Clin. exp. Immunol. (1986) **64**, 606–614.

Role of antigenic charge and antibody avidity on the glomerular immune complex localization in serum sickness of mice

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(Accepted for publication 9 December 1985)

SUMMARY

Passive injection of mice with preformed immune complexes (IC) made from cationized bovine serum albumin (BSA) and anti-native BSA antibody gave immune deposits along the glomerular capillary walls at predominantly subepithelial sites, while similar quantities of complexes made with native, anionized BSA did not deposit. Peripheral localization could be obtained also using low avidity antibody and a great excess of native BSA. Ultracentrifugation analysis showed that the size of IC in the animals given complexes containing cationized BSA was a little larger than 7 S, whereas those formed with the native or anionized BSA were around 19 S. The anti-native BSA antibody had a low avidity for cationized BSA *in vitro*, and thus all the IC which could deposit peripheral capillary walls were small and contained low avidity antibody. Chemical cationization of BSA alters the precipitability of the antibody and also the size and stability of the complexes formed. In an active model, injection of cationized BSA into mice preimmunized with cationized BSA caused localization of the BSA and its antibody in the peripheral capillary walls. Analysis of the circulating IC formed in this model also revealed low avidity of antibody and small-sized IC. From these results, it is clear that chemical cationization of antigen changes the characteristics of the antigen-antibody interaction, e.g. low precipitating efficiency and the formation of small-sized IC. Therefore, in addition to interaction of cationized IC with the polyanion layer of the glomerular basement membrane (GBM), the properties of antigen–antibody interaction play an important role in the deposition of IC along the peripheral capillary walls in a model of membranous glomerulonephritis.

Keywords antigenic charge glomerular IC localization avidity low-precipitating antibody size of IC

INTRODUCTION

Formation of immune deposits within the glomerulus is believed to result from two possible mechanisms: glomerular deposition of circulating, soluble IC formed by combination of antibody and antigen within the circulation (Dixon, Feldman & Vasquez, 1961, Germuth, Senterfit & Dreesman, 1972), and combination of antibody with structual or 'planted' antigens within the glomerular capillary walls itself (Couser & Salant, 1980). Recent evidence that the glomerular capillary wall is highly anionic (Kanwar & Farquhar, 1979) has led to the realization that charge is a

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major determinant of antigen localization, especially at subepithelial sites. Repeated injection of cationic antigen readily leads to deposition of antigen at subepithelial sites, together with specific antibody. Present theory suggests that these IC result predominantly or exclusively from *in situ* combination of cationic antigen and specific antibody (Border *et al.*, 1982).

In the model of passive serum sickness in mice, a number of properties, especially size, have been shown to influence localization, but in general injected complexes localize predominantly within the mesangium, or in the subendothelial space (Koyama *et al.*, 1978). Only occasionally have subepithelial deposits been noted, except when small complexes prepared with low-avidity antibody are used (Germuth *et al.*, 1979, Lew *et al.*, 1984b). However, when complexes are prepared from cationized antigen, their injection is followed by a predominantly subepithelial localization (Gallo, Caulin-Glaser & Ramm, 1981).

In this study we have examined the effects of antigenic charge on both the localization of preformed IC made with BSA, and complexes formed after active immunization with BSA.

MATERIALS AND METHODS

Preparation of antigen. Crystalized BSA (Sigma Chemical Co., St Louis, Missouri, USA) was used unmodified as native BSA, and as substrate to prepare charge-modified cationic and anionic BSA. Chemical cationization; replacement of carboxyl-group of BSA with ethylendiamine using water-soluble carbodiimide (amidation), was carried out according to a modification of Hoare & Koshland (1967), and by methylation (esterification) as described by Ram & Maurér (1959). Charge-modified anionic BSA was prepared by succinylation as described by Klotz (1976).

Preparation of antibodies. Rabbits were immunized with native BSA in complete Freund's adjuvant. Antisera were taken 10 days after the first injection as a source of low-avidity antibodies. Animals were then hyperimmunized and bled as a source of high-avidity antibodies. These rabbit antibodies (IgG) to native BSA were isolated by DEAE cellulose column chromatography (0·01 M phosphate buffer, pH 8·0) and affinity chromatography using native BSA-coated Sepharose. Antibodies were eluted with glycine buffer, pH 2·4.

Characterization of antigens. The pI of each BSA preparation was measured in thin layers of polyacrylamide gel, pH range 3·5–9·5 (Ampholine, Pageplate, LKB Instruments, Rockville, MD, USA).

Preparation of IC. The equivalence point of anti-native BSA was determined by the quantitative precipitation test. Complexes were prepared mainly at 40-fold antigen excess by weight based on equivalence point with native, charge-modified BSA and anti-native BSA which contained 600 μg of precipitating antibodies in each case.

Characterization of antibody. The quantitative precipitation test was performed by the method of Kabat & Mayer (1961), with native, charge-modified BSA and anti-native BSA. Antigen binding capacity-33 (ABC-33) was measured by the method of Minden & Farr (1978). Antibody avidity was measured by the antigen dilution effect on ABC-33.

Radiolabeling. Native, charge-modified BSA and anti-native BSA antibodies were labeled with ^{125}I and ^{131}I, respectively, by the chloramine-T method.

Sucrose density gradient ultracentrifugation studies of antigens and IC. The sizes of antigens and IC were measured by sucrose density gradient ultracentrifugation (5–35% linear sucrose density gradient in PBS, pH 8·0). Antigens and IC were run at 100,000 *g* for 24 h in a Beckman L-5 series Ultracentrifuge (Beckman Instrument, California, USA). BSA, human IgG and IgM were used as 4·6 S, 7 S and 19 S markers and radioactivity assayed in each fraction in a gamma spectrometer.

Experimental design. C57BL/6J mice were used throughout. Each group or subgroup consisted of seven to ten mice.

Passive serum sickness. Five experimental groups were used. The first group of mice was given preformed IC composed of cationized BSA and anti-native BSA (cationic-BSA IC); the second preformed IC composed of methylated BSA and anti-native BSA (methylated-BSA IC); the third preformed IC composed of anionized BSA and anti-native BSA (anionic-BSA IC); the fourth preformed IC composed of native BSA and anti-native BSA (high-avidity Ab native-BSA IC). All

these IC were made using the same high-avidity (avidity = 0·94) anti-native BSA antibodies. The fifth group was given preformed IC composed of native BSA and low-avidity (avidity = 0·32) anti-native BSA (low-avidity Ab native-BSA IC). In each case, the IC injected contained 600 μg of precipitating antibody. In addition, a final group was given IC composed of native BSA, and containing 3,000 μg of precipitating antibodies. Animals were killed at 6 h after the injection of IC and the localization and amounts of IC deposition were examined.

Active serum sickness. Four experimental groups of 20 mice were used. The first group was immunized with 500 μg of cationized BSA plus complete Freund's adjuvant intraperitoneally. At 14 days after immunization, 10 mice were bled and killed for measurement of antibody and histological examination. The remaining 10 mice were injected intravenously with 150 μg of ^{125}I-cationized BSA. 15 min after the antigen injection, the mice were bled for examination of the size of circulating IC, and killed for histological examination. The second and third groups of mice were immunized with anionized and native BSA, and a fourth group was injected with saline plus complete Freund's adjuvant. Controls were killed at day 14.

Histological examination. The histological examinations were performed as described previously (Koyama *et al.*, 1978).

RESULTS

Characteristics of antigen

The pI values of the antigens were as follows: cationized BSA, above 9·5; methylated BSA, 8·5–9·0; native BSA, 4·5–5·0; anionic BSA; 3·5–4·5. When analyzed by ultracentrifugation, the sizes of these antigens were almost identical (data not shown).

Passive serum sickness

In mice given preformed cationic-BSA IC or preformed methylated-BSA IC, antibody was localized mainly along the peripheral capillary walls in subepithelial and subendothelial sites (Fig. 1a, b and e, Table 1). In mice given the anionic-BSA IC and high-avidity Ab native-BSA IC, glomerular fixed antibody was almost undetectable, but in the mice given five times the quantity of anionic-BSA, high-avidity Ab native BSA-IC, antibody was localized in the mesangium (Fig. 1c). Similarly, in the group given five times the quantity (3,000 μg) of low-avidity Ab native-BSA IC, antibody was localized along the peripheral capillary walls at subepithelial and subendothelial sites (Fig. 1d and f).

Characterization of IC and effect of cationization of antigen on antigen-antibody interaction in vitro

Quantitative precipitation test. Precipitating efficiency was very low between cationized BSA and anti-native BSA. In addition, antibody prepared using native BSA as antigen had low avidity to cationized BSA, but high avidity toward anionized and native BSA (Table 1).

Size of IC. The ultracentrifugation gradients of circulating complexes *in vivo* 5 min after injection (Fig. 2) showed that high avidity Ab native-BSA IC were large (Fig. 2a, b and c, closed circle), ranging 7 S to 25 S with a peak at around 19 S. In contrast, the cationic or methylated-BSA IC were only 7 S or little larger (Fig. 2a and b, open circle), similar to low-avidity Ab native-BSA IC (Figure 2c, open circle). Fig. 2d and e showed the ultracentrifugal profiles of plasma from animals given double-labelled antigen and antibody. The distribution of the complexed antibody was mainly around 19 S in the case of IC composed of high-avidity anti-native BSA and native BSA, and those of the antigen was between 7 S and 19 S (Fig. 2d). The anti-native BSA-cationized BSA complexes ran at a peak only just greater than 7 S, virtually no 19 S material was present, and the ^{125}I-labelled antigen showed two peaks, one of free BSA at 4·6 S and another around 7 S, presumably Ag1Ab1 IC (Fig. 2e).

Active serum sickness

In the group immunized with cationized BSA, at 14 days after the immunization, small amounts of mouse IgG were observed in the mesangium (Fig. 3a), as found also in the control group (Fig. 3b).

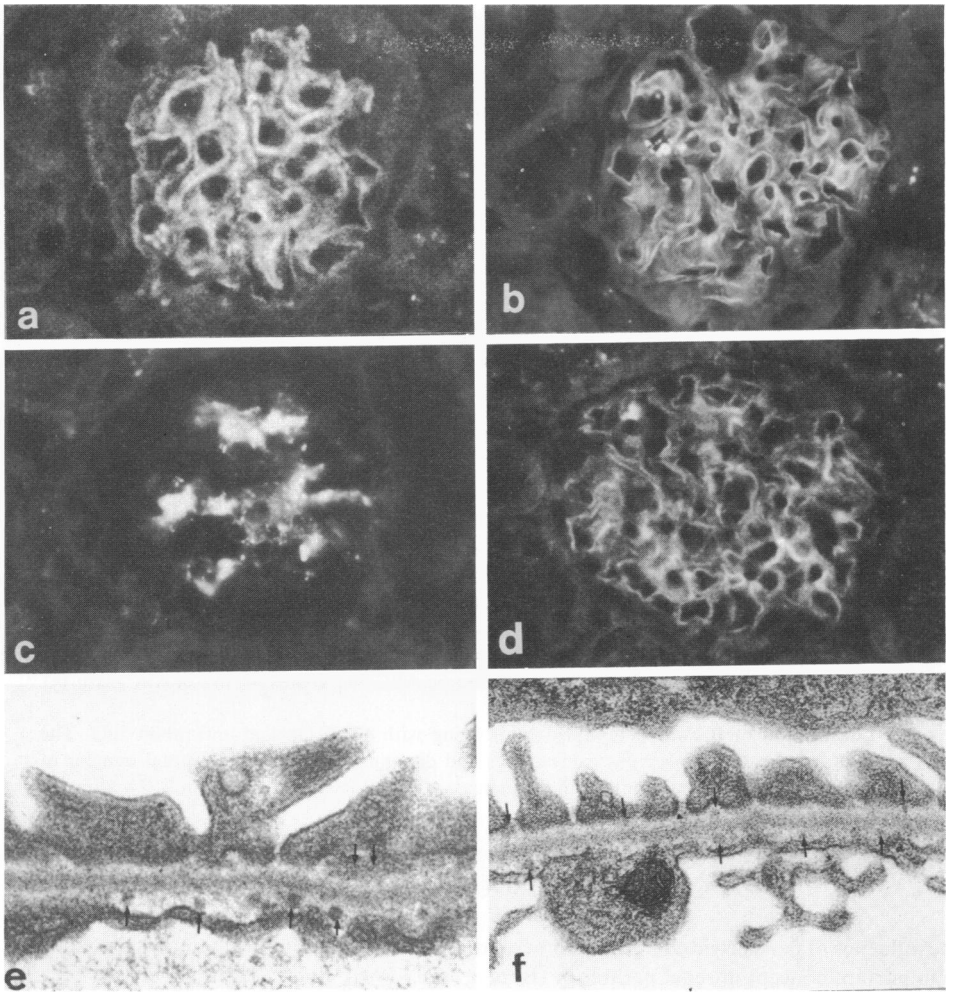

Fig. 1. Immunofluorescence patterns after staining with fluoresceinated anti-rabbit IgG, in mice given preformed IC formed with anti-native BSA antibodies and various charged BSA. Cationized BSA–anti-native BSA IC (a), methylated BSA–anti-native BSA IC (b), native BSA–high-avidity anti-native BSA IC (3,000 μg of antibodies) (c) and native BSA–low-avidity anti-native BSA IC (3,000 μg of antibodies) (d). Electron microscopic findings in the group given preformed cationized BSA–anti-native BSA IC (e) (× 36,800) and native BSA–low-avidity anti-native BSA IC (f) (× 29,600). Arrows indicate subepithelial and subendothelial deposits.

At 15 min after the intravenous injection of the radio-labelled antigen, diffuse deposition of mouse IgG was detected along the peripheral capillary walls (subepithelial and subendothelial deposition) (Fig. 3c, d). IC formed in the cationic group were small, nearly 7 S (Fig. 2f). In the group immunized with anionized and native BSA, at 14 days after the intravenous injection of the antigens, minor mesangial deposition was observed.

In the group immunized with cationized BSA, at 14 days after the immunization the level of antibodies measured by ABC-33 was significantly lower than those in the group immunized with native BSA, and the level of antibody avidity was also significantly lower than in the group immunized with native BSA (Table 2).

Table 1. Effect of chemical modification of antigen on antigen-antibody interaction and localization of IC

IC (Added BAS)	Anionic-BSA IC (Anionic)	High-avidity native BSA IC (Native)	Low-avidity native-BSA IC (Native)	Methylated-BSA IC (Methylated)	Cationic-BSA IC (Cationized)
PPt Ab (mg/ml)	7·7	10·4	—	0	0
ABC-33 (μg/ml)	1386	2970	—	—	1419
Avidity	0·69	0·94	0·32*	0·12	0·11
Peripheral deposition	0/7	0/10	9/10	7/7	10/10
Mesangial deposition	6/7	8/10	0/10	0/7	0/10
Trace or no deposition	1/7	2/10	1/10	0/7	0/10

* Using high avidity antibody against native BSA except for low avidity antibody.

PPt Ab. The levels of precipitating antibodies were measured by quantitative precipitation test between native, charge-modified BSA (1·25 mg) and anti-native BSA antibodies.

ABC-33: Antigen binding capacity-33 was carried out between native, or charge modified BSA (antigen concentration was 1 μg) and anti-native BSA antibodies.

Avidity was measured by antigen dilution effect on ABC-33 (Avidity = value of ABC-33 @ 0·1 μg BSA/value of ABC-33 @ 1 μg BSA).

Localization of IC was determined by staining with FITC-labelled anti-rabbit IgG. The numerator indicates number of positive cases and denominator indicates the total number of experimental animals. As a control, in the animals given antigen or antibody only 6 h after the injection, no rabbit IgG was detected.

DISCUSSION

The influence of net electric charge on glomerular localization of immune materials has emerged as an important concept in renal pathology (Border *et al.*, 1982, Gallo *et al.*, 1981, Oite *et al.*, 1982, Germuth, Rodriguez & Wise, 1982) and the idea of *in situ* assembly of antigen and specific antibody has become predominant, particularly in the case of subepithelial deposits. To define further the role of charge in the pathogenesis of IC nephritis, we examined the characteristics of charge-modified antigens and IC in the systems of passive and active serum sickness in mice.

We injected preformed IC using cationized, native and anionized antigens. These experiments gave similar results to those of Gallo *et al.* (1981) who used BGG complexes: in the case of cationic BSA IC the deposition of IC along the glomerular capillary wall was visible by immunofluorescence. While in contrast, injection of IC composed of native and anionized BSA gave mesangial deposition of IC. Under certain conditions, IC composed of native BSA, could also deposit along the peripheral capillary walls (Fig. 1d and f).

We examined also the effect of charge modification of antigen on the interaction between antigen and antibody *in vitro*. Anti-native BSA antibodies were of low avidity to cationized BSA, compared with their binding to native and anionized BSA, and the precipitating efficiency was very low between cationized BSA and anti-native BSA (Table 1). Sucrose density gradient analysis showed that cationic-BSA IC were smaller than those of anionic and high-avidity Ab native-BSA IC (Fig. 2). There was no evidence that the cationic-BSA IC dissociated *in vivo* (Fig. 2d and e) and both Caulin-Glaser, Gallo & Lamb (1983) and Lew *et al.* (1984a) have reported recently that the injection of covalently linked (and hence non-dissociable) cationic complexes leads to deposition at subepithelial sites.

Clearly the chemical modification of carboxyly-group of BSA molecule has affected its antibody

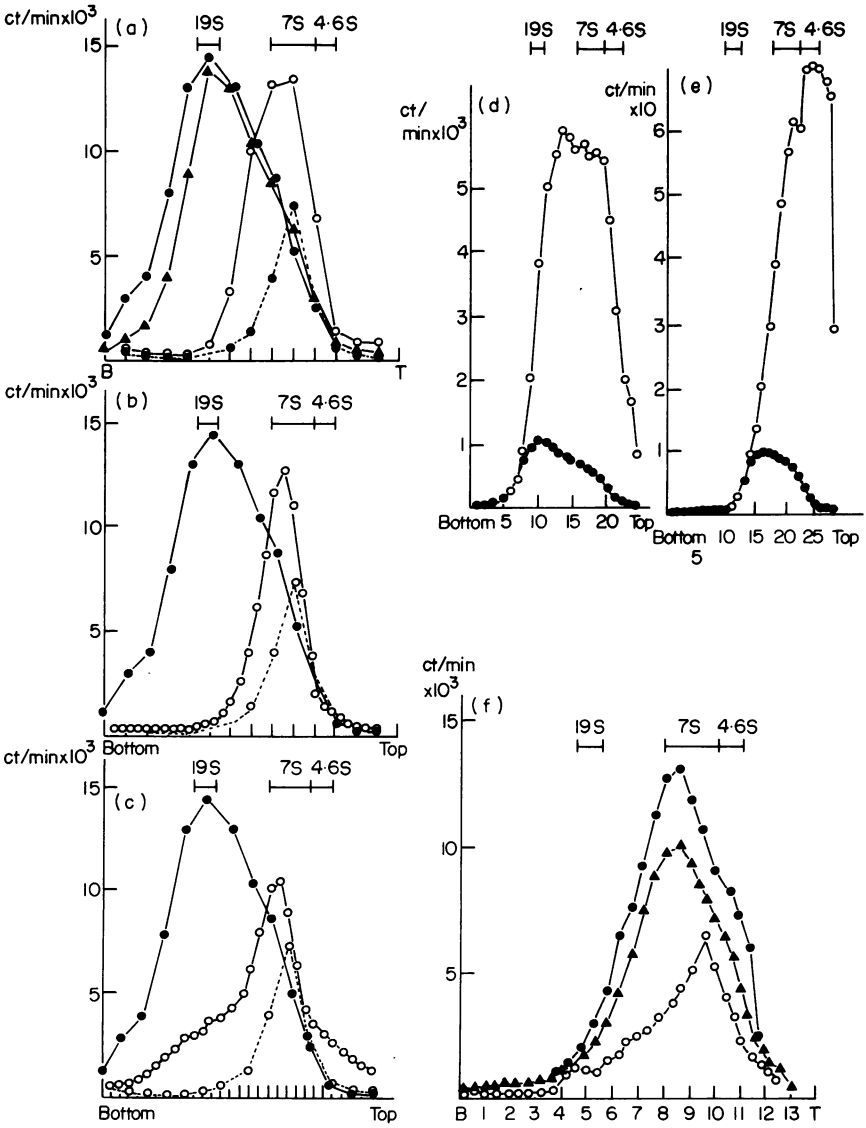

Fig. 2. Sizes of IC formed between rabbit anti-native BSA and various charged BSA species *in vivo*. (a) Shows the sizes of IC formed by cationized BSA–anti-native BSA-^{125}I (○), anionized BSA–anti-native BSA-^{125}I (▲) and native BSA–high-avidity anti-native BSA-^{125}I (●). (b) Shows similar data for methylated BSA–anti-native BSA-^{125}I (○). (c) Shows native BSA–low-avidity anti-native BSA-^{125}I (○). Dashed lines (- - -) indicate anti-native BSA and closed circles (●) indicate the native BSA–high-avidity anti-native BSA IC in each figure. (d) Shows the distribution of ^{125}I-native BSA (○) ^{131}I-high-avidity anti-native BSA (●). (e) Shows the distribution of ^{125}I-cationized BSA (○) anti-native BSA-^{131}I (●). (f) Shows the distribution of ^{125}I-cationized BSA at 15 min after the second injection of antigen in active model ($n = 3$, ●, ▲, ○). All sera were taken at 5 min after the preformed IC injection. BSA, human IgG and IgM were used as 4·6 S, 7 S and 19 S markers.

binding and precipitability with anti-native BSA antibody, whether amidation or methylation are used, even though the pI of methylated BSA was only 8·5. Thus, small IC form when cationized BSA reacts with anti-native BSA antibody. It is interesting that Gallo *et al.* (1981) did not obtain

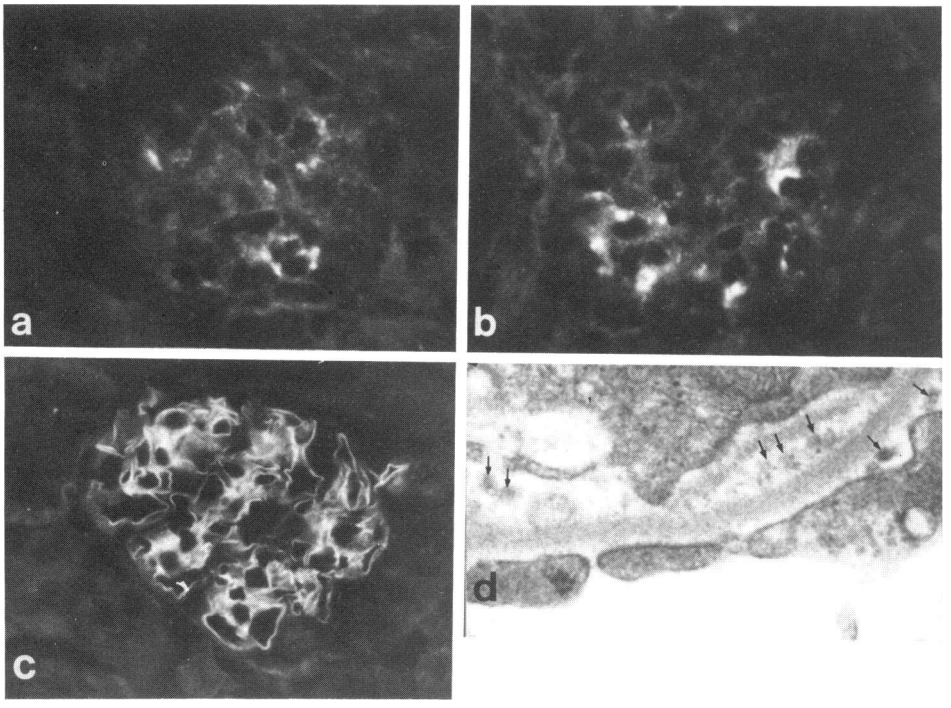

Fig. 3. Immunofluorescence patterns after staining with FITC-anti-mouse IgG, in mice immunized with cationized BSA, before (a) and after (c) the second injection of the antigen. Control (b). Electron microscopic finding in mice immunized with cationized BSA after the second injection of the antigen (d). Arrows indicate subepithelial, intramembranous and subendothelial deposits.

Table 2. Antigen binding capacity-33 and antibody avidity in mice immunized with cationized and native BSA at 14 days after the immunization.

Mice immunized with	N	ABC-33 (μgBSA/ml)	Avidity
Native BSA	9	9·9±8·4	0·37±0·16
Cationic BSA	5	2·7±2·1	0·13±0·04
		$P<0·05$	$P<0·01$

ABC-33: antigen binding capacity-33 at 1 μg BSA was carried out between native, cationized BSA and rabbit anti-native BSA antibodies.

Avidity was measured by antigen dilution effect on ABC-33. Values are expressed as mean±s.d.

peripheral capillary wall localization of complexes made from cationic BGG prepared by isoelectric focusing, whereas they did so with chemically modified BGG complexes.

Repeated immunization with cationized BSA in rabbits (Border *et al.*, 1982) or BGG in mice (Gallo *et al.*, 1983) shows that such modified antigens are more nephritogenic than their native or anionized counterparts, and that predominantly subepithelial deposits are formed when cationized antigens are used. In mice, using BSA as an antigen, we found that in the presence of free circulating

antibody during the primary response there was no peripheral deposition. However, soon after a second injection of cationized antigen, predominant localization in the peripheral capillary wall occurred. The size of IC in the circulation was very small, slightly larger than 7 S. Our results suggest that subepithelial deposition can derive from circulating IC during long-term immunization because the antibody produced in the group immunized with cationized antigen is of low avidity and poor precipitating capacity (Table 2), and thus forms small IC.

From these observations, we speculate that if the IC are small enough they can deposit on the subepithelial site of GBM even without modification of carboxyl side chains. In the mice given a higher dose of IC made with low-avidity antibody and native BSA, antibody was localized along the peripheral capillary walls (Germuth *et al.*, 1979).

We conclude that the charge of antigen and the size of IC are important factors determining the localization of IC within the glomerulus. This conclusion supports the proposal that appearance of IC in the glomerulus, even in the subepithelial space, may in part be due to deposition of circulating IC of small size formed from antibody of low avidity and probably cationically charged, especially if the antigen is cationic, as well as the formation of deposits *in situ* (Fleuren *et al.*, 1978; Van Demme *et al.*, 1978., Couser *et al.*, 1978). Probably, both mechanisms operate.

The authors are grateful for helpful discussions with Dr T. Fujita and thank Ms H. Sakurai, A. Uchinokura, S. Isawa and Mr S. Abe for excellent technical assistances and Ms H. Sukegawa who typed the manuscript. This work was supported by a Research Grant for the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, Japan.

REFERENCES

BORDER, W.A., WARD, H.J., KAMIL, E.S. & COHEN, A.H. (1982) Induction of membranous nephropathy in rabbits by administration of an exogenous cationic antigen, demonstration of a pathogenic role for electrical charge. *J. clin. Invest.* **69,** 451.

CAULIN-GLASER, T., GALLO, G.R. & LAMM, M.E. (1983) Nondissociating cationic immune complexes can deposit in glomerular basement membrane. *J. exp. Med.* **158,** 1561.

COUSER, W.G., STEINMULLER, D.R., STILANT, N.M., SALANT, D.J. & LOWENSTEIN, L.M. (1978) Experimental glomerulonephritis in the isolated perfused rat kidney. *J. clin. Invest.* **62,** 1275.

COUSER, W.G. & SALANT, D.J. (1980) *In situ* immune complex formation and glomerular injury. *Kidney Int.* **17,** 1.

DIXON, F.J. JR., FELDMAN, D. & VASQUEZ, J.J. (1961) Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis. *J. exp. Med.* **113,** 899.

FLEUREN, G.J., LEE, R.V.D., GREGEN, H.A., VAN DAMME, B.J.C. & HOEDMAEKER, PH. J. (1978) Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. IV. Investigations into the pathogenesis of the model. *Lab. Invest.* **38,** 496.

GALLO, G.R., CAULIN-GLASER, T. & RAMM, M.E. (1981) Charge of circulating immune complexes as a factor in glomerular basement membrane localization in mice. *J. clin. Invest.* **67,** 1305.

GALLO, G.R., GAULIN-GLASER, T., EMANCIPATOR, S.N. & LAMM, M.E. (1983) Nephritogenicity and differential distribution of glomerular immune complexes related to immunogen charge. *Lab. Invest.* **48,** 353.

GERMUTH, F.G., SENTERFIT, L.B. & DREESMAN, G.R. (1972) Immune complex disease. V. The nature of the circulating complexes associated glomerular alterations in the chronic BSA-rabbit system. *Hopkins Med. J.* **130,** 344.

GERMUTH, F.G., RODRIGUEZ, E., LORELLE, C.A., TRUMP, E.I., MILANO, L.L. & WISE, O. (1979). Passive immune complex glomerulonephritis in mice: models for various lesions found in human disease. II. Low avidity complexes and diffuse proliferative glomerulonephritis with subepithelial deposits. *Lab. Invest.* **41,** 366.

GERMUTH, F.G., RODRIGUEZ, E. & WISE, O.L. (1982) Passive immune complex glomerulonephritis in mice. *Lab. Invest.* **46,** 515.

HOARE, D.G. & KOSHLAND, E.D. JR. (1967) A model for the quantitative modification and estimation of carboxylic acid groups in proteins. *J. biol. Chem.* **242,** 2447.

KABAT, E.A. & MAYER, M.M. (1961) *Experimental Immunochemistry.* 2nd ed. (ed. by Charles C. Thomas), p. 72. Springfield, Illinois.

KANWAR, Y.S. & FARQUHAR, M.G. (1979) Presence of heparan sulfate in glomerular basement membrane. *Proc. natn. Acad. Sci. USA*, **76,** 1303.

KLOTZ, I.M. (1976) Succinylation. *Methods Enzymol.* **11,** 576.

KOYAMA, A., NIWA, Y., SHIGEMATSU, H., TANIGUCHI, M. & TADA T. (1978) Studies on passive serum sickness. II. Factors determining the localization of antigen-antibody complexes in the murine renal glomerulus. *Lab. Invest.* **38,** 224.

LEW, A.M., TOVEY, D.G. & STEWARD, M.W. (1984a) Localization of covalent immune complexes on the epithelial side of the glomerular basement membrane in mice. *Int. Archs Allergy appl. Immun.* **75,** 242.

LEW, A.M., STAINES, N.A. & STEWARD, M.W. (1984b) Glomerulonephritis induced by pre-formed immune complexes containing monoclonal antibodies of defined affinity and isotype. *Clin. exp. Immunol.* **57,** 413.

MINDEN, P. & FARR, R.S. (1978) Ammonium sulfate method to measurement antigen binding capacity. In *Handbook of Experimental Immunology* 3rd edn (ed by D.M. Weir) Blackwell,Oxford, UK.

OITE, T., BATSFORD, R., MIHATSCH, M.J., TAKAMIYA, H. & VOGT, A. (1982) Quantitative studies of *in situ* immune complex glomerulonephritis in the rat induced by planted, cationized antigen. *J. exp. Med.* **155,** 460.

RAM, J.S. & MAURER, P.H. (1959) Modified bovine serum albumin. VII. Studies on the role of free carboxyl groups of the protein in the interaction with its antibodies. *Arch. Bioch. Biophys.* **83,** 223.

VAN DAMME, B.J.C., FLEUREN, G.J., BECKER, W.W., VERNIER, R.L., & HOEDMAEKER, PH.J. (1978) Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab. Invest.* **38,** 502.