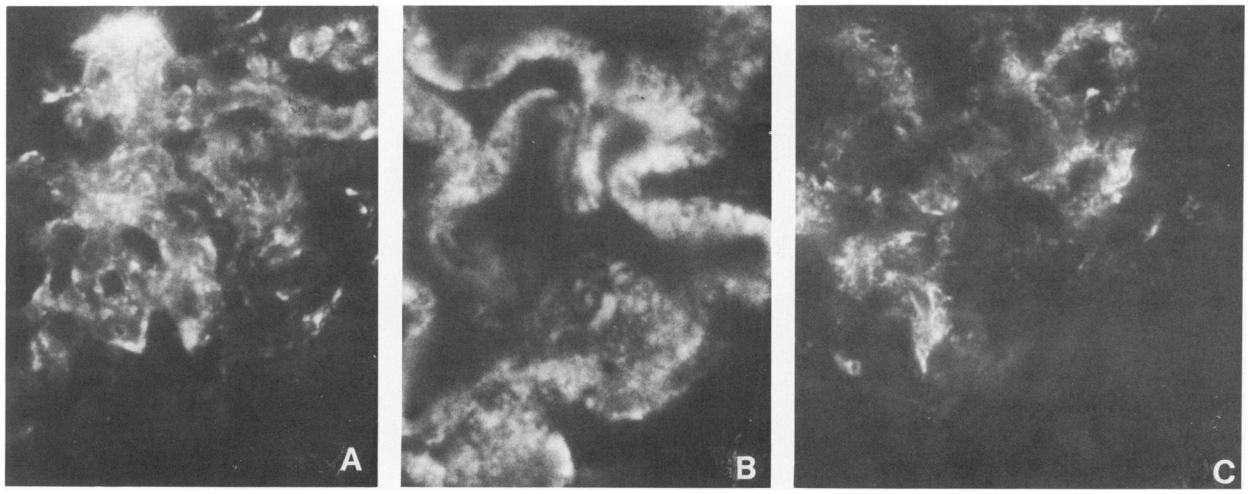

Figure 4 — Immunofluorescence micrographs of kidney sections from mice treated with preformed immune complexes. **A** — Mouse treated with immune complexes containing 10,000-dalton DEAE-dextran; note the membranous deposition. Stained with rabbit anti-mouse IgA. (×2800) **B** — Segment of a glomerular capillary loop from the animal in **A**, enlarged to show granular deposits. **C** — Mouse treated with immune complexes containing 70,000-dalton neutral dextran. Note the heavy mesangial deposition. (×1400)

Discussion

The work presented represents the first study of passive immune complex administration employing polysaccharide antigens. The study is noteworthy in suggesting the possibility that this class of antigenic materials might be important in the pathogenesis of glomerulonephritis.

The ability of such complexes, whose charge has been altered by modification of the antigen, to show varied patterns of localization, is of great interest. This observation supports the prior work of Gallo

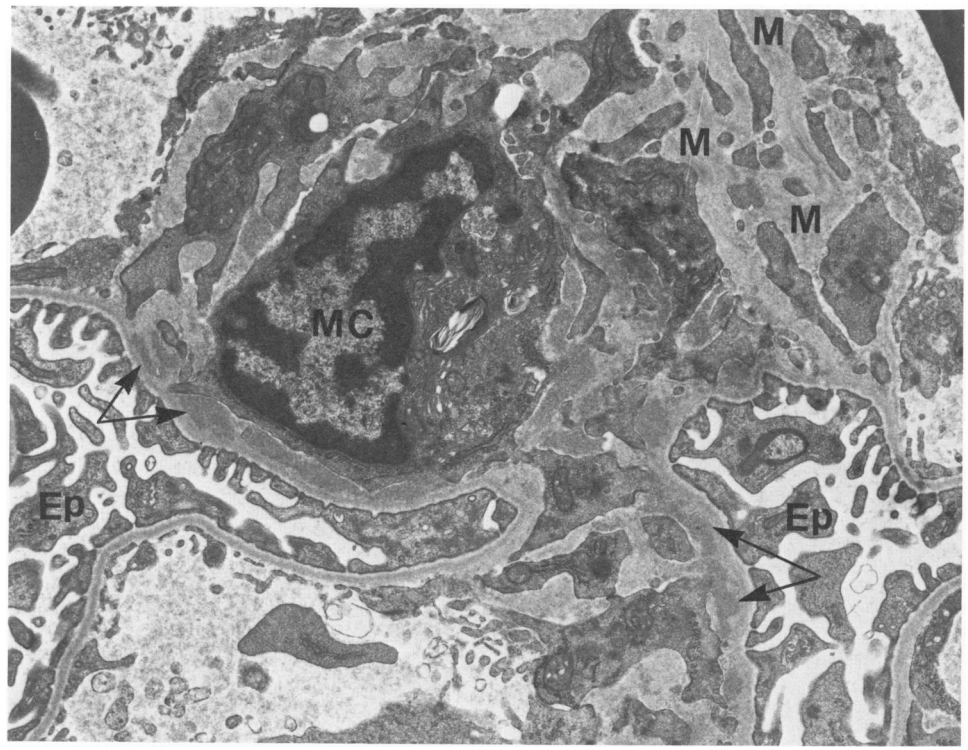

Figure 5 — Electron micrograph of a portion of a glomerulus from a mouse treated with preformed immune complexes containing 70,000-dalton neutral dextran. Note the intramembranous mesangial dense deposits that extend into capillary loops (*arrows*). *Ep*, epithelial cell; *MC*, mesangial cell; *Mi*, mesangial matrix. (×11,000)

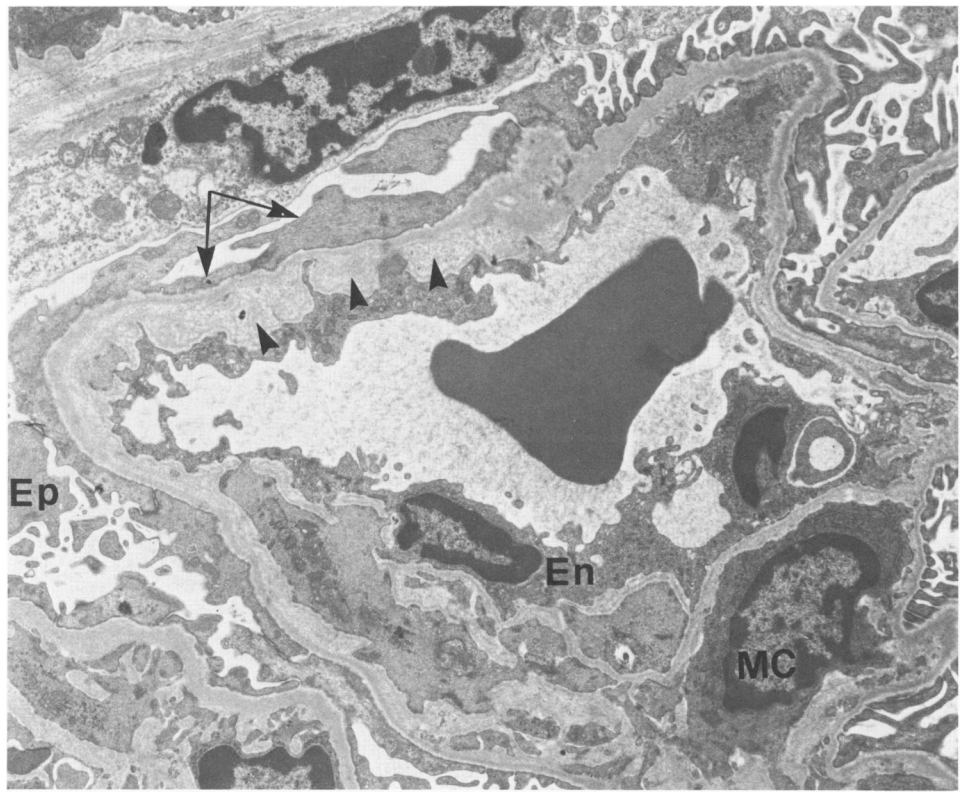

Figure 6 – Electron micrograph of a portion of a glomerulus from a mouse treated with preformed immune complexes containing 10,000-dalton DEAE-dextran. Note the accumulation of material within the peripheral capillary loops in a subendothelial location (*arrowheads*) and fusion of foot processes (*arrows*). *Ep*, epithelial cell; *En*, endothelial cell; *MC*, mesangial cell. (×6800)

and collaborators[20] as well as the reports of Border's group.[18,19] To be emphasized is the mesangiocapillary (subendothelial) deposition seen with the 10,000-dalton DEAE-dextran–anti-dextran complexes and the mesangial pattern characterizing the larger molecules and the neutral and anionic complexes. We believe, as stated by Gallo et al,[20] that the cationic dextran complexes interact with anionic sites on endothelial cells and within the membrane proper. Such electrostatic interactions have been well documented,[39] particularly in the case of the kidney.[13,16] Our studies differ from those noted above in that we were clearly able to define size as a factor in site deposition. It may well be that it is really axial ratio, much higher for linear polysaccharides than globular proteins, and not mass that, with charge, is critical. That would explain why dextran complexes using DEAE-dextran antigens of 10,000, and not 70,000, daltons showed subendothelial deposition, while derivatized bovine gamma globulin (BGG) (an antigen of much greater mass)–anti-BGG showed a pattern more like the smaller dextran.[19] Such comparisons are difficult because they do not consider the many other factors which may play a role: affinity, isotype of antibody, and/or role of complement and other mediators.[9,10,11,40] Of importance is the possibility, despite the injection of preformed complexes, that we may be dealing with an *in situ* complex model. The use of antigen excess creates the probability that initial clearance of the DEAE-dextran and subsequent interaction with intramembranous or perimembranous groups then permits interaction with antibody or even complex. In addition, it should be noted that this is a relatively low affinity system, a fact that might favor entry of complexes into the membrane. Preliminary clearance studies indicate that this system will allow definitive exploration of the circulating versus *in situ* models.

Our studies explored only a scant fraction of the perturbations possible. We have previously shown that active immunization leads to overt glomerulonephritis.[23] The heterogeneity of the immune reaction under the protocol employed made determination of the role played by charge, size, and affinity very difficult. The availability of anti-dextran myelomas and hybridomas (Table 3), the ease of determining association constants,[41] the existence of antibodies of several isotypes, the ability to easily modify the antigen, and the capacity to cross-link[42] the determinants to mimic complexes make it feasible to construct virtually any simulation of the active immune response desired. It must be pointed out (with regard

Table 3—Myeloma Proteins With Antidextran Activity

Myeloma	Type	Anti-dextran	Reference
W3129	IgA κ	Alpha 1, 6	28
W3434	IgA κ	Alpha 1, 6	28
QUPC 52	IgA κ	Alpha 1, 6	28
UPC 102	IgA λ	Alpha 1, 3	28
J 558	IgA λ	Alpha 1, 3	46
MOPC104E	IgM λ	Alpha 1, 3	46
CAL 20 TEPC 1035	IgG2b	Alpha 1, 3	47

to the last point) that the 500,000-dalton antigen given alone is an ideal control for the 10,000- or 70,000-dalton antigen–antibody complex. No renal deposition was observed, suggesting that the formation of larger aggregates, possibly within the membrane, might be required and that covalent cross-linking would preclude dissociation (to a filterable size) and reassociation.

One major concern has been the possible interaction of the dextrans with serum protein. While we cannot exclude this contribution, the failure of dextrans alone to deposit or induce symptoms at comparable or greater doses is reassuring. We have previously shown that the elution profile of serum from a molecular seiving medium was not altered by the presence of dextran derivatives under physiologic conditions (pH and ionic strength).[23] It has been reported that cationic (DEAE-) dextran can cause acute renal failure in rats; that the effect is dose-dependent and related to a deleterious effect on mitochondria; and that charge is an important determinant.[43] In mice, at the doses examined, we did not observe any anatomic changes; however, as pointed out by Simmons et al, low-level effects require assay of mitochondrial function.[43] On the other hand, dextran sulfate has been associated with several biologic effects that could have an influence on its action within the glomerulus.[44] Again, the absence (at least in our short-term study) of localization of the antigen alone would mitigate against the importance of such a contribution.

Our studies have focused on the potential renal basement-membrane-seeking properties of immune complexes (or antigen) as an explanation for localization. The recent report of Finbloom and co-workers[45] suggests that extrarenal factors may also be charge-dependent and could thus influence renal deposition. Increased hepatic clearance would presumably result in diminished load to the kidneys. The role of such considerations, the significance of varying antigen/antibody ratios, and other parameters previously mentioned, remain to be explored. The role of alternate pathway of complement activation or other mediators in explaining the systemic toxicity of the complexes is likewise deserving of additional study. It is noteworthy that both anionic *or* cationic, as opposed to neutral, species may interact with the filtration barrier to facilitate complex deposition. The basis for each has been discussed at length elsewhere.[19,20]

The use of an IgA system was based on our studies employing active immunization.[23] In that investigation mice preferentially produced IgA (and essentially no IgG) to dextran immunization regardless of modification. Since myelomas and hybridomas with anti-dextran IgG and IgM isotypes exist (Table 3), we are currently exploring their use in this model. The importance of polysaccharides as antigens in glomerulonephritis has not yet been studied despite their wide occurrence in nature.

In summary, a passive immune-complex system employing polysaccharides (modified dextrans) that allows for analysis of size and charge factors in deposition within the glomerulus is presented. We have shown localization with low-molecular-weight (ie, 10,000 daltons) polycationic antigens and an IgA (myeloma) antibody. We believe this system lends itself to the study of parameters governing immune-complex deposition, including antigenic charge as a determinant in interactions with groups in or about the basement membrane.

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