Characterization of Human Anti–Glomerular Basement Membrane Antibodies Eluted from Glomerulonephritic Kidneys

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ABSTRACT Eluates from glomerulonephritic kidneys of nine patients with anti–glomerular basement membrane (anti-GBM)–mediated nephritis were studied to define their antigenic specificity and content of kidney-fixing antibodies. Five of these patients had Goodpasture's syndrome with pulmonary and renal involvement clinically; four patients did not. All had in vivo fixation of IgG in the characteristic linear pattern by direct immunofluorescence, and eluted IgG fixed to normal human kidney sections. Eluates from kidneys of patients with Goodpasture's syndrome fixed more frequently to homologous nonglomerular renal and extrarenal antigenic sites and to heterologous GBM than did non-Goodpasture eluates over a hundredfold range of antibody concentrations; both could be blocked by prior absorption with soluble GBM antigens. By radial immunodiffusion and precipitation tests the content of IgG in the eluates was measured to range from 2 to 20% of the total protein eluted. By paired label isotopic fixation studies with some of the eluates the per cent of IgG that was kidney fixing ranged from 0.6 to 23.4%. Although the in vivo fixation studies with radiolabeled eluates failed to indicate significant fixation to monkey lung, the observations define quantitative as well as qualitative differences between anti-GBM antibody populations mediating the Goodpasture syndrome compared to those causing glomerulonephritis without lung involvement.

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INTRODUCTION

Extensive studies have led to the elucidation of the two animal models of immunologically mediated glomerular injury, one due to specific anti–glomerular basement membrane (anti-GBM) antibody (1–3) and the other resulting from the deposition in the glomeruli of circulating, macromolecular, soluble, nonglomerular antigen-antibody complexes (4–7). These pathogenetic mechanisms of experimental glomerulonephritis have been validated in human disease. From kidneys observed to have linear fixation of host immunoglobulins by direct immunofluorescent examinations, anti-GBM antibodies have been eluted and transferred passively to normal heterologous recipients, causing an immediate glomerulonephritis (8). In these transfers the characteristic linear fixation to GBM of the antibodies and their pathogenicity have been demonstrated. In contrast, studies by Koffler, Schur, and Kunkel (9) and Krishnan and Kaplan (10) on cases of systemic lupus erythematosus glomerulonephritis with granular deposits of host immunoglobulins along GBM, have shown the eluted immunoglobulins to be directed towards deoxyribonucleic acid and other nuclear antigens rather than the GBM.

It was the purpose of the present experiments to attempt further characterization of eluted immunoglobulins from kidneys of patients with glomerulonephritis and linear fixation of host immunoglobulin G (IgG) along the GBM (Fig. 1). These eluates were tested by direct immunofluorescence on normal homologous kidney sections and certain nonrenal tissue sections, as well as on heterologous organs. The specificity of these in vitro fixation studies was confirmed both by absorption studies using solubilized human GBM antigens as well as by

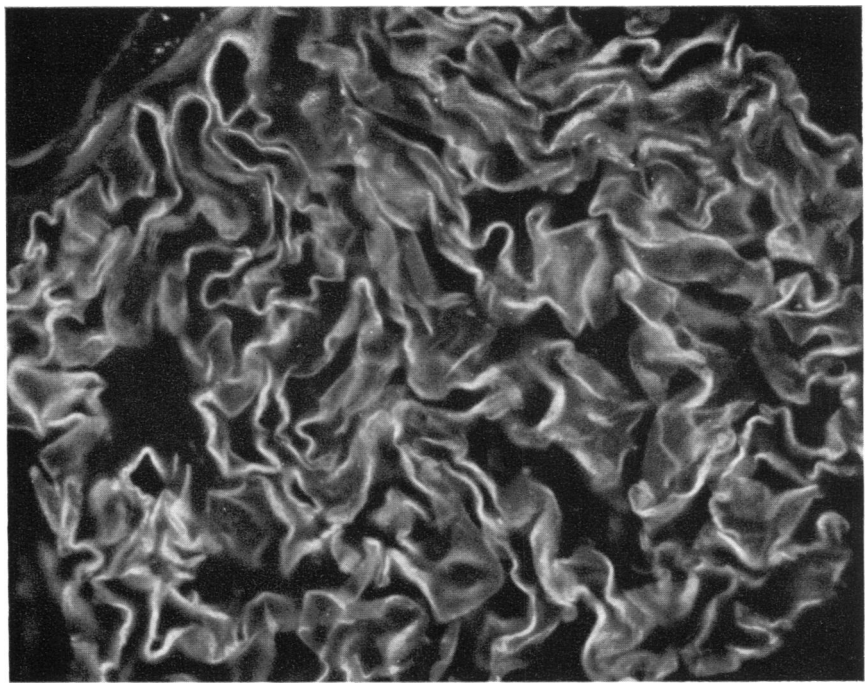

FIGURE 1 Photomicrograph illustrating typical linear fixation of IgG along glomerular basement membranes of native, diseased kidney of patient with Goodpasture's syndrome, visualized by direct immunofluorescence. × 350

isotopically labeled antibody fixation studies in monkeys. The latter studies allowed precise quantification of the anti-GBM antibody content of the selected eluates so tested.

METHODS

Organs eluted

Elutions were performed from kidney tissue[1] from five patients with Goodpasture's syndrome and part of one lobe of a lung from one of the same patients. In addition, kidney tissue from four patients showing no clinical stigmata of Goodpasture's syndrome was eluted. These tissues were frozen at autopsy or at nephrectomy and stored at −20°C until study. Quick-frozen biopsies of these same organs were stored and shipped separately and examined by direct immunofluorescence as detailed below. All showed linear fixation of immunoglobulin G characteristic of fixation of anti-GBM antibodies.

Elution procedure

The elution protocol was essentially that described previously (8). Kidneys were homogenized in isotonic phosphate-buffered saline (PBS), and the fast-sedimenting debris washed extensively in cold PBS until supernatant fluid was clear. Subsequently, the sediment was washed in distilled water, transferred to 100–200 ml of 0.02 M citrate buffer, pH 3.2, and stirred at 37°C for 2½ hr. After this elution procedure, the debris was discarded, the supernate neutralized with 0.5 M NaOH, and dialyzed overnight against PBS. The eluates subsequently were concentrated by ultrafiltration and precipitated at half saturation with ammonium sulfate in the cold; ammonium sulfate precipitates were resuspended in PBS, dialyzed, and concentrated to 10–30 mg/ml protein concentration. The ammonium sulfate–precipitable eluates were studied by immunoelectrophoresis (IEP) and radioimmunoelectrophoresis (radio-IEP). Representative eluates were examined also for rheumatoid factor (RF) activity and immunoglobulin G subtypes by Dr. Howard Grey; rheumatoid factor activity was tested by a slide test utilizing latex particles coated with fraction II of pooled human serum.[2]

Reagents

Antisera. Antisera were made in rabbits to human 7S IgG (RAHIgG) and human serum albumin (HSA) by immunizing with the purified antigens in incomplete Freund's adjuvant at 10 mg/ml and 0.5 mg/ml protein concentrations respectively. Initially, animals were injected with 0.5 ml of the emulsions into each hind footpad; subsequent injections of 0.5 ml at two separate subcutaneous sites were made on days 14, 28, and 40. Animals were bled and antisera harvested 10 days after the last injection. Monospecificity of antisera was assured by examination by double diffusion in 0.5% agarose plates and by IEP against anti–whole human serum antiserum. Before conjugation with fluorescein isothiocyanate, antisera were fractionated at half saturation with ammonium sulfate and then chromatographed on diethyl-

[1] We are indebted to the many physicians who have made these studies possible: H. A. Bloomer, E. Ginn, R. Glassock, C. Huddleston, K. Popowniak, R. Rieselbach, G. Schmitt, G. Schreiner, J. Maher, T. Starzl, and G. M. Williams.

[2] Hyland Laboratories, Los Angeles, Calif.

aminoethyl cellulose (DEAE-cellulose) equilibrated with 0.0175 M phosphate buffer, pH 6.5. The IgG fractions of these antisera isolated by DEAE chromatography were fluoresceinated by the dialysis method of Clark and Shepard (11), and subsequently rechromatographed on DEAE at pH 7.4. The conjugate fraction eluting at 0.05 M phosphate buffer concentration was concentrated before use.

Soluble antigens. Particulate human GBM from kidneys obtained at autopsy and prepared by the method of Krakower and Greenspon (12) was solubilized by enzymatic digestion using highly purified collagenase from *Cl. histolyticum*[3] as described by Spiro (13) or bovine pancreatic trypsin[3] as described by Cole, Cromartie, and Watson (14). Urinary basement membrane antigens (UA) were prepared from normal human urine (15). Bovine serum albumin was obtained commercially[4] and sheep tropocollagen was the gift of R. A. Lerner, M. D.

Radiolabel procedure. Streptococcal cell membrane antigens (PGT-CM), and eluates and control proteins used in paired-label isotopic studies were labeled with ^{125}I or ^{131}I by the method of McConahey and Dixon (16).

Measurement of IgG concentrations

Immunoglobulin G concentration in eluates was measured by radial immunodiffusion by the technique of Fahey and McKelvey (17) or by quantitative precipitation of radiolabeled immunoglobulin G using an excess of rabbit anti-7S human IgG.

Immuno- and radioimmunoelectrophoresis

These procedures were performed on glass slides in 1% agarose in barbital buffer, pH 8.2, $\mu = 0.04$, at eluate protein concentrations of 10–20 mg/ml, utilizing normal human serum diluted 1 : 2 as control; the conditions for study were 5.5 volts/cm^2 for 35 min at room temperature. Anti-whole human serum made in rabbits and obtained commercially[2] as well as RAHIgG was used to develop precipitin lines. Gels were incubated in a moist chamber at room temperature for 18–36 hr. To determine whether eluted IgG would react with a well characterized streptococcal cell membrane antigen, radio-IEP was performed in the following manner: IEP was done with each eluate as just described and developed with anti-whole human serum; then gels were washed extensively for 48–72 hr with PBS, and ^{125}I-labeled streptococcal cell membrane (PGT-CM)[5] was added to the antibody trough. Incubation with the labeled antigen again was carried out in a moist chamber at room temperature for 36 hr, refilling the trough twice; slides were subsequently washed in PBS for 48–72 hr, dried in an oven at 37°C, and exposed to a photographic plate for 24–72 hr.

Immunofluorescent microscopy

Direct immunofluorescent examination of frozen tissue sections of human organs for presence of IgG fixed in vivo and of frozen tissue sections for in vitro fixation of IgG as detailed below were performed by the method of Coons and Kaplan (18) as already described (19). Specificity of fluorescent reagents was tested by absorption of antisera with their specific antigens, and by blocking fluorescence in tissues by prior incubation with unconjugated antisera. All fluorescent microscopic examinations were performed in duplicate; controls for fluor examinations included fluorescein-conjugated anti-HSA and known positive and negative sections. For immunofluorescent studies of fixation of eluate IgG to normal frozen tissue sections, controls included direct staining of tissue sections with fluorescein-conjugated anti-human IgG, unstained sections, and use of acid citrate–treated normal human globulins prepared by the same technique as the eluates, at comparable protein concentrations, as the intermediate reagent instead of eluates. The target substrates for all eluate IgG fixation immunofluorescent tests will be detailed for each experiment. Human tissues were obtained at autospy or from cadavers at time of nephrectomy for renal allografting; animal organs were obtained immediately upon sacrifice using deep pentobarbital anesthesia. All tissues were flash-frozen and stored at −20°C or −70°C until use.

Immunofluorescent microscopy experiments to detect fixation of eluted IgG to normal tissue sections

Testing for homologous renal antigenic specificity. Normal human kidney sections were incubated with each eluate at varying protein concentrations: neat, 10 and 1 mg/ml, 500, 100, and 50 μg/ml. The amount of IgG contained in each undiluted eluate ranged from 150 μg to 2.2 mg/ml; IgG concentrations for each test are detailed in Table I. Each eluate was scored (0 to 3+) at each concentration for its fixation to homologous GBM, Bowman's capsule (BC), tubular basement membrane (TBM), and nonglomerular renal capillary basement membranes (VBM).

Testing for homologous nonrenal antigen specificity. Four Goodpasture (GP) and two non-Goodpasture (non-GP) eluates were tested at 1 mg/ml concentration on human lung, liver, heart, and adrenal sections and scored for evident fixation to any structural determinants. The IgG content of the eluates at that concentration ranged from 30 to 146 μg/ml. Additional studies on human lung sections were done with the non-GP eluates at IgG concentrations of 300–450 μg/ml.

Testing for heterologous renal antigenic specificity. Each eluate, at concentrations of 100 μg/ml and IgG concentrations of 3–20 μg/ml, was incubated with human, chimpanzee, rabbit, rat, and mouse kidney sections and scored for evidence of fixation to GBM.

Testing for heterologous nonrenal epithelial basement membrane antigenic specificity. All eluates were tested at 100 μg/ml on rhesus monkey choroid plexus and chimpanzee salivary gland; two GP and two non-GP eluates were tested also at 1 mg/ml (IgG concentrations 30–65 μg/ml) on the same tissues.

Absorption experiments

In order to test the reactivity of these eluates with solubilized human GBM antigens and urinary basement membrane antigens, three GP eluates and one non-GP eluate were each absorbed in separate experiments with each of the following reagents: collagenase-digested human GBM, trypsin-digested human GBM, human urinary basement membrane antigens, BSA, and solubilized sheep tropocollagen. These absorption experiments were done as follows: 10 mg of the soluble antigens was added to eluates containing 30–146 μg IgG/ml, then incubated at 37°C for 1 hr, allowed to stand at 4°C overnight, and centrifuged before study. The immunofluorescent intensity of each was then compared to the BSA and sheep tropocollagen controls and to comparable concentrations of eluates to which no absorbent

[3] Obtained from Worthington Biochemical Corp., Freehold, N. J.

[4] Armour Pharmaceutical Co., Chicago, Ill.

[5] Gift of A. S. Markowitz, Ph.D.

was added. Where blocking of immunofluorescence was not achieved with at least one of the antigens, experiments were carried out at 20 mg/ml and 30 mg/ml antigen concentrations.

Paired label isotopic experiments

In order to confirm in vivo the specificity of these eluted immunoglobulins and to measure the per cent of kidney-fixing antibodies (KFAB), paired-label studies were done by a modification of the technique of Pressman, Day, and Blau (20). Eluates were chromatographed on DEAE equilibrated with 0.0175 M phosphate buffer, pH 6.5, as described before; IEP confirmed that IgG was the only serum protein isolated. Radiolabeling with ^{125}I was performed by the technique of McConahey and Dixon (16) of 250-μg aliquots of the fractionated proteins; IgG contents of these labeled isolates ranged from 18 to 45% of the total labeled protein. Control IgG was obtained by treating normal human serum with 0.02 M citrate buffer, pH 3.2, for 2½ hr at 37°C, subsequent fractionation with ammonium sulfate at half saturation in the cold, and DEAE chromatography. The control protein was labeled with ^{131}I. Simultaneous intravenous injections of 250 μg of labeled eluate and control proteins were made into previously unimmunized squirrel monkeys; a pair was used to test each eluate. Animals were given potassium iodide in their drinking water and were sacrificed 72 hr after injection. After perfusion with PBS, individual organs were removed, and ^{131}I and ^{125}I radioactivity in each was measured in a well counter with sodium iodide crystal using a dual channel spectrometer. Per cent KFAB was calculated as per previously published reports (2, 8). In one experiment, half the labeled test protein was absorbed with a 10:1 (w/w) excess of squirrel monkey liver powder before injection; after ultracentrifugation at 25,000 *g* for 45 min the supernate of the absorbed and unabsorbed aliquots was injected into separate squirrel monkeys and results compared. A further control consisted of test labeling and injecting an eluate from a human kidney shown by direct immunofluorescent examination not to have linear fixation of IgG and which would not fix to normal human kidney sections.

TABLE I

Comparison of Goodpasture and Non-Goodpasture Anti-GBM Eluates with Respect to Specificity for Renal Antigens

Eluate	Protein	IgG*	GBM‡	TBM	BC	VBM
	mg/ml	*mg/ml*				
Goodpasture eluates						
B	26	2.2	3+	2+	2+	2+
	10	0.850	2+	1+	2+	2+
	1	0.085	3+	2+	trace	2+
	0.5	0.043	2+	2+	0	1+
	0.1	0.009	2+	1+	0	1+
F	30	0.720	3+	2+	0	0
	10	0.240	2+	2+	0	0
	1	0.024	2+	2+	0	0
	0.5	0.012	2+	2+	0	0
	0.1	0.003	1+	±	0	0
O lung	2.5	0.150	3+	2+	1+	2+
	1	0.060	2+	1+	1+	1+
	0.5	0.030	3+	3+	2+	2+
	0.1	0.006	1+	trace	0	trace
O kidney	15	0.975	3+	2+	2+	2+
	10	0.650	2+	1+	±	1+
	1	0.065	2+	2+	1+	2+
	0.1	0.007	2+	2+	1+	2+
H	13	1.900	3+	1+	1+	0
	10	1.460	2+	1+	0	0
	1	0.146	2+	2+	0	0
	0.5	0.073	2+	1+	0	0
	0.1	0.015	1+	0	0	0
W	10	0.280	3+	1+	3+	0
	1	0.028	2+	0	0	0
	0.5	0.014	1+	0	0	0
	0.1	0.003	1+	0	0	0
Non-Goodpasture eluates						
DE	15	0.675	2+	1+	1+	1+
	10	0.450	2+	1+	0	1+
	1	0.045	2+	1+	0	1+
	0.1	0.005	2+	±	0	0
C	16	0.480	1+	0	0	0
	10	0.300	1+	0	0	0
	1	0.030	2+	0	0	0
	0.5	0.015	2+	0	0	0
	0.1	0.003	1+	0	0	0
MC	2	0.400	3+	0	1+	0
	1	0.200	1+	0	0	0
	0.5	0.100	1+	0	0	0
	0.1	0.020	trace	0	0	0
MA	3	0.438	1+	0	0	0
	1	0.146	2+	1+	0	0
	0.5	0.073	2+	1+	0	0
	0.1	0.015	2+	1+	0	0

* IgG = Immunoglobulin G.

‡ GBM = glomerular basement membrane; TBM = tubular basement membrane; BC = Bowman's capsule; VBM = nonglomerular capillary basement menbrane.

Testing the elution procedure

To test the efficiency of the elution procedure another non-GP anti-GBM antibody containing eluate was test labeled as above. This eluate was injected into four squirrel monkeys: two were sacrificed 3 days and the others 8 days after injection. The organs were perfused and counted; subsequently the kidneys of each pair were pooled, homogenized, and washed extensivey with PBS and then distilled water, and eluted as described already. The latter eluates were neutralized, dialyzed, and the centrifuged supernate injected into separate monkeys. The latter were sacrificed 1 hr later, perfused and the organs counted.

RESULTS

The predominant class of immunoglobulins in these eluates was shown by IEP to be IgG, although trace amounts of other immunoglobulins were contained also. The per cent of total protein eluted and precipitable by ammonium sulfate that was IgG was shown by radial immunodiffusion to range from 2.4 to 20%; there was no systematic difference observed between GP and non-GP eluates. Double diffusion analyses indicated that IgG types 1, 2, and 3 were represented in the eluates in normal proportions. No eluate tested had rheumatoid factor activity. Radio-IEP studies of the 10 eluates using labeled streptococcal cell membrane antigen demonstrataed binding between eluted IgG of one GP kidney and PGT-CM; one non-GP eluate showed weak antinuclear antibody activity as tested on human kidney sections while the other nine had no antinuclear activity.

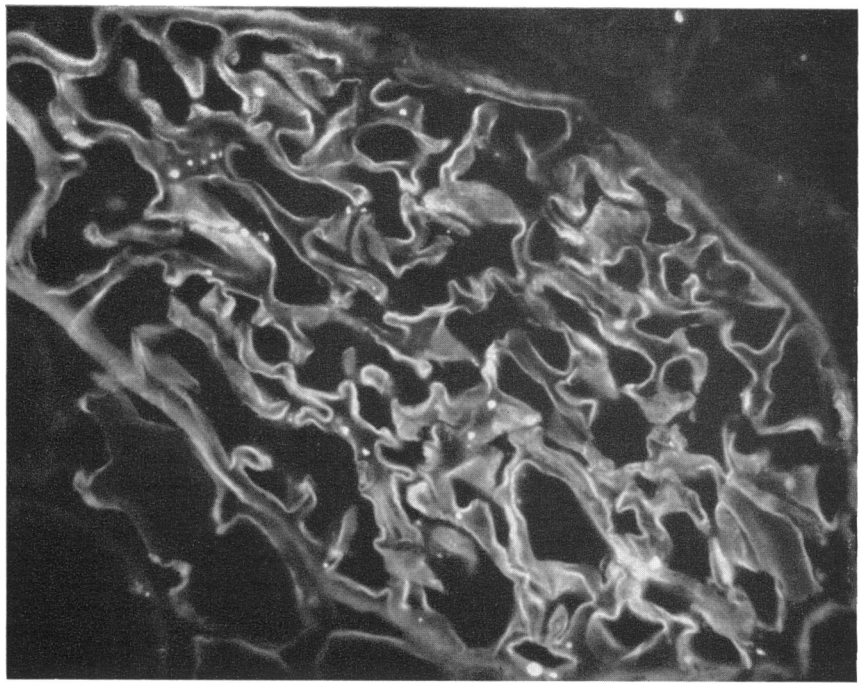

FIGURE 2 Photomicrograph of immunofluorescence of eluted IgG from a diseased human kidney fixed to glomerular basement membranes and Bowman's capsule of a normal, homologous kidney section (case F). × 350

Homologous renal antigenic specificity. At all protein concentrations tested from neat to 100 μg/ml, every eluate demonstrated fixation to homologous GBM (Fig. 2); this represented a range of minimal IgG concentrations of 3–20 μg/ml (mean 8.5 μg/ml). As is demonstrated in Table I and Fig. 3, Goodpasture eluates more frequently tended to fix to nonglomerular antigenic sites than did non-Goodpasture eluates, at all concentrations tested. Particularly noticeable was the frequency with which eluted IgG from GP organs fixed in a focal manner (Fig. 4) to certain of the TBM, failing to fix to adjacent TBM.

Homologous nonrenal antigenic specificity. Three of the four GP eluates tested fixed to human lung sections at the 1 mg/ml test concentration; two of the three positives were from the same patient, eluted from lung and kidney respectively. Neither of the two non-GP eluates tested demonstrated such fixation at 1 mg/ml; however, when tested at 10 mg/ml, one non-GP eluate did fix to human alveolar basement membranes. None of these eluates fixed to sections of homologous adrenal, liver, or heart.

Heterologous renal antigenic specificity. Although all eluates were demonstrated to fix to human GBM at IgG concentrations of 3–20 μg/ml, important differences were apparent when tested on heterologous kidney sections at the same concentrations (Table II). Goodpasture eluates usually fixed to GBM of kidney sections of most species used. Non-GP eluates, however, frequently failed to fix to GBM of rabbit, rat, and mouse.

Heterologous nonrenal epithelial basement membrane antigenic specificity. All eluates failed to fix either to rhesus monkey choroid plexus or chimpanzee salivary

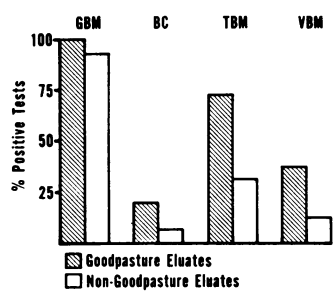

FIGURE 3 Comparison of frequency with which eluted anti-GBM antibodies from diseased kidneys of patients with Goodpasture's syndrome and patients without Goodpasture's syndrome fix to glomerular basement membranes, tubular basement membranes, Bowman's capsule, and nonglomerular renal capillary basement membranes. Plotted are the results of immunofluorescent fixation studies in the eluate protein ranges 100 μg/ml to 10 mg/ml, comparing kidney eluates only (see Table I). A positive test denotes uneqivocal fixation of eluate IgG to kidney substrate test sections; equivocal (±) and trace immunofluorescent reactions were scored as negative.

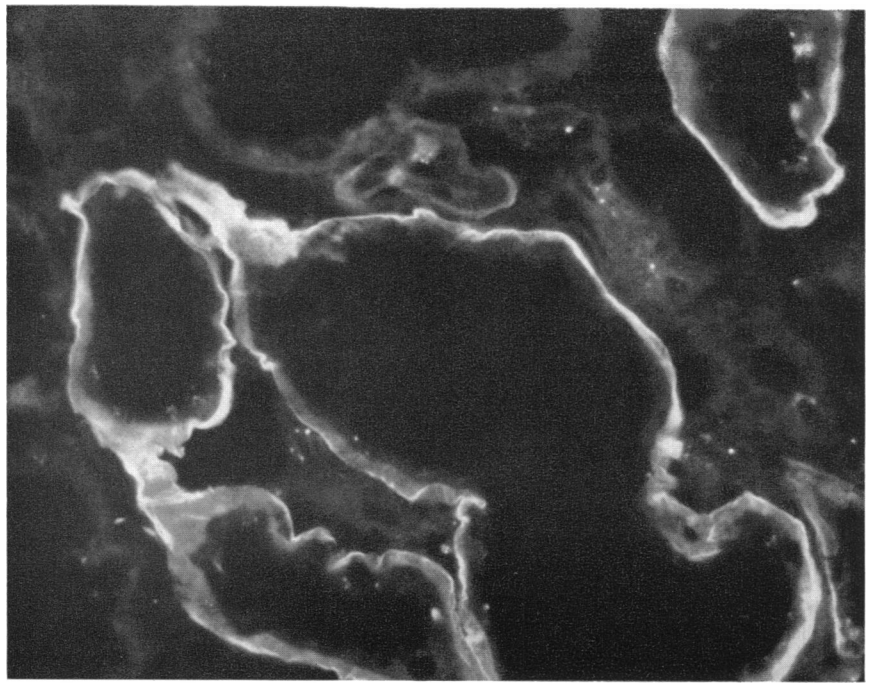

FIGURE 4 Photomicrograph of immunofluorescent fixation of eluted IgG to tubular basement membranes of normal human kidney sections. Note that fixation is focal, sparing adjacent tubules to those which it binds (case F). ×350

gland sections at the 30–65 μg/ml IgG test concentrations. At a tenfold increase in eluate concentrations, both GP eluates tested showed fixation to rhesus choroid plexus and one to chimp salivary gland; both non-GP eluates fixed to neither.

Absorption experiments. Table III demonstrates that blocking of fluorescence on normal human kidney sections could be achieved using soluble GBM and urinary basement membrane antigens to absorb out homologous anti-GBM antibodies. The amount of added antigens

TABLE II

Comparison of Human Anti-GBM Antibodies from Goodpasture and Non-Goodpasture Eluates for Their Specificity for Heterologous GBM

		Species from which kidney sections were used for direct testing of IgG fixation in vitro				
	IgG	Human	Chimpanzee	Rabbit	Rat	Mouse
	mg/ml					
Goodpasture eluate						
B	0.009	2+	4+	trace		1+
F	0.003	2+	3+	1+	1+	1+
O lung	0.006	2+	3+	1+	2+	2+
O kidney	0.007	3+	4+	1+	2+	2+
H	0.015	1+	0	trace	0	0
W	0.003	1+	1+	0	0	0
Non-Goodpasture eluate						
DE	0.005	2+	2+	0	trace	0
C	0.003	1+	1+	0	0	0
MC	0.020	1+	0	0	0	0
MA	0.015	2+	3+	1+		

required varied between the different soluble antigens employed. BSA and solubilized sheep tropocollagen failed to inhibit the fixation of anti-GBM antibodies from any of the eluates tested.

Paired-label isotopic experiments. Six eluates (four GP and two non-GP) used for tests of fixation to normal tissue sections were tested also in the paired-label experiments; two of the GP eluates were from a single patient, eluted from diseased lung and kidney respectively. As indicated in Table IV, the kidney-fixing antibody content in these eluates ranged from 0.6 to 23% of the injected IgG, and the GP eluates contained three- to tenfold greater specific activity than non-GP eluates. In contrast, eluted IgG from the kidney of a patient not demonstrating anti-GBM antibody activity by direct examination of native kidney and attempted eluate fixation on tissue sections did not localize to the monkey kidneys. Although the preponderant site of in vivo fixation of these injected eluate fractions was to the kidney, a substantial fraction of some eluates also fixed to liver and spleen (Table V). In order to test whether the latter might be due to cross-reacting antibodies directed at hepatic and splenic determinants, one eluate was tested by injecting into one monkey without prior absorption with monkey liver powder, and into another monkey after such absorption. There was no difference between patterns of fixation or total kidney-fixing antibody content between these two preparations. In none of the animals injected with eluted IgG was there significant fixation in vivo to squirrel monkey lungs, and there was no systematic difference between GP and non-GP eluates in this regard. Similarly, no differences were noted between the two eluates from lung and kidney of the same patient (O). Both contained high percentages of kidney-fixing antibodies, but no localization to lungs was noted.

Tests of the elution procedure. The efficacy of the technique was demonstrated by elution of 60% of the labeled protein (Du) from the particulate kidney debris under the conditions of the procedure. After neutralization and dialysis against PBS, 5% of the eluted labeled kidney-fixing protein precipitated. As shown in Table IV, when these eluates were reinjected into new host animals, 17–26% of the injected labeled material fixed to host kidneys; this amount represented 20–36 times greater fixation than was measured in the first monkey hosts at 3 or 8 days respectively. However, there was no difference in the in vivo patterns of fixation to the tertiary hosts' organs compared to the secondary hosts' organs (Table V).

DISCUSSION

These experiments demonstrate in vitro more precisely antibodies on homologous tissue sections, as well as their reactivity with tissues from heterologous species. Tests over a hundredfold range of concentrations indicated

TABLE III

Results of In Vitro Absorption Studies Performed Using Eluates and Soluble Antigens Derived from Human GBM by Enzymatic Digestion and from Human Urine

Eluate, IgG concentrations	Soluble antigen	Antigen concentration, *mg/ml*	GBM*	TBM	BC
Non GP-DE, 45 mg/ml	O		2+	trace	0
	Collagenase GBM	10	1+	0	0
	Trypsinized GBM	10	trace	0	0
	Urinary antigens	10	0	0	0
GP-B, 85 mg/ml	O		3+	2+	0
	Collagenase GBM	10	2+	1+	0
	Trypsinized GBM	10	1+	trace	0
	Urinary antigens	10	0	0	0
GP-O (kidney), 65 mg/ml	O		3+	3+	1+
	Collagenase GBM	30	0	0	0
	Trypsinized GBM	30	trace	0	0
	Urinary antigens	30	trace	trace	0
GP-H, 146 mg/ml	O		3+	2+	2+
	Collagenase GBM	30	trace	0	0
	Trypsinized GBM	30	trace	0	0
	Urinary antigens	30	0	0	0

* GBM = glomerular basement membranes; TBM = tubular basement membranes; BC = Bowman's capsule.

that both GP and non-GP anti-GBM antibodies are comparable in their specificity for fixation to homologous GBM. There are, however, definite differences between the eluted, kidney-fixing antibodies of these two clinical glomerulonephritic groups. Goodpasture eluates tend to fix to a broader spectrum of renal, antigenic sites, binding to Bowman's capsule, renal tubular basement membrane, and occasionally to capillary basement membranes not in the glomerulus. This same in vivo fixation of host IgG has been observed in the nephritic kidneys from some of these patients by direct immunofluorescent examination. In contrast, eluted anti-GBM antibodies from non-Goodpasture nephritic patients less frequently fixed to nonglomerular antigenic sites. Nonetheless, these differences were not absolute, and overlap occurred.

Differences between these eluates were more apparent when tested on heterologous renal tissue sections. Although tested at antibody concentrations at which binding to homologous GBM was demonstrated on normal human kidney sections by immunofluorescence, several eluates failed to bind to GBM of the heterologous species tested. Although the latter was more characteristic of non-Goodpasture eluates, some Goodpasture eluates also failed to bind.

Despite the cross-reactivity for heterologous GBM these eluted antibodies demonstrated, they had markedly restricted specificity for nonrenal antigens, as inferred from immunofluorescent studies of these eluates on other normal homologous tissue sections and selected heterologous tissues. Although three of four GP eluates, including the eluate from lung, reacted in vitro with septal membranes of homologous lung sections, neither of the two non-GP eluates did so at concentrations tenfold greater than was necesary to demonstrate fixation to GBM. However, one of these same non-GP eluates did bind to lung sections at a hundredfold greater antibody concentration.

In contrast to the fixation to homologous lung basement membranes, no eluate antibody fixation to homologous heart, adrenal, or liver sections was observed. These observations are in accord with those of Koffler, Sandson, Carr, and Kunkel (21) who studied two GP eluates and observed immunofluorescent fixation to homologous and heterologous lung and kidney substrates the antigenic specificity of eluted human anti-GBM but not to other organs. Nevertheless, two GP eluates in the present series reacted with heterologous choroid differences between eluted antibodies from the two cliniplexus and one with chimpanzee salivary gland basement membranes whereas the non-GP eluates did not. Such limited, common antigenic specificity shared by

TABLE IV

Immunoglobulin G Contents of DEAE-Fractionated Eluates Used in Paired Label Isotopic Studies, and Their Percentage Fixation in Squirrel Monkey Organs

Eluate	Per cent of eluate total protein* as IgG	Percentage fixation of labeled protein in all organs	Percentage fixation to kidneys (KFab)‡	Percentage bound to washed, particulate kidneys
Control§	58	0.9	0.2	0.0
Non-Goodpasture				
DE	23	4.9	4.4	—
C	29	3.4	3.1	—
Du	43	1.2	0.8	0.6
3 day transfer		39.0	25.7	21.8
8 day transfer		30.8	16.8	14.3
Goodpasture				
B	29	11.9	9.3	7.6
F	18	15.2	12.8	11.0
O kidney	29	33.8	30.0	23.4
O lung	45	24.7	17.7	15.5
S absorbed‖	19	4.5	2.0	1.4
S nonabsorbed	18	4.5	2.3	1.6

* 0.25 mg labeled eluate protein injected per monkey.

‡ KFab: kidney-fixing antibody

$$\text{counts } {}^{125}\text{I in kidneys} - \left[\frac{\text{counts } {}^{125}\text{I}}{\text{ml blood} \times \text{in kidneys}} \Big/ \frac{\text{counts } {}^{131}\text{I}}{\text{ml blood}}\right] = \frac{}{\text{total counts } {}^{125}\text{I eluate IgG injected}}$$

§ Control eluate: eluate from nephritic human kidney, with IgG fixed in a granular, discontinuous pattern, and which failed to fix to human kidney sections in vitro.

‖ Absorbed: eluate protein absorbed with a 10:1 (w/w) excess of dry squirrel monkey liver powder (see Methods).

TABLE V

Organ Fixation of Radiolabeled Human Eluates Containing Anti-Glomerular Basement Membrane Antibodies Injected into Squirrel Monkeys

Eluate	Percentage fixation* to: Kidney	Lungs	Heart	Spleen	Liver
Control*	17	6	2	4	71
Goodpasture					
B	78	2	2	3	15
F	85	1	0	2	12
O lung	71	1	0	4	23
O kidney	89	1	1	4	6
S absorbed*	44	3	2	2	50
S unabsorbed	51	3	1	3	42
Non-Goodpasture					
DE	90	7	2	1	0
C	92	5	3	0	0
Du	53	5	2	2	36
Du 3 day transfer	66	2	1	3	28
Du 8 day transfer	55	3	1	2	40

* Control, absorbed, percentage fixation: as in Table IV.

lung and renal basement membranes has been the subject of several investigations in animal models and is well documented (22–24), but definition of the restricted specificity of human anti-GBM antibodies, comparing GP and non-GP eluates on homologous substrates has not been done previously.

The specificity of these immunofluorescent observations is confirmed by absorption studies performed with soluble antigens derived from enzymatic digestion of homologous GBM. Although the amount of such antigens required to block specific immunofluorescence varied between different eluates as well as with the antigen preparation employed, each eluate tested could be blocked successfully. Failure to do so with comparable concentrations of a serum protein and a solubilized fibrillar protein indicates that the results of absorption experiments were not due to nonspecific absorption of antibodies to the substrates used.

Studies of in vivo fixation of these eluates define their content of specific kidney-fixing antibodies and permit greater confidence in the preceeding analyses of antigenic specificity by fixation to tissue sections. Although the paired-label studies showed that GP eluates tended to contain several times greater concentrations of anti-GBM antibodies than did non-GP eluates, this disparity is insufficient to explain the systematic differences observed between their fixation to homologous antigens on normal tissue sections over the hundredfold range tested. Moreover, the high degree of in vivo binding to monkey kidneys confirm the studies reported by Lerner, Glassock, and Dixon (8) on the binding and pathogenicity of comparable eluates from other patients with anti-GBM antibody–mediated glomerulonephritis.

These transfer experiments with eluted antibodies, however, failed to demonstrate significant antibody fixation to squirrel monkey lungs. Although such might have been expected, particularly of the GP eluates, there are several possible reasons for their failure to do so. First, basement membranes of pulmonary alveolar septae may not be so readily available to intravascular antibodies as the glomerular basement membranes, and some adjunctive circumstances may be required to expose them to circulating antibodies. Comparative studies by electron microscopy of lung and kidney define an anatomical difference compatible with this suggestion (25). Secondly, since these experiments were carried to a 72 hr termination after injection, it is possible that antibodies which may have fixed in the lungs within the first few hours subsequently dissociated and translocated to the readily available GBM. Such a phenomenon could explain an apparent lack of fixation to lung at the time sampled. Significant dissociation of heterologous nephrotoxic antibody from one antigenic site and reassociation to another in vivo has been demonstrated in injected rats (26), in parabionts, and in normal isologous recipients of a kidney from a nephritic donor animals (27).

A further possibility is that the patients' kidneys had selected a primarily kidney-fixing antibody population not containing antibodies directed toward pulmonary alveolar basement membranes. Strong argument against this hypothesis is offered by the lung eluate tested which fixed to kidney in an almost quantitative manner compared to the kidney eluate from the same patient. Lastly, the monkey lung may contain far fewer antigens in common with homologous kidney than does human lung.

Despite failure to demonstrate lung fixation in monkeys in vivo, substantial uptake of labeled protein into liver and spleen did occur. That this uptake was not immunologic is suggested by failure to block it by preabsorption of antibody-containing preparations with an excess of squirrel monkey liver powder. Moreover, an even higher relative percentage uptake into liver was measured with the control eluate not containing anti-GBM antibody. More likely, such fixation of labeled test proteins may reflect phagocytosis of eluted antigen-antibody complexes and (or) denatured IgG resulting from the elution procedure, or localization of labeled non-IgG tissue proteins eluted from the native, diseased human kidney. The unchanging percentage of eluted radiolabeled proteins taken up into liver and spleen of both secondary and tertiary hosts in retransfer experiments with non-GP eluate Du (Tables IV and V) speaks in favor of the former possibilities. The identity of non–serum protein components of these eluates is not known, but the elution method, employing mechanical agitation and warm, low molarity acid buffer, undoubtedly is capable of disrupting cells and extracting soluble components.

The retransfer experiments also gave measure of the quantitative success of elution under the conditions of these studies, with a 60% recovery of specifically labeled protein from the washed, particulate portion of homogenized kidneys of secondary hosts. Upon neutralization and dialysis of these eluates against saline, 5–10% loss of labeled protein occurred in the flocculent precipitate that formed. The latter precipitate, probably largely histones (28), may have trapped antibodies nonspecifically; alternatively, the small amount of labeled antibody precipitated may have been complexes of anti-GBM antibody and GBM antigens solubilized by the elution buffer.

Despite inability to demonstrate preferential in vivo fixation of anti-GBM antibodies to lungs of squirrel monkeys, these experiments show important general cal groups of patients with anti-GBM antibody–mediated glomerulonephritis. Qualitatively, eluted antibody from GP patients possessed greater cross-reactivity with homologous and heterologous renal and nonrenal antigens than did non-GP eluates. Quantitatively, these GP eluates

contained a substantially greater percentage of kidney-fixing antibodies than did non-GP eluates. Although the quantitative considerations may result indirectly from a more acute course and precipitate loss of renal function in GP patients, both of these differences are concordant with the clinical differences observed between patients with Goodpasture's syndrome and patients with anti-GBM–mediated nephritis without the Goodpasture syndrome. The data indicate that the Goodpasture syndrome is usually mediated by potent anti-GBM antibodies with significant cross-reactivity with nonglomerular vascular and epithelial basement membranes. However, the factors responsible for elicitation of these autoantibodies and the initiation of the pulmonary lesions in the Goodpasture syndrome are not clear.

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