Specificities of Antibodies Eluted from Human Cadaveric Renal Allografts

MULTIPLE MECHANISMS OF RENAL ALLOGRAFT INJURY

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ABSTRACT The purpose of the present experiments was to evaluate the role of circulating antibodies in the rejection of human renal allografts and to study the apparent target(s) for antibody binding. Eluates obtained from surgical biopsy and nephrectomy specimens of rejecting, cadaveric human renal allografts were tested for antibodies directed to structural antigens of normal kidney and for cytotoxic antibody activity against mononuclear cell populations. By indirect immunofluorescence 23 of 35 eluates contained immunoglobulin that bound to normal kidney. Staining was in smooth muscle only in 10 patients, in smooth muscle and other structures such as tubular basement membranes, proximal cells, or brush border in 9 patients, and in structures other than smooth muscle in 4 patients. All 16 eluates tested contained antibodies cytotoxic for cells derived from a panel of normal volunteers. Six were cytotoxic to T cells and 10 to B cell and monocyte-enriched preparations. Absorption of eluates with pooled buffy coat cells, platelet concentrates and packed, cultured B cells removed antibodies reactive with vascular wall smooth muscle and endothelium, but not antibodies to tubular basement membranes, proximal or distal tubular cells, brush border, or other structures of kidney sections. Two of five eluates containing antikidney antibodies were found to bind to rat kidneys in vivo. These results suggest that circulating antibodies participate in cadaveric renal allograft destruction and demonstrate that they can be recovered directly from the allograft. Moreover, the data indicate that there are different antibody populations involved: some clearly directed to allo-specific differences and others that are apparently kidney-specific.

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INTRODUCTION

Although participation of antibodies in the destruction of initial renal allografts in some experimental models is well demonstrated (1, 2), the importance of antibodies in human renal allograft rejection has not been defined clearly (2). They are thought to play a pivotal role in hyperacute rejection, associated with recipient presensitization and demonstrable reactivity with donor cells (3–5). Moreover, their participation has been implicated in the development of vascular lesions conspicuous in later rejections (6, 7). Data establishing the relationship of circulating antibodies to graft fate in man are largely circumstantial, and include disappearance from circulation of preexisting antibodies when graft is placed, the appearance or rise in titer of antibodies after graft removal, development of rheumatoid factor and heterophile antibodies (8, 9) and deposition of immunoglobulin in vascular lesions, glomeruli, and tubulo-interstitial areas (7, 10).

Stronger evidence has been adduced by analysis of eluates from renal allografts at nephrectomy or autopsy (11–18). Nevertheless, results have not been consistently positive for evidence of antibodies bound to grafts, and no studies of specificities of these eluates toward intrarenal structures have been reported. Antiglomerular basement membrane (GBM)[1] anti-

[1] *Abbreviations used in this paper*: GBM, glomerular basement membrane; IF, immunofluorescence; NHK, normal human kidney; TBM, tubular basement membrane.

bodies have been eluted from allografts placed into patients with circulating GBM antibodies (19), and tubular basement membrane (TBM) antibodies have been eluted from occasional allografts with apparent in vivo fixation of immunoglobulins (Ig) seen by direct immunofluorescent (IF) tests on renal tissue (20–22). The frequency of anti-TBM antibodies has not been studied, and there has been no systematic investigation searching for other structurally-specific antibodies in a group of rejecting (rejected) renal allografts.

The present studies of eluates from cadaveric renal allografts with marked deterioration of renal function or irreversible clinical rejection were undertaken for three purposes: (*a*) to determine the presence of antitissue antibodies in eluates from such grafts; (*b*) to characterize apparent antitissue antibodies for structural and organ specificity; and (*c*) to test these eluates concomitantly for their content of cytotoxic antibodies to different mononuclear cell populations (T cell, B cell, and monocytes).

Our results indicate that all eluates from rejecting cadaveric human renal allografts tested contained antibodies cytotoxic to allogeneic mononuclear cell populations. In addition, most contained antibodies with apparent specificity for structural antigens of normal, human kidney; some of these could be absorbed with peripheral blood buffy coat, platelets, and/or B cell-rich preparations. However, several of the eluates tested contained substantial concentrations of antibodies which appear to bind only to kidney. Absorption experiments and paired label, in vivo, fixation studies in rats indicated that these antibodies against specific kidney structures are a population of antibodies distinct and separable from the antibodies cytotoxic for defined mononuclear cell populations.

METHODS

Kidney specimens were obtained from cadaveric allografts placed into 35 recipients (group I). The specimens investigated included 17 surgical biopsies obtained from grafts showing serious deterioration of function and 25 nephrectomies obtained after irreversible rejection. All patients were still on standard immunosuppression at the time of biopsy or nephrectomy. No patients had received antilymphocyte globulin. No instances of "hyperacute" rejection are included and neither are any apparent cases of technical failure, tubular necrosis, etc. As controls, native kidneys were obtained by nephrectomy from 11 patients anticipating renal allografts (group II); 3 of the 11 later had allograft nephrectomy and are included in group I. These 11 patients had: glomerulonephritis (seven cases), focal sclerosing glomerulopathy, interstitial nephritis, nephrosclerosis, and reflux nephropathy (one case each); 9/11 had glomerular Ig deposits. None of the tissues tested were from patients with GBM antibody-mediated disease as inferred from direct IF, and tests of sera and eluates for GBM antibodies. One eluate of group II was known to contain antibodies specific for vascular smooth muscle.

Antibodies. Antisera to human immunoglobulins were made in rabbits using fractions of normal human serum or myeloma proteins as described previously (23). Antisera to human renal tubular antigen and Tamm-Horsfall protein used as localization markers for tissue studies were made similarly, using antigens isolated as described by Edgington and associates (24) and Hoyer et al. (25), respectively. Fluoresceination of globulin fractions was done by the method of Clark and Shepard (26).

Elution procedure. Cortical segments were minced and washed extensively with phosphate-buffered saline, pH 7.4; the 1,000 *g* sediment was eluted with 0.02 M citrate pH 3.2 at 37°C for 2.5 h with constant stirring (23). After centrifugation at 4,000 *g* for 30 min in the cold, the supernate was neutralized, dialyzed overnight against phosphate-buffered saline, and concentrated by ultrafiltration before testing.

Quantitative Ig concentrations. Quantitative Ig concentrations were measured by radial immunodiffusion using commercial kits (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.; Meloy Laboratories, Inc., Springfield, Va.). Total Ig concentrations (text and Table I) comprise the sum of individually measured IgG, IgM, and IgA.

Radiolabeling. Proteins were labeled with ^{131}I or ^{125}I by the technique of McConahey and Dixon (27); counting was done in a well detector with thallium-activated crystal.

Anti-GBM antibodies. Anti-GBM antibodies were assayed in serum and eluates by indirect IF and radioassay (28).

IF. Direct IF tests of tissue were done using incident-light fluorescence microscopy and conventional controls (23, 28). For indirect IF using sera and eluates, samples were tested at known Ig concentrations. Cryostat sections were treated as described (28), first with serum or eluates and subsequently with fluorescein-conjugated anti-human IgG and FII fraction of human serum proteins reagent (anti-human Ig). Controls for indirect IF included incubation of sections (*a*) with normal human serum, (*b*) with sham eluates made from incubating normal human autopsy kidney with normal human serum followed by elution procedure, and (*c*) with acid-citrate treated human IgG. The binding specificity of all eluates was tested by IF individually on multiple normal kidneys (NHK) including kidney from blood group O donors; in comparative experiments in which all, or a representative number of eluates were tested simultaneously, the same target substrate was used.

The apparent amount of antibodies contained in eluates and binding to structural components of NHK by IF was estimated by two methods: (*a*) titer of antibody binding per microgram of IgG compared with concomitantly collected serum titer per microgram of IgG; such ratios always refer to antibodies with the same binding pattern; (*b*) minimal absolute eluate IgG concentration associated with demonstrable binding by IF.

To determine organ specificity, binding to nonrenal, human tissues was investigated. Eluates were incubated with sections of human tissues obtained freshly from surgical biopsy and/or autopsy including: heart, thyroid, lung, liver, spleen, testicle, skeletal muscle, adrenal, pancreas, stomach, gut, and lymph node. The IF staining technique was the same as that used with kidney sections.

A number of eluates known to bind to NHK were absorbed with either pooled whole human leukocytes (buffy coat), pooled, washed human platelets, or cultured human B cells. For absorption the eluates were incubated with an equal volume of packed cells, at room temperature for 1 h. After removal of the cells by centrifugation the eluates were concentrated back to their original volume before testing.

Paired label, in vivo binding studies of radiolabeled eluates. Test eluates were radiolabeled with ^{125}I or ^{131}I, dialyzed free of inorganic iodine, and concentrated by diafiltration. Control

proteins (either acid-citrate treated human FII fraction or an apparently inert transplant eluate) were labeled with the alternate isotope, and prepared similarly to the test eluates. Approximately equal amounts of labeled test eluate and control protein were mixed and injected simultaneously ($1-10 \times 10^6$ dpm of each) by tail vein into two lightly anesthetized Sprague-Dawley rats. Rats were treated with saturated solution of potassium iodide in drinking water, housed in metabolism cages, and killed after 72 h. At sacrifice the carcasses were perfused with saline and excised organs counted (liver, spleen, kidneys, skeletal muscle): kidneys were homogenized in a mechanical blendor, debris washed with phosphate-buffered saline; and the pellet after centrifugation at 4,000 *g* for 30 min was counted. The results were expressed as percent kidney-fixing antibody (KFab), which refers to the radioactivity fixed to the washed, particulate kidney debris, and is calculated as described by Lerner and Dixon (29): KFab = counts per minute cpm kidney-fixed ^{125}I-(blood ^{125}I × kidney ^{131}I/blood ^{131}I) ÷ cpm ^{125}I protein injected. Nine different transplant eluates were studied. Three eluates of native kidneys known to contain anti-GBM antibody were used as positive controls.

Cell preparations for cytotoxicity tests. Venous blood obtained from normal volunteer donors, members of the tissue typing laboratory reference panel, was anticoagulated with preservative-free heparin and the mononuclear cells were isolated on Ficoll-Hypaque (30). After centrifugation at 400 *g* for 30 min at room temperature the mononuclear cells obtained from the interface were washed once with RPMI 1640 medium containing 20% pooled human serum and suspended in the same.

Monocytes were isolated by adherence. For this, $20-30 \times 10^6$ mononuclear cells were cultured in 25 ml of medium in a 10-cm Diam dish at 37°C in a humid atmosphere containing 5% CO_2, 95% air. After about 2 h the nonadherent cells were removed by washing with culture medium, checking for completeness of their removal with an inverted phase microscope. It usually required five or six washes of 8–10 ml each for removal of lymphocytes. The adherent cells were then released from the dish by gently scraping the surface with a sterile rubber policeman. The cells were collected in a conical centrifuge tube containing RPMI 1640 with 20% pooled human serum and washed once. The resulting cell preparations were usually 75–95% viable. About 80% of the cells showed characteristics of monocytes upon staining with euchrysine and ingested latex particles.

B lymphocytes were obtained from the nonadherent cells by removal of T lymphocytes with neuraminidase-treated sheep erythrocytes: nonadherent lymphocytes were mixed with an equal volume of 2% neuraminidase-treated sheep erythrocytes (31), incubated at 37°C for 15 min in a water bath with shaking, underlayered with Ficoll-Hypaque solution and refrigerated at 4°C for 30 min to allow the formation of rosettes. The preparations were then centrifuged at 400 *g* for 30 min and the B lymphocytes removed from interface were washed once and adjusted to 3×10^6 per ml. The B lymphocyte preparations usually contained <5% rosette-forming cells. Less than 10% reacted with a rabbit anti-human T cell serum; 70–95% were killed by a rabbit anti-human Ia serum.

T lymphocytes were prepared from the sheep cell rosettes by hypotonic lysis. Having discarded the supernatant fluid, the erythrocyte pellets containing the T lymphocytes were suspended in 1–2 ml of Tris-ammonium chloride solution (32) and incubated at 37°C for 10 min. The tubes were filled with saline solution and the T lymphocyte recovered by centrifugation. T lymphocyte preparations usually showed <10% killing with a rabbit anti-human Ia serum and 80–85% were killed by a rabbit anti-human T cell serum.

Cytotoxicity tests. Cytotoxicity tests were performed using the Standard National Institutes of Health procedure for microcytotoxicity (33). 16 different allograft eluates, selected because of the differing specificities they had in IF binding assays and sufficient eluate was available were tested. In these tests we used 2,000 T cells, and 3,000 B cells or monocytes and 0.001 ml of eluate or serum. The T cell preparations were incubated for 30 min with eluate or serum and 60 min with rabbit complement, both at room temperature. The B cell and monocyte cytotoxicity tests were incubated at 20°C for 60 min with eluate or serum and for an additional 120 min after addition of rabbit complement; rabbit complement was pretested to insure potency and lack of toxicity to human lymphocytes and monocytes. After the final incubation the reaction droplets were stained with eosin and fixed with formalin. The reading of the results were performed in an inverted phase contrast microscope. The coding was as follows: <10% dead cells, negative; 10–30% cells killed, questionable negative; 30–50% cells dead, questionable positive, 50–80%, definite positive; and >80% killed, strong positive. Each eluate was tested against cell preparations from 14-18 different donors. As control, 11 native kidney eluates were tested similarly.

RESULTS

Binding to NHK. 12 allograft eluates had no descernible Ig binding to NHK by IF assay; 7 were from biopsy specimens and did not contain detectable Ig (<50 μg/ml). The other five were nephrectomy eluates with Ig concentrations of 150–270 μg/ml. Eluates from 23 allografts contained Ig which bound to specific structural components of homologous kidney (Table I). At least seven different patterns of IF staining were defined by these tests (Figs. 1–5). They consisted of Ig binding to: smooth muscle, endothelium, proximal tubular cells, proximal tubular brush border, a granular cytoplasmic antigen associated with basal lamina of proximal tubular cells, distal tubule and/or collecting duct cells, and cortical TBM. No instances of GBM binding were seen. The positive eluates comprised three groups: (*a*) binding to vascular smooth muscle only (10 patients), (*b*) binding to vascular smooth muscle and to other structural components (9 patients), and (*c*) binding to structures other than vascular smooth muscle only (4 patients). In contrast, only 1 of 11 eluates from native, diseased kidneys (group II) contained antibodies specific for vascular smooth muscle. The other 10 eluates from native kidneys were negative. Moreover, all 11 were negative against all other NHK target sites. In three patients eluates from native kidneys were negative for smooth muscle antibodies, whereas their allografts contained them.

Quantitation of antibodies against specific kidney structures. Sera drawn from 18 patients at time of graft nephrectomy were tested along with their graft eluates. Serum from five patients contained antibodies with specificities similar to their graft eluates and eluates/serum ratios could be estimated: titration results of serum and eluate suggested preferential uptake of structurally-specific antibody by allografts in four of the

TABLE I

Antibodies against Tissue Structures in Allograft Eluates and Serum Specimens Tested on NHK Sections

Eluate No.	Eluate Ig concentration	Structural specificity of eluate		Serum specificity	Eluate/serum antibody ratio	Minimal Ig binding concentration	Graft* duration	Rejection pattern	Ig deposits by direct IF tests		
		SM	Other						Glomerular	Vascular	Tubules
	μg/ml	reciprocal titer			per μg Ig	μg/ml	d				
Group A											
1	400	10		EN		40	120	AVR	+	+	
2	670	1		NEG		670	20	AVR		+	
3	270	1		NEG		270	35	AVR	Essentially negative		
4	490	10		NEG		49	5	AVR	Technically inadequate		
5	590	5		NEG		115	120	AVR	Essentially negative		
6	290	10		NT		29	180	CVR	Technically inadequate		
7	47	20		NT		2.7	70	AVR		+	
8	1360	10		NEG		136	180	CVR	Essentially negative		
9	55	5		NT		11	42	AVR		+	
10	1360	80		NT		<17	90	CVR		+	
Group B											
11	620	10	EN(10)TBM(20)	SM	130	31	150	ACR	+		+
12	2900	10	EN(5)PTC(80)PTBB(1)	SM, PTC	7:53	<36	660	CVR	Essentially negative		
13	2040	20	PTC(20)PTBB(10)	PTC, PTBB	86:43	102	42	AVR		+	
14	1010	10	PTC(20)PTBB(10)	SM, PTC	163:35	51	63	AVR	+	+	
15	1160	1	DC(10)	SM	1	116	17	AVR	Essentially negative		
16	380	10	PTC(1)	NT		38	120	CVR	+	+	+
17	2170	10	PTC(10)	NEG		217	43	AVR		+	+
18	620	10	EN(10)	NEG		62	30	AVR	Essentially negative		
19	710	10	PTC(5)PTBB(1)TBM(10)	EN		71	420	AVR		+	+
Group C											
20	1155		PTC(10)PTBB(10)PTAG(10)	NEG		<115	20	AVR		+	+
21	100		TBM(10)	NEG		10	180	CVR			+
22	569		TBM(10)	NEG		<56	105	AVR		+	+
23	370		TBM(1)	NEG		370	134	AVR	+		+

Abbreviations used in this table: SM, smooth muscle, EN, endothelium, PTC, proximal tubular cells, PTBB, proximal tubular brush border, PTAg, proximal tubular granular antigen, DC, distal cells, TBM, tubular basement membrane; NT, not tested; AVR, acute vascular rejection; CVR, chronic vascular rejection; ACR, acute cellular rejection; NEG, negative; no specificity detected by indirect IF.

* All grafts were initial allografts, except patients 22 and 23.

five (eluates 11–14). Eluates from 14 patients contained antibodies not found in their serum. Titration of all eluates to the minimal Ig concentration at which specific staining could be seen confidently showed it to vary from 2.7 to 670 μg/ml (median 62 μg/ml); in 14 of the 25 eluates tested binding was observed at Ig concentrations <100 μg/ml (11 < 50 μg/ml) indicating that major amounts of structurally specific antibodies were present. IgG titers were not significantly different than FII fraction of human protein titers.

Binding to nonrenal, human tissue sections. 10 eluates were tested. Eluates which contained antibodies to vascular smooth muscle of homologous kidney bound also to smooth muscle of stomach and gut, and to cardiac but not skeletal muscle. On the other hand, eluates containing antibodies to tubule cells and TBM did not stain other tissues, with one exception: Ig in one eluate bound also to pancreatic duct cells (not muscle cells). The single native kidney eluate with smooth muscle antibodies bound only to vascular smooth muscle, and did not cross-react with visceral smooth, cardiac, or skeletal muscle.

Absorption experiments. Whole buffy coat, pooled platelet concentrates, and pooled, cultured B-cell concentrates were equally effective in absorption of antibodies with apparent specificity for smooth muscle cells of homologous kidney and endothelium. However, none of these cell preparations diminished or eliminated binding to other structural antigens such as tubule cells, brush border, or TBM (Table II).

Paired label, in vivo studies of radiolabeled eluates. Nine allograft eluates were tested: two showed clear

binding to renal tissue in excess of 1% (eluates 15 and 17, Table III). Precipitation of immunoglobulin from the eluates before injection diminished renal binding substantially, but absorption of eluates with pooled human platelets and B-cell rich concentrates did not alter binding. Positive-control eluates used in these studies were three different native kidney eluates from patients with anti-GBM antibody-mediated disease; they had binding of 0.8–4.4% to renal sediment. Two other transplant eluates (eluates 11 and 22) showed smaller binding of uncertain significance.

Cytotoxic antibody reactivity. All eluates tested had cytotoxic reactivity toward one or more cell populations. In general, those eluates (eluates 4, 5, 12, 14, 20, 27) that contained antibodies reactive with T cells also were cytotoxic to B cells, but seldom with a predominantly monocyte-enriched cell population (Table IV), and tended to be more reactive. In contrast, those eluates essentially unreactive with T cells tended to be as reactive with monocytes as with B cell preparations. Cytotoxic antibodies against the B cell-enriched preparations coexisted with structural antibodies of all specificities, particularly smooth muscle antibodies. Serum from nine patients taken at the time of nephrectomy were tested also and the specificities differed from the eluates in seven (Table IV). The 11 eluates from native kidneys contained only two reactors: one was T + B + M +, and the other had mild reactivity to monocytes only.

FIGURE 1 Eluate Ig binding to arterial vascular smooth muscle of NHK is seen using indirect immunofluorescence (×400).

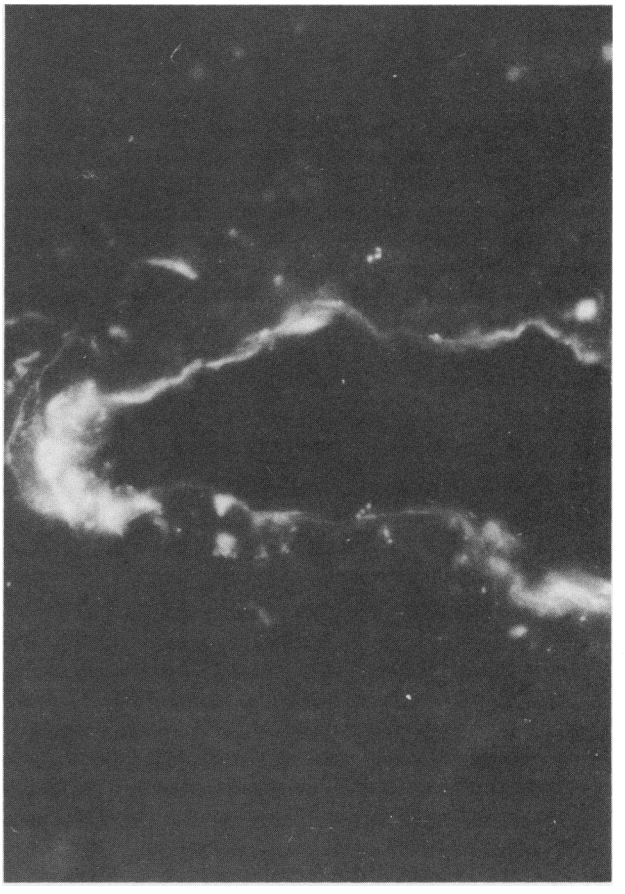

FIGURE 2 Eluate Ig binding to endothelium of vein is demonstrated (×400).

Direct immunofluorescent tests of allograft biopsy and nephrectomy specimens. Tissue from two specimens was technically faulty, presumably due to improper freezing and hence, could not be evaluated. Vascular Ig deposits were detected in 15 grafts by direct IF; eluates from 10 of them contained antibodies to smooth muscle, whereas 5 did not. Conversely, eluates from 19 patients contained smooth muscle antibodies; direct IF of kidney tissue showed vascular Ig infiltrates in 10, glomerular staining in 4, no staining of vessels in 7 and was technically faulty in 2 (above). Staining detected Ig fixed to tubules in eight allografts: TBM (five cases), proximal tubule cells (four cases), brush border (two cases); all eluates from these kidneys contained antibodies that fixed to comparable structures when tested by indirect IF on NHK sections. Significant glomerular Ig deposits were detected by direct IF tests of seven renal allografts; no eluate contained specific glomerular-fixing antibodies.

DISCUSSION

In these experiments we have shown the presence of two kinds of antibodies in eluates from rejecting (or

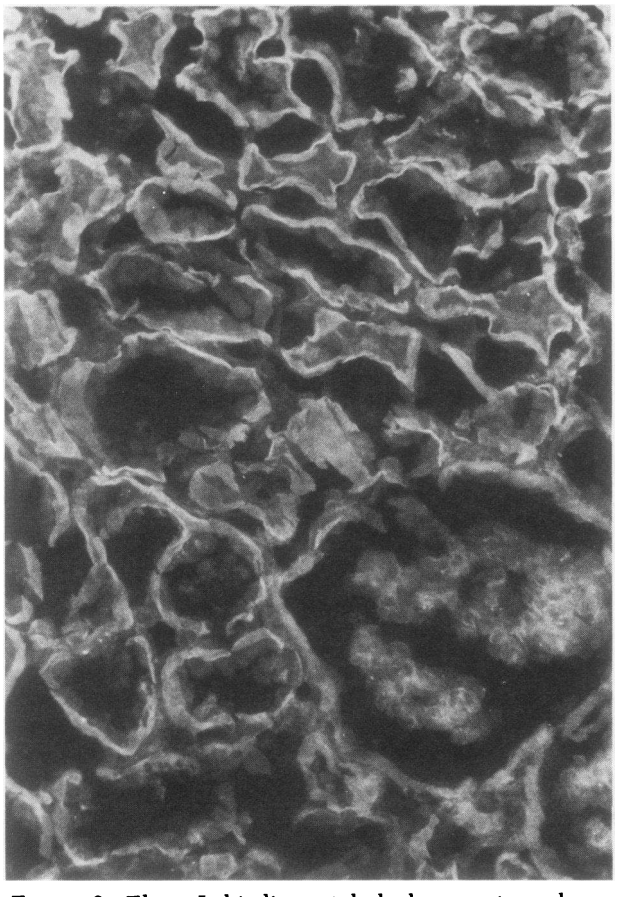

FIGURE 3 Eluate Ig binding to tubular basement membrane of cortical tubules of NHK is seen; there is no Ig fixed to Bowman's capsule or glomerulus. (×100)

rejected) cardaveric human renal allografts: cytotoxic antibodies to mononuclear cells and antibodies with specificity for structural kidney antigens demonstrable by immunofluorescence. Cytotoxic antibodies against isolated mononuclear cells were detected in every eluate tested. Specificities of the eluted antibodies often differed substantially and unpredictably from serum antibodies. Presumably eluates reflect a subset of antibodies that bound to donor kidney over a period of time, whereas serum contains antibodies that did not bind to donor kidney and only those present at the time the sample was obtained. Thus, differences in the reactions of eluates and serum samples are not surprising.

In two thirds of eluates (23/35) antibodies to structural kidney antigens were detected by immunofluorescence; most common were antibodies to smooth muscle (19 of 23 eluates). These antibodies bound to vascular smooth muscle on homologous kidney sections, but reacted also with smooth muscle of other organs, binding to stomach, gut, and heart, but not to skeletal muscle. Thus, they were not organ-specific. Additionally, these antismooth muscle antibodies were absorbed by viable mononuclear cells and by platelets, suggesting that they are directed towards determinants also represented on cells other than those of blood vessels.

Organ-specific antibodies binding structural antigens not shared with vascular components were detected in 12 eluates: they included tubule cells, brush border, and TBM. These antibodies did not react with sections of tissues other than kidney and were therefore thought to be organ-specific. They were not absorbed by concentrates of platelets or mononuclear cells; hence, they do not seem to be HLA or Ia-related. TBM antibodies can be absorbed, and their binding blocked, by solubilized TBM antigen in vitro, attesting to their relative structural specificity (21, 22).

To define the pathophysiologic significance of antitissue antibodies we tried to estimate their relative concentration in serum and tissue eluates. On the basis of dilutional indirect IF binding tests on homologous kidney sections, we estimated that half of the eluates contained anti-structural antibodies at concentrations <50 μg of Ig per ml, suggesting an important concentration of antibodies. For comparison, eluates from native kid-

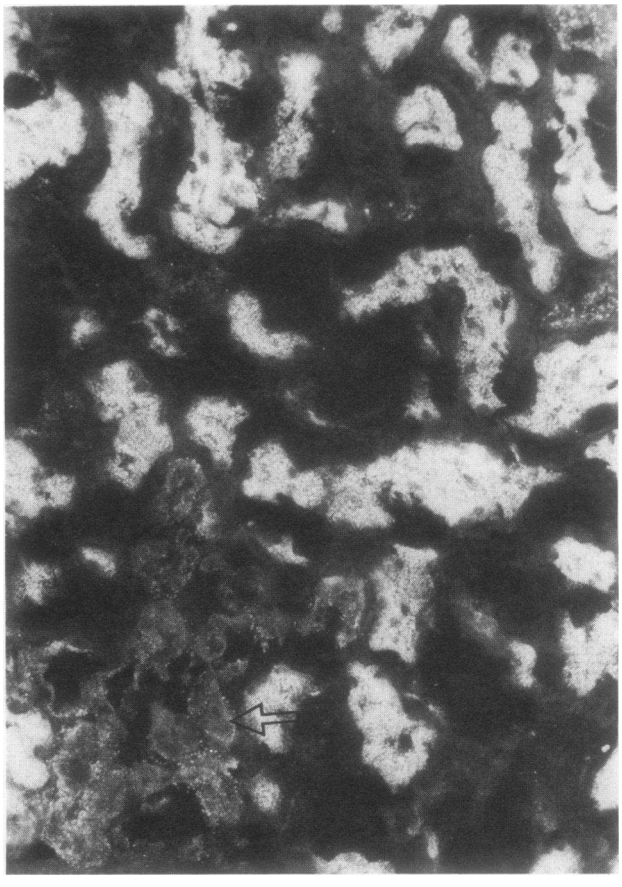

FIGURE 4 Eluate Ig shows binding to cells of proximal tubules of NHK; there is also binding to discrete granular antigens associated with basal lamina of tubule cells. (arrow)

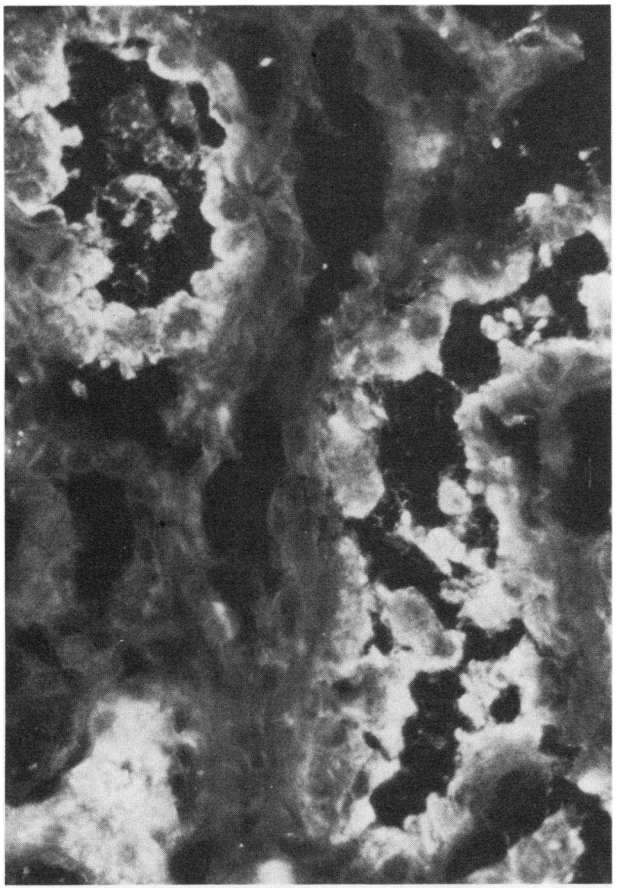

FIGURE 5 Staining demonstrates binding of eluate Ig to brush border antigens of NHK proximal tubule cells (×400).

neys of patients with GBM-antibody-induced glomerulonephritis, contained antibodies demonstrable to a concentration of approximately 9 μg of Ig per ml by the same technique (23). The existence of substantial antibody gradients between eluates and serum at the time of nephrectomy or biopsy in 4 out of 5 patients, and antibodies eluted without detectable serum antibodies in 14 of the others tested, indicated that eluted antibody was not present from nonspecific trapping.

Next, we attempted to show that antibodies extracted from rejecting allografts were capable of binding to kidney tissue in vivo. Two of five allograft eluates containing structural antibodies that might be expected to bind in vivo (specificities to tubule cells, BB, TBM) fixed with net binding of 0.8–1.4%; three other eluates

TABLE III

Paired-label In Vivo Binding of Radiolabeled Eluate Antibodies to Normal Rat Kidney Compared to Simultaneously Injected Control Protein

Allograft eluate No.	Structural antibody detected by IF	Percent kidney-fixing antibodies bound to renal sediment of rat		
			Absorption or treatment	
		Not absorbed	Platelets and B-cells	Anti-Ig
11*	SM, E, TBM	0.37	0.26	ND
		0.44	ND	ND
15	SM, DC	1.03	1.04	0.31
		1.13	ND	ND
17	SM, PTC	1.8	2.43	0.8
22	TBM	0.35	0.45	0.24
		0.35	ND	ND
Eluates from native kidneys‡				
H		4.42	ND	ND
L		1.08	ND	ND
D		0.80	ND	0.00

Abbreviations used in this table: SM, smooth muscle; E, endothelium; TBM, tubular basement membrane; DC, distal cells; PTC, proximal tubular cells; ND, not tested.

* Five other eluates, three containing anti-tissue antibodies (eluates 4, 12, 18), showed no in vivo binding.

‡ Eluates H, L, and D, were known to contain anti-GBM antibodies, and were used as positive controls for kidney-fixing antibodies.

TABLE II

Immunofluorescent Binding on NHK after Cell Absorptions of Eluates

Eluate No.	Preabsorption antigenic specificity	Absorptions		IF specificity after absorption
		Platelets	B cells	
8	SM	+	ND	negative
11	SM, EN, TBM	+	ND	TBM
12	SM, EN, PTC, PTBB	+	ND	PTC, PTBB
15	SM, DC	+	+	DC
18	SM, EN	+	ND	negative
22	TBM	+	+	TBM

Abbreviations used in this table: SM, smooth muscle; EN, endothelium; TBM, tubular basement membrane; PTC, proximal tubular cells; PTBB, proximal tubule brush border; DC, distal cells; ND, not tested.

TABLE IV

Cytotoxic Reactions of Antibodies Eluted from Rejecting Human Cadaveric Renal Allografts

Eluate No.	Number of cytotoxic reactions						
	T cell (T+B+M+)		B cell and monocyte (T−B+M+)		Monocyte (T−B−M+)		Negative tests (T−B−M−)
	Eluate	[Serum]	Eluate	[Serum]	Eluate	[Serum]	Eluate
1	0	[5]	6	[7]	2	[4]	9
2	0	[2]	2	[2]	4	[1]	11
3	1		1		3		13
6	0		4		3		10
7	1		1		5		10
11	0	[0]	3	[3]	2	[1]	13
13	0	[1]	5	[4]	2	[5]	10
15	1	[2]	7	[4]	6	[3]	3
22	0		2		5		10
23	0		4		1		12
4	4	[1]	5	[2]	0	[0]	8
5	9	[3]	3	[5]	0	[3]	6
12	17	[17]	0	[1]	0	[0]	1
14	7	[18]	2	[0]	0	[0]	5
20	4		2		3		9
27	3		3		0		12

Cytotoxic reactions were coded positive if at least 50% of the cells were killed. For statistical purposes an eluate was considered positive for a certain type of reactivity only when such reactivity was seen with cells from at least two donors.

with structural antibody content did not show convincing binding. In the present experiments, when labelled GBM-antibody eluates were tested in rats, the range of binding was from 0.8 to 4.4% KFab (Table III). Importantly, absorption of the eluates with human platelets and B cell-rich preparations did not alter their kidney binding in vivo.

There is little doubt that these antitissue antibodies resulted from transplantation; only one eluate from native nephritic kidneys (group II) contained antivascular smooth muscle antibodies, and three allograft eluates which contained antitissue antibodies were from recipients whose native kidney eluates had not contained them. Nevertheless, the precise mode of elicitation of the structurally-specific antibodies is not clear. Three major possibilities exist: (*a*) The allograft may carry an array of structural antigens in which the recipient is deficient. A similar mechanism has been demonstrated when Brown Norway (BN) rat kidneys were grafted into Lewis (LE) recipients (34). Because LE strain lacks some structural antigens carried by BN donors, recipients develop anti-TBM antibodies which fix to BN (graft) kidney but not to LE (recipient) kidneys. In other experiments, Wilson and associates (21) reported studies of TBM antibodies eluted from a human renal allograft which did not react with the patient's native kidney-a situation analogous to the rat allografts. Further, Mancilla-Jimenez and co-workers (22) have described an eluate containing TBM antibodies that bound to TBM of 39/40 human kidneys tested. (2) Widespread damage to renal cortical structures occurring in the course of allograft rejection may give rise to altered structural antigens that elicit production of antitissue antibodies. Such a sequence has been inferred in native kidneys in unusual, but well-documented nephritis complicated by anti-TBM antibodies (35-37) and antibodies reactive with proximal tubular antigens (38). (3) The immune response, involving production of cytotoxic antibodies towards various cell populations and organ-specific, antistructural antibodies, may be more characteristic of the repertoire of available responses of the host than due to specific antigenic challenge.

The frequency of structurally-specific antibodies participating in pathophysiologic events characterized clinically as allograft "rejection" cannot be estimated accurately from our data for two principal reasons: (*a*) our observations derive from analyses of organs whose deterioration was sufficiently serious and sustained that biopsy or nephrectomy was done; (*b*) our data are derived from cadaveric grafts. The extent to which similar mechanisms participate in living, related-donor graft injury is not clear.

These observations are important in several regards.

First, cytotoxic antibodies were eluted successfully in every instance in this series of rejecting renal allografts. Moreover, two thirds of the eluates contained antibodies apparently reactive against smooth muscle of vascular walls, and some with endothelium, suggesting the involvement of humoral antibody in vascular lesions observed in renal allografts. Additionally, the eluates contained a variety of structurally-specific antibodies. Although TBM antibodies have been reported before in renal allografts recipients (20, 22, 34), in the present series 22% of the eluates with antitissue antibodies contained TBM antibodies alone or in association with other structural specificities—a surprising frequency.

Antibodies with apparent specificity for smooth muscle were detected in seven eluates, although no vascular Ig was detected by direct IF examination of the same kidneys. This disparity may be due to the segmental variation of pathologic processes which characterizes modified graft rejections. Another possibility is that elution, concentrating available antibodies into a small volume for testing, enhanced the likelihood of their detection. Mean minimal Ig concentrations at which smooth muscle antibodies were detected was 127 μg/ml (median concentration 71 μg/ml) in eluates from kidneys where direct IF was positive and 299 μg/ml (median concentration 136 μg/ml) in eluates from IF negative kidneys, suggesting that lesser amounts of smooth muscle antibody were present in the latter eluates. On the other hand, vascular Ig deposits were seen by direct IF without smooth muscle or endothelial antibodies in eluates in five cases. Similar vascular Ig deposits have been seen in renal biopsies from patients with arterial hypertension, where they have been attributed to insudation by passive forces or nonspecific vascular injury and not to immunological mechanisms.

There has not been any previous report of allografts with specific uptake of antiproximal cell antibodies, although an instance with Fanconi syndrome may have occurred in native kidney disease (38). Autoantibodies to cells of Henle's loop have been detected in serological surveys and in serum of patients reported to have renal tubular acidosis (39, 40), but have not been demonstrated immunopathologically to bind in vivo. Our results, indicating binding in vivo, suggest that such antibodies may have a pathophysiologic role in human disease, and may be relevant to renal tubular functional derangements reported in patients with renal allografts (41).

The high frequency of eluted antibodies reacting mostly with B lymphocytes is of considerable interest. It has been reported that antibodies to B lymphocytes appear in the serum of patients rejecting kidney allografts but their role in rejection has not been established (42). Garavoy and coworkers (13) eluted kidney allografts undergoing rejection and reported finding a factor capable of inhibiting Fc receptors, thought to be antibodies against Ia-like antigens, but the specificity of the factor(s) involved was not established. Moraes and Stastny (43) have reported that certain antibodies against alloantigens of endothelial cells found in sera from transplant recipients react also with monocytes. However, since both cells also appear to have HLA-DR antigens, the specificity of the monocyte cytotoxic antibodies observed in the present study is not yet known.

We infer from our investigations that there was evidence of multifaceted humoral antibody participation in the pathogenic events involved in renal allograft deterioration of the patients we have studied. The relative impact of two kinds of antibody systems (cytotoxic and antitissue antibodies), contrasted to primary cellular immune mechanisms, cannot be established from our observations. The more consistent detection of cytotoxic antibodies in the eluates tested suggests that they may be the more important antibody expression of graft-directed humoral immunity. Further studies will be needed to characterize the antigens involved and to quantify the in vitro activity and pathogenic significance of antibodies that can be eluted from allografts undergoing rejection.

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