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Administration of an Exogenous Cationic Antigen:  
*DEMONSTRATION OF A PATHOGENIC ROLE FOR ELECTRICAL  
CHARGE***

Wayne A. Border, ... , Elaine S. Kamil, Arthur H. Cohen

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# Induction of Membranous Nephropathy in Rabbits by Administration of an Exogenous Cationic Antigen

## DEMONSTRATION OF A PATHOGENIC ROLE FOR ELECTRICAL CHARGE

WAYNE A. BORDER, HARRY, J. WARD, ELAINE S. KAMIL, and ARTHUR H. COHEN,  
*Departments of Medicine and Pathology, University of California at Los  
Angeles School of Medicine, Harbor-UCLA Medical Center, Torrance,  
California 90509*

**ABSTRACT** We examined the role of antigenic electrical charge as a determinant of glomerular immune complex localization in the rabbit. Serum sickness nephritis was induced in groups of New Zealand white rabbits by daily 25-mg intravenous injections of bovine serum albumin (BSA) chemically modified to be cationic ( $pI > 9.5$ ) or more anionic ( $pI$ , 3.5–4.6); an additional group received unmodified native BSA ( $pI$ , 4.5–5.1). Factors known to influence immune complex localization, e.g., molecular size of the administered antigen and resulting circulating immune complexes, immunogenicity, and disappearance time from the circulation were examined and found to be similar for both anionic and cationic BSA. Charge modification did increase the nonimmune clearance of cationic and anionic BSA compared with native BSA. Injected cationic BSA was shown in paired label experiments to bind directly to glomeruli compared with native BSA. The renal lesion produced by cationic BSA was markedly different from that found in rabbits given anionic or native BSA. Animals receiving cationic BSA uniformly developed generalized diffuse granular capillary wall deposits of IgG, C3, and BSA detected after 2 wk of injections and increasing until death at 6 wk. Qualitatively similar deposits were produced by the administration of low doses of cationic BSA of only 1

or 10 mg/d. In contrast, the injection of both anionic and native BSA resulted in mesangial deposits at 2 and 4 wk with capillary wall deposits appearing by 6 wk. Ultrastructural examination of animals receiving cationic BSA revealed pure, extensive formation of dense deposits along the lamina rara externa of the glomerular basement membrane whereas such deposits were absent or rare in animals injected with the anionic or native BSA. Albuminuria was significantly greater at 6 wk in the groups receiving cationic BSA with a mean of 280 mg/24 h compared with 53 mg/24 h in the combined groups injected with anionic or native BSA. Blood urea nitrogen values were similar in all groups at 2 and 4 wk but higher in the animals receiving cationic BSA at 6 wk.

These experiments describe the reproducible induction of epimembranous immune deposits by administration of an exogenous cationic antigen. They suggest that antigenic charge can play an important role in the pathogenesis of membranous nephropathy by permitting direct glomerular binding of an antigen and predisposing to *in situ* immune complex formation.

## INTRODUCTION

Membranous nephropathy (MN)<sup>1</sup> is one of the commonest forms of glomerular disease in man (1) and the most frequent cause of the adult idiopathic nephrotic syndrome (2). The renal lesion is characterized by gen-

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Dr. Ward is a Research Fellow of the National Kidney Foundation. Dr. Kamil is the Arthur Gordon Fellow of the National Kidney Foundation of Southern California.

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<sup>1</sup> *Abbreviations used in this paper:* ABC-33, antigen binding capacity-33; BUN, blood urea nitrogen; EDA, anhydrous ethylenediamine; EDC, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride; GBM, glomerular basement membrane; IC, immune complex; Ig, immunoglobulin; IV, intravenous; MN, membranous nephropathy; PBS, phosphate buffered saline; RTE and Fx1A, renal tubular epithelial antigen.

eralized, diffuse granular deposits of immunoglobulin (Ig) and C3 that, as shown by ultrastructural examination, are confined to the subepithelial space, which is composed of the lamina rara externa of the glomerular basement membrane (GBM) and the area of the filtration slits (3). The immune deposits impart a thickness to the glomerular capillary wall that, in the absence of glomerular hypercellularity, gave early observers the impression of a primary change in the GBM, hence the term MN. Functionally, these patients have nephrotic syndrome and experience an average 10% reduction in glomerular filtration rate each year (2). MN is regarded as a prototype of immune complex mediated glomerulonephritis (4) but the mechanism(s) by which the immune complexes (IC) form in man is unproven and currently is a subject of considerable scientific interest and debate (5, 6). Chronic serum sickness nephritis in the rabbit (7, 8) and Heymann nephritis (autologous immune complex nephropathy) in the rat (9) have served as experimental models of MN. Historically the pathogenesis of MN in both models was attributed to the formation of circulating IC that deposited in the glomerular capillary wall, localizing in the subepithelial space (4, 9). Couser and Salant (6) have thoroughly reviewed the circulating IC hypothesis and in light of the extensive new findings in passive Heymann nephritis, have convincingly argued that subepithelial IC deposits in this model are formed *in situ* by interaction of renal tubular epithelial antigen (RTE, Fx1A) present in the GBM and corresponding antibody molecules. Despite the striking functional and pathological similarities between Heymann nephritis and human MN and the occasional demonstration of RTE in human glomeruli, it is unlikely that the pathogenesis of MN in man is due to an RTE-anti-RTE IC. When human kidneys were perfused with antibody to human RTE, no GBM deposits were found (6); two extensive searches (10, 11) involving 131 patients with MN have revealed no evidence of circulating antibody to RTE or the presence of RTE in glomerular deposits. Although the study of Heymann nephritis has been crucial to elaborating the mechanism of *in situ* IC formation, it is possible that an alternative model using either an endogenous or exogenous antigen, as described in this report, may prove more relevant to human MN. Indeed, in a study in rats (12), the renal perfusion of alternating cycles of antigen and antibody has been shown to produce subepithelial deposits.

Simultaneously with the description of experimental *in situ* IC formation has been the elucidation of the glomerular ultrafilter as both a charge and size selective barrier (13–15). Fractional clearance studies performed using either dextran (16–18) or protein molecules (19) of comparable size, but different net elec-

trical charge, have shown a greater clearance for cationic molecules than for anionic molecules. The degree to which macromolecules penetrate the glomerular wall is also dependent on charge. By modifying the net electrical charge of ferritin, Rennke and Venkatachalam (20, 21) have shown an almost linear relationship between increasing positive charge and depth of entry into the glomerular wall. Anionic ferritin, pI 3.5–4.5, is repelled at the lamina rara interna, whereas cationic molecules, pI 9–10, reach the subepithelial space. Using cationic probes in the rat, Kanwar and Farquhar (22) have demonstrated a network of fixed anionic sites along the lamina rara interna and externa of the GBM and within the mesangial matrix; additional sites are located on the endothelial and epithelial cell coats. The GBM sites are composed of glycosaminoglycans uniquely rich in heparan sulfate (23), whereas the epithelial cell sites contain sialic acid (24). It is therefore readily apparent that the electrical properties of the glomerulus might be important in the immunopathogenesis of glomerulonephritis, particularly MN. It is possible that differences in the electrophysical interactions of antigens and/or antibodies with the glomerular wall is a determinant of the site of IC localization; this is true whether IC form in the circulation or *in situ*. To test this hypothesis we investigated the effect of altering the charge of the experimental antigen, bovine serum albumin (BSA), in an animal model patterned after chronic serum sickness nephritis. Our results indicate that the administration of an exogenous cationic antigen uniformly induced subepithelial IC formation and the renal lesion of MN. The data suggest that electrical charge plays a role in the pathogenesis of experimental MN by predisposing to *in situ* IC formation.

## METHODS

**Preparation of antigens.** Crystalline fraction V BSA (Miles Laboratories, Inc., Elkhart, Ind.) was used unmodified as native BSA and as substrate to prepare charge-modified cationic and anionic BSA. Cationization was carried out according to a modification of the method of Hoare and Koshland (25) using 1-ethyl-3-[(3-dimethylaminopropyl)-carbodiimide hydrochloride] (EDC) (Pierce Chemical Co., Rockford, Ill.) and anhydrous ethylenediamine (EDA) (Fisher Scientific Company, Pittsburgh, Pa.). An EDA solution was prepared in a 1-liter glass flask by mixing 67 ml of EDA and 500 ml of distilled water. The pH was adjusted to 4.75 with 350 ml of 6 N HCl and the solution cooled to 25°C in an ice bath. 5 g of native BSA dissolved in 25 ml of distilled water was added to the EDA solution followed by 1.8 g of EDC. With continuous stirring the reaction was continued for 120 min maintaining temperature and pH constant and stopped by adding 30 ml of 4-M acetate buffer, pH 4.75. The product was dialyzed 48 h against distilled water at 4°C, lyophilized, and stored at –70°C. Charge-modified anionic BSA was prepared by succinylation as described by Klotz (26). 5 g of native BSA was dissolved in 250 ml of distilled

water in a flask and the pH adjusted to 8.65 with 1 N NaOH. Succinic anhydride (Eastman Kodak Co., Rochester, N. Y.) was added in nine 50-mg increments (total 450 mg) over 60 min with continuous stirring at constant pH. The product was dialyzed 48 h against distilled water at 4°C, lyophilized, and stored at -70°C.

**Characterization of antigens.** The pI of each BSA was measured in thin layers of polyacrylamide gel, pH range 3.5 to 9.5 (PAG plates, LKB Instruments, Inc., Rockville, Md.), using an LKB flatbed electrofocusing unit and accompanying power supply. The pH gradient was determined directly from the gel with an LKB surface glass pH electrode and Beckman pH meter (Beckman Instruments, Inc., Palo Alto, Calif.). The molecular size of cationic, anionic, and native BSA was measured by Sepharose 6B gel filtration using a standard molecular weight calibration kit containing albumin, aldolase, catalase, and ferritin (Pharmacia, Div. Pharmacia Fine Chemicals, Piscataway, N. J.). The nonimmune clearance of the three BSA species was quantitated in groups of six rabbits each as follows: 1 mg of each BSA was labeled with  $^{125}\text{I}$  (cationic BSA sp act, 140  $\mu\text{Ci}/\text{mg}$ ; anionic BSA 158  $\mu\text{Ci}/\text{mg}$ ; native BSA, 160  $\mu\text{Ci}/\text{mg}$ ) and mixed with 24 mg of unlabeled BSA of the same species and injected into each animal to simulate the dose used to induce nephropathy. Blood was obtained from the contralateral ear artery before injection and at 15 min and 2, 3, 4, 5, and 6 d postinjection and placed in a tube containing EDTA. Duplicate samples of whole blood, plasma, and serum (after addition of  $\text{CaCl}_2$ ) were counted in a Beckman automatic gamma counter (Beckman Instruments, Inc.). Plasma and serum samples contained >95% of  $^{125}\text{I}$  counts indicating insignificant binding of any of the BSA antigens to formed elements in the blood. The immunogenicity of the cationic and anionic antigens was assessed in each of the experimental nephritis groups by the antigen-binding-capacity-33 (ABC-33) technique performed as described by Minden and Farr (27). Five serial dilutions of each sample were prepared in duplicate and mixed with 0.5 ml of the respective  $^{125}\text{I}$  BSA solution. Controls included normal serum, buffer, and the remaining two BSA forms not administered to the particular experimental group. Results were plotted on semilog paper and the dilution corresponding to 33% BSA binding determined. The potential for cationic BSA to bind directly to the glomerulus was studied by the paired label method (28) using fully radiolabeled doses of 100, 250, 500, and 1,000  $\mu\text{g}$  of BSA (composed of 0.5  $^{125}\text{I}$  cationic BSA and 0.5  $^{131}\text{I}$  native BSA) in groups of three nonimmunized rabbits for each dosage (cationic BSA sp act, 210  $\mu\text{Ci}/\text{mg}$ ; native BSA, 230  $\mu\text{Ci}/\text{mg}$ ). After general anesthesia, the left kidney was exposed, the renal artery cannulated, and the kidney flushed with 3 ml phosphate-buffered saline (PBS), pH 7.4. The paired labels contained in 3 ml of PBS were infused by syringe followed by a repeat flush with 3 ml of PBS with the effluent collected from a puncture site in the renal vein. The renal circulation was reestablished for 15 min at which time the kidney was removed, 3 ml of blood obtained systemically by arterial puncture, and the animal was killed. Kidneys from each experimental group were decapsulated and the cortex was separated, diced, and generously washed three times in PBS. The renal cortices were repeatedly passed through graduated sieves (W. S. Tyler, Inc., Mentor, Ohio) until each preparation consisted of >85% glomeruli as determined by visual inspection; the glomeruli were allowed to air dry  $\times 120$  min, were weighed, and counted in an automatic gamma counter. The data were analyzed according to the formula (29): specific glomerular binding = renal  $^{125}\text{I}$  - (serum  $^{125}\text{I}$   $\times$  renal  $^{31}\text{I}$ ) / serum  $^{131}\text{I}$  and ex-

pressed as micrograms of cationic BSA per milligram of glomeruli. All iodination was performed using the chloramine T method (30); all radiolabeled proteins used were >90% trichloroacetic acid precipitable.

**Experimental animals.** Male New Zealand white rabbits (2 kg) were divided into experimental groups that received injections of charge-modified cationic ( $n = 20$ ) or anionic BSA ( $n = 10$ ) or unmodified native BSA ( $n = 5$ ). All animals received a primary intravenous immunization consisting of 1 mg of the respective antigen, containing 1  $\mu\text{g}$  of endotoxin (Difco Laboratories, Detroit, Mich.) as adjuvant (31), dissolved in PBS, pH 7.4; 1 wk later daily intravenous injections (six times per week) of 25 mg of cationic, anionic, or native BSA were begun. After 4 wk the BSA dose was increased to 50 mg and 2 wk later the animals were killed. Before each injection, BSA (100 mg/ml) was dissolved in PBS and centrifuged at 15,000 rpm for 30 min in a RC-2B Sorval centrifuge (Sorval Biomedical Div, DuPont Co., Wilmington, Del.). The animals were housed in laboratory cages and fed a standard diet. A blood sample and a 24-h urine collection were obtained weekly. Renal biopsies were performed on all animals before immunization and thereafter at 2, 4, and 6 wk. Additional groups of rabbits were treated in an identical manner except that the daily dose remained constant at 1 or 10 mg of the respective BSA.

**Laboratory procedures.** For light microscopy, tissue from each biopsy was initially fixed in alcoholic Bouin's solution for 24 h, processed in the usual fashion, and stained with periodic acid-Schiff and periodic acid silver methenamine reagent. A portion of each specimen was immersed in chilled isopentane and frozen in liquid nitrogen for immunofluorescence microscopy. After fixation in acetone the 4- $\mu\text{m}$  tissue sections were stained in a standard manner (31) using fluorescein isothiocyanate-conjugated (FITC) antisera (Meloy Laboratories Inc., Springfield, Va.) to rabbit IgG, C3, albumin, and BSA. The antisera were shown to be monospecific by Ouchterlony analysis and immunoelectrophoresis. The FITC-anti-BSA reagent did not cross-react with rabbit albumin but reacted equally well with cationic, anionic, and native BSA in double diffusion in gel and on tissue sections containing deposits of each species of BSA. A semi-quantitative grading scale was used with 0 = negative and  $\geq 4$  = maximal intensity (31). At least 20 glomeruli were evaluated in each biopsy by two observers and a mean score was given for both mesangial and capillary wall deposits. For electron microscopy (three glomeruli per specimen were examined), cubes of tissue were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, and further processed as described (32). Albuminuria was quantitated by radial immunodiffusion (33) using monospecific antirabbit albumin antiserum (Meloy Laboratories Inc.). Blood urea nitrogen (BUN) values were measured on an automated Beckman BUN analyzer (Beckman Instruments, Inc.). The molecular size of circulating IC occurring in animals following the daily injection of cationic or anionic BSA was determined at 2 wk as follows: the daily BSA dose was trace labeled with the respective  $^{125}\text{I}$  BSA as described for the clearance experiments and injected; 30 min later blood was obtained from the artery of the contralateral ear. The blood was allowed to clot at 37°C to avoid loss of cryoglobulins. Duplicate 100- $\mu\text{l}$  serum aliquots were applied to linear 10-40% sucrose gradients. Molecular weight markers included thyroglobulin, aldolase, and ferritin. The samples were placed in a SW 50.1 rotor and spun in a Beckman ultracentrifuge (Beckman Instruments, Inc.) at 100,000  $g$  for 16 h. 10-drop fractions were collected in 12  $\times$  75-mm plastic tubes and checked at 280 OD and counted in a Beckman

automatic gamma counter. Refractive index of representative samples was measured to assure a linear gradient.

**Statistical analysis.** Differences between groups in values of glomerular binding of  $^{125}\text{I}$  cationic BSA, BUN, albuminuria, and glomerular immune deposits were analyzed by *t* test and ABC-33 values by rank-sum test (34).

## RESULTS

The pI of each experimental antigen remained constant with serial measurements and was: cationic BSA,  $>9.5$  (although the standard pH range of the gel was pI 3.5 to 9.5, it was possible to extrapolate and estimate a pI of 9.5–10.0 for cationic BSA); native BSA, 4.5–5.1, and anionic BSA, 3.5–4.6. It is important to note that succinylation only slightly enhanced the anionic properties of native BSA. The elution profiles of the charge-modified BSA species (Fig. 1) were identical

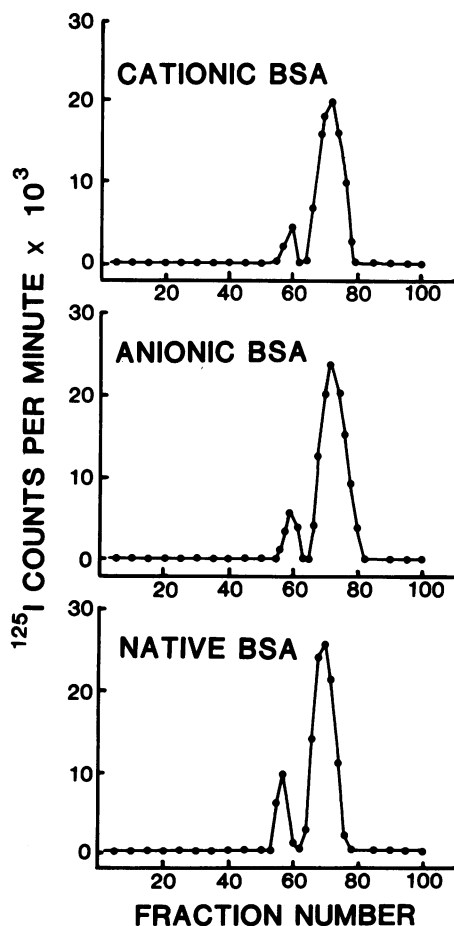


FIGURE 1 Elution patterns of cationic, anionic, and native BSA on a  $2.5 \times 86$ -cm column of Sepharose 6B. Total bed volume = 421 ml, void volume = 167 ml, eluent = PBS (0.01 M  $\text{PO}_4$ , pH 7.4), fraction volume = 5 ml. The top and center panels show that charge modification did not alter the molecular weight of native BSA, 66,000 or increase the small subpopulation of dimers that is normally present in BSA preparations.

to those observed and reported for native BSA (35), indicating that charge modification did not alter the molecular size of the BSA. Rennke and Venkatachalam have shown similar results with charge modification of ferritin (21) and horseradish peroxidase (36).

To be certain that differences in nonimmune elimination times would not confuse interpretation of the renal lesions produced by the three BSA antigens, the disappearance times were determined. Both charge-modified proteins rapidly disappeared from the circulation compared with native BSA (Fig. 2). Cationic BSA levels were slightly higher after day 3 but the range of values overlapped with those for anionic BSA. Immunogenicity of the charge-modified antigens was not significantly different as reflected by serial ABC-33 measurements (Table 1). Of the variables characterized the only difference between the BSA species was the demonstrated specific glomerular binding of  $^{125}\text{I}$  cationic BSA relative to  $^{131}\text{I}$  native BSA. As shown in Table II, with increasing doses greater amounts of glomerular uptake of specific  $^{125}\text{I}$  cationic BSA was

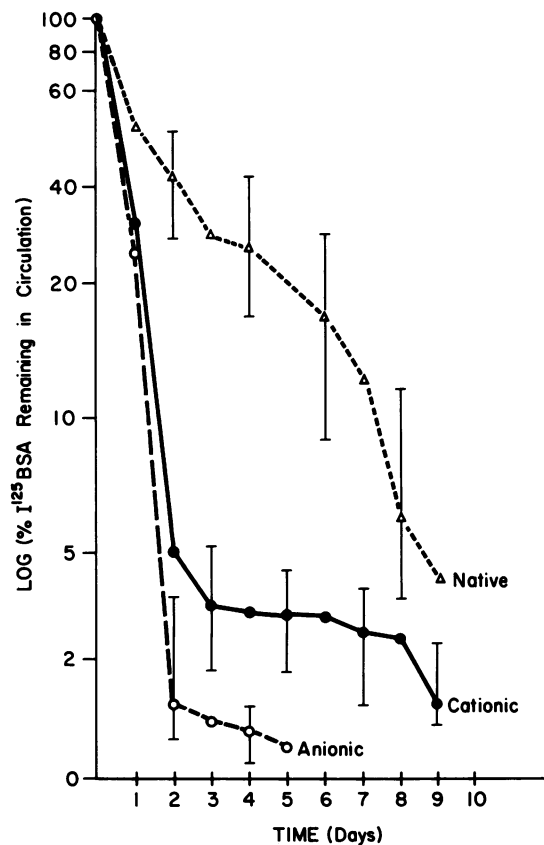


FIGURE 2 Disappearance time from the circulation of cationic, anionic, and native BSA measured in nonimmunized groups of six rabbits each. Both charge-modified BSA antigens were cleared more rapidly than native BSA. Values are mean  $\pm$  SEM.

TABLE I  
Antibody Response to Cationic and Anionic BSA Measured by  
Antigen Binding Capacity-33

BSA injected	n*	Anti-BSA antibody weeks of injections			
		0	2	4	6
			mg/dl		
Cationic	5	0	8.5 (0-15.3)†	19.0 (4.2-31.7)	9.4 (2.9-32.5)
Anionic	6	0	6.7 (0.8-26.4)	15.1 (2.4-31.7)	10.9 (1.3-47.5)
P value		NS‡	NS	NS	NS

\* Number of animals studied.

† Values are mean and range.

‡ Differences between cationic and anionic groups at each time interval are not significant ( $P > 0.05$ ).

observed. This binding occurred at a level below the detectability of direct immunofluorescence because all tissue samples examined by this technique were negative.

A comparison was made of the molecular size of circulating IC detected at 2 wk in animals receiving cationic or anionic BSA. At this time all animals were receiving daily 25-mg BSA injections which, based on ABC-33 measurements, would have produced a state of antigen excess resulting in small, soluble IC (4, 37). As shown in Table III, no qualitative differences in IC size were observed between the two groups and no IC larger than 500,000 daltons were found.

*Renal lesions produced by charge-modified and native BSA.* The renal lesions induced by the administration of cationic BSA were significantly differ-

TABLE II  
Results of Paired Label Renal Artery Injection of  $^{131}\text{I}$  Native  
BSA and  $^{125}\text{I}$  Cationic BSA

BSA injected*	n†	Specific cationic BSA binding‡	
$\mu\text{g}$			
100	3	$1.02 \pm 0.009^{\text{  }}$	$2.04 \pm 0.2^{\text{¶}}$
250	3	$1.43 \pm 0.320$	$2.15 \pm 0.25$
500	3	$13.49 \pm 3.25$	$5.39 \pm 1.28$
1,000	3	$37.43 \pm 7.83$	$7.42 \pm 1.52$

\* Total dose of BSA injected composed of 0.5 native BSA and 0.5 cationic BSA.

† Number of animals studied.

‡ Values are mean  $\pm$  SEM.

<sup>||</sup> Absolute amount of cationic BSA specifically bound in micrograms per milligrams of isolated, air-dried glomeruli.

<sup>¶</sup> Percentage of injected cationic BSA specifically bound per milligrams of isolated, air-dried glomeruli.

TABLE III  
Molecular Size of Immune Complexes Isolated  
from the Circulation of Rabbits Injected  
with Cationic or Anionic BSA

BSA injected*	n‡	Free BSA- 66,000	100,000- 300,000§	300,000- 500,000	500,000
Cationic	8	8 <sup>  </sup>	6	4	0
Anionic	5	5	5	5	0

\* Experiments performed after 2 wk of injections.

† Number of animals studied.

§ Molecular weight.

<sup>||</sup> Number of animals demonstrating immune complexes in each molecular weight range.

ent, both quantitatively and qualitatively from those in animals receiving anionic or native BSA (Fig. 3). After 2 wk of injections all animals in the cationic groups developed uniform granular capillary wall deposits of IgG, C3, and BSA. The IgG and C3 deposits were increased at 4 wk (BSA staining declined after

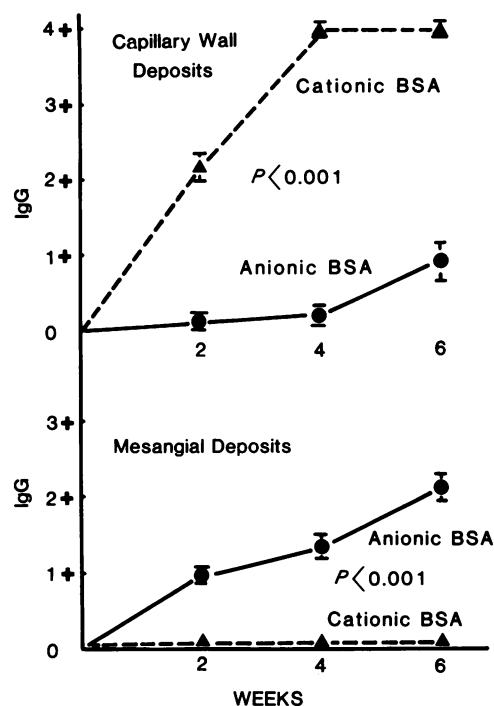


FIGURE 3 Semiquantitative grading of glomerular IgG deposits by immunofluorescence microscopy after 2, 4, and 6 wk of BSA injections. Data from animals receiving anionic ( $n = 10$ ) and native ( $n = 5$ ) BSA were combined into one anionic BSA group ( $n = 15$ ) and compared with animals receiving cationic BSA ( $n = 20$ ). Animals injected with cationic BSA ( $\Delta$ ) developed heavy granular capillary wall deposits ( $P < 0.001$ ), whereas administration of anionic or native BSA ( $\bullet$ ) produced mesangial deposits ( $P < 0.001$ ). Values are mean  $\pm$  SEM.

4 wk) and by 6 wk all animals showed advanced immunological lesions consisting of heavy, nearly confluent IgG granular deposits localized in the glomerular capillary wall (Fig. 4B) in a pattern resembling human MN. Ultrastructural examination revealed the presence of numerous, uniform electron-dense deposits in the lamina rara externa of the GBM (subepithelial space) (Fig. 4C). Small, sparse subepithelial deposits were first seen at 4 wk and by 6 wk were uniformly enlarged. Rare mesangial deposits were present in two of the animals, no deposits were encountered in the lamina rara interna. Light microscopy revealed numerous slightly thickened capillary walls with scattered short subepithelial basement membrane projections, as well as the presence of polymorphonuclear leukocytes and monocytes in some capillary lumens (Fig. 4A). The additional groups receiving lower daily doses of 1 or 10 mg of cationic BSA developed qualitatively similar lesions as the higher dose group. Quantitatively the IgG deposits were less intense at 2 and 4 wk, but by 6 wk were comparable and at this time similar subepithelial deposits were present.

The renal lesions in animals receiving anionic or native BSA were indistinguishable, but were strikingly different from those produced by cationic BSA. Because the glomerular lesions were identical, for purposes of presentation the data from animals receiving anionic or native BSA were combined into one anionic group and compared with the animals receiving cationic BSA. After 2 wk of injection, animals in the anionic group demonstrated by immunofluorescence, sparse granular deposits of IgG, C3, and BSA located predominantly in the mesangium (Fig. 3). By 4 and 6 wk the mesangial deposits had increased in quantity and scattered capillary wall deposits were beginning to appear (Fig. 4E). Ultrastructural examination verified the immunofluorescence observations showing increasing mesangial deposits at 2, 4, and 6 wk with

the majority of capillary walls appearing normal (Fig. 4F). Rare isolated subepithelial deposits were seen at 6 wk. Animals receiving low doses of 1 or 10 mg of anionic BSA showed qualitatively similar mesangial deposits as animals receiving the higher doses; however, no subepithelial deposits were present in the low-dose groups after 6 wk of injections. When examined by light microscopy, the renal lesions in the anionic group showed mild to moderate mesangial hypercellularity affecting all lobules equally (Fig. 4D). No crescents were observed.

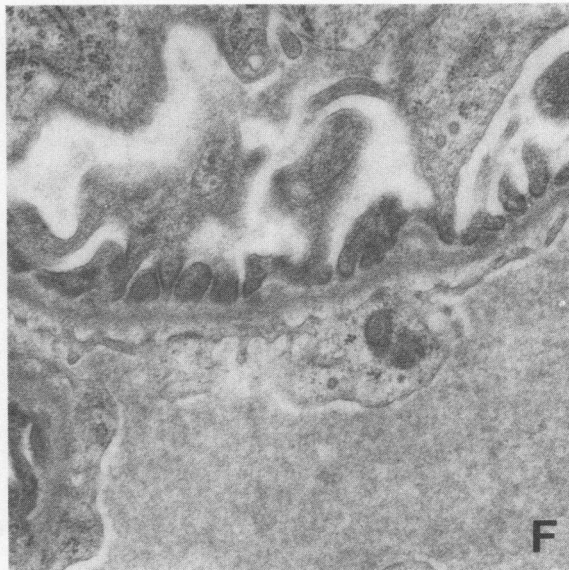
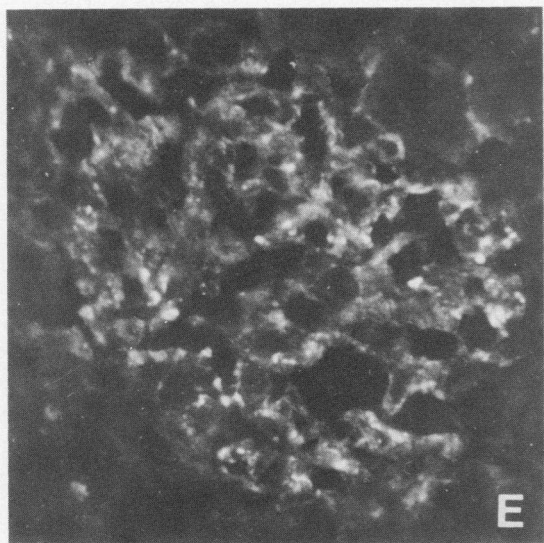
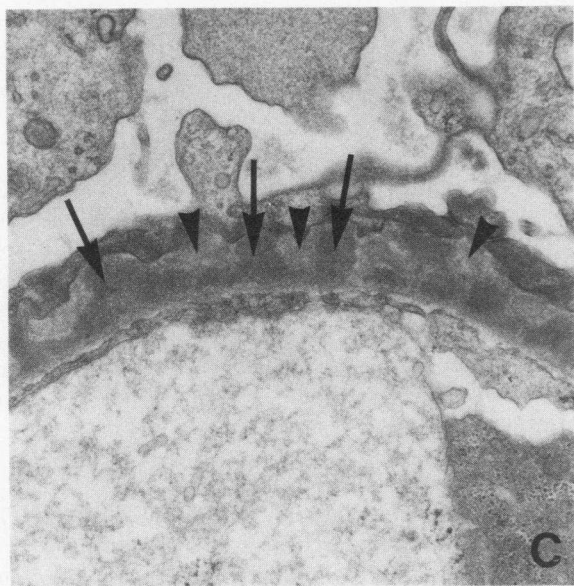
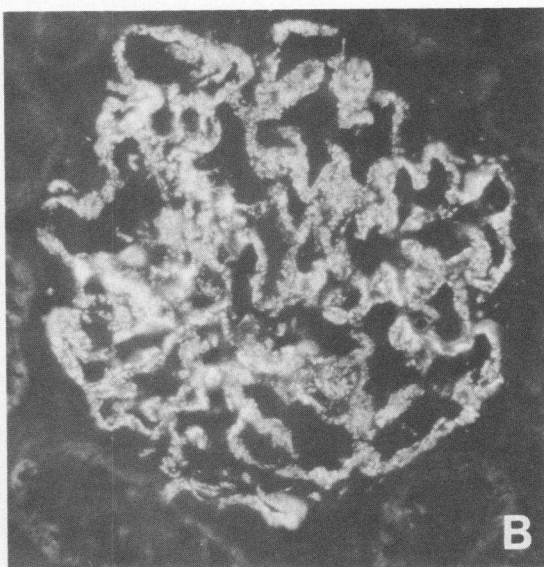
Abnormal amounts of albuminuria ( $>20$  mg/24 h) were detected after 2 wk of injections of cationic BSA and increased until the time of death at 6 wk (Fig. 5). In contrast, albuminuria appeared after 4 wk of administration of anionic BSA but did not progressively increase as in the cationic group. Rabbit albumin was detected by immunofluorescence in the 4- and 6-wk biopsies of animals in all groups as protein reabsorption droplets within renal tubular cells; glomerular staining did not occur at any time. Renal function as measured by BUN levels was similar in all groups until 6 wk when a significant increase was observed in the animals receiving cationic BSA (Fig. 6).

## DISCUSSION

Electrical charge, as a factor influencing the glomerular localization of IC, is emerging as an important new concept in renal immunopathology. The classic studies of chronic serum sickness by Dixon et al. (7, 37) and Germuth et al. (4, 8) were performed before the elucidation of the glomerular wall as a charge-selective barrier; thus, the potential importance of electrical charge was not investigated. To define more critically the role of charge in the pathogenesis of IC glomerulonephritis and to extend the historical information, we deliberately chose for study a model pat-

**FIGURE 4** Representative light (LM), immunofluorescence (IF), and electron (EM) micrographs of glomerular lesions after 6 wk of injections of cationic BSA (A, B, and C) or anionic BSA (D, E, and F). (A) LM of a glomerulus from a rabbit receiving cationic BSA showing the capillary walls and basement membranes to be thin. There is a mild increase in cellularity, primarily resulting from the accumulation of circulating leukocytes in several capillaries. (Periodic acid silver methenamine,  $\times 440$ ). Inset: Higher magnification of a capillary wall revealing short irregular projections of basement membrane material (arrow) that are seen beneath the epithelial cell cytoplasm. (Periodic acid silver methenamine,  $\times 1725$ ). (B) IF demonstrating  $\geq 4$  granular capillary wall deposits of IgG ( $\times 500$ ). (C) EM of same animal shown in A and B. EM indicates that the capillary wall is thickened and has numerous, regular subepithelial electron-dense deposits (arrows) and associated basement membrane projections (arrow heads). The foot processes of the epithelial cells are completely effaced ( $\times 13,000$ ). (D) LM of a glomerulus from a rabbit receiving anionic BSA. There is an expansion and increase in cellularity affecting mesangial regions of all lobules to a more or less equal degree. (Periodic acid silver methenamine,  $\times 440$ ). (E) IF showing  $\geq 2$  coarse, irregular mesangial and occasional capillary wall deposits of IgG ( $\times 500$ ). (F) EM of same animal shown in D and E. The EM depicts foot processes that are largely discrete and a lack of deposits. There were, however, mesangial and very rare solitary subepithelial "hump" deposits ( $\times 13,000$ ).







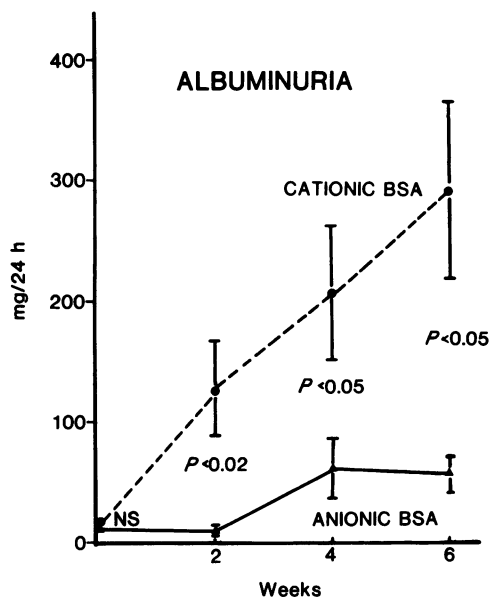


FIGURE 5 Proteinuria measured as excretion of rabbit albumin in animals receiving cationic (●) or anionic (▲) BSA. Preinjection values were not different (NS) but at 2, 4, and 6 wk, albuminuria was greater in the cationic group. Values are mean±SEM.

terned after chronic serum sickness. Our results indicate that the charge of the administered antigen has a profound effect on the nature and severity of the renal lesion and that it plays a decisive role in the formation of subepithelial immune deposits and the production of membranous nephropathy.

It is important to note that in our model all of the animals receiving cationic BSA developed a renal lesion uniformly consisting of capillary wall deposits of granular IgG, BSA, and C3. The deposits were observed as early as 2 wk and increased in intensity after 4 and 6 wk of injections. Electron-dense deposits, confined to the subepithelial space, were detected uniformly in all animals in the 6-wk biopsies. The delayed appearance of the ultrastructural lesion, relative to the immunofluorescence findings, also occurs in active Heymann nephritis and presumably represents the time required for the antigen-antibody lattice to achieve sufficient size to become electron dense (38). On the other hand, if an electron-dense antigen, e.g., ferritin, is used to induce IC glomerulonephritis (39, 40), then no delay is seen. Changes by light microscopy were minimal, consisting of thickening of capillary walls with early basement membrane "spike" formation and a mild increase in circulating leukocytes. The renal lesion produced by cationic BSA thus fits the generally accepted definition of MN. Animals receiv-

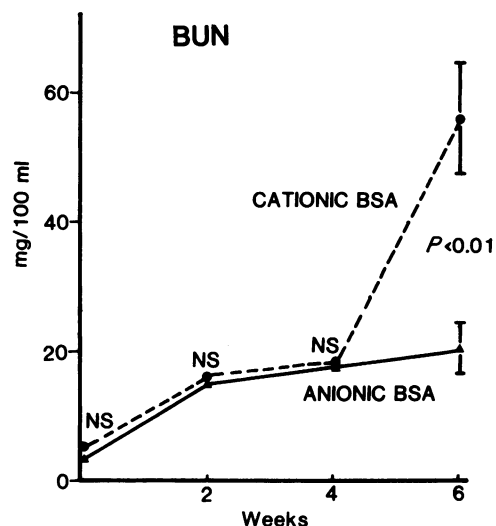


FIGURE 6 Renal function in rabbits injected with cationic (●) BSA ( $n = 20$ ) or anionic (▲) BSA ( $n = 10$ ). Differences in BUN values at 0, 2, and 4 wk were not significant (NS); unlike the difference at 6 wk ( $P < 0.01$ ). Values are mean±SEM.

ing either anionic BSA or native BSA showed mesangial hypercellularity. At 6 wk the deposits observed by immunofluorescence and electron microscopy were located principally in the mesangium; these findings would best be described as mesangial proliferative glomerulonephritis and are consistent with other studies of traditional serum sickness (31, 41, 42) in which renal tissue was examined after a similar period of injections.

Membranous nephropathy has been observed in chronic serum sickness induced by native BSA, but it appears to be an uncommon finding. Dixon et al. (7), injected rabbits for 1.5 to 7.5 mo and induced proteinuria and glomerulonephritis. At autopsy large and irregularly distributed hump-shaped subepithelial electron-dense deposits were found frequently but usually in association with a diffusely hypercellular, lobular glomerular lesion, often showing inflammation, scarring, capillary obliteration, and/or crescent formation. Germuth et al. (41) selected from a group of 56, 18 rabbits for their ability to clear the daily BSA dose of 12.5 mg within 24 h. Of the 18, 9 developed epimembranous deposits, 2 developed mesangial proliferative glomerulonephritis, and the remaining 7 showed no evidence of renal disease. In one of the few serum sickness studies in which serial renal biopsies were performed (42), only 1 of 11 animals was found to have membranous nephropathy. Thus, the universal occurrence of membranous nephropathy in our ex-

perimental group receiving cationic BSA is striking compared with its low incidence reported in the literature and its absence in our groups receiving anionic or native BSA.

We believe that the induction of epimembranous deposits by injections of cationic BSA is due to *in situ* IC formation. Cationic BSA, relative to anionic BSA, was shown to bind directly to the glomerulus, providing a necessary precondition for *in situ* formation; this binding was likely due to electrical attraction between the cationic BSA and the anionic glomerular capillary wall (a similar GBM binding of cationic human albumin in the rat has been reported by Purtell et al.) (43). We have not excluded the possibility that circulating IC produced the membranous deposits in the cationic group, but we think this is unlikely for the following reasons: (a) In serum sickness, circulating IC size has been thought to be the most important factor governing glomerular localization with IC of 300,000–500,000 daltons, producing subepithelial IC deposits (4, 41). Such IC are formed in animals administered excessive antigen, producing low levels of antibody and/or possibly antibody of low avidity (4). On the other hand, larger IC formed in the presence of antibody excess and/or highly avid antibody are thought to deposit principally in the mesangium. In our experiments differences in IC size did not explain the strikingly different renal lesions found in the cationic and anionic groups. At 2 wk, the time of the first renal biopsy, animals in both groups receiving 25-mg daily injections were in antigen excess based on the ABC-33 measurements and at 2 wk measurement of the size of circulating IC in both groups showed a similar spectrum of sizes, all under 500,000 daltons. Two additional factors, quantity of circulating IC and anti-BSA antibody avidity were not determined in this study and may have contributed to the morphologic differences between the cationic and anionic groups. (b) Epimembranous deposits also formed in animals receiving as little as 1 or 10 mg of cationic BSA daily. Although circulating IC size was not directly measured in these low dose animals, they were in antibody excess based on ABC-33 values, and would have been expected to form mesangial deposits as did occur in parallel groups given anionic BSA. In support of our conclusion is the work of Couser and Salant (6); they reviewed the experimental evidence in serum sickness and other studies in which preformed IC were injected and have concluded that there is no direct evidence that soluble IC can cross the GBM to form subepithelial deposits. Future experiments are planned to elucidate the potential of the cationic BSA system to form IC *in situ*.

It is entirely possible that both mechanisms of IC formation, *in situ* and soluble deposition, can coexist and even act in a synergistic manner. A role for *in situ* IC formation in native BSA chronic serum sickness had been historically excluded by the failure to detect glomerular bound BSA before antibody formation (7). We found no evidence of direct glomerular binding of cationic BSA by immunofluorescence microscopy, but radioisotope techniques verified that it had occurred. Fleuren et al. (12) demonstrated in the isolated, perfused rat kidney that native BSA can induce *in situ* IC formation. In our animals injected with anionic or native BSA, early *in situ* IC formation may have increased GBM permeability and facilitated deposition of circulating IC. Such a sequence may theoretically occur in human glomerulonephritis.

Electrical charge as a pathogenic factor was not originally considered to play a role in described models of *in situ* IC glomerulonephritis induced by IgG aggregates (44), concanavalin A (29), or anti-RTE antibody (45, 46). Subsequently, it has been shown that cationic anti-RTE antibody binds in greater quantity than anionic antibody (47) suggesting that a role for charge in one or more of these models must remain a possibility. The importance of charge is suggested by a recent report by Batsford et al. (40) who gave rats a single injection of cationized ferritin followed by antiferritin antibody. Both ferritin and IgG were found deposited along the GBM; however, no anionic ferritin was included as an experimental control and administration of cationized ferritin alone produced subepithelial dense deposits. Furthermore, when cationized ferritin was given to produce active serum sickness, ferritin was ultimately detected principally in a mesangial pattern (40). In previous work in the rat we found that the administration of cationic BSA (50-mg injections, three times per week for 2 wk) produced mesangial glomerulonephritis (48). The different injection schedule used in the rat experiments makes it impossible to directly compare those results with the current findings in the rabbit. A role for the net charge of circulating IC in producing GBM deposits has been proposed by Gallo et al. (49) based on a study in which preformed, putative cationic or anionic IC were administered to mice; however, the injection of the cationic antigen alone resulted in GBM deposits, suggesting that *in situ* IC formation may also have occurred.

We have demonstrated the reproducible induction of subepithelial IC deposits by administration of an exogenous cationic antigen. These experiments suggest a mechanism by which antigenic charge can predispose to *in situ* IC formation and the production of

MN. It is hoped that this new model will provide important insights into the pathogenesis and treatment of MN in man.

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