THE DETECTION AND CHARACTERIZATION OF IMMUNE COMPLEXES IN GLOMERULONEPHRITIS

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SUMMARY

The detection of granular deposits of immunoglobulin (Ig) and complement (C) in the glomeruli of over 70% of patients with glomerulonephritis (GN) suggests that most GN in man is immune complex (IC) mediated. However, final confirmation of the concept and diagnosis of ICGN requires the detection of IC in the serum of such patients and the identification of antigen in the glomerular IC deposits. These two objectives are pursued in the present study.

I. The detection of circulating IC in patients with GN. Four assays for the detection of IC were evaluated in vitro and then used to test sera from patients with a spectrum of renal diseases. The Raji cell radioimmune assay (*IRCA) and the solid phase Clq assay (ClqSPA) were found to be more sensitive and specific than the radiolabeled Clq binding assay (*IClqBA) or the microcomplement consumption test (MCT). In general, the assays detected large amounts of circulating IC in patients with systemic lupus erythematosus (SLE) and GN associated with other systemic diseases. The prevalence of IC detection in primary GN was much lower; IC were detected more frequently in acute GN than chronic (membranous, membranoproliferative, etc.) GN, and in patients with low C3, C4, and properdin factor B (C3PA) levels. The inability of the assays to detect IC in all patients with immunofluorescence (IF) evidence of ICGN may simply reflect the limited sensitivity of the assays, but may imply that some patients with GN have as yet undefined abnormalities in the handling of more or less physiologic amounts of IC.

II. The identification of IC antigen and antibody in GN. Improved techniques are required for the identification of antigen and antibody

in glomerular IC deposits. Special IF and elution techniques were, therefore, evaluated using the bovine serum albumin (BSA) induced serum sickness model of ICGN in the rabbit. Optimal conditions for the elution and recovery of glomerular IC antibody were defined in these studies, and similar techniques were then applied to identify antigen-antibody systems in (i) spontaneous ICGN in rabbits, and (ii) human GN.

(i) IgG eluted from the kidneys of rabbits with spontaneous GN reacted with the glomerular capillaries and blood vessels of normal kidney sections. It is proposed that a structural antigen, possibly derived from the glomerular epithelial glycocalyx, participates in the formation of nephritogenic circulating IC in this form of GN.

(ii) Eluates were obtained from nephrectomy or autopsy kidneys from patients with various forms of GN. These eluates were screened for antibodies to a panel of endogenous antigens (glomerular basement membrane (GBM), renal tubular antigen (RTA), nucleic acids, and IgG), and viral antigens (measles virus, Epstein-Barr virus (EBV), and C-type virus). Anti-measles virus antibodies were selectively concentrated over serum levels in four eluates (three SLE, and one primary ICGN) suggesting a role for measles virus antigen-antibody IC in these patients.

It is concluded that multiple antigen-antibody systems can be involved in individual patients with ICGN, and the participation of ubiquitous antigens again suggests that these patients have underlying abnormalities which cause them to handle a "normal" antigen and IC load in a nephritogenic manner.

This thesis contains no material which has been submitted for the award of any other degree or diploma from any university. To the best of my knowledge and belief, this thesis contains no material published or written by another person, except where due reference is made in the text of the thesis.

Andrew J. Woodroffe May 2, 1977

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PUBLICATIONS

The following is a list of publications arising from studies incorporated in this thesis.

Woodroffe, A. J., and Wilson, C. B.: Improved techniques in the identification of antigen (Ag) and antibody (Ab) in immune complex glomerulonephritis (ICGN) and the detection of anti-measles virus antibody in eluates from human ICGN kidneys. Fed. Proc. 35:574, 1976 (abstr.).

Woodroffe, A. J., Border, W. A., Theofilopoulos, A. N., Götze, O., Glassock, R. J., Dixon, F. J., and Wilson, C. B.: Detection of circulating immune complexes (IC) in glomerulonephritis (GN). Amer. Soc. of Nephrology, 9th Annual Meeting, November 1976, Washington, D. C. Kidney Int. 101:552, 1976 (abstr.).

Woodroffe, A. J., and Wilson, C. B.: An evaluation of elution techniques in the study of immune complex glomerulonephritis. J. Immunol. (in press).

Woodroffe, A. J., Border, W. A., Theofilopoulos, A. N., Götze, O., Glassock, R. J., Dixon, F. J., and Wilson, C. B.: The detection of circulating immune complexes in patients with glomerulonephritis. Evaluation and application of the Raji cell radioimmune assay, the radiolabeled C1q binding assay, and microcomplement consumption test. Kidney Int. (in press).

Tung, K. S. K., Woodroffe, A. J., Ahlin, T. D., Williams, R. C., and Wilson, C. B.: Application of the solid phase Clq and Raji cell radioimmune assays for the detection of circulating immune complexes in glomerulonephritis. J. Clin. Invest. (in press).

INTRODUCTION

Background. The role of immune mechanisms in the pathogenesis of human glomerulonephritis (GN) was recognized in the 1950's when renal biopsy (1) and immunofluorescence (IF) (2) techniques were introduced. The detection by IF of immune reactants in the glomeruli of patients with GN (2-5) strongly suggested an immunological process, and these observations were supported by studies with experimental models of GN. In these studies it was recognized that GN could be mediated either by antibodies reactive with glomerular basement membrane (GBM) antigens to produce nephrotoxic or anti-GBM antibody GN (6-10), or by glomerular trapping of immune complexes (IC) formed in the circulation with non-glomerular antigens, to produce ICGN (11-17). The two mechanisms could be identified and differentiated one from the other by IF studies. Thus anti-GBM antibody GN is characterized by linear deposits of immunoglobulin (Ig) and complement (C) along the GBM, and ICGN by the presence of granular deposits of Ig and C in the mesangium or glomerular capillary loops. These conditions could not be differentiated by conventional morphological techniques, and the importance of IF in the evaluation of renal biopsies was recognized.

IF findings in a series of 2,540 renal specimens have recently been reported by Wilson and Dixon (18). Granular deposits of Ig generally accompanied by C were present in approximately one half of these specimens; linear deposition was much less frequently observed (5%). The incidence of granular deposits in patients with clinico-pathological evidence of GN was even greater, between 70 and 80%. These data support the view that the majority of human GN is IC mediated, a premise which will be explored in this thesis.

Experimental Models of ICGN. Much of our knowledge regarding the role of circulating IC in GN is derived from experimental models of artificially induced GN (e.g. serum sickness in the rabbit and autologous ICGN in the rat) or spontaneous GN (e.g. "autoimmune" IC disease in New Zealand (NZ) mice). Some of these models will now be reviewed.

(i) Serum sickness following the administration of foreign serum proteins was reported by Von Pirquet in 1911 (19), but the IC nature of the disease was not recognized until the 50's. The detection of circulating and tissue IC in the bovine serum albumin (BSA) induced rabbit model of serum sickness by Germuth (11, 12) and Dixon (14-17) established the role of IC in this disease, and the model has subsequently been used to study the factors involved in IC mediated GN.

In acute (one-shot) serum sickness the sequence of events is as follows (17). After intravenous administration, BSA disappears from the circulation in three phases. The first (24 to 48 hours) is due to equilibration with intra and extravascular fluids, and the second to catabolism (half disappearance time of 4.2 days). Antibody can be detected on day 4 or 5, and circulating IC are formed. Initially the IC are in antigen excess, are too small to be phagocytosed by the reticuloendothelial system (RES), and continue to circulate. As antibody production increases, the circulating IC increase in size and by about day 10 are eliminated from the circulation (third phase). During this immune elimination phase, small amounts of IC (BSA, antibody, and C) are deposited in vascular structures to produce arteritis and proteinuric GN. Free antibody appears in the circulation after immune elimination increasing the size of the IC deposits and "masking" the BSA. The disease, however, is self-limiting and proteinuria is

transient. The model closely resembles classical acute post-streptococcal GN (PSGN) in man.

In chronic (daily injection) serum sickness (CSS) (15), rabbits given BSA in amounts to balance antibody production develop proteinuric ICGN after two to three months. Like chronic GN in man, the histologic lesions are variable, but a mixed membranous and proliferative form of GN is most commonly observed.

(ii) The autologous ICGN model in rats is induced by immunization with extracts of rat kidney containing renal tubular antigen (RTA). Morphologically the lesion closely resembles membranous GN in man. The model, originally described by Heymann in 1959 (20), has since been studied extensively by Edgington and others (21-23). Antibodies reactive with the proximal convoluted tubule cell brush border can be eluted from the glomerular IC deposits and the antigen has been identified as a high molecular weight (MW) lipoprotein (RTE-alpha 5). Small amounts of this antigen are normally present in the circulation to form IC with antibody induced by immunization, and the constant availability of antigen maintains the disease. Quantitative studies using radiolabeled antigen (24) have recently confirmed these observations.

(iii) Spontaneous GN in NZ black (NZB) and NZB-white hybrid mice (NZB x NZW) F_1 (25, 26) is similar to human systemic lupus erythematosus (SLE). These mice develop anti-nuclear antibodies (ANA), Coomb's positive haemolytic anaemia, and progressive ICGN. The role of C-type virus infection in the pathogenesis of this disease is suggested by the detection of murine leukaemia virus antigens in the glomerular IC deposits (27), and by the presence of anti-viral

antibodies in eluates from GN kidneys (28, 29). However, the relative contributions of the different antigen-antibody systems (nuclear antigen-antibody, and virus antigen-antibody) is still not clear. Finally, these mice (as in human SLE) have a defect in thymus-dependent T cell function (30) which might allow the formation of antibodies to self-antigens.

(iv) Other viral infections are also associated with ICGN. These include lymphocytic choriomeningitis (LCM) (31-35) and lactic dehydrogenase (LDV) (36, 37) viral infections in mice, Aleutian disease in mink (38), equine infectious anaemia (39), and hog cholera (40). Typically these viruses are not highly cytopathic but produce a persistent infection with a constant supply of viral antigen which effectively leads to chronic virus antigen-antibody IC formation and glomerular deposition. In LCM the IC are in a state of antigen excess, and no free antibody can be detected in the circulation. However, anti-LCM antibody can be eluted from the glomeruli, and LCM viral antigen can be identified in the glomerular IC deposits by IF (33, 35).

(v) Finally, there is a background incidence of spontaneous ICGN in many species; guinea pigs (41), subhuman primates (42-44), rabbits (45), rats (Wilson, C. B., personal communication), dogs (46, 47), cats (48), horses (49), and sheep (50, 51). These conditions, like spontaneous GN in man, are for the most part still poorly understood, and in view of their possible similarity to human ICGN are worthy of more extensive study.

Glomerular Deposition of IC and the Mediation of ICGN. Factors which are known or believed to influence the glomerular deposition of circulating IC include (i) the size and composition of the IC, (ii) the

function of the RES and of the glomerular mesangium, and (iii) the role of vaso-active substances.

(i) Studies with pre-formed IC have shown that large IC (greater than 19S) are cleared from the circulation much more rapidly than smaller (antigen excess) complexes (52-56). Germuth (57) postulates three categories of circulating IC: class I, small (less than 1,000,000 daltons) soluble complexes formed in antigen excess which deposit along the GBM and are highly nephritogenic; class II, intermediate size, poorly soluble complexes which are taken up by the mesangium and may produce GN; and class III, large (greater than 1,000,000 daltons) insoluble complexes formed in antibody excess which are rapidly removed from the circulation by the RES and do not produce GN. This conceptual approach is useful rather than exacting, and although the avidity of the antibody may also be important (57, 58) the ratio between the amount of IC antigen and antibody is probably the most critical factor in determining the potential nephritogenicity of the IC (59).

(ii) The RES effectively removes large IC from the circulation and conceivably "exhaustion" of this function could lead to glomerular IC deposition. A similar role for the mesangium has been suggested (60, 61) but recent studies in rabbits with CSS indicate that mesangial function (as measured by the uptake of aggregated human gamma globulin) is in fact increased (62).

(iii) Finally, the role of vaso-active substances (histamine, serotonin) and other mediators must be considered. By increasing the permeability of the glomerular capillary wall, these substances increase glomerular deposition of IC (63-65), and antagonists of histamine or serotonin have been found to reduce the severity of acute serum sickness

in rabbits (63), autologous ICGN in rats (66), and spontaneous GN in NZ mice (67). Although C is present in the glomerular IC deposits and may participate in mediation of the inflammatory response, there is no evidence that the fixation of C by circulating IC in any way alters their ability to deposit in blood vessels (55, 56).

The granular deposits of Ig and C in the glomeruli of the experimental models discussed above are so characteristic that this has become the hallmark of the disease. However, certain recent studies demand that this concept be qualified. Thus Mauer et al (68) have described *in situ* formation of glomerular mesangial IC in rats given aggregated human gamma globulin followed by antibody. Similarly, Izui et al (69) induced the *in situ* formation of glomerular deoxyribonucleic acid (DNA)-anti DNA IC in mice; in this model, endogenous DNA released into the circulation following administration of bacterial lipopolysaccharide (LPS) binds to the GBM where it is then accessible to antibody. Finally, fixation of antibody to conconavalin A (after it has bound to GBM associated polysaccharides) produces *in situ* glomerular IC in rats (Golbus, S. M., personal communication). This Arthus-like mechanism has also been identified in experimental thyroiditis (70) and tubulo-interstitial nephritis (71), and a similar mechanism could conceivably be involved in certain forms of human GN, particularly PSGN and mesangial IgA disease (18). As a corollary, it is clear that in serum sickness the glomerular bound IC are in dynamic equilibrium with antigen and antibody from the circulation and that once initiated the disease could be maintained by glomerular deposition of non-complexed antigen or antibody (18).

Finally, two other mechanisms for the deposition of glomerular immune reactants have recently been proposed. C receptors have been identified in human glomerular epithelial (72, 73) and/or mesangial (74) cells, and these may be involved in glomerular trapping of IC from the circulation. These receptors have not been identified in the glomeruli of experimental animals, and their precise role, if any, in the mediation of ICGN requires further detailed evaluation. Similarly, there is some evidence for a "non-immune C activation" mechanism in some forms of GN (75). Such patients have GN with glomerular deposits of C in the absence of Ig. This is accompanied by a depression in serum C levels, and may be mediated by the non-immune (non-Ig induced) activation of the alternative C pathway.

The precise mechanism of glomerular IC deposition is less important in considering the mediation of IC induced glomerular injury. Common mediator pathways are involved with interaction between the C, kinin, and coagulation systems (65, 76, 77). The final morphologic expression of injury depends on the tempo of the disease rather than the nature of the underlying immunopathogenic mechanism, and this limits the value of traditional morphologic classifications of GN.

Human (presumed IC mediated) GN. The inadequacies of classification are even more acute in human GN. Clinical, morphological, IF, and ultrastructural features have been variously used to categorize human GN, and there has been much confusion in this area. The problem will not be resolved until a definitive aetiologic classification becomes available. In the meantime the best approach is a combination of the various indices.

Table 1 depicts the key morphologic, IF, and ultrastructural features of the major types of human GN. This classification identifies the two major immunologic mechanisms (IC mediated GN, and anti-GBM antibody GN) and allows recognition of the various clinico-pathological forms of GN. Thus IF findings differentiate ICGN from anti-GBM antibody GN,and clinical and serological information separate ICGN secondary to systemic diseases from primary ICGN. The clinical presentation also helps to differentiate the primary ICGN subtypes, i.e., classical acute PSGN with acute diffuse proliferative GN, recurrent haematuria and proteinuria with mesangial IgA disease, and the nephrotic syndrome with focal sclerosing (FSGN), membranous (MGN), or membranoproliferative GN (MPGN).

Morphologic lesions are sometimes characteristic, e.g. in FSGN, MGN, and MPGN, but more often are not diagnostic and require qualification. In general the IC deposits detected by IF or electron microscopy (EM) correspond to the site of the morphologic lesions, but in some instances, glomerular IC deposits are found in the absence of (and presumably precede) morphologic change. The localization of deposits to the mesangium or to the subendothelial or subepithelial aspects of the GBM, and the preponderance of one or more of the Ig subclasses in the deposits (as depicted in Table 1) are useful pointers in diagnosis. Thus the IF findings are characteristic in MGN and in SLE, and are of diagnostic value in mesangial IgA disease, minimal lesion nephrotic syndrome, (and anti-GBM antibody GN). EM findings are characteristic in MPGN (and its subtype, dense deposit disease), MGN and classical acute PSGN, and are of diagnostic value in minimal lesion nephrotic syndrome and in some forms of hereditary GN.

TABLE 1

CHARACTERISTIC MORPHOLOGIC, IF, AND ULTRASTRUCTURAL FEATURES OF THE MAJOR FORMS OF HUMAN GN

CATEGORY	MORPHOLOGY	IF (Ig AND C3)	EM
PRESUMED ICGN			
Primary GN			
Focal sclerosing GN	Focal and segmental areas of sclerosis	Granular Ig (IgM) and C3 in areas of sclerosis	Subendothelial and mesangial deposits and sclerosis
Focal mesangial proliferative GN (mesangial IgA disease)	Focal mesangial proliferation	Granular Ig (IgA) and C3 in mesangium	Mesangial deposits
Acute diffuse proliferative GN (PSGN)	Exudation and proliferation of endothelial and mesangial cells	Diffuse granular Ig and C3	Subepithelial "humps"
Rapidly progressive GN	Extracapillary proliferation with crescents	Variable granular Ig and C3	Variable
Membranous GN	Diffuse thickening of GBM	Diffuse granular Ig (IgG) and C3 along GBM	Extramembranous "spikes"
Membranoproliferative (mesangiocapillary) GN	Thickening of GBM with mesangial proliferation and interposition	Granular Ig and C3 in mesangium and GBM	Subendothelial and intramembranous sub-types
Chronic (end-stage) GN	Variable proliferative and sclerotic lesions	Residual granular Ig and C3	Variable
GN secondary to systemic disease			
Systemic lupus erythematosus	Membranous, focal, or diffuse proliferative GN	Granular Ig (IgG, IgA, IgM) and C3 in mesangium or GBM and interstitium. In vivo ANA.	Mesangial, subendothelial, or subepithelial deposits. Microtubular structures.
Henoch-Schönlein purpura	Focal or diffuse proliferative GN	Granular Ig (IgA) and C3 in mesangium and GBM	Mesangial and subendothelial deposits
Wegener's granulomatosis	Proliferative and crescent forming GN	Variable granular Ig and C3	Variable
Polyarteritis nodosa	Proliferative GN and arteritis	Variable granular Ig and C3 in vessels and glomeruli	Variable
Subacute bacterial endocarditis	Focal or diffuse proliferative GN	Granular Ig and C3 in mesangium and GBM	Subendothelial, subepithelial deposits
ANTI-GBM ANTIBODY GN			
Goodpasture's syndrome	Extracapillary proliferation with crescents	Linear Ig (IgG) and C3 along GBM and TBM	Gaps and discontinuity in GBM
IMMUNOLOGIC MECHANISM UNCERTAIN			
Minimal lesion	No or minimal microscopic abnormalities	Negative	Fusion of foot processes
Hereditary GN	Proliferative GN and interstitial nephritis	Occasional segmental Ig and C3	Splitting of GBM
Coagulopathies (haemolytic uraemic syndrome, thrombocytopaenic purpura)	Variable ischaemic and proliferative lesions	Occasional segmental Ig and C3	Fibrin deposition

The routine evaluation of GN by renal biopsy has contributed greatly to precision in diagnosis and prognosis, but in most instances the aetiologic process remains obscure. The thrust of GN research should be directed toward this latter objective.

Aims of the Present Studies. As we have seen, the diagnosis of ICGN in man rests largely on the IF detection of characteristic granular deposits of Ig and C within the glomeruli. In some patients this is supported by the presence of depressed serum C levels (78), C breakdown products in the circulation (79), and cryoglobulins (80, 81), or rheumatoid factor (82), which also suggest IC formation. However, final confirmation of the concept and diagnosis of ICGN requires I. the detection of IC in the circulation, and II. the identification of antigen in the glomerular IC deposits.

These are the objectives of the present study. The first offers the simplicity and convenience of a serum instead of a tissue diagnosis of IC disease which should help to further define the role of circulating IC in the pathogenesis of GN and could be used as a rational serologic parameter in guiding management. The second holds the key to an understanding of the antigen-antibody systems involved in ICGN, and with it the potential of specific therapy in such patients. Recent advances in technology have made these objectives feasible.

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I. THE DETECTION OF CIRCULATING IC IN PATIENTS WITH GN.

INTRODUCTION

The technology in this area is still new and, although many different IC assay systems have been proposed, it is clear that none are infallible and that each has its own special reactivity, sensitivity, and idiosyncrasies. Assays which are based on the physico-chemical properties of IC include analytical or sucrose gradient ultracentrifugation (65, 83), precipitation with polyethylene glycol (84), and the detection of cryoglobulins (80, 81). Assays based on the immunochemical properties of IC are, in general, more sensitive. These rely on the altered Fc reactivity of complexed Ig, which causes fixation of C (85, 86), binding of Clq (87-91), and binding of RF (90, 92); or on the special properties of IC bound C components, which cause binding to bovine conglutinin (93), and the production of serum immunoconglutinins (94). Finally, there are assays based on the cellular reactivities of IC: IC induced platelet aggregation (95), phagocytosis of IC by peritoneal macrophage cells (96), inhibition of C dependent lymphocyte EAC rosette formation (97), and binding of IC to the C receptors of certain lymphoblastoid cell lines (98).

In the present study, four IC assays were used to test sera from patients with GN. These are the Raji cell radioimmune assay (98) in which the binding of serum IC to cell surface C receptors is quantitated, the radiolabeled Clq binding assay (88) which quantitates the binding of Clq to serum IC, a solid phase Clq binding assay (91) in which serum IC are bound to Clq coated tubes, and the microcomplement consumption test (85, 86) which measures serum anticomplementary activity by quantitating consumption of added C by the test serum. In this way,

the individual reactivities of different assays could be evaluated and exploited. Sera from patients with a spectrum of renal diseases were tested in three separate studies, and the results correlated with clinico-pathological information. Renal IF detection of granular deposits of Ig and C was used as the criterion of ICGN.

METHODS

IC Assays

The Raji cell radioimmune assay (*IRCA) (98). Raji cells, a lymphoblastoid cell line derived from Burkitt's lymphoma, were cultured in Eagles minimal essential medium (MEM) and were used in the assay when a concentration of 1 x 10^6 cells per ml was reached. Cell viability as determined by trypan blue exclusion was greater than 95%. Each test was performed in triplicate. 2 x 10^6 Raji cells in 200 μl 1% BSA-MEM were reacted with 25 μl of a 1:4 dilution of test serum for 45 min at 37° C. The cells were washed three times with 1% BSA-MEM and then reacted with approximately 2.5 μg ^{125}I goat anti-human IgG in 30 μl 1% BSA containing ^{131}I BSA for 30 min at 4° C. The cells were washed once in 1% BSA-MEM and radioactivity in the cell pellet was determined in a gamma-counter. The uptake of ^{125}I goat anti-human IgG was calculated after correcting for non-specific binding of ^{131}I BSA and the result referred to a standard curve of the uptake of antibody by cells incubated with aggregated human gamma globulin (AHG) in normal human serum (NHS). The amount of complexes in the test serum was expressed as μg AHG equivalent (equiv) per ml serum.

The radiolabeled C1q binding assay (*IC1qBA). The assay was modified from Nydegger et al (88). C1q was isolated from fresh NHS using

the method of Yonemasu and Stroud (99) except that relative salt concentrations of 0.04 M and 0.078 M were used for the first and second precipitation steps, respectively. The purity of the isolate was confirmed by immunoelectrophoresis using rabbit antisera to whole human serum and to human C1q. The C1q was stored at -70° C in aliquots at a concentration of 1 mg per ml. 500 μg quantities were radiolabeled with 0.5-1.0 mCi ^{125}I using chloramine T (100) and specific activities of 0.3-1.4 μCi per μg were obtained. 1% BSA was added and aliquots were stored at -70° C. For use in the assay, an aliquot was thawed, diluted in isotonic Veronal-buffered saline (VBS) (101) containing 1% BSA, and centrifuged at 10,000 x G for 30 min at 4° C. The upper one half of the supernatant was used for the assay. Each test was performed in duplicate. 200 μl serum in 100 μl VBS was heated at 56° C for 30 min, cooled to 25° C, and approximately 0.1 μg ^{125}I C1q in 100 μl volume was added. After 30 min at 25° C, 2.5 ml of polyethylene glycol (PEG) (Union Carbide MW 6000) in 0.1 M borate buffer pH 8.4 was added to a final concentration of 2.6% and reacted for 30 min at 25° C. The precipitate obtained after centrifugation at 1000 x G for 30 min was washed once with 2.6% PEG and then counted in a gamma-counter. The result was expressed as percent binding of added ^{125}I C1q.

The solid phase C1q binding assay (C1qSPA). The method was modified from Hay et al (91). 12 x 75 mm polystyrene tubes (Falcon Ca.) were coated with freshly isolated C1q (99) by incubation with approximately 4 μg C1q in 1.0 ml 0.15 M phosphate buffered saline (PBS) pH 7.2 for 20 hours at 4° C. The tubes were then washed three times with 5.0 ml PBS, incubated with 2.0 ml 1% BSA in PBS for 2 hours at 25° C, washed a further three times with PBS, and then stored at -70° C

until use. Using the above protocol, 500 to 1000 tubes could be coated with the Clq isolated from 100 ml serum. In preliminary studies it was found that the tubes were stable with -70° C storage for at least two months.

The assay was performed as follows. 50 µl test serum was incubated with 100 µl 0.2 M ethylenediaminetetraacetic acid (EDTA) pH 7.5 for 30 min at 37° C. 60 µl of the EDTA treated serum (equivalent to 20 µl whole serum) was added to Clq coated tubes in 1.0 ml PBS. Each test was performed in duplicate. The tubes were incubated for 60 min at 37° C and for 20 hours at 4° C, and then washed three times with PBS. Approximately 2.5 µg ^{125}I goat anti-human IgG antibody was added in 1.0 ml 1% BSA-PBS and incubated for 60 min at 37° C and 30 min at 4° C. The tubes were then washed three times with PBS and radioactivity bound to the tubes was counted in a gamma-counter. The result was referred to a standard curve of the binding obtained with AHG in NHS, and expressed as µg AHG equiv per ml.

The microcomplement consumption test (MCT). A modification of the assays described by Mayer et al (85) and by Mowbray et al (86) for the detection of anti-complementary activity in sera was employed. Each test was performed in duplicate using glass tubes. 100 µl test serum was heated at 56° C for 30 min and then cooled for 15 min at 0° C. Fresh NHS diluted to contain 2-2.5 $C'H_{50}$ was added in 300 µl VBS and reacted for 30 min at 37° C. 200 µl of a 3% suspension of sheep red blood cells optimally sensitized with rabbit anti-sheep red cell IgM antibody was added and incubated for 60 min at 37° C. The mixture was then diluted with 1.0 ml ice-cold VBS, centrifuged at 1500 x G for 10 min, and cell lysis quantitated by measuring the released haemoglobin (412 nM) using

a Gilford spectrophotometer. Background absorption with the test serum blank (100 μl serum and 1.5 ml VBS) was subtracted and the result expressed as percent inhibition of control lysis with C alone (100 μl VBS replacing the test serum).

A panel of 66 control sera was obtained from healthy laboratory personnel and stored in aliquots at -70° C. Sera from the panel were used to determine the 95% confidence limit for normal serum in each of the assays. This was 11.0% binding of Clq in the *IClqBA, 29 μg AHG equiv per ml in the Clq SPA, and 30% inhibition of control lysis in the MCT. The background binding in the *IRCA, however, varied considerably from day to day and, in order to control this variation, four standard normal sera, selected to statistically represent the total control group, were tested in each day's assay. The binding obtained with these sera was used to determine the upper limit of normal for each day, and quantitation of the test sera was expressed as μg AHG equiv per ml above this value. Using this system, the average standard error of the mean values obtained on 6 different days with 9 sera was 12.4%.

In Vitro Evaluation of IC Assays.

Detection of AHG and the effect of heat inactivation of serum. The sensitivity of the assays was assessed by determining the minimum amount of AHG detected in NHS. AHG was prepared from Cohn Fraction II using the method described by Mauer et al (102), which employs an ultracentrifugation step to remove unaggregated gamma globulin. Sucrose density gradient fractionation of ^{125}I labeled AHG showed the aggregates to be 40-100 S. Protein concentration was quantitated by micro-Kjeldahl nitrogen determination and the AHG was stored in aliquots at -70° C. Prior to use in the assays, each aliquot was centrifuged at 10,000 x G

for 5 min. Serial dilutions of AHG were incubated in NHS for 30 min and then tested in the assays.

Detection of in vitro formed complexes. Complexes of varying antigen-antibody ratios were formed *in vitro* with human serum albumin (HSA) and rabbit anti-HSA antibody, BSA and rabbit anti-BSA antibody, and with tetanus toxoid (Wyeth) and tetanus immune human serum. Tubes were incubated for 60 min at 37° C and then for 18 hours at 4 ° C. Precipitates obtained by centrifugation at 2000 x G were washed with PBS, and the protein content (micro-Kjeldahl nitrogen determination) used to define the equivalence point. The supernatants were then tested in the IC assays.

The effects of storage and handling conditions of sera. A panel of sera were tested in the assays after repeated freeze-thawing or after storage at 25°, 4°, -20°, or -70° C.

The influence of non-IC reactants. The effects of heparin, DNA, and endotoxin were studied by testing NHS to which heparin (Lipo-Hepin, Riker Laboratories), DNA (calf thymus DNA, Worthington Biochemical Corp.) or E. coli endotoxin (Difco Laboratories) had been added. The influence of serum anti-lymphocyte antibodies on the *IRCA was studied by a Raji-cytotoxicity assay. A modification of the microcytotoxicity assay described by Terasaki and McClelland (103) was employed. 1 μl test serum which had been heat inactivated at 56° C for 30 min was added to a microdroplet of 1000 Raji cells under mineral oil and reacted for 30 min at 25° C. 2 μl rabbit C {1:2 normal rabbit serum (NRS)} was added and incubated for 3 hours at 25° C. Percent cytotoxicity was determined by eosin dye exclusion under phase contrast microscopy. 33 sera from patients with SLE were tested and the results were correlated with those of the *IRCA.

Sucrose density gradient fractionation of test sera. 300 μl of 1:2 dilutions of selected test sera were applied to 5 ml 10-37% sucrose gradients in PBS with a 200 μl 60% sucrose "cushion". 7S (^{131}I IgG) and 19S (^{125}I IgM) markers were applied to identical gradients. The gradients were centrifuged at 250,000 x G for 6.2 hours in an SW 50.1 rotor and 30 drop fractions were collected. Each fraction was then dialyzed against PBS for 48 hours, and tested in the *IRCA. The IgG concentration of each fraction was quantitated by radioimmunoassay for human IgG (104).

Patients and Sera.

Sera from three groups of patients were studied separately.

Group A (*IRCA). Sera from 213 patients referred by contributors for IF evaluation of renal disease were selected for study. Selection was based upon renal IF findings so that only patients with unequivocally positive or negative IF were included. Patients were then sub-categorized according to available clinical and pathological information. No protocol was followed for the collection and handling of the sera in this group, all of which had been thawed and refrozen at least once before IC determination.

Group B (*IRCA, *IClqBA, and MCT). Sera from 85 patients with GN were obtained in a prospective study performed in collaboration with Dr. R. J. Glassock and Dr. W. A. Border, UCLA Harbor General Hospital, Torrance, Ca. In each patient appropriate investigations were performed to establish a definitive clinical or pathological diagnosis. Renal biopsy with light microscopic and IF findings was performed in 69 patients. Serum C3, C4, and properdin factor B (C3PA) determinations (105) were carried out in all patients and when indicated,

sera were tested for C3 nephritic factor activity (106), ANA, and anti-GBM antibodies (107). Sera for IC determinations were obtained as follows: blood was collected in glass vacutainer tubes, allowed to clot at 25° C for 60 min, and the serum separated by centrifugation at 2000 rpm for 10 min at 25° C. The serum was stored in 0.5 ml aliquots at -70° C until being tested in the *IRCA, the *IC1qBA, and the MCT. Control sera from 31 healthy laboratory personnel were collected and tested in the same way.

Group C (*IRCA and C1qSPA). This study was performed in collaboration with Dr. K. S. K. Tung, University of New Mexico, Alberquerque, N. M. with sera obtained from 57 patients with GN and other renal diseases. Each patient was fully evaluated by serological testing and renal biopsy. Sera for IC determinations were stored in aliquots at -70° C until being tested in the *IRCA and the C1qSPA. Control sera from 29 healthy laboratory personnel were collected and tested in the same way.

RESULTS

In Vitro Evaluation of IC Assays.

Detection of AHG and the effect of heat inactivation of serum (Figs. 1-4). All four assays were able to detect 5-10 μg AHG per ml NHS which is in accord with sensitivities previously reported with these assays (88, 91, 98, 108). However, in the *IC1qBA (Fig. 3) and the MCT (Fig. 4) this sensitivity was only obtained if AHG was added to pre-heat inactivated NHS. When AHG was added to fresh NHS and then heat inactivated, which more closely resembles the situation *in vivo*, the minimum amount of AHG detectable by the *IC1qBA or the MCT was 50-100 μg per ml. These findings suggest that heat inactivation

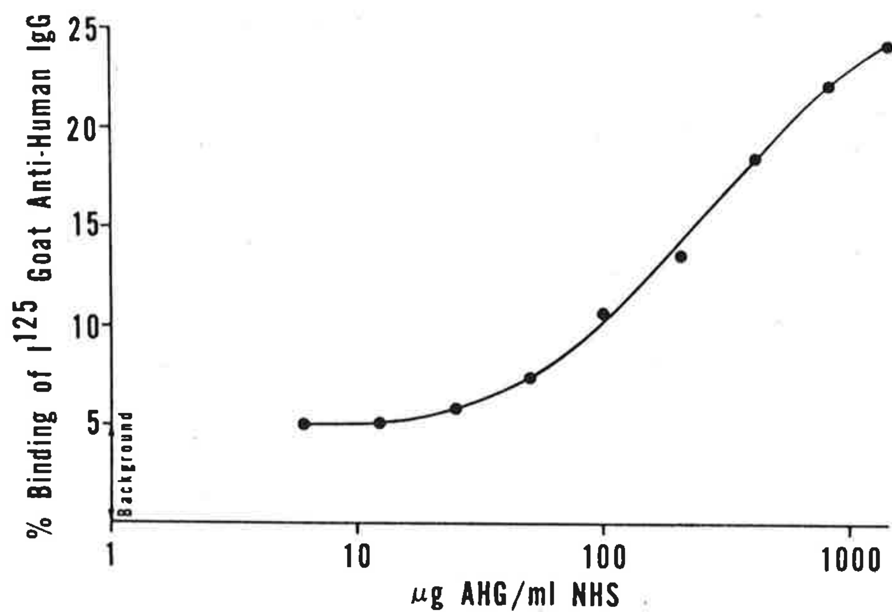

Figure 1. *IRCA. Binding of AHG in NHS.

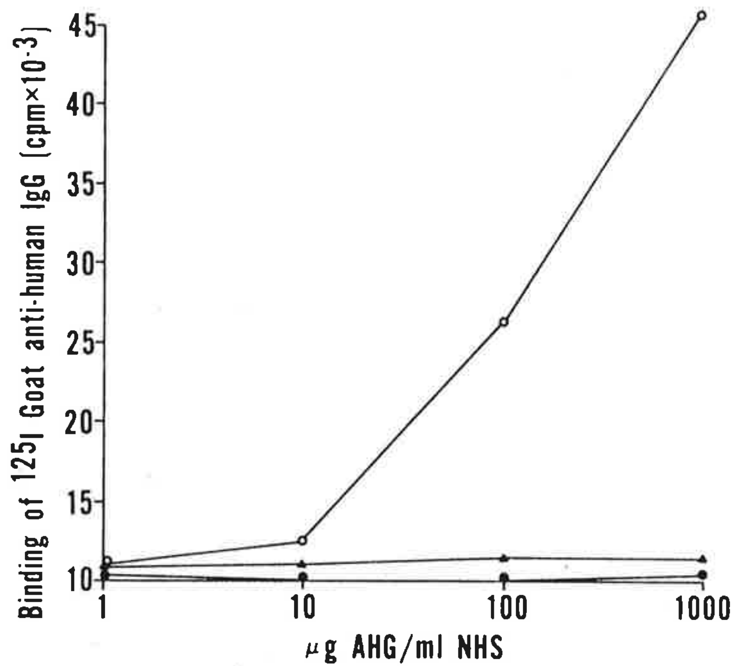

Figure 2. ClqSPA. Binding of AHG (or 7S IgG) in NHS.

- ○——○ Binding of AHG
- ▲——▲ Binding of 7S IgG
- ●——● Binding of AHG to heat-inactivated Clq coated tubes

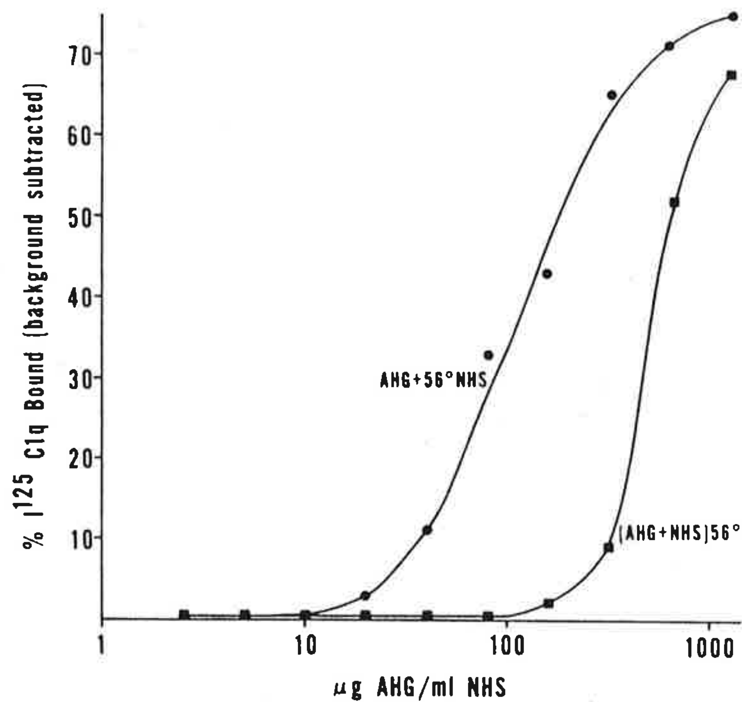

Figure 3. *IC1qBA. Binding of AHG in NHS and the effect of preliminary heat inactivation of the test serum.

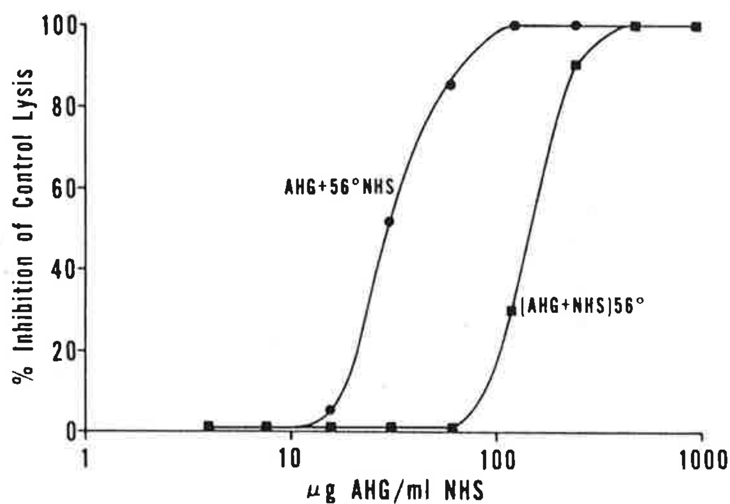

Figure 4. MCT. Binding of AHG in NHS and the effect of preliminary heat inactivation of the test serum.

is not very effective in removing bound C, and that this factor may limit the detection of in vivo formed IC by these assays. On the other hand, there was no evidence for heat-induced aggregation of serum gamma globulin (the background ^{125}I Clq binding was slightly less with heat inactivated NHS than with fresh NHS), or for thermolabile IC (known positive sera remained positive in the *IRCA after heat inactivation). These problems were not encountered with the *IRCA which uses fresh test sera, or the ClqSPA which uses EDTA treated sera.

Detection of in vitro formed IC. All four assays were able to detect IC formed in vitro near equivalence. The assays were much less sensitive with low MW, antigen excess complexes. Fig. 5 illustrates the detection of HSA-anti-HSA complexes with the *IClqBA.

The effects of storage and handling conditions of sera. The effects of repeated (10x) freeze-thawing of test sera and of standing at 25° C for 4 hours were evaluated. Under these conditions, NHS became anticomplementary in the MCT, but no effect was noted in the *IClqBA, the ClqSPA, or the *IRCA. The anticomplementary activity was primarily a storage-induced phenomenon; all of 12 NHS became anticomplementary after 2 days at 25° C, 7 of 12 became anticomplementary after 3 days at 4° C, and 5 of 12 after 3 days at -20° C. Similar findings by Nielsen and Svehag (109) were attributed to aggregation of serum gamma globulin. The effect was not enhanced by repeated freeze-thawing cycles and was not observed in NHS stored at -70° C for up to 21 days. Optimal handling of test sera and -70° C storage are thus particularly critical in the MCT.

The influence of non-IC reactants (Table 2). Heparin, when added to NHS, produced anticomplementary activity in the MCT and increased

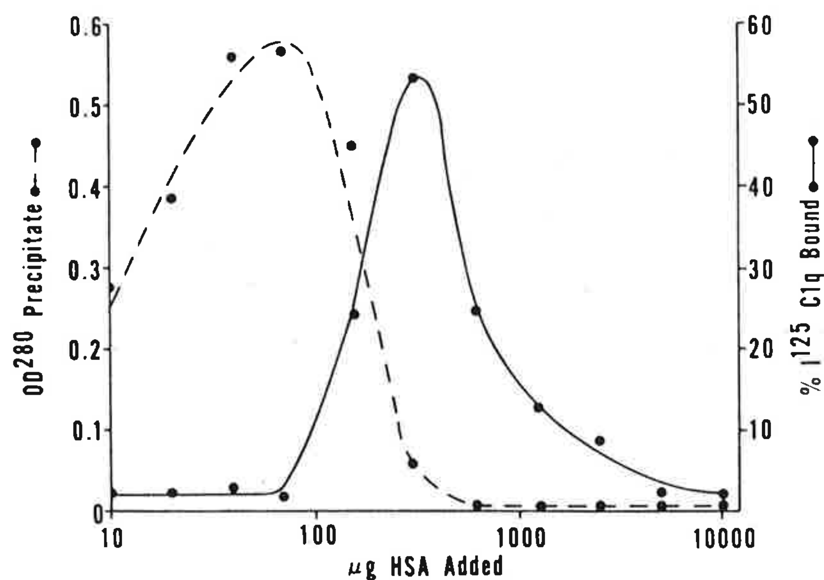

Figure 5. Detection of in vitro formed HSA-anti HSA complexes by the *IClqBA.

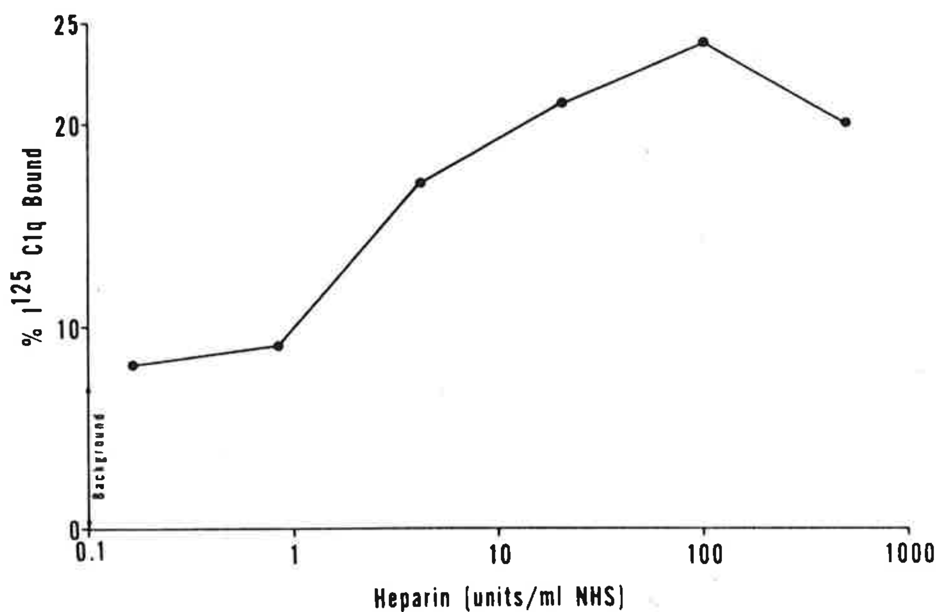

Figure 6. The effect of heparin (added to NHS in vitro) on the *IClqBA.

binding of ^{125}I Clq in the *IClqBA. In the *IClqBA (Fig. 6) the effect was maximal with 100u heparin per ml NHS but was still detected with less than 1u per ml. Heparin had no effect on the *IRCA or the ClqSPA. Similarly, DNA, both native and denatured, caused an increase in ^{125}I Clq binding when added to NHS. The effect, however, was less marked than that of heparin; maximum specific ^{125}I Clq binding was 2.7% with 50 μg DNA per ml. DNA was anticomplementary in the MCT but had no effect on the *IRCA or the ClqSPA. E. coli endotoxin (100 μg per ml NHS) had no significant effect on the *IClqBA, the ClqSPA, or the *IRCA, but was anticomplementary in the MCT. Finally, 9 of 33 SLE sera (27%) were found to have cytotoxic activity (>10% cells killed) when tested with Raji cells in the microcytotoxicity assay. 8 of these 9 sera (89%) were positive in the *IRCA (mean 150 μg AHG equiv per ml), compared with 19 of 24 (79%) and 123 μg AHG per ml, respectively, for the cytotoxic negative group. Analysis of the individual sera showed some correlation between cytotoxic activity and the degree of *IRCA positivity. Although not conclusive, these findings suggest that serum anti-lymphocyte antibodies may contribute to *IRCA positivity.

TABLE 2

THE INFLUENCE OF NON-IMMUNE COMPLEX REACTANTS ON

MCT, *IClqBA, *IRCA, AND ClqSPA

	Heparin	DNA	Endotoxin	Serum Anti-Lymphocyte Antibody	Suboptimal Storage Conditions
MCT	++	++	++	0	++
*IClqBA	++	+	0	0	0
*IRCA	0	0	0	?	0
ClqSPA	0	0	0	0	0

Sucrose density gradient fractionation of test sera (Fig. 7). NHS, serum from a patient with acute GN and glomerular IC deposits, and serum from a patient with SLE were fractionated by sucrose density gradient ultracentrifugation, and the fractions tested in the *IRCA. 13 to 19S peaks of *IRCA binding were detected in the two nephritic sera, presumably representing the IC containing fractions, but the majority of the *IRCA binding in the SLE serum was in fact associated with the 7S fraction. After correction for IgG content, the binding observed with the SLE 7S fraction was 2.3 times greater than with the NHS 7S fraction. This monomeric reactant in SLE serum may represent anti-lymphocyte antibody or antibodies that bind to cell components, but is not yet fully characterized (Eisenberg, R. A., personal communication).

Results of IC Study

Group A (*IRCA). (Tables 3 and 4). Of the 213 patients, there were 41 with SLE, 111 with non-lupus GN and granular glomerular deposits of Ig and C, 31 with IF negative renal diseases, 7 with anti-GBM antibody GN, and 23 renal transplant patients (Table 3). Sera in the IF negative renal disease group (end-stage GN, hypertensive renal disease, chronic pyelonephritis, etc.) were used as an internal control for this study since these sera had been handled and stored in the same manner as the entire group. However, it was recognized that circulating IC may still be present in patients with end-stage GN in whom glomerular IC can no longer be detected. 3 of these control sera (10%) were positive. In contrast, 68% of the SLE sera, 23 % of the non-SLE IF positive GN sera, and 26% of the renal transplant sera were positive. None of the anti-GBM antibody GN sera was positive. The results indicate that the prevalence of *IRCA positivity is much greater in SLE than in

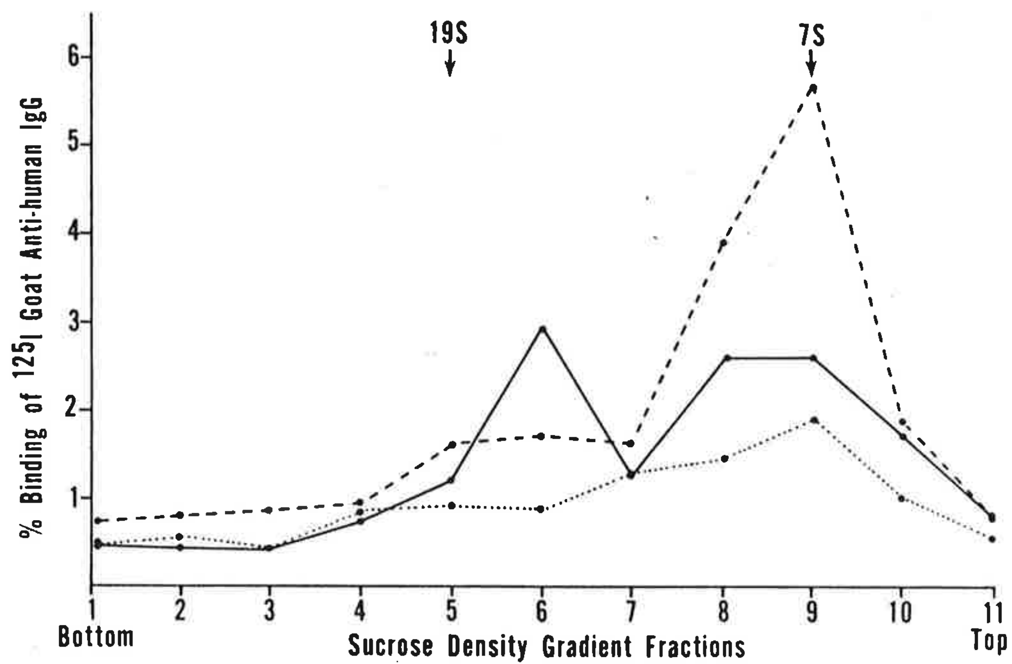

Figure 7. Sucrose density gradient fractions of sera tested by the *IRCA.

- ●· · ·● NHS
- ●——● Acute GN
- ●– –● SLE

any other group. However, the prevalence in both the non-SLE IF positive GN group and in the transplant group was greater than in the control group.

TABLE 3

RESULTS OF IC STUDY. GROUP A (*IRCA)

	*IRCA positive/total studied	%
SLE	28/41	68
IF positive renal disease	25/111	23
IF negative renal disease	3/31	10
Anti-GBM antibody GN	0/7	0
Transplant sera	6/23	26

Of the 38 patients in the non-SLE IF positive GN group with definitive histologic diagnoses (Table 4), the *IRCA was most often positive in acute GN (AGN) (5/7). In particular, none of the 12 patients with rapidly progressive GN (RPGN), none of the 5 with MPGN, and only 1 of the 5 with MGN were positive. Transplant patients were sub-categorized by IF evaluation of the grafted kidneys into those with granular deposits of Ig and C (suggesting glomerular IC deposits) and those with IF negative grafts (Table 4). 6 of the 14 patients with IF positive grafts were positive in the *IRCA, and 3 of these 6 patients had clinical and pathological evidence suggesting recurrence of their original GN in the transplant kidney. None of the 9 patients with IF negative kidney grafts was positive in the *IRCA.

TABLE 4

SUBCLASSIFICATION OF PATIENTS IN GROUP A

IF positive renal diseases with histologic diagnosis	*IRCA positive/total studied
Acute GN (AGN)	5/7
Focal GN	2/5
Rapidly progressive GN (RPGN)	0/12
Membranoproliferative GN (MPGN)	0/5
Membranous GN (MGN)	1/5
Focal sclerosing GN (FSGN)	0/1
Chronic GN (CGN)	1/3
Transplant sera	
IF positive graft	6/14
IF negative graft	0/9

Group B (*IRCA, *IC1qBA, and MCT). (Tables 5 and 6). The patients were categorized into three major groups: SLE (n=23), GN associated with other systemic diseases (n=17), and primary ICGN, i.e., GN with granular deposits of Ig and C in the absence of systemic disease (n=36). The prevalence of positivity of sera in these groups and in the control group (n=31) is given for each assay, and for the panel of assays (Table 5). Each group of test sera had a higher prevalence of positivity with each test than did the control group. However, this was more marked in the SLE group (particularly with the *IRCA, 74%), and in the group with GN associated with other systemic diseases (particularly with the *IC1qBA, 41%), than in the primary GN group. The amounts of IC detected

in patients with primary GN were also less than in SLE or GN associated with other systemic diseases (Fig. 8). The correlation between the three assays was not statistically significant, and when the three assays were used in combination, the prevalence of positivity in each of the three patient categories was further increased, i.e., SLE 87%, GN with other systemic disease 65%, primary GN 39%, (control 10%).

TABLE 5

RESULTS OF IC STUDY. GROUP B (*IRCA, *IC1qBA, MCT)

	percent positive			
Patients (number studied)	*IRCA	*IC1qBA	MCT	ANY TEST
SLE (23)	74	35	27	87
Other systemic GN (17)	29	41	18	65
Primary GN (36)	14	17	14	39
Control (31)	3	3	6	10

Patients were sub-categorized according to clinico-pathologic criteria. Data from patients with primary GN, GN associated with non-SLE systemic diseases, and from 9 additional patients {anti-GBM antibody GN (n=3), minimal lesion nephrotic syndrome (n=5), pre-eclamptic toxaemia (n=1)}, is given in Table 6. In the primary GN group, 4 of 7 patients with AGN, 3 of 8 with mesangial injury GN, and 4 of 11 with MPGN were positive in one or more of the assays. In the group with systemic diseases, 4 of 6 patients with subacute bacterial endocarditis (SBE), 3 of 4 with polyarteritis nodosa (PAN), and 2 patients with Sjögren's syndrome associated GN were positive. None of the 3

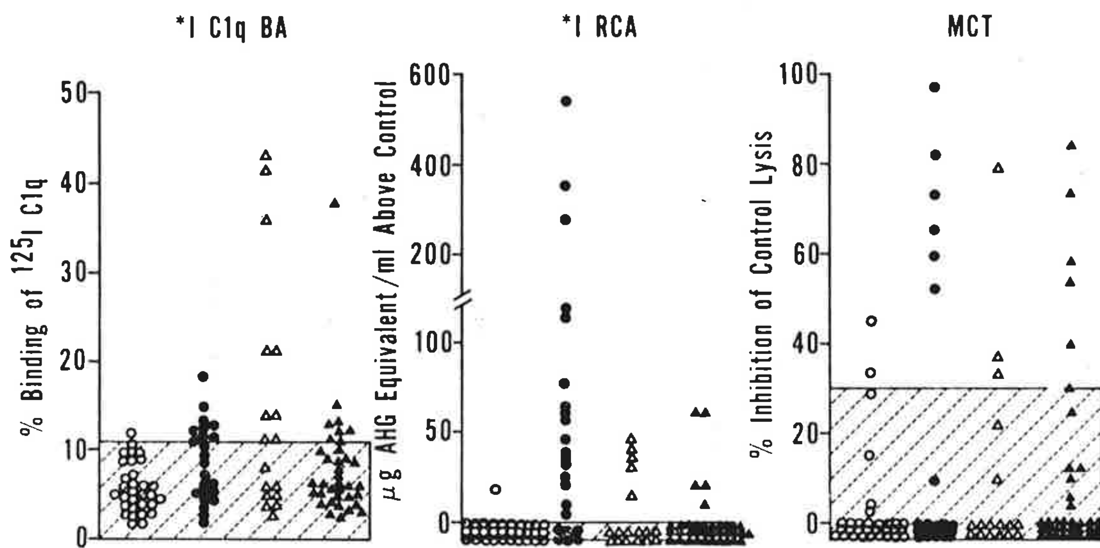

Figure 8. Quantitation of circulating IC in prospective study of GN sera with the *IC1qBA, *IRCA, and MCT. (Group B).

Shaded area indicates 95% confidence limit for each assay.

- o Control group
- ● SLE
- △ Other systemic GN
- ▲ Primary GN

TABLE 6

SUBCLASSIFICATION OF PATIENTS IN GROUP B

Clin-path Subcategory	*IRCA	*IC1qBA	MCT	ANY TEST
	number positive			
Primary GN				
7 AGN	1	2	1	4
8 Mesangial injury GN	1	2	2	3
2 RPGN	0	0	1	1
11 MPGN	3	0	1	4
2 MGN	0	0	0	0
4 FSGN	0	2	0	2
2 CGN	0	0	0	0
GN associated with systemic disease				
6 SBE	2	3	1	4
4 PAN	1	2	0	3
2 HSP	0	0	0	0
2 HUS	0	1	1	2
1 Vasculitis	0	0	0	0
2 Sjögren's disease	2	1	1	2
Other GN				
3 Anti-GBM antibody GN	0	0	0	0
5 Minimal lesion	0	0	3	3
1 PET	0	0	1	1

SBE, subacute bacterial endocarditis; PAN, polyarteritis nodosa; HSP, Henoch-Schönlein purpura; HUS; haemolytic-uraemic syndrome; PET, pre-eclamptic toxaemia.

patients with anti-GBM antibody GN was positive. Serum anticomplementary activity was detected in 3 of the 5 patients with minimal lesion nephrotic syndrome, a condition not thought to be mediated by circulating IC, but in which positive results have been reported with the macrophage uptake assay (110), the C1q deviation test (111), and the lymphocyte EAC rosette test (112). These sera were negative in the *IRCA and the *IC1qBA. Anticomplementary activity was detected in the one patient with pre-eclamptic toxaemia (PET) in this study, a condition in which glomerular immune deposits have been reported (113), and in 9 of 9 women in the third trimester of pregnancy (Border, W. A., personal communication). Again, these sera were negative in the *IRCA and the *IC1qBA. Thomson et al (114) reported serum anticomplementary activity in 5 of 14 patients with PET, but found no other corroborative evidence for circulating IC.

C data on the sera from the three groups of patients in group B is given in Table 7. Overall, there was an inverse correlation between

TABLE 7

SERUM C DATA ON PATIENTS IN GROUP B

Serum C levels * (number in each category)	Prevalence of positivity in IC assays (%)		
	*IRCA	*IC1qBA	MCT
C3 low (40)	45	28	26
C3 normal (45)	20	22	18
C4 low (27)	59	30	35
C4 normal (58)	19	22	16
C3PA low (21)	52	33	30
C3PA normal (64)	25	22	19

*Normal range (μg/ml) for C3 is 1104-1373, C4 456-745, and C3PA 153-226.

C levels (C3, C4, C3PA) and positivity in the assays. This correlation was more marked with the *IRCA and the MCT than with the *IClqBA. C3 nephritic factor was detected in two patients with MPGN; one was positive in the MCT only, and the other was negative in all three assays.

Serial IC determinations by the *IRCA and the *IClqBA were performed with 55 sera from 7 patients, 3 with SLE, 2 with Henoch-Schönlein purpura (HSP), 1 with MPGN, and 1 with mesangial IgA disease. Of the 3 SLE patients, one was repeatedly negative in the *IRCA, one was repeatedly positive, and one converted from positive to negative. Serial *IRCA determinations in the patients with HSP, MPGN, and IgA disease were consistently negative. There was considerably more variation using the *IClqBA. Thus, sera from the 3 patients with SLE and from the patient with IgA disease were positive on some occasions and negative on others. Sera from the patients with HSP and MPGN were consistently negative in the *IClqBA.

Group C (*IRCA and ClqSPA). The results of this study are summarized in Table 8. The overall prevalence of circulating IC, as determined by both assays was 47%; 52% in patients with presumed ICGN and 31% in those with non-ICGN renal disease. The prevalence differed greatly according to the type of GN. Associated with a high prevalence of circulating IC were patients with SLE (100%), RPGN (80%), and AGN (71%). In contrast circulating IC were not detected in patients with mesangial IgA disease (0%) or MGN (0%). A similar correlation existed when the data from either the *IRCA or the ClqSPA alone was considered, though as a rule a higher prevalence was detected by the *IRCA. Once again the highest levels of circulating IC were in sera from patients with SLE.

TABLE 8

RESULTS OF IC STUDY. GROUP C (*IRCA AND C1qSPA)

Diagnostic category	(number studied)	number positive *IRCA	C1qSPA	EITHER
Controls	(29)	3	0	3
Presumed ICGN				
SLE	(9)	9	6	9
AGN	(7)	4	5	5
RPGN	(5)	3	3	4
CGN	(5)	2	1	3
Mesangial IgA disease	(7)	0	0	0
MGN	(8)	0	0	0
Wegener's granulomatosis	(1)	1	1	1
HSP	(1)	0	0	0
Diabetic nephropathy with glom IC	(1)	0	1	1
	(44)	19	17	23
Non ICGN diseases				
Minimal lesion	(4)	0	1	1
Anti-GBM antibody GN	(3)	2	0	2
Monoclonal gammopathy	(2)	0	0	0
Renal amyloid	(1)	0	0	0
Medullary cystic disease	(1)	0	0	0
Acute renal failure ? cause	(1)	1	0	1
Renal infarct	(1)	0	0	0
	(13)	3	1	4

Follow-up sera (obtained after an interval of 13 to 583 days) were studied in 27 patients. In 20 of these patients, the repeat IC determination remained either positive (8 patients) or negative (12 patients). One SLE patient converted from positive to negative. This was associated with improving renal function and a decrease in the amount of glomerular IC in serial renal biopsies. Two other patients converted from positive to negative in both the *IRCA and the ClqSPA; one RPGN patient who improved clinically without haemodialysis, and a patient with AGN who made a complete clinical recovery. However, the number of patients in this study was too small to permit any definite conclusion regarding the correlation between clinical activity and circulating IC.

36 patients were studied for circulating IC within one month of their renal biopsy. Granular deposits of Ig and/or C3, consistent with glomerular IC, were as frequent in patients without circulating IC (58%) as those with circulating IC (46%). This overall lack of correlation between glomerular and circulating IC was largely due to the consistently negative IC determinations in patients with MGN and mesangial IgA disease. Tha absence of circulating IC in MGN has been noted previously (115) but is still unexplained, whereas the failure to detect circulating IC in mesangial IgA disease must be qualified since both assays have IgG specific end points. Circulating IC were detected in two patients without glomerular deposits. One of these was a patient with minimal lesion nephrotic syndrome, while the second had acute renal failure of uncertain origin. In contrast to the other studies, circulating IC were also found in 2 of 3 patients with anti-GBM antibody GN. GBM antigens have been demon-

strated in serum (116), and it is, therefore, conceivable that circulating GBM-anti-GBM IC could be formed in patients with anti-GBM antibody GN.

Attempts were made to correlate the results of the IC studies with the ultrastructural localization of electron-dense deposits within the glomerulus. As a rule, circulating IC were absent when IC were exclusively subepithelial. However, this observation is based on findings in idiopathic MGN, and it would be necessary to study patients with the membranous form of SLE GN before any conclusion is made. Mesangial IC may or may not be associated with circulating IC. Thus, 7 patients with mesangial IgA disease were negative for circulating IC, whereas 3 patients with AGN, 4 patients with SLE, and 1 patient with diabetic nephropathy whose glomerular IC were exclusively mesangial in location, were positive. These results indicate that the detection of circulating IC correlates better with the type of GN than with the site of glomerular IC.

Finally, there was an inverse correlation between serum C3 levels and positivity in either the *IRCA or the ClqSPA (Table 9).

TABLE 9

CORRELATION BETWEEN SERUM C3 LEVELS

CIRCULATING IC, AND GLOMERULAR IC IN GROUP C

Serum C3 levels (number)	% positivity in IC assays		% glomerular IgG and/or C3
	*IRCA	ClqSPA	
C3 low (28)	67	52	82
C3 normal (21)	21	14	80

There was no such correlation between C3 levels and the presence or absence of glomerular IC.

DISCUSSION

The sensitivity of IC assays has generally been evaluated by the in vitro detection of AHG. Extrapolation from sensitivities reported in the literature are limited by the fact that no two batches of AHG are identical, and estimation of sensitivity will be lower with preparations that have not been freed of monomeric gamma globulin than with totally aggregated preparations. Finally, the sensitivity should refer to the detection of AHG in serum rather than buffer. In this study the *IRCA and ClqSPA were more sensitive (5 to 10 μg AHG per ml serum) than the *IClqBA and the MCT (50 to 100 μg AHG per ml). Studies with in vitro formed IC showed that the assays can readily detect large complexes near equivalence, but that they are less sensitive with smaller, antigen excess complexes.

The sensitivity of the *IClqBA and the MCT with AHG added to fresh serum and subsequently heat inactivated was less than with AHG added to pre-heat inactivated serum. This reduced sensitivity is probably due to inhibition by pre-fixed C, which imples that the ability of these assays to detect IC which have fixed C in vivo might be impaired relative to detection with the *IRCA. The heat inactivation step has been previously criticized (117). On one hand, heat-induced aggregation of serum gamma globulin could produce false positive results and, on the other hand, false negatives might be obtained with thermolabile IC reactants. In this study we found no evidence for heat-induced aggregation; in fact, the background binding of Clq in the *IClqBA was slightly less with heat inactivated sera than with fresh

serum. (This observation was also made by Zubler et al (118) and has been attributed to the incorporation of C1q into the C1qrs complex in fresh but not in heated serum). Similarly, heat inactivation of test sera did not affect *IRCA positivity. However, heat-labile anticomplementary activity has been reported with certain in vitro reactants (109) and sera (119), and it is clear that assays which utilize unheated test sera are at an advantage in this regard.

The specificity of the assays must also be established. In particular, C1q is known to bind endotoxin (120) and polyanionic substances (121, 122) and, since neither the fluid phase *IC1qBA nor the MCT have IgG specific end-points, such substances could produce false positive results. In this study, the in vitro addition of endotoxin, heparin, or DNA to NHS produced anticomplementary activity in the MCT. The *IC1qBA was affected by heparin, and to a lesser extent DNA, but not by endotoxin. The *IC1qBA detects macromolecular C1q reactants which are insoluble in 2.6% PEG. Endotoxin and DNA are PEG soluble (118), while heparin (MW 6000 to 20000) is presumably bound to serum proteins to form PEG insoluble complexes. The effects of sub-optimal handling and storage conditions of test sera were also evaluated. The MCT was particularly susceptible to such factors, and NHS rapidly became anticomplementary unless stored at -70° C. The *IRCA and C1qSPA were not affected by these conditions and were not influenced by heparin, DNA, or endotoxin. There is, however, some evidence that serum anti-lymphocyte antibodies can influence the *IRCA. Warm, reactive IgG type serum anti-lymphocyte antibodies have been identified in SLE (123), and it is likely that these contribute to *IRCA positivity in some lupus sera.

The above considerations are important in the interpretation of data generated by IC assays and wherever possible the data should be corroborated by independent serologic and tissue (IF) studies. The specificity of the MCT is particularly questionable, and this assay is best regarded as a screening test. In these studies, the *IClqBA was less sensitive than the ClqSPA or the *IRCA, and detected IC in only 8 of 23 patients with SLE. This may be due in part to the deleterious effects of preliminary heat-inactivation of test sera (118), and in using EDTA-treated sera in the ClqSPA, IC were detected in 6 of 9 patients with SLE. The *IClqBA, however, did detect large amounts of IC in some *IRCA negative patients (particularly those with GN and systemic diseases other than SLE). The reactivities of the assays are different with the *IRCA detecting IC which have fixed C3 _in vivo_, and the Clq assays detecting IC which can fix additional Clq _in vitro_. These different reactivities can be exploited by using the assays in combination.

In this study and in others (88, 91, 95, 96, 110, 117, 118, 124), IC were detected most frequently in SLE. Similarly, circulating IC were detected more frequently in GN associated with other systemic diseases than in primary GN, and in acute GN more frequently than in chronic GN. The poor correlation between glomerular IC and detectable circulating IC requires explanation. Quantitative differences in the load of circulating IC may account for these findings. Thus, in group C, large amounts of circulating IC were detected in systemic (SLE) and acute or fulminant (AGN, RPGN) GN. It is possible that the more indolent forms of GN (MGN, MPGN, etc.) are mediated by much smaller

quantities of circulating IC and that the limited sensitivity of the assays precludes their detection. In addition, such patients may have inherent abnormalities causing them to handle more or less physiologic amounts of circulating IC in a nephritogenic manner.

Alternatively, circulating IC may be present only intermittently or with changing composition in some forms of GN. The detection of circulating IC would then be influenced by the timing of such determinations. Thus, circulating IC may be present only during the acute phase of PSGN or during exacerbation in mesangial IgA disease. Glomerular IC deposits are probably more persistent and could, therefore, be present in the absence of randomly detectable circulating IC. In experimental serum sickness it is clear that the glomerular IC deposits are in dynamic equilibrium with IC in the circulation, and that once initiated, the disease could be perpetuated by "layering" of free (non-IC) antigen or antibody onto existing binding sites in the glomerular IC deposits (18). More extensive studies with serial IC determinations may identify a similar process in human GN.

The size and composition of circulating IC may influence both their detection and their site of localization within the glomerulus. Thus, most IC assays detect large IC more readily than small IC, and it has been suggested that small IC are responsible for MGN (57). This concept could explain the absence of detectable IC in MGN (115), but requires further evaluation.

Finally, it is possible that the glomerular immune deposits in some forms of GN in fact represent _in situ_ formed IC (see page 14) and in this situation glomerular IC would be present in the absence of circulating IC.

The failure to detect circulating IC in all patients with presumed IC mediated GN is shared by others. Thus, Johnson et al (117) described serum anticomplementary activity in 19 of 51 (37%) patients with GN, Stülinger et al (110) using the macrophage uptake assay detected circulating IC in 29 of 60 (48%) patients with GN, Rossen et al (125) using the *IClqBA detected circulating IC in 18 of 100 (18%) patients with GN, Gluckman et al (126) using the lymphocyte EAC rosette test reported inhibition in 32 of 67 (48%) patients with GN, and Sobel et al (111) using the Clq deviation test detected circulating IC in approximately 40% of patients with GN. Some of the current difficulties may be resolved as more sensitive and specific IC assays become available. In the meantime, serial IC determinations may prove to be of clinical and prognostic value in some patients, but it is clear that these serologic studies can not replace renal IF in GN diagnosis.

CONCLUSION

Four IC assays were used in this study to test sera from patients with GN: the Raji cell radioimmune assay (*IRCA), the radiolabeled Clq binding assay (*IClqBA), a solid phase Clq binding assay (ClqSPA), and the microcomplement consumption test (MCT). The sensitivity and specificity of each assay was evaluated in preliminary studies, and the greater sensitivity {5 to 10 μg of AHG per ml serum} and IgG specificity of the *IRCA and the ClqSPA were apparent. Problems related to the preliminary heat inactivation of test sera, the interaction of Clq with substances other than IC, and the effects of suboptimal storage of test sera were experienced with the MCT and, to a lesser extent, the *IClqBA. However, the individual reactivities of the

different assays could be exploited by using them in combination. In these studies large amount of circulating IC were detected in SLE and in GN associated with other systemic diseases. IC were detected more frequently in patients with acute than chronic GN, and in patients with low serum C3, C4, and C3PA levels. Serial IC determinations may be of clinical and prognostic value in some patients, but the poor correlation between circulating IC and glomerular IC limits the value of such studies in the diagnosis of ICGN.

II. THE IDENTIFICATION OF IC ANTIGEN AND ANTIBODY IN GN.

Identification of the antigen and of the specificity of the antibody in the glomerular deposits of GN would confirm their IC nature, and could, by providing an aetiologic diagnosis, permit the introduction of more specific therapeutic measures.

Numerous antigens have already been demonstrated in human ICGN. These include the specific infectious agents responsible for ICGN secondary to nephritogenic streptococcal infection (PSGN) (127-132), SBE (133, 134), infected ventriculoatrial shunts (135), pneumococcal pneumonia (136), typhoid fever (137), syphilis (138), malaria (139, 140), schistosomiasis (141), toxoplasmosis (142), and hepatitis B infection (143-146). Oncornavirus related antigen has been detected in glomerular IC deposits of patients with leukaemia (147), and a human C-type virus antigen (HEL 12) has recently been identified in the glomerular IC deposits of patients with SLE (148). Measles virus has been detected in glomerular IC in subacute sclerosing panencephalitis (SSPE) (149), and in one patient with SLE (150), and Epstein-Barr virus (EBV) has been incriminated in GN associated with Burkitt's lymphoma (151) and infectious mononucleosis (152-154). Finally, a number of endogenous antigens have been identified in glomerular IC deposits: nuclear antigens in SLE (155-157); RTA in MGN (158), sickle cell anaemia (159), and renal carcinoma (160); thyroglobulin in ICGN associated with thyroiditis (161); carcinoembryonic antigen (CEA) and tumour antigens in ICGN associated with malignancies (162-165), and Ig in cryoglobulinaemia (80, 81) and possibly other forms of GN (82).

In general the identification of IC antigens has been successful only in patients in whom the nature of the putative antigen could be reasonably predicted on other grounds. However, in most patients with GN, there are no clinical or serological clues with which to direct a search for antigen, and such attempts are probably made even more difficult by "masking" of the IC bound antigen by antibody in excess. These problems were addressed in the following studies: A. Techniques for the detection of glomerular IC antigen and antibody were explored in the CSS rabbit model of ICGN. These techniques were then used to study the antigen-antibody systems in B. spontaneous GN in rabbits, and C. human GN.

A. EVALUATION OF TECHNIQUES FOR THE DETECTION OF GLOMERULAR IC ANTIGEN AND ANTIBODY IN THE CSS MODEL OF ICGN.

INTRODUCTION

Attempts to identify antigen in glomerular deposits by immunohistochemical techniques have been successful in some instances, but are limited, in part, by masking of the antigen by antibody in excess. Alternatively, the specificity of glomerular bound antibody can be studied after dissociation of the glomerular IC with appropriate elution buffers. Freedman and Markowitz (166) introduced the elution technique in 1959, demonstrating the presence of ANA in a renal eluate from a patient with SLE. The technique has been of particular value in studies with anti-GBM antibody GN (167-169), in which the antigen (GBM) is tissue fixed, and in lupus nephritis (155, 157, 170), in which the identity of at least one of the antigenic components (DNA) is known.

Application of these techniques to the study of primary ICGN with unknown antigen-antibody systems is more difficult, and optimal elution conditions are needed if this approach is to be successful. The elution of IC antigen and antibody from kidneys of rabbits with BSA induced CSS was quantitated in order to define these conditions.

METHODS

Chronic Serum Sickness Model.

CSS GN was induced in 3 kg male New Zealand White rabbits by repeated injection of BSA (171). BSA was administered intravenously (IV) six days per week and anti-BSA antibody production was determined weekly by the radioactive precipitin method (P-80) (172). The initial BSA dosage was 10 mg per day; subsequently, the dosage was adjusted

weekly to balance antibody production. Rabbits making a sustained precipitating antibody response developed proteinuric CSS GN after 4 to 10 weeks of injection. IF studies of the kidneys demonstrated typical granular glomerular IC deposits of IgG, BSA, and C3 (Fig. 9). IV injections of ^{125}I BSA or ^{131}I rabbit anti-BSA antibody were given 24 hours before sacrifice in order to radiolabel a portion of the antigen and/or antibody in the IC deposits (171).

Elution Technique and Quantitation of Eluted BSA and Anti-BSA Antibody.

Portions of single (^{125}I BSA) or paired (^{125}I BSA and ^{131}I anti-BSA antibody) labeled CSS kidneys were weighed, cut into small pieces while still frozen, suspended in PBS, and then homogenized in a chilled Waring Blendor. The homogenate was washed repeatedly with PBS by centrifugation at 2000 x G, counted, and then suspended in the eluting buffer and stirred for a specified time and temperature. The suspension was then centrifuged, and radioactivity in the eluted homogenate was counted. Elution of BSA and anti-BSA antibody was quantitated by calculating the percent of counts eluted from the washed homogenate. Protein bound radioactive iodine in the PBS washes and in the eluate was determined by precipitation in trichloroacetic acid (TCA) at a final concentration of 10%. In some experiments, the eluates were either neutralized or dialyzed against PBS, centrifuged, and then recounted to determine the percentage of the eluted counts that precipitated on return to physiologic conditions.

A radiolabeled BSA binding assay was used to quantitate functional anti-BSA antibody activity in the eluates. 3 x 2 ml aliquots of the eluate (still dissociated) were added to ^{131}I BSA (1-200 μg) in 50 μl volume. Each aliquot was then either neutralized or dialyzed against

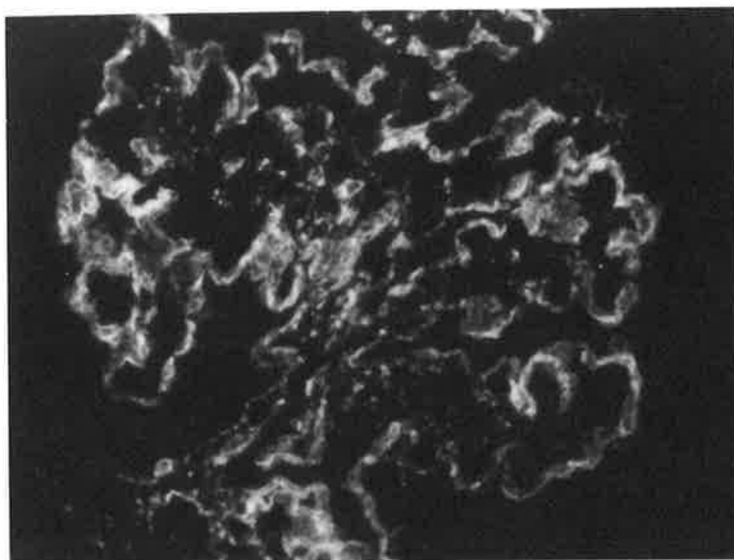

Figure 9. IF detection of rabbit IgG in the glomerular IC deposits of CSS GN. (CSS kidney section stained with FITC conjugated goat anti-rabbit IgG).

PBS and incubated for 18 hours at 4° C. The precipitates obtained after 50% saturated ammonium sulphate (SAS) precipitation were counted, and the percent "insertion" of ^{131}I BSA was determined for each quantity of added ^{131}I BSA. The assay was standardized with BSA anti-BSA complexes formed _in vitro_: 1 to 2071 μg anti-BSA antibody (determined by the quantitative precipitin method) was added to BSA at equivalence (Ab:Ag = 5.5:1 by weight) in PBS and allowed to react for 18 hours at 4° C. The resultant complexes were then dissociated in 0.02 M citrate buffer pH 3.2 for 60 min at 25° C and then combined with 1 to 5000 μg ^{131}I BSA. The solutions were neutralized with 0.1 N NaOH and incubated for 18 hours at 4° C. The precipitates obtained with 50% SAS were counted, and the amount (μg) of ^{131}I BSA required for 50% insertion was calculated for each quantity of anti-BSA antibody. A standard curve (Fig. 10) was obtained and this was used to quantitate eluted antibody. In a typical experiment, 3 x 2 ml aliquots of a 20 ml citrate buffer eluate from a 4.12 gm portion of CSS kidney were added to 10, 100, and 200 μg ^{131}I BSA, and 50% ^{131}I BSA insertion was achieved with 38 μg ^{131}I BSA which, by extrapolation from the standard curve, represents 110 μg anti-BSA antibody per ml (Fig. 10), and total anti-BSA antibody eluted = 267 μg per gm kidney. Since the avidity of the dissociated antibody could influence the apparent amount of antibody recovered, the test was used to compare recovery of antibody from portions of homogenates from the same kidney so that the values obtained would be relative.

<u>Immunochemical Characterization of CSS Kidney Eluates.</u>

Eluates were concentrated to 0.5-1.0 ml by negative pressure ultrafiltration.

Protein concentration was quantitated by micro-Kjeldahl nitrogen determination with an assumed 16% nitrogen content.

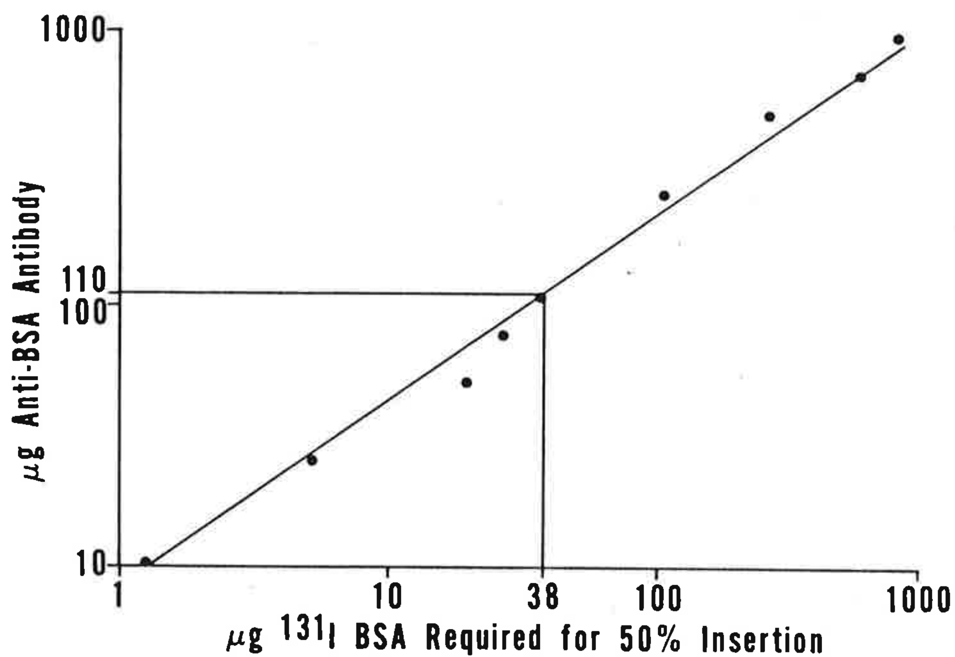

Figure 10. Quantitation of eluted anti-BSA antibody activity was achieved by comparison with the amount of ^{131}I BSA required for 50% insertion in citrate buffer dissociated in vitro formed BSA anti-BSA antibody IC containing increasing amounts of anti-BSA antibody. An example of the use of this assay is shown for an eluate in which 50% insertion was achieved with 38 μg ^{131}I BSA which represents 110 μg anti-BSA antibody per ml.

IgG concentration was determined by radioimmunoassay (104).
(Exposure of antibody to 0.02 M citrate buffer at pH 3.2 did not appreciably affect IgG quantitation.)

Gel diffusion was performed in 1% agarose in PBS, and immunoelectrophoresis in 1% agarose in barbital buffer pH 8.2, ionic strength 0.04, with goat antisera to whole rabbit serum, IgG, IgM, and albumin; with sheep antiserum to C3; and with rabbit antiserum to BSA.

SAS precipitation was done using the appropriate concentration of SAS for 30 min at 4° C, followed by centrifugation at 2000 x G.

Isolation of IgG was accomplished by chromatography with diethylaminoethyl cellulose (DEAE) equilibrated with 0.01 M phosphate buffer pH 7.5.

Sucrose density gradient fractionation: 300 μl aliquots of a concentrated unneutralized citrate buffer eluate were placed on 5 ml 10-37% sucrose gradients in 0.02 M citrate buffer pH 3.2. ^{125}I BSA (4.5 S), ^{131}I IgG (7 S), and ^{125}I IgM (19 S) were used as markers in a separate tube. The gradients were centrifuged at 250,000 x G for 6.2 hours, and drained; then the IgG fractions were collected, pooled, and dialyzed against PBS.

Studies with Eluted Anti-BSA Antibody.

IF: Eluted anti-BSA antibody activity was detected by indirect IF. Rat liver or kidney obtained 60 min after IV infusion of 125 mg of heat-aggregated BSA was used as a target. BSA deposits were present in the Kupffer cells of the hepatic sinusoids (Fig. 11), in the glomerular mesangium and, to a

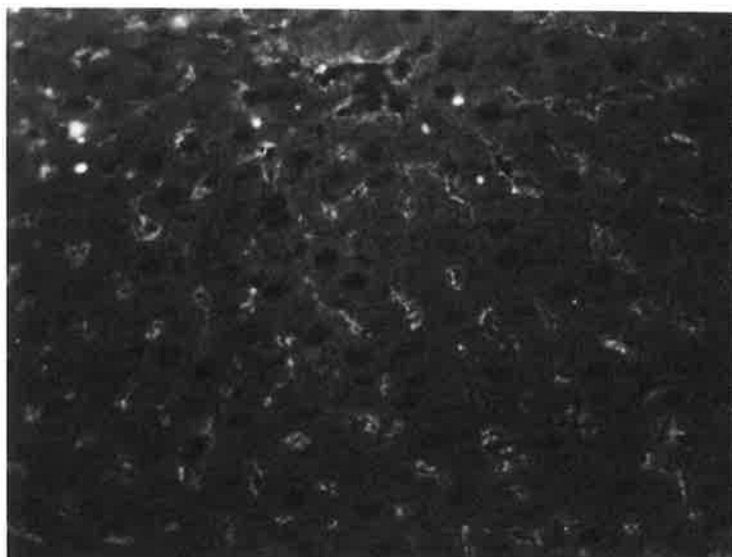

Figure 11. BSA deposits in the Kupffer cells of the hepatic sinusoids of a rat after IV administration of heat aggregated BSA. (Rat liver section reacted with rabbit anti-BSA antibody and then FITC conjugated goat anti-rabbit IgG).

lesser extent, within the glomerular capillary lumens. The eluate was incubated with the target section which was then reacted with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG.

C fixing capacity (173): Eluates were dialyzed against isotonic VBS and heated at 56° C for 30 min before being tested by indirect IF using a BSA-infused rat kidney target, fresh NRS (1:10) as a source of C, and FITC conjugated sheep anti-rabbit C3 which had been absorbed with rabbit IgG.

Elution of Cryostat Sections of CSS Kidney.

Six 5 μ cryostat sections from a CSS kidney were mounted side by side on glass slides, surrounded by a ring of embroidery paint (Lee Wards, Elgin, Ill.), and washed in PBS for 30 min. The sections were covered with 200 μl of the elution buffer and incubated for a specified time and temperature. The "eluate" was then aspirated from each slide and dialyzed against PBS. IgG concentrations in the eluates were determined by radioimmunoassay, and anti-BSA antibody activity was tested by IF. The efficiency of elution was assessed by IF of the eluted sections for IgG, BSA, and C3.

RESULTS

Quantitative Evaluation of Different Elution Conditions.

Homogenization time (Table 10).

Portions of a ^{125}I BSA and ^{131}I anti-BSA antibody labeled CSS kidney were homogenized for times ranging from 15 sec to 10 min, washed 3 times with PBS, and then eluted with 0.02 M citrate buffer at pH 3.2 for 60 min at 25° C. The amount of ^{125}I BSA and ^{131}I anti-BSA antibody in the

TABLE 10

THE EFFECT OF HOMOGENIZATION TIME ON THE

ELUTION OF ^{125}I BSA AND ^{131}I ANTI-BSA ANTIBODY

FROM CSS KIDNEY

Time of Homogenization (min)	^{125}I BSA (CPM* washed homogenate)	^{125}I BSA Eluted (%)	^{131}I Anti-BSA Antibody (CPM washed homogenate)	^{131}I Anti-BSA Antibody Eluted (%)
0.25	25268	(77)	6827	(76)
0.5	23800	(81)	6306	(78)
1	20180	(77)	5780	(79)
2	16948	(75)	4655	(66)
10	12855	(72)	4223	(61)

* counts per minute

washed homogenate and in the eluate decreased with increasing homogenization times. The percent elution of the residual BSA and anti-BSA antibody was much less affected. Presumably, prolonged homogenization dislodges the least firmly bound BSA-anti-BSA complexes from the kidney tissue and this reduces the amount of antigen and antibody available for elution. Homogenization should, therefore, be restricted to the minimum time required for adequate dispersion of the tissue (generally 10-30 sec).

Washing the homogenate (Fig. 12).

The washing procedure was evaluated by recording protein bound ^{125}I BSA or ^{131}I anti-BSA antibody counts in each of four consecutive PBS washes and in the final eluate. Free iodine and most of the unbound ^{125}I BSA and ^{131}I anti-BSA antibody were removed during the first PBS wash, and 3 or 4 washes were found to be adequate. In studies with paired labeled CSS kidneys, it was found that ^{125}I BSA and ^{131}I anti-BSA antibody counts paralleled each other during washing and elution of the homogenate.

The effect of a final water wash of the homogenate, previously employed (168) to lower ionic strength prior to elution, was evaluated and was found to reduce the elution of ^{125}I BSA by approximately 60%. This step was subsequently omitted.

Volume of elution buffer (Fig. 13).

Elution of ^{125}I BSA from washed homogenates of CSS kidney was quantitated using citrate buffer volumes varying from 2.5 to 20 ml per gm of homogenate. A ratio of at least 10 ml of citrate buffer per gm of homogenate was required for optimal elution of ^{125}I BSA. With ratios less than this, the end-elution pH of the suspension rose above 3.50, and the elution yield decreased.

pH of elution buffer (Fig. 14).

Pooled, washed homogenates from ^{125}I BSA labeled CSS kidneys were eluted for 60 min at 25° C with 0.15 M NaCl (20 ml per gm homogenate) adjusted with HCl or NaOH to pH values from 1.5 to 11.0. The results are the mean of triplicate studies. Dissociation and elution of ^{125}I BSA increased sharply with pH values less than 4.0 or greater than 10.0, and the effective pH zones for elution are thus critically restrictive.

Molarity of chaotropic ion elution buffers (Fig. 15).

Pooled, washed homogenates from ^{125}I BSA labeled CSS kidneys were eluted for 60 min at 25° C with 20 ml per gm of homogenate of 0.5 to 3.0 M sodium chloride, potassium thiocyanate, or potassium iodide, in PBS at pH 7.0. The results are the mean of triplicate studies. Dissociation and elution of ^{125}I BSA increased with increasing molarity of the chaotropic ion buffers. Potassium iodide was the most effective of these buffers, but difficulty in separating (by centrifugation) homogenates eluted with potassium iodide at 2 M and above is a technical limitation.

Elution with standard buffers.

Two separate studies were performed: in one, (Table 11), aliquots of pooled, washed homogenates from ^{125}I BSA labeled CSS kidneys were eluted for 30 min at 25° C with six different buffers: 0.02 M citrate buffer at pH 3.2, 3 M sodium chloride at pH 5.3, 2 M sodium thiocyanate in acetate buffer at pH 4.5, 0.15 M sodium chloride at pH 1.5, 0.15 M sodium chloride at pH 11.0, and PBS. The percent of ^{125}I BSA eluted was determined for each buffer. The eluates were then either neutralized (citrate and 0.15 M sodium chloride buffers) or dialyzed against PBS

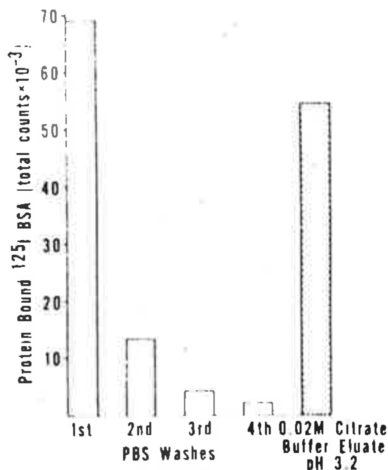

Figure 12. The protein bound ^{125}I BSA counts per minute found in four consecutive PBS washes of CSS kidney homogenate and in its subsequent citrate buffer eluate are shown.

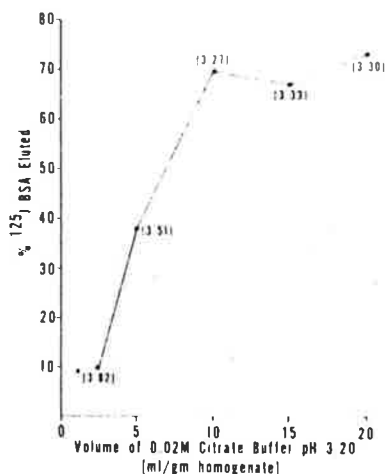

Figure 13. The percent of ^{125}I BSA which eluted from washed CSS kidney homogenates using citrate buffer volumes from 2.5 to 20 ml per gm homogenate is shown.

* The number in parentheses is the pH of suspension after elution.

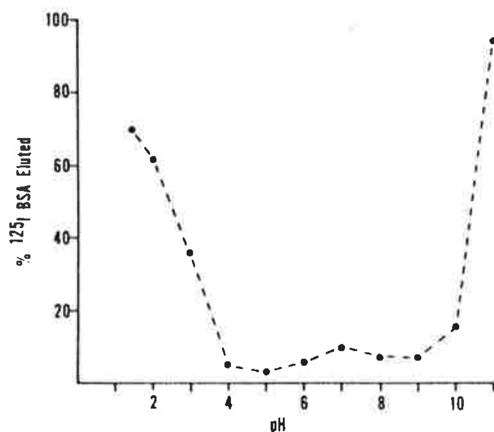

Figure 14. The percent ^{125}I BSA which eluted from washed CSS kidney homogenates with 0.15 M NaCl at pH values varying from 1.5 to 11.0 is shown.

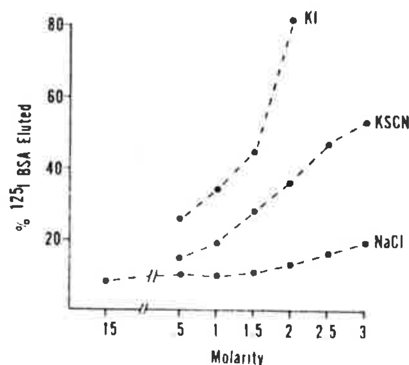

Figure 15. The percent ^{125}I BSA which eluted from washed CSS kidney homogenates with 0.5 to 3 M chaotropic ion buffers (sodium chloride, potassium thiocyanate, potassium iodide) in PBS pH 7.0 is shown.

(3 M sodium chloride and sodium thiocyanate buffers), and the percentage of the eluted ^{125}I BSA that was precipitated by centrifugation after return to physiologic conditions was determined. The results are the mean of seven individual experiments. Some variation in the ease of elution was noted for different pooled homogenates; it was felt that kidneys with more advanced nephritis and scarring generally eluted less well, but this was not quantitated.

TABLE 11

THE EFFECT OF DIFFERENT ELUTION BUFFERS ON THE ELUTION OF ^{125}I BSA FROM CSS KIDNEY

Elution Buffer	pH	^{125}I BSA Eluted (%)	Precipitation of Eluted ^{125}I BSA * (%)
0.02 M Citrate Buffer	3.2	66	72
3 M Sodium Chloride	5.3	25	73
2 M Sodium Thiocyanate	4.5	68	43
0.15 M Sodium Chloride	1.5	58	58
0.15 M Sodium Chloride	11.0	85	56
PBS	7.2	7	10

* Percentage of the eluted ^{125}I BSA which precipitated on return of the eluate to physiologic conditions.

The best elution of ^{125}I BSA in this study was achieved with 0.15 M sodium chloride pH 11.0, with intermediate amounts using 2 M sodium thiocyanate pH 4.5, 0.02 M citrate buffer pH 3.2, or 0.15 M sodium

chloride pH 1.5. Elution with 3 M sodium chloride pH 5.3 was much less efficient. Precipitation of eluted ^{125}I BSA on return to physiologic conditions, a measure of the recombination of eluted BSA and anti-BSA antibody (vida infra), was greatest with citrate buffer and 3 M sodium chloride.

In the other study (Table 12), aliquots of washed homogenate from a ^{125}I BSA labeled CSS kidney were eluted for 60 min at 25° C with seven different buffers: 0.02 M citrate buffer at pH 3.2, 0.1 M borate buffer at pH 11.25, 2.5 M potassium iodide at pH 7.0, 2.5 M potassium thiocyanate at pH 7.0, 1 M proprionic acid at pH 2.7, 3 M sodium chloride at pH 5.3, and PBS. Eluted ^{125}I BSA and functional anti-BSA antibody (radiolabeled BSA binding assay) were quantitated for each buffer. The results are the mean of duplicate studies.

TABLE 12

THE EFFECT OF DIFFERENT ELUTION BUFFERS ON THE ELUTION OF ^{125}I BSA AND ANTI-BSA ANTIBODY FROM CSS KIDNEY

Elution Buffer	pH	^{125}I BSA Eluted (%)	Anti-BSA Antibody Eluted* (μg per gm kidney)
0.02 M Citrate Buffer	3.2	55	344
0.1 M Borate Buffer	11.25	80	143
2.5 M Potassium Thiocyanate	7.0	35	142
1 M Proprionic Acid	2.7	86	270
3 M Sodium Chloride	5.3	13	97
PBS	7.2	4	---

* Quantitated by the radiolabeled BSA binding assay.

Maximal elution of ^{125}I BSA was achieved with 1 M proprionic acid at pH 2.7 and 0.1 M borate buffer at pH 11.25. These buffers, however, yielded less functional antibody than did citrate buffer. 3 M sodium chloride and 2.5 M potassium thiocyanate gave low and intermediate results, respectively. 2.5 M potassium iodide (not shown in Table 12) eluted homogenates could not be separated completely, and this precluded quantitation of the elution of ^{125}I BSA. Using the radiolabeled BSA binding assay, which is not greatly affected by the presence of kidney homogenate, 2.5 M potassium iodide eluted 202 µg anti-BSA antibody per gm kidney.

Elution time (Fig. 16).

Portions of washed homogenates from ^{125}I BSA labeled CSS kidneys were eluted with citrate buffer at 25° C for times varying from 15 min to 24 hrs. Elution of functional anti-BSA antibody was maximal at 4¼ hrs, and the reduction in antibody activity seen in the 24 hr eluate was attributed to the deleterious effects of prolonged exposure of antibody to acid pH. Elution of ^{125}I BSA decreased with elution times longer than 60 min. This may have been due to progressive denaturation of ^{125}I BSA since the percentage of the eluted counts which precipitated after neutralization (a measure of antigen-antibody recombination and, therefore, of the integrity of the eluted ^{125}I BSA) also decreased with elution time.

Elution temperature (Table 13).

Portions of washed homogenates from ^{125}I BSA labeled CSS kidneys were eluted with citrate buffer for 60 min at 4°, 25°, 37°, or 56° C. Elution of both ^{125}I BSA and anti-BSA antibody was significantly

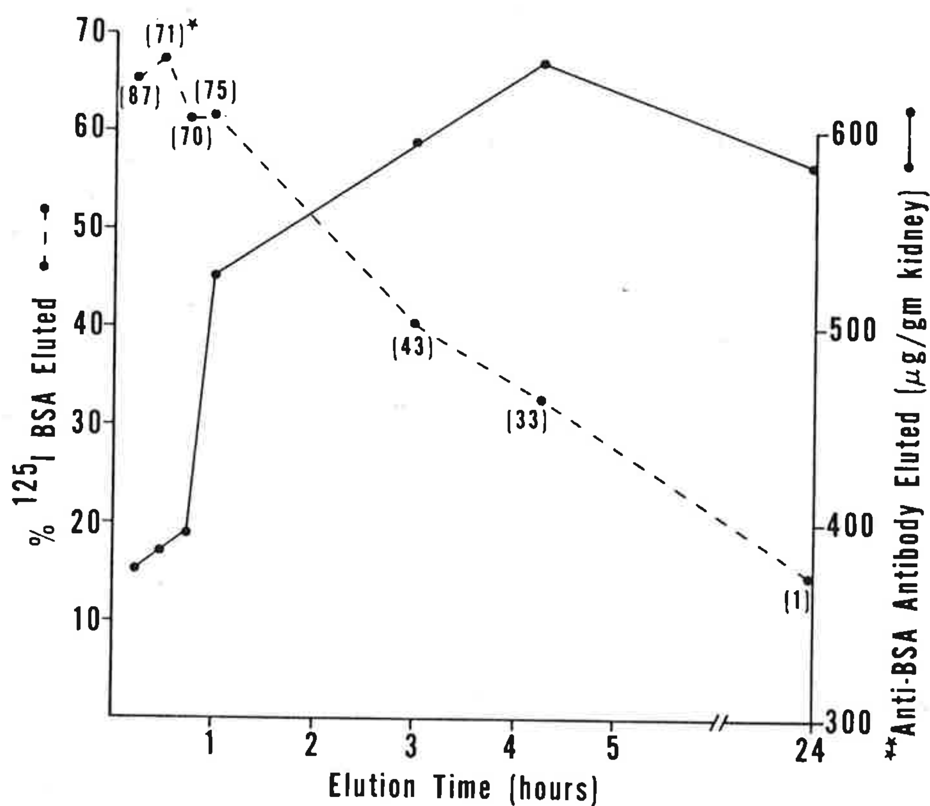

Figure 16. The elution of ^{125}I BSA and anti-BSA antibody from washed CSS kidney homogenates with citrate buffer at times varying from 15 min to 24 hours is shown.

* The number in parentheses shows the percentage of the eluted ^{125}I BSA which precipitated on return of the eluate to physiologic conditions.

** The anti-BSA antibody activity was quantitated by the radiolabeled BSA binding assay (see Figure 10).

greater at either 25° C or 37° C than at 4° C, but less functional antibody eluted at 56° C, suggesting a deleterious effect on antibody activity under these conditions.

TABLE 13

THE EFFECT OF TEMPERATURE ON
THE ELUTION OF ^{125}I BSA AND ANTI-BSA
ANTIBODY FROM CSS KIDNEY

Elution Temperature (°C)	^{125}I BSA Eluted (%)	Anti-BSA Antibody Eluted* (μg per gm kidney)
4	58	205
25	78	264
37	81	253
56	68	146

* Quantitated by the radiolabeled BSA binding assay.

Immunochemical Characterization of CSS Kidney Eluates.

The unfractionated CSS kidney eluate was found to be a relatively crude preparation with IgG accounting for only 10% of the total protein content. Contamination by serum and tissue proteins could be reduced by further purification; the IgG concentration after 50% SAS precipitation and 0.01 M DEAE fractionation was 43%.

Immunoelectrophoresis of unfractionated eluates revealed IgG and IgM, together with faint lines in the pre-albumin and β-globulin regions (Fig. 17). Rabbit serum albumin and C3 could be demonstrated by gel diffusion, as could anti-BSA antibody (Fig. 18). However, BSA

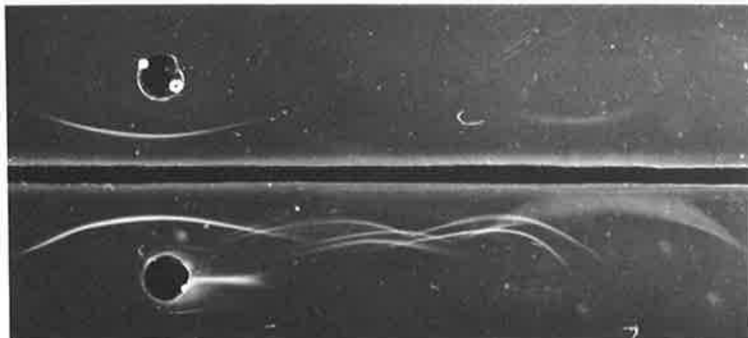

Figure 17. Immunoelectrophoresis of concentrated citrate buffer CSS kidney eluate (top well) and serum from the same animal (bottom well). Trough contains goat antiserum to whole normal rabbit serum. Anode is to the right.

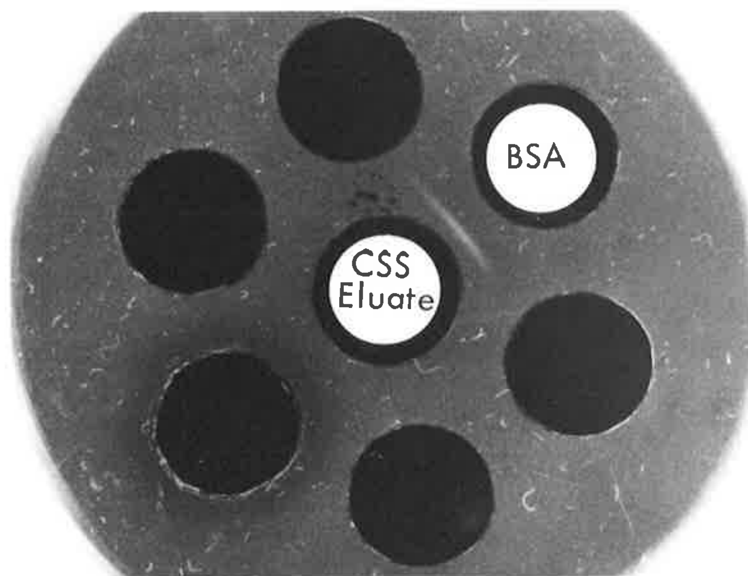

Figure 18. Gel diffusion; the detection of precipitating anti-BSA antibody in a concentrated citrate buffer CSS kidney eluate.

could not be detected, presumably because it was bound to antibody in excess. Free BSA was identified by gel diffusion in the concentrated supernatant obtained after 33% SAS precipitation of an unneutralized citrate buffer CSS kidney eluate. In this situation, advantage was taken of the different relative solubilities of IgG and BSA at a final concentration of 33% SAS in an acid medium. The total recovery of IgG was less than at 50% SAS; however, the 33% SAS concentration allowed partial separation of IgG from BSA, the latter being insoluble in 50% SAS in the acid conditions of this study.

Recombination of eluted anti-BSA antibody with eluted BSA occurs following return of the eluate to physiologic conditions, and this effectively reduces the yield of free antibody. Up to 75% of eluted ^{125}I BSA could be precipitated after dialysis or neutralization of the eluates. This phenomenon was abrogated by the addition of excess unlabeled BSA, and was, therefore, attributed to, and used as an index of, recombination. The loss of eluted anti-BSA antibody by recombination could be prevented if steps were taken to partition eluted antigen (BSA) and antibody prior to return of the eluate to physiologic conditions. Thus, the pooled IgG fractions obtained after sucrose density gradient ultracentrifugation of dissociated citrate buffer eluates had up to four times more anti-BSA antibody activity (radiolabeled BSA binding assay) per μg IgG than the unfractionated eluate. Recovery of total IgG with this technique was 35%.

Finally, the concentration of anti-BSA antibody in the renal eluates was compared with that in serum. Anti-BSA antibody activity in citrate buffer eluates and in their corresponding sera were titrated by indirect IF, and IgG concentrations were determined by radioimmuno-

assay. The minimum amount of IgG required for positive IF was used to determine the specific antibody concentrations. It was found that anti-BSA antibody was selectively concentrated in the eluates. Even at 24 hours after the last injection of BSA when the serum is in great antibody excess, a two to five-fold increase in specific antibody concentration was detected in the eluates.

Studies with Eluted Anti-BSA Antibody.

In this study, citrate buffer eluted anti-BSA antibody was reactive in conventional gel diffusion, indirect IF, and radiolabeled BSA binding test systems. In these systems, the quantity of antibody was more often limiting than any qualitative functional impairment of antibody activity.

In contrast, an impairment of C fixation was observed in preliminary studies with citrate buffer treated anti-BSA antibody. When 0.02 M citrate buffer pH 3.2, 0.1 M borate buffer pH 11.25, 1 M proprionic acid pH 2.7, 2.5 M potassium iodide pH 7.0, and 2.5 M potassium thiocyanate pH 7.0 CSS kidney eluates, each containing approximately 50 μg IgG per ml, were tested for their ability to fix C in a modified Burkholder technique (173), only the chaotropic ion (2.5 M potassium iodide and thiocyanate)-eluted antibodies effectively retained C fixing activity. This factor limits the application of C fixation tests in the detection of eluted antibody.

Directly labeled antibody is required when studying the reactivity of eluates with glomerular IC containing homologous IgG. Therefore, techniques for FITC conjugation and radioiodination of eluted anti-BSA antibody were devised. Initial attempts to conjugate eluted anti-BSA antibody with FITC were frustrated by aggregation of the conjugated

protein. However, it was possible to FITC conjugate SAS precipitated or 0.01 M DEAE fractionated citrate buffer eluates at 100-200 μg IgG per ml by dialysis against 10 volumes of 0.025 M carbonate buffer, pH 9.5, with 0.01 mg FITC per ml. The conjugated eluate was then fractionated by mixing with a suspension of DEAE in phosphate buffer in ratios adjusted to yield a final molarity of 0.05 to 0.1. Such FITC conjugated eluates reacted with the glomerular IC deposits in CSS kidney sections, and this staining could be blocked by prior absorption of the conjugate with BSA or by pre-incubation of the sections with unconjugated anti-BSA antibody, thus confirming its immunologic specificity. It was found that weak staining with these conjugates could be greatly enhanced by pretreatment of the CSS kidney sections with 1% BSA (Fig. 19). Presumably, the added BSA is bound to glomerular IC by anti-BSA antibody present in excess. The effect is to increase the quantity and the accessibility of glomerular bound BSA, and to thus augment the subsequent reaction with FITC conjugated anti-BSA antibody.

Citrate buffer CSS kidney eluates were radiolabeled with ^{125}I by using chloramine T (100). Very poor uptake of ^{125}I was achieved with unfractionated eluates; in contrast, 0.01 M DEAE fractionated eluates were successfully radiolabeled and retained anti-BSA antibody activity as shown by IF testing.

Elution of Cryostat Sections of CSS Kidney (Table 14).

Submicrogram IgG concentrations, at the limit of sensitivity of the IF test system for anti-BSA antibody, were eluted with this technique. Elution at 25° C or 37° C was more efficient than at 4° C. The best results were obtained with 0.1 M borate buffer at pH 11.25, 2.5 M potassium iodide at pH 7.0, and 0.02 M citrate buffer at pH 3.2. IF

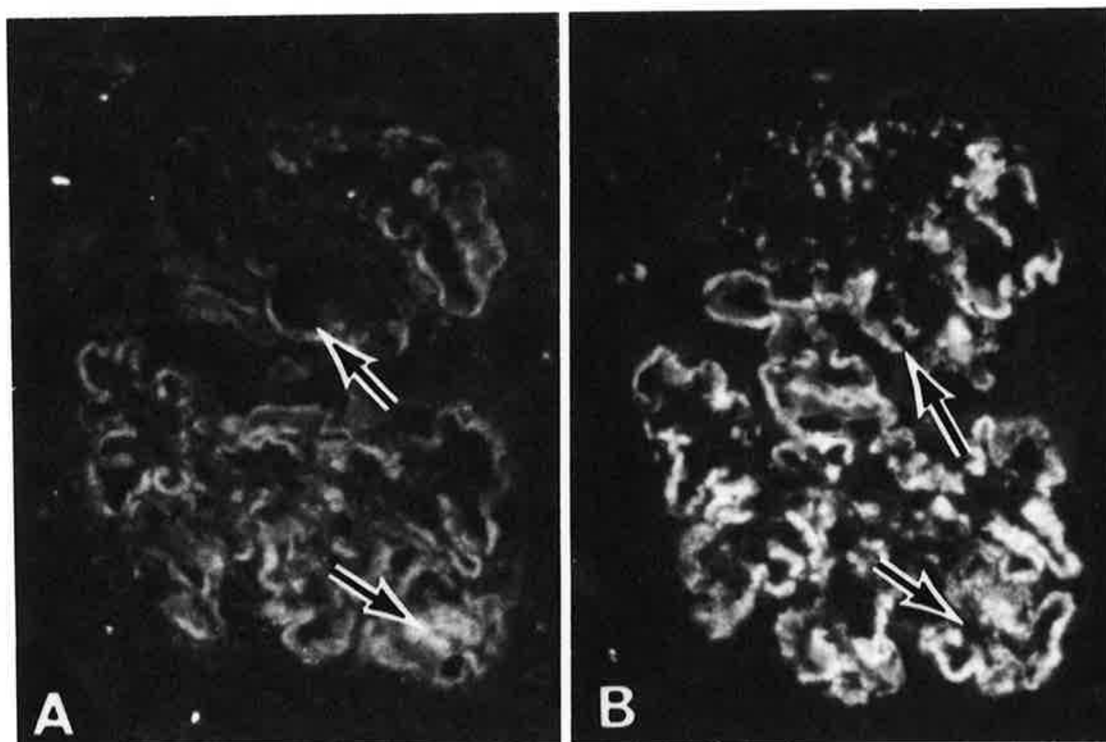

Figure 19. The IF detection of BSA in glomerular IC deposits (arrows) is shown on serial sections of the same glomerulus from a CSS kidney using FITC conjugated anti-BSA antibody eluted from a CSS kidney. The binding of the eluate noted in (A) is greatly enhanced by the pretreatment of the section with 1% BSA as shown in (B).

of the eluted sections showed that IgG, BSA, and C3 were eluted in parallel, and that this estimate of the efficiency of elution correlated well with the amount of IgG recovered in the eluate.

TABLE 14

ELUTION OF CRYOSTAT SECTIONS
OF CSS KIDNEY (60 min, 25° C)

Elution Buffer	pH	IF of Eluted Sections for IgG*	Eluted Anti-BSA Antibody**	IgG Eluted (ng per ml)
0.02 M Citrate Buffer	3.2	2+	+	416
2.5 M Potassium Iodide	7.0	2+	+	632
2.5 M Potassium Thiocyanate	7.0	2+	0	216
0.1 M Borate Buffer	11.25	1+	+	826
3 M Sodium Chloride	5.3	3+	0	33
PBS	7.2	4+	0	68

* BSA and C3 were eluted in parallel with IgG.

** Detected by indirect IF.

DISCUSSION

Factors which adversely affected the quantity or quality of eluted antibody were identified at each step in the elution process. In this way, optimal conditions for homogenization, washing, buffer volume, and elution time and temperature were defined. It was found that prolonged homogenization of the kidney tissue reduced the elution yield. This effect was minimized if homogenization was restricted to 30 sec, which was sufficient time for effective dispersion of the tissue. Removal

of free BSA and anti-BSA antibody was effectively achieved with three or four PBS washes of the homogenate; however, attempts to lower the ionic strength of the homogenate by a final water wash resulted in a decrease in the elution yield. The volume of the elution buffer was also found to be critical. Optimal elution with citrate buffer was achieved with a buffer volume of 10 ml or more per gm of homogenate, the ratio required for maintenance of an effective pH in the suspension. The amount of functional anti-BSA antibody eluted with citrate buffer increased with elution time up to at least 4 hours. There was a slight decrease in eluted antibody activity at 24 hours, presumably because prolonged exposure to acid pH has a deleterious effect on antibody activity. Elution at 25-37° C was more efficient than at 4° C and functional antibody activity decreased with elution at 56° C.

Significant quantitative and qualitative differences in eluted antibody were also apparent with various standard elution buffers. In general, the most efficient buffers also tended to have the most pronounced deleterious effects on the functional activity of the eluted antibody. In this study, elution of functional antibody was optimal with 0.02 M citrate buffer at pH 3.2, but it is recognized that each antigen-antibody system may well be different. In particular, elution with chaotropic ions has been advocated in studies with _in vitro_ IC (174), in the dissociation of antibody fixed to erythrocytes (175), and in elution of human ICGN kidneys (159). Good preservation of functional antibody activity with these elution agents is further supported in the present study, in which antibodies eluted with chaotropic ion retained the ability to fix C. Impairment of the C fixing capacity

of acid eluted antibodies has been previously reported (176-178) and attributed to structural alterations in the Fc portion of the immunoglobulin molecule (178).

The problem of recombination of eluted antibody with dissociated antigen was recognized and quantitated in this study. It was found that up to 75% of the eluted antibody is lost to further study by recombination unless steps are taken to partition eluted antigen and antibody before return of the eluate to physiologic conditions. This can be achieved, for example, by sucrose density gradient fractionation of the dissociated eluate. In another approach, Milgrom et al (179) employed immunoelectrophoretic techniques to separate eluted antigen and antibody. The problem may be simpler if the antigen is known. For example, deoxyribonuclease can be used to elute ANA from IC in SLE kidneys (155, 157) by destroying DNA.

Immunochemically, the eluate was a rather crude mixture of a number of proteins that included not only specific antibody (free and in combination with antigen), but C, albumin, and unidentified tissue proteins. SAS precipitation of citrate buffer eluates prior to neutralization proved to be a convenient one-step technique in the concentration and purification of eluted antibody. Further purification and IgG fractionation was possible, but, of course, reduced the final amount of antibody recovered.

Methods suitable for the detection of antibody activity in eluates were evaluated. Simple gel precipitation techniques lacked sensitivity, and methods based upon C fixation were limited by the impairment of C fixing capacity of eluted antibody. A binding assay in which eluted anti-BSA antibody could be quantitated by its binding

with ^{131}I BSA proved to be highly satisfactory. Similarly, indirect IF was shown to be a sensitive and semi-quantitative technique for the detection of eluted anti-BSA antibody. By comparing the concentrations of IgG in serum and eluates minimally required for positive IF, it was possible to demonstrate an increased concentration of specific antibody in the eluates over that in the corresponding sera, a prerequisite for interpretation of the significance of eluted antibody.

The increased sensitivity of indirect (double antibody) assays is generally advantageous, but these techniques cannot be used for the identification of antigen in glomerular IC deposits which contain homologous IgG. In this situation direct techniques using labeled antibody are required. Thus, it was possible to FITC conjugate SAS precipitated or IgG fractionated eluted anti-BSA antibody for use as IF reagents with CSS kidney sections. Such FITC conjugates reacted specifically with BSA in the glomerular IC. It was found that this staining could be greatly augmented by pre-incubation of the sections with unlabeled BSA. This observation is of particular interest. Presumably, the added BSA is bound to glomerular IC by anti-BSA antibody present in excess, thus increasing the amount and accessibility of glomerular bound BSA. This technique of "antigen IF" has wide application, and may facilitate the identification of antigen in other types of ICGN.

Studies with elution of antibody from cryostat sections of CSS kidney confirmed the previous reports (138, 169, 180, 181) that these micro-elution techniques can be used successfully, but indicated, as expected, that the quantity of antibody eluted is very small. Similar

procedures have been advocated for use prior to staining cryostat sections for specific antigen (155, 159, 160, 182-184). This has been done in an attempt to "unmask" antigen by the partial elution of antibody; however, in the CSS model of ICGN elution of antigen and antibody appeared to occur in parallel. (The quantity of eluted antibody can profitably be increased by collecting and eluting multiple cryostat kidney sections in a small tube, and using this technique, it was possible to elute ANA and anti-GBM antibody from kidney biopsy specimens from patients with SLE and anti-GBM antibody GN, respectively.

CONCLUSION

Antigen and antibody from glomerular IC deposits in rabbits with experimental BSA induced CSS were quantitated in eluates from kidneys in which a portion of the antigen and antibody had been radiolabeled. The largest quantities of ^{125}I BSA eluted with 1 M proprionic acid at pH 2.7 (86%) and 0.1 M borate buffer at pH 11.25 (80%). However, these buffers yielded less functional anti-BSA antibody than 0.02 M citrate buffer at pH 3.2 (344 μg per gm kidney). Citrate buffer-eluted anti-BSA antibody was reactive in immunodiffusion, IF, and radiolabeled BSA binding test systems, but C fixation was impaired relative to chaotropic ion-eluted antibody. It was found that up to 75% of the eluted antibody was lost to further study by recombination with eluted BSA unless steps were taken to fractionate the dissociated eluate prior to neutralization. Elution of cryostat sections of CSS kidney was also studied; BSA, IgG, and C3 eluted in parallel, and sub-microgram quantities of anti-BSA antibody were recovered with this technique.

B. THE IMMUNOPATHOGENESIS OF SPONTANEOUS GN IN RABBITS.

INTRODUCTION

The occurrence of spontaneous GN in New Zealand white rabbits has been described by Verroust et al (45). In this study 3 of 25 laboratory New Zealand white rabbits (age 14 to 52 weeks) were found to be proteinuric. These rabbits had features of a mixed membranous and proliferative form of GN, and IF studies revealed granular deposits of IgG and C3 along the GBM consistent with glomerular IC deposits. Similar features were found in 5 to 10% of New Zealand white rabbits by Wilson and Abinanti (Wilson, C. B., personal communication). The disease is generally indolent, and proteinuria may be intermittent. However, some rabbits, particularly peripartum does, become frankly nephrotic and may succumb to the disease.

The present study was designed to explore the immunopathogenesis of this form of GN.

METHODS

Experimental animals. 42 New Zealand white rabbits were studied (21 8 to 12 week old animals, and 21 adults).

Proteinuria. 24 hour urine protein determinations were performed on three consecutive days by the sulphosalicylic acid method (185). Abnormal proteinuria was defined by two measurements above 20 mg/24 hours.

Pathological studies.

(i) Histology. Tissue samples were fixed in Bouin's solution, post-fixed in 50% ethyl alcohol, and stained with haematoxylin-eosin or periodic acid Schiff (PAS).

(ii) IF. Tissue samples were snap-frozen in liquid nitrogen. 2μ thick cryostat sections were fixed (50% ether-alcohol followed by 95% alcohol) and reacted with FITC conjugated goat anti-rabbit IgG and sheep anti-rabbit C3.

(iii) EM. Tissue fragments were diced in 2% glutaraldehyde and post-fixed in 1% osmic acid. The sections were stained with 2% uranyl acetate and 0.5% lead citrate.

Detection of circulating IC. Sera from these animals were tested for IC by the *IRCA using ^{125}I goat anti-rabbit IgG antibody and heat aggregated rabbit gamma globulin for the standard curve.

Elution studies. Kidneys from rabbits with proteinuric GN and from control rabbits were homogenized, washed six times with PBS, and eluted with 0.02 M citrate buffer pH 3.2 for 2 hours at 25° C. The eluted Ig was concentrated by precipitation with 50% SAS and then dialyzed against PBS. The IgG content of the concentrated eluates was quantitated by radioimmunoassay for rabbit IgG (104), and the reactivity of the eluates (and sera) with sections of normal rabbit kidney (NRK) was tested by indirect IF using FITC conjugated goat anti-rabbit IgG.

Additional studies to determine the specificity of the eluted antibody.

(i) Glomeruli were isolated from the cortices of NRK using the method of Krakower and Greenspon (9), and a renal tubule preparation (Fx 1A) was prepared as described by Edgington et al (21). The ability of these preparations to absorb the reactivity of the eluted antibody was tested by incubating aliquots of the eluates with equal

volumes of packed glomeruli or Fx 1A respectively for 18 hours at 4° C. The supernatants (after centrifugation at 8,400 x G for 10 min) were then tested by indirect IF with sections of NRK.

Extracts were obtained from the glomerular suspension by sonication, treatment with 8 M urea, and various enzymes {pronase (K & K Laboratories), trypsin (Worthington Biochemical Corp.), and neuraminidase (ICN Pharmaceutical Inc.)}. The ability of these various extracts to absorb the IF reactivity of the eluted antibodies was tested as above. The extracts were also tested by gel diffusion (1% agarose in PBS) with the eluates and sera from rabbits with GN.

(ii) The eluates were tested for anti-GBM antibody by radioimmunoassay (107).

(iii) Eluates and sera were studied for anti-smooth muscle antibodies by indirect IF using unfixed cryostat sections of fresh rabbit uterus (186). In addition, highly purified rabbit skeletal muscle F-actin (obtained from Dr. M. Hegeness, Department of Biology, University of California, San Diego, Ca.) was tested by absorption IF and gel diffusion with rabbit GN eluates and sera, and with high titre human smooth muscle antibody (HuSMA) sera (obtained from Dr. D. G. Taylor, Department of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, Ca.). The absorption IF studies were performed with F-actin as in (i) above; gel diffusion studies were carried out with depolymerized actin in 1% agarose with 0.0005 M KCl, 0.002 M Tris HCl buffer, and 0.0005 M 2-mercaptoethanol pH 7.9.

(iv) NRK sections were pre-treated with 100 μl collagenase (Worthington Biochemical Corp.), pronase, or neuraminidase for 60 min at 37° C and then reacted with the eluates by indirect IF.

(v) Human β_2 glycoprotein III (Hu β_2 GP III) (obtained from Prof. H. G. Schwick, Behringwerke, Marburg, West Germany) was tested by absorption IF and gel diffusion with the rabbit GN eluates and sera, and with rabbit antiserum to Hu β_2 GP III (Behring Diagnostics).

RESULTS (Table 15)

Proteinuria. Proteinuria (range 44 to 338 mg/24 hours) was detected in 5 of the 42 rabbits (12%). All but one of these were adult.

Pathological studies. Histological examination of the kidneys from these rabbits revealed irregular thickening of the GBM, variable mesangial proliferative change, and areas of tubulo-interstitial damage. IF studies showed finely granular deposits of IgG and C3 along the GBM or in the mesangium; no deposits were present in the tubulo-interstitial areas. EM examination revealed "saw-tooth" irregularities of the GBM, and electron-dense deposits predominately in the subepithelial aspect of the GBM. Figs. 20-22 illustrate some of these features.

Detection of circulating IC. Serum IC were detected by the *IRCA in one of the 5 rabbits with GN.

Elution studies. IgG eluted from the kidneys of rabbits with GN was reactive by indirect IF with the glomeruli and blood vessels of NRK sections (Fig. 23). The glomerular staining was particularly striking. The staining was quite diffuse and could not be clearly localized to any recognizable glomerular substructural component. The distribution was quite unlike that of anti-GBM antibody and appeared to involve glomerular cell cytoplasm. A similar pattern of staining

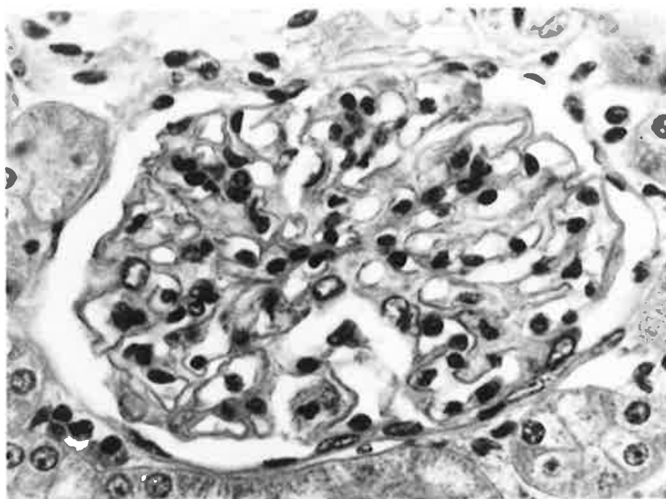

Figure 20. Light microscopic appearance of the glomerular lesions seen in rabbit #2 with spontaneous GN. There is pronounced thickening of the GBM. Haematoxylin and eosin.

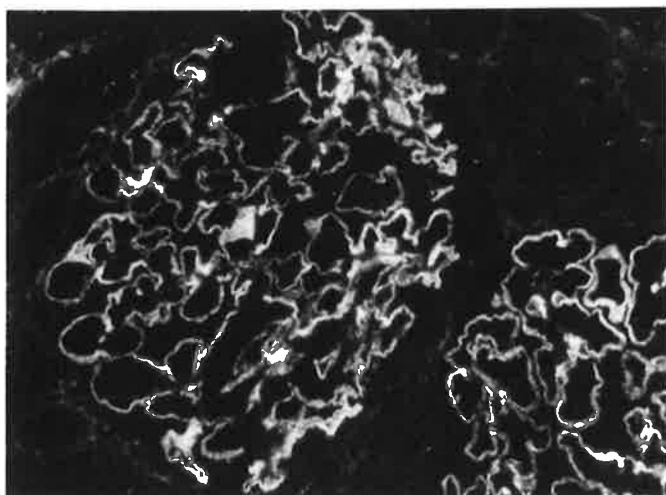

Figure 21. Detection of finely granular deposits of IgG along the GBM. (Rabbit #2 with spontaneous GN). FITC conjugated goat anti-rabbit IgG.

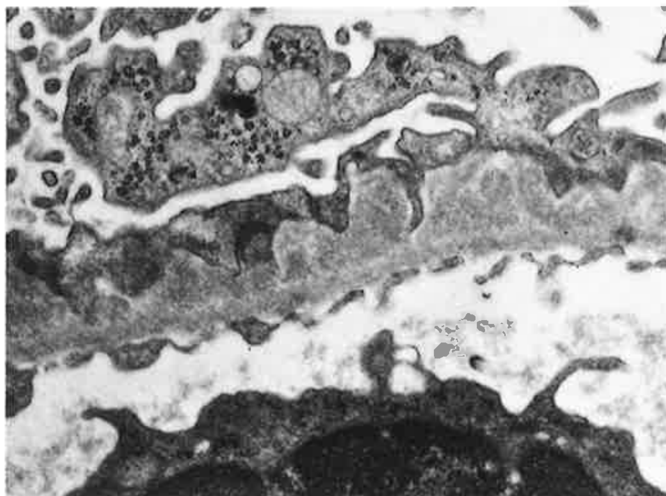

Figure 22. Electron dense deposits in the subepithelial aspect of the GBM with fusion of the epithelial cell foot processes (Rabbit #2 with spontaneous GN). (x 20,000).

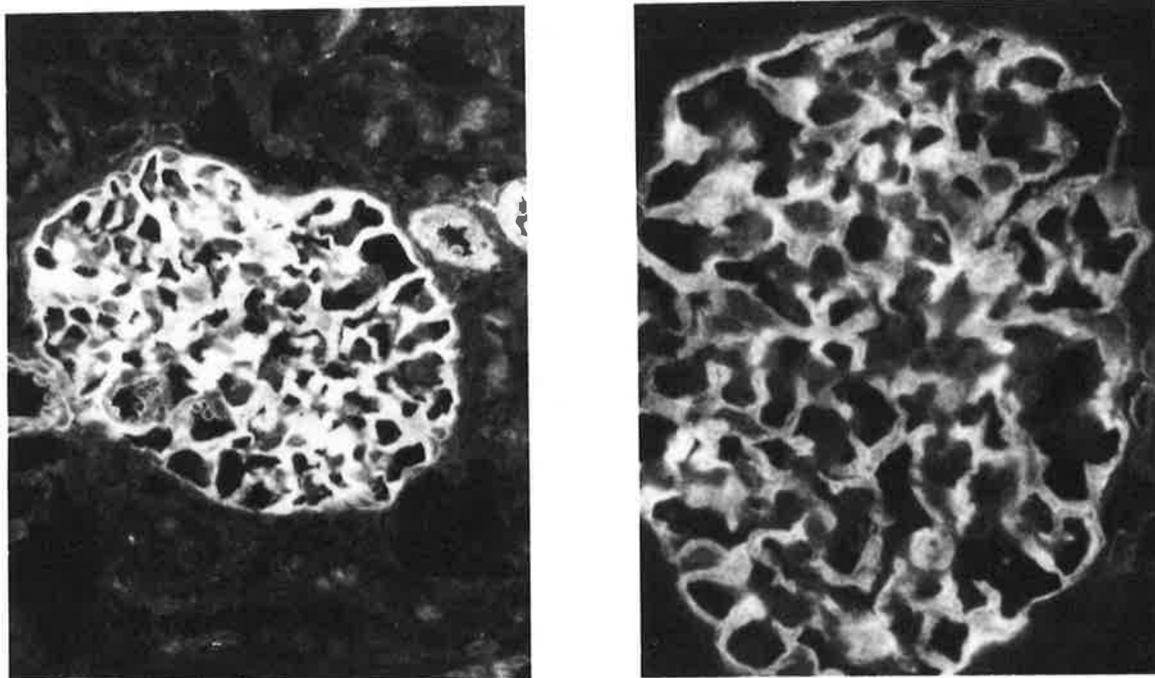

Figure 23. Staining of the glomerular capillary and blood vessel walls by IgG eluted from the kidney of a rabbit (#2) with spontaneous GN. (Sections of NRK reacted with the eluate and then with FITC conjugated goat anti-rabbit IgG).

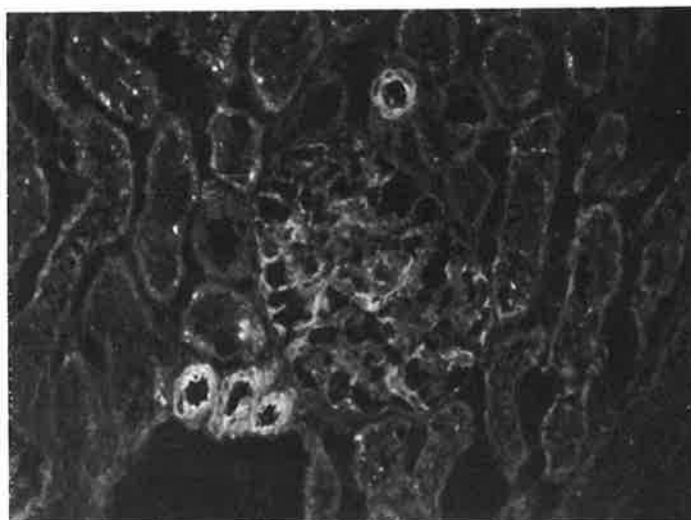

Figure 24. Indirect IF with the serum of a rabbit (#2) with spontaneous GN. The pattern of staining of the glomerular and blood vessel walls is similar to that seen with the corresponding eluate (Figure 23), but is much less intense.

TABLE 15

RENAL PATHOLOGY AND ELUTION STUDIES

IN RABBITS WITH SPONTANEOUS GN

Number	Age	Proteinuria mg/24 hours*	Light Microscopy	Immunofluorescence (IgG and C3 0 to 4+)	IgG eluted (μg/gm kidney)	E/S ratio of anti-glomerular antibody
1	Adult	183	GBM thickening and mesangial proliferation	4+ granular deposits along GBM	85	5
2**	Adult	153	GBM thickening. Focal areas of tubulo-interstitial damage	3-4+ finely granular deposits along GBM	92	2
3	12 wk	44	Focal mesangial hypertrophy	1-2+ segmental granular deposits (predominantly mesangial)	25	20
4	Adult	338	Focal mesangial proliferation	1-3+ segmental granular deposits (predominantly mesangial)	6	10
5	Adult	61	GBM thickening, mesangial proliferation, and focal areas of tubulo-interstitial damage.	2-3+ finely granular deposits along GBM	19	51

* mean of three determinations

** circulating IC detected by *IRCA

was found with rat, baboon, and human kidney target sections. No staining was detected with eluates from non-GN control kidneys.

Sera from the 5 rabbits with GN (and from some of the control rabbits) stained NRK glomeruli and blood vessels in a similar manner albeit much less brightly (Fig. 24). The sera and eluates were tested at serial dilutions to determine the IF end point titre, and IgG was quantitated by radioimmunoassay (104) to obtain eluate to serum (E/S) ratios of "anti-glomerular" antibody per μg IgG. An increased concentration of this antibody was demonstrated in each of the 5 eluates (Table 15).

Studies to determine the specificity of eluted antibody.

(i) The IF reactivity of the eluted anti-glomerular antibody was totally abolished by absorption with isolated glomeruli. However, none of the glomerular extracts (obtained by sonication or by treatment with 8 M urea, pronase, trypsin, or neuraminidase) was able to absorb this reactivity, and none reacted with the eluates or sera by gel diffusion. The renal tubule preparation (Fx 1A) did not absorb the IF reactivity of the eluates.

(ii) Anti-GBM antibody activity was not detected by radioimmunoassay (and pre-incubation of kidney sections with anti-GBM antibody did not prevent subsequent staining of the glomerular capillary wall by the eluates).

(iii) The eluates (and sera) stained smooth muscle in blood vessels and myometrium but the pattern of reactivity differed significantly from that seen with HuSMA. Thus, HuSMA selectively stained the glomerular mesangium of human and rabbit (Fig. 25) kidneys. This staining was unlike that seen with the rabbit GN associated antibody.

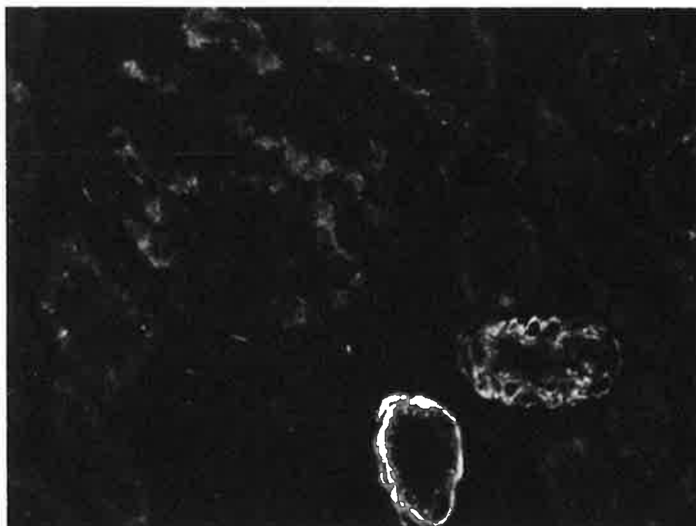

Figure 25. Staining of the glomerular mesangium and blood vessel walls of NRK by HuSMA. {Indirect IF with HuSMA serum then FITC conjugated goat anti-human IgG (absorbed with NRS)}.

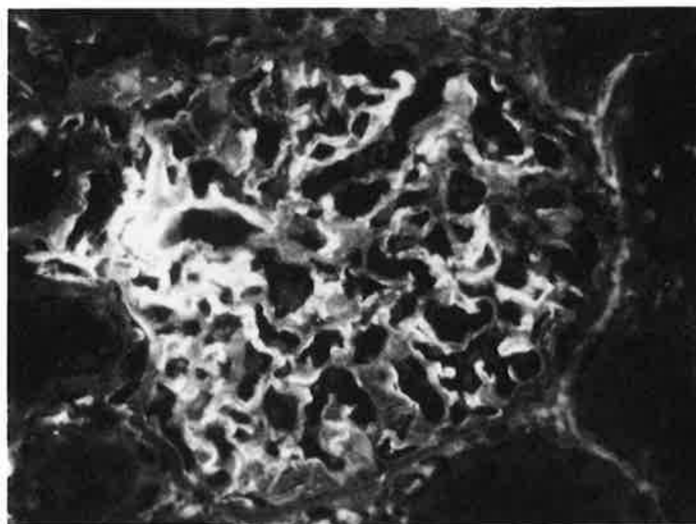

Figure 26. Glomerular localization of rabbit anti-serum to Hu β_2 GP III. (Indirect IF; normal human kidney reacted with rabbit anti-Hu β_2 GP III then FITC conjugated goat anti-rabbit IgG).

Finally, absorption with F-actin abolished the IF reactivity of the HuSMA but not that of the rabbit GN associated antibody, and there was no reaction with actin in gel diffusion.

(iv) The IF reactivity of the rabbit GN eluates with NRK glomeruli and blood vessels was unaffected by pre-treatment of the target sections with collagenase or pronase, but was totally abrogated by pre-treatment with neuraminidase. This suggested that the antigen might be a sialoprotein (187). Polyanionic sialoproteins can be demonstrated in the glomerular epithelial glycocalyx by colloidal iron staining (188) and are thought to be important to the functional integrity of the glomerular filter (189, 190).

Neuraminidase did not dissociate the glomerular deposits of IgG and C3 from the kidney sections of rabbits with spontaneous GN.

(v) Hu β_2 GP III is a serum glycoprotein which can also be detected in normal glomeruli (191). IF studies with rabbit antiserum to Hu β_2 GP III showed that this antibody stained human (Fig. 26) but not rabbit glomerular capillary walls. The anti-glomerular antibody eluted from the kidneys of rabbits with spontaneous GN did not react in absorption IF and gel diffusion studies with purified Hu β_2 GP III or NRS.

DISCUSSION

Approximately 10% of laboratory New Zealand white rabbits spontaneously develop proteinuric GN. The lesions vary from focal areas of mesangial hypertrophy to frank membranous or proliferative GN. The presence of granular deposits of IgG and C3 in the glomeruli and of dense deposits along the GBM suggest that this disease is IC mediated.

The detection of anti-glomerular and blood vessel antibodies in citrate buffer eluates from these kidneys suggests that the IC antigen is a structural protein. The deposits might, therefore, represent IC formed in situ. However, the structural location of the antigen is different from the site of the immune deposits, and it is more likely that the antigen, by gaining access to the circulation, forms circulating IC which are then deposited in the glomeruli. Indeed, circulating IC were detected in one of the 5 GN rabbits in this study. If this concept is correct, then the renal origin of the antigen is fortuitous (as it is in experimental (21) and human (158) RTA mediated ICGN).

The precise nature of the antigen remains obscure. HuSMA stains glomeruli (192-194), and this staining can be blocked by absorption with purified actin preparations (194). The spontaneous GN rabbit antibody did not react with actin, and the pattern of staining differed significantly from that seen with HuSMA. It is, therefore, unlikely that this antigen-antibody system is involved in the disease.

The glomerular localization of a serum β globulin in mice (195) and of a serum β_2 glycoprotein fraction (Hu β_2 GP III) in man (191) raises the possibility that the putative antigen might be present in the circulation and in the glomeruli. Of interest, the amount of glomerular Hu β_2 GP III is greatly increased in patients with GN. However, the rabbit GN associated antibody did not react with NRS or with Hu β_2 GP III.

Finally, the antigen may be a structural glycoprotein associated with the glomerular epithelial glycocalyx and blood vessel walls.

Pre-treatment of target kidney sections with neuraminidase blocked the reactivity of the eluted antibody, a finding consistent with this hypothesis. In this regard Shibata et al (196) induced a membranous form of GN in rats with a glomerular sialoprotein containing extract, and it is possible that this experimental model is analagous to the spontaneous disease in the rabbit.

Sera from occasional patients with GN have been found to produce diffuse (non-anti-GBM) staining of normal human glomerular capillaries (Wilson, C. B., personal communication), and it is, therefore, quite conceivable that a similar mechanism might be operative in some forms of human GN. This requires further study.

CONCLUSION

IgG eluted from kidneys of rabbits with spontaneous GN reacted with the glomerular capillaries and blood vessels of normal kidney sections. It is proposed that this form of GN is mediated by circulating IC formed with an antigen derived from or cross-reacting with a structural constituent present in glomeruli and blood vessels. The precise nature of the antigen is unknown, but it may be a determinant linked with the glomerular epithelial glycocalyx. It is possible that a similar mechanism might be involved in some forms of human ICGN.

C. THE SPECIFICITY OF ANTIBODIES ELUTED FROM HUMAN GN KIDNEYS.

INTRODUCTION

A panel of potential antigens was used in this study to determine the specificity of antibodies eluted from the kidneys of patients with GN. The eluates were screened for antibodies to endogenous antigens (GBM, RTA, nucleic acids, and IgG), and viral antigens (measles virus, EBV, and C-type viruses). IF test systems were used for the detection of anti-viral antibodies. This method was used in preference to conventional C fixation assays because the C fixing capacity of eluted antibodies may be impaired, and because IF studies with virus-infected cells also allow the expression of viral neo-antigens. The patients in this study were unselected in the sense that none had clinical or serological information pointing towards a specific antigen (other than nucleic acids in patients with SLE, and GBM antigens in patients with anti-GBM antibody GN).

The methods were adapted from those used in studying the rabbit models of GN.

METHODS

Patients. Nephrectomy or autopsy kidneys from 28 patients were supplied by contributing centres. There were 9 patients with SLE, 13 with primary ICGN, and 6 with anti-GBM antibody GN. Serum was available in 20 of these patients. 5 "normal" human kidneys (NHK), which had originally been obtained from cadavers for transplantation purposes, were supplied by Dr. W. M. Le Fore, V. A. Hospital, Indianapolis, Indiana.

IF. The kidneys were examined for IgG, IgA, IgM, C3, fibrin, albumin, and RTA using standard direct IF techniques (197).

Elution studies. Renal cortical tissue was homogenized, washed six times with PBS, and then eluted with 0.02 M citrate buffer pH 3.2 for 1 to 2 hours at 25° C. The supernatant eluate was neutralized with NaOH and allowed to stand for 18 hours at 4° C. The precipitate obtained by centrifugation was discarded and the clarified eluate was then concentrated by negative pressure ultrafiltration to approximately 1.0 ml. The eluates were stored in aliquots at -20° C.

Studies to determine the reactivity of the eluted antibodies.

(i) Indirect IF with NHK sections. The eluates (and sera) were tested by indirect IF with sections of NHK and FITC conjugated goat anti-human IgG antibody. ANA, anti-GBM antibodies, and antibodies to RTA can be detected by this technique.

(ii) The eluates (and sera) from patients with anti-GBM antibody GN were tested for anti-GBM antibodies by radioimmunoassay (107).

(iii) Anti-globulin activity. The eluates were tested for anti-human IgG (rheumatoid factor) antibodies using the latex RA test (Hyland).

(iv) Anti-viral antibody activity:

Measles virus. He La cells persistently infected with measles virus (Edmonston strain) were cultured (198) and supplied by Dr. M. B. A. Oldstone, Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, Ca. Cell monolayers were prepared on glass cover-slips; these were then washed with PBS, fixed for 10 min in acetone, and used as targets in testing the

eluates (and sera) for anti-measles virus antibodies by indirect IF using FITC conjugated goat anti-human IgG. FITC conjugated rabbit anti-measles virus antibody (supplied by Dr. M. B. A. Oldstone) was used as a positive control.

EBV. EBV (and EBNA) (199) positive human B lymphocytes were obtained from Dr. F. C. Jensen, Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, Ca. Cytocentrifuge preparations of these cells were made on glass slides which were then fixed in acetone and used as targets in testing the eluates (and sera) for anti-EBV antibodies by indirect IF using FITC conjugated goat anti-human IgG. Known EBV positive and negative human sera (supplied by Dr. F. C. Jensen) were used as controls.

C-type virus. The eluates (and sera) were tested by radioimmunoassay for antibodies to the p30 and gp70 murine C-type virus antigen determinants by Ms. P. J. McConahey, Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, Ca. (200).

IgG quantitation and determination of E/S ratios of specific antibody concentration. When positive in one of the above assays, the eluates and sera were tested at serial dilutions to determine the end point titre of antibody activity, and the IgG content was determined by radioimmunoassay (104) to obtain the eluate and serum antibody concentration per μg IgG.

RESULTS (Table 16).

Antibodies to endogenous antigens. ANA was detected in each of the eluates from the 9 patients with SLE (Fig. 27). Sera were available

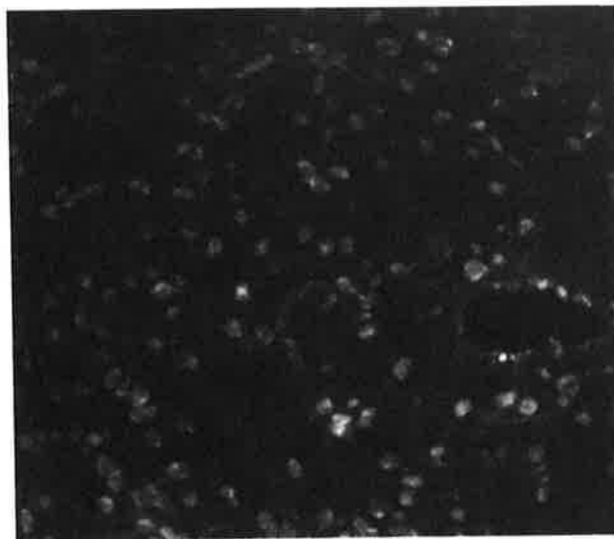

Figure 27. Detection of ANA in an eluate from the kidney of a patient with SLE. (Indirect IF; NHK reacted with the SLE kidney eluate and then FITC conjugated goat anti-human IgG).

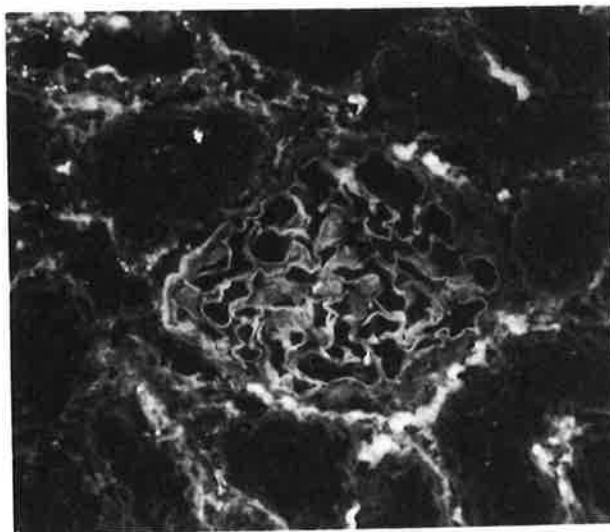

Figure 28. Detection of anti-GBM antibody in an eluate from the kidney of a patient with anti-GBM antibody GN. (Indirect IF; NHK reacted with the anti-GBM kidney eluate and then FITC conjugated goat anti-human IgG).

in 4 of these patients, and in 3 the eluted ANA was concentrated over that in serum.

Anti-GBM antibodies were detected by IF (Fig. 28), and by radioimmunoassay in each of the eluates from the 6 patients with anti-GBM antibody GN. The eluted anti-GBM antibody was greatly concentrated over serum in the 5 patients where serum was available.

None of the eluates reacted with RTA (as detected by indirect IF with NHK), and none was positive in the latex test for rheumatoid factor.

Anti-viral antibodies.

(a) Anti-measles virus antibodies were detected in 11 eluates (Fig. 29); 5 SLE, 4 primary ICGN, 1 anti-GBM antibody GN, and 1 NHK eluate. Sera were available in 7 of these patients, and an increased concentration of eluted anti-measles virus antibody was detected over serum in 4 (3 with SLE, and 1 with primary ICGN). Attempts were made to identify measles virus antigen in the glomerular IC deposits in these patients using rabbit anti-measles virus antibody in indirect IF studies. No staining was detected.

(b) Anti-EBV antibodies (Fig. 30) were detected in 8 eluates; 3 SLE, 3 primary ICGN, and 2 anti-GBM antibody GN eluates. Sera were available in 5 of these patients, and in none of these was there an increase in the concentration of eluted anti-EBV antibody over serum.

(c) Anti-C-type virus antibodies. None of the eluates (or sera) was positive in the radioimmunoassay for antibodies to p30 or gp70.

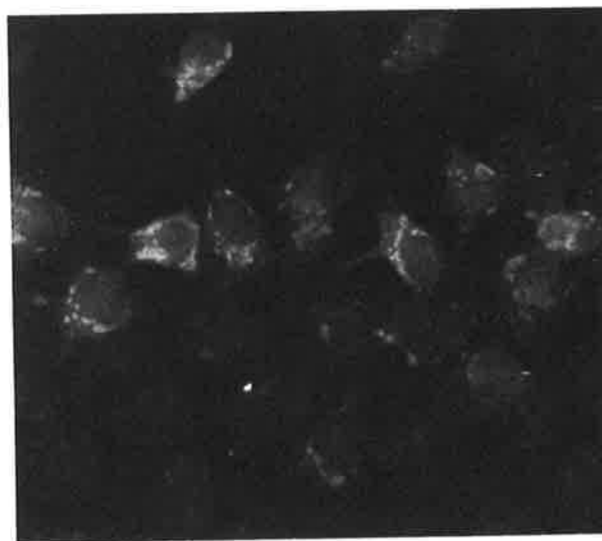

Figure 29. Detection of anti-measles virus antibody in the eluate from the kidney of a patient with SLE. (Indirect IF; measles virus-infected monolayer of He La cells reacted with the eluate and then with FITC conjugated goat anti-human IgG).

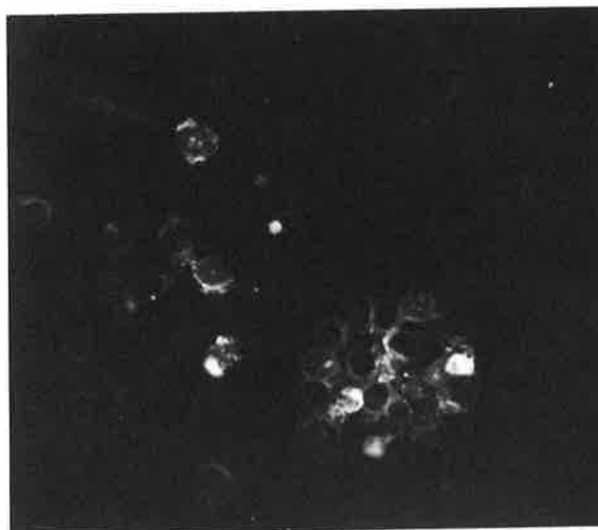

Figure 30. Detection of anti-EBV antibody in the eluate from the kidney of a patient with primary ICGN. (Indirect IF; cytocentrifuge preparation of EBV positive human B lymphocytes reacted with the eluate and then with FITC conjugated goat anti-human IgG).

TABLE 16

IDENTIFICATION OF THE SPECIFICITY OF

ANTIBODIES ELUTED FROM HUMAN GN KIDNEYS

KIDNEYS ELUTED		REACTIVITY OF ELUTED Ig							
Category	Number	ANA	anti-GBM antibody	anti-RTA	anti-IgG	anti-measles virus	anti-EBV	anti-C type virus p30	gp70
SLE	1	+ (1)	0	0	0	+ (5)	0	0	NT
	2	+ (4)	0	0	0	+ (2)	0	0	0
	3	+ (3)	0	0	0	+ (1)	+ (1)	0	0
	4*	+	0	0	0	+	0	0	0
	5*	+	0	0	0	0	+	0	0
	6*	+	0	0	0	0	+	0	0
	7*	+	0	0	0	0	0	0	0
	8*	+	0	0	0	0	0	0	0
	9	+ (2)	0	0	0	+ (2)	0	0	0
PRIMARY ICGN	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	+ (1)	0	0
	3	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0
	7	0	0	0	0	+ (5)	+ (1)	0	0
	8	0	0	0	0	0	0	0	0
	9*	0	0	0	0	+	0	0	0
	10*	0	0	0	0	+	+	0	0
	11	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0
	13	0	0	0	0	+ (1)	0	0	0
ANTI-GBM ANTIBODY GN	1	0	+ (270)	0	NT	0	0	0	0
	2	0	+ (100)	0	0	0	0	0	0
	3	0	+ (240)	0	0	0	+ (1)	0	0
	4	0	+ (115)	0	0	0	0	0	0
	5*	0	+	0	NT	0	0	NT	NT
	6	0	+ (90)	0	0	+ (1)	+ (1)	0	0
NHK	1*	0	0	0	0	0	0	0	0
	2*	0	0	0	0	0	0	0	0
	3*	0	0	0	0	0	0	0	0
	4*	0	0	0	0	+	0	0	0
	5*	0	0	0	0	0	0	0	0

NT not tested

* serum not available

( ) E/S ratio of specific antibody concentration

DISCUSSION

This study raises the possibility that measles virus antigen-antibody IC might be involved in some patients with SLE and primary ICGN. The evidence for this is suggestive rather than conclusive, and identification of measles virus antigen in the glomerular IC deposits is required to confirm the hypothesis. This has, in fact, been reported in SSPE (149), and in one patient with SLE (150). The participation of measles virus antigen in IC formation, and disease has been based on the model of SSPE (149, 201), but is now being proposed in SLE (202, 203), multiple sclerosis (204, 205), and other conditions (206). Serum anti-measles virus antibodies are frequently elevated in SLE (203, 207-210). This may reflect the hypergammaglobulinaemic status of patients with SLE, but not all anti-viral antibodies are correspondingly elevated, and a specific impairment of cell mediated immunity to measles virus has been detected in SLE (211).

The possibility of C-type virus expression in SLE has also been proposed recently (148, 202, 212, 213). This is an extension of studies with the NZB model of SLE in the mouse (25-29, 200), and of studies with canine lupus (214-216). The detection of C-type viral antigens in the glomerular IC deposits of human SLE has been the subject of two reports (148, 213). These findings need further confirmation. In the present study, and in others (217), no anti-C-type viral antibody activity was detected.

Finally, antibodies to RTA or IgG were not detected in this study, and none of the eluates demonstrated the "anti-glomerular" reactivity of the rabbit ICGN associated antibody.

It is very likely that multiple antigen-antibody systems are involved in individual patients with ICGN. This is certainly the case in SLE (218). If this is indeed correct, the elucidation of such systems will be a highly complex task. The antigen(s) may, in fact, be quite ubiquitous (e.g. measles virus, EBV, etc.) which would help to explain the absence of clinical and serologic "clues" in most patients with ICGN. This would imply that such patients have an as yet undefined abnormality which causes them to handle a "normal" antigen and IC load in a nephritogenic manner. Peters and Lachman (219) have proposed an immune deficiency state in the pathogenesis of human ICGN. Thus, low affinity or non-precipitating antibodies, which might predispose to ICGN by allowing the chronic formation of IC, have been demonstrated in experimental models of ICGN (57, 58, 220), and Friend et al (221) have recently reported non-precipitating anti-DNA antibodies in the sera of patients with membranous SLE GN. This concept should be evaluated further.

CONCLUSION

The reactivity of antibodies eluted from the kidneys of patients with GN was investigated. ANA and anti-GBM antibodies were detected in eluates from patients with SLE and anti-GBM antibody GN respectively, and were found to be selectively concentrated over the levels in serum. Anti-measles virus and anti-EBV antibodies were detected in some of the eluates. Anti-measles virus antibodies were selectively concentrated over serum in 4 of the eluates (3 from patients with SLE and 1 from a patient with primary ICGN). Measles antigen could not be detected in the glomerular IC deposits, and so the evidence for

participation of measles antigen-antibody IC in these patients is suggestive rather than conclusive.

The concept of multiple and ubiquitous antigens in IC disease is discussed in relation to the immune-deficiency theory of GN.

ABBREVIATIONS

AGN	acute GN
AHG	aggregated human gamma globulin
ANA	antinuclear antibody
BSA	bovine serum albumin
C	complement
C1qSPA	the solid phase C1q binding assay
C3PA	properdin factor B
CEA	carcinoembryonic antigen
CGN	chronic GN
CSS	chronic serum sickness
DEAE	diethylaminoethyl cellulose
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra-acetic acid
EM	electron microscopy
equiv	equivalent
E/S	eluate to serum ratio of specific antibody activity per μg IgG
FITC	fluorescein isothiocyanate
FSGN	focal sclerosing GN
GBM	glomerular basement membrane
GN	glomerulonephritis
HSA	human serum albumin
HSP	Henoch-Schönlein purpura
Hu β_2 GP III	human β_2 glycoprotein III
HUS	haemolytic-uraemic syndrome

HuSMA	human smooth muscle antibody
IC	immune complex
*IClqBA	the radiolabeled Clq binding assay
IF	immunofluorescence
Ig	immunoglobulin
*IRCA	the Raji cell radioimmune assay
IV	intravenous
LCM	lymphocytic choriomeningitis
LDV	lactic dehydrogenase virus
LPS	bacterial lipopolysaccharide
MCT	microcomplement consumption test
MEM	minimal essential medium
MGN	membranous GN
MPGN	membranoproliferative (syn: mesangiocapillary) GN
MW	molecular weight
NHK	normal human kidney
NHS	normal human serum
NRK	normal rabbit kidney
NRS	normal rabbit serum
NZ	New Zealand
PAN	polyarteritis nodosa
PAS	periodic acid Schiff
PBS	0.15 M phosphate buffered saline pH 7.2
PEG	polyethylene glycol
PET	pre-eclamptic toxaemia
PSGN	post-streptococcal GN

RES	reticuloendothelial system
RPGN	rapidly progressive GN
RTA	renal tubular antigen
SAS	saturated ammonium sulphate
SBE	subacute bacterial endocarditis
SLE	systemic lupus erythematosus
SSPE	subacute sclerosing panencephalitis
TCA	trichloroacetic acid
VBS	Veronal-buffered saline

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