Experimental Autoimmune Glomerulonephritis Induced by Anti-Glomerular Basement Membrane Antibody

II. Effects of Injecting Heterologous, Homologous, or Autologous Glomerular Basement Membranes and Complete Freund's Adjuvant Into Sheep

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The effects of injecting human, rabbit, rat, or single-kidney homologous glomerular basement membrane (GBM) or autologous GBM, each in complete Freund's adjuvant (CFA), into 15- to 18-month-old sheep are compared. All sheep receiving heterologous GBM and 3 of 6 sheep receiving homologous GBM had anti-GBM nephritis, but such sheep did not bind autoantibodies or have Goodpasturelike lesions in their lungs. Sheep given injections of human GBM had autoantibodies to antigenic determinants shared by fetal or adult sheep and human GBM, by lung basement membranes, and by certain nonvascular basement membranes. Sheep given homologous GBM had two populations of autoantibodies: one was neither species- nor organ-specific; the other was sheep-specific. No sheep given autologous GBM had any evidence of anti-GBM autoantibodies or nephritis. Their kidneys were indistinguishable by histologic, immunohistologic, and functional studies from CFA-treated controls. Thus, sheep seem very tolerant to autologous GBM. These findings suggest that human anti-GBM nephritis may occur if the GBM is altered so that it becomes cross-reacting and induces autoantibodies, as does homologous GBM. (Am J Pathol 1983; 113:125–133)

IN SHEEP given injections of heterologous glomerular basement membrane (GBM) and complete Freund's adjuvant (CFA) progressive glomerulonephritis and anti-GBM antibodies develop.[1,2] Some of the antibodies react with the sheeps' own GBM,[3] and these autoantibodies may induce transient[3,4] or progressive[5,6] anti-GBM glomerulonephritis. For theoretic reasons, we assumed that sheep would be tolerant to their own GBM,[1] so we first used heterologous GBM to break tolerance. Later, in a preliminary abstract,[7] we reported that pooled homologous GBM and CFA can also induce anti-GBM nephritis. Because sheep easily develop fulminating nephritis[8] and because their large kidneys provide ample GBM antigen, we tested the ability of autologous GBM to induce anti-GBM autoantibodies and nephritis in sheep. We now describe the relative nephritogenic efficacy of autologous GBM, single-kidney homologous GBM, and equivalent weights of heterologous GBM, injected with CFA in each sheep, and we compare the specificity of autoantibodies induced by heterologous or homologous GBM. Moreover, we show that nephritic sheep do not bind autoantibodies or develop Goodpasturelike lesions in their lungs.

Materials and Methods

General Experimental Plan

Healthy 15- to 18-month-old sheep of mixed breeds, free of proteinuria, were housed on a farm

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and divided into 7 groups: 6 sheep received autologous GBM preparations and CFA (Group I); 6 sheep received single-kidney homologous GBM preparations and CFA (Group II); 3 sheep received rabbit GBM, and 2 received rat GBM, all with CFA (Group III); 2 sheep received only CFA (Group IV); 2 sheep received human GBM and CFA (Group V); 13 sheep were age-matched, CFA-treated controls (Group VI); 10 sheep were age-matched, untreated controls (Group VII). Prior to immunization all sheep in Groups I–V had a left nephrectomy. The excised kidneys served as controls for kidney changes in the immunized sheep and provided the autologous and homologous GBM antigens used to immunize Groups I and II. The experiment was terminated on Day 190.

Preparation of Antigens and Immunization of Sheep

Human, rabbit, rat, and sheep GBMs were prepared and emulsified with CFA as described.[1] To increase the amount of autologous GBM antigen, the supernatant of the low-speed (550*g*)-centrifuged, sonicated glomeruli was centrifuged at high speed (28,400*g*) for 20 minutes. This high-speed sediment contained small fragments of GBM. The high-speed supernatant contained still smaller and lighter fragments and possibly solubilized GBM. The GBM and high-speed sediment were each made up to 15 mg (wet weight)/ml of emulsion. The high-speed supernatant was dialyzed against water, lyophilized, and made up to 1.5 mg (dry weight)/ml of CFA emulsion. Each sheep in Group II was likewise given the GBM, high-speed sediment, and supernatant from a *single* homologous kidney. In order to compare equivalent amounts of heterologous GBM, 15 mg (wet weight) of rat or rabbit GBM/ml of emulsion was used. As a positive control 25 mg (wet weight) of human GBM/ml of emulsion was used because this antigen concentration was always effective.[1] The sheep were immunized every 2 weeks with 4 ml of CFA emulsion, with or without GBM antigen, intradermally in the axillary, inguinal, or nuchal region. Groups I and II received successively GBM, then high-speed sediment, and finally high-speed supernatant until the sheep died of nephritis or nonrenal causes or used up all of the antigens. Sheep receiving only CFA were given 12 injections.

Blood Urea Nitrogen (BUN) and Urine Protein Determinations in Sheep

Serial BUN determinations were done by standard automated procedures. Clean urine specimens were easily obtained by holding the sheep's nose until it became hypoxic and reflexly urinated. The urine was examined for protein by heat and acetic acid and for hematuria by microscopy.

Light Microscopy

All kidney and lung tissues were fixed in formalin, sectioned at 4μ, and stained with hematoxylin and eosin, periodic acid–Schiff (PAS) and alcian blue, Congo red, Verhoeff's elastic tissue stain, or silver methenamine. The fixed glomerular cells per glomerular cross-section were counted in the 20 glomeruli having the largest diameter in each kidney of each sheep in Groups I, II, and IV before immunization and at the termination of the experiment.

Elution Studies

GBM of sheep with anti-GBM nephritis was eluted with citric acid buffer, pH 3.2, as described.[9] Dialyzed eluates were lyophilized and made up to a 1% solution in phosphate-buffered saline (PBS). Lyophilized material from kidneys with no evidence of anti-GBM nephritis was made up to a 2% solution in PBS. Lung tissues from sheep with anti-GBM nephritis were eluted with citric acid buffer, as described.[10] Supernatants of the centrifuged eluates were dialyzed against water, lyophilized, and made up to a 5% solution in PBS.

Immunofluorescence Studies

Unfixed 4-μ cryostat sections of sheep kidney or lung were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit antiserums to sheep IgG and complement component 3 (C3), prepared as described.[9] The intensity of fluorescence was graded on a scale of 0, or trace, to 4+.

Autoantibodies were sought in serum or kidney and lung eluates of sheep injected with heterologous or sheep GBM by indirect immunofluorescence (IF) on a preinjection autologous or homologous kidney overlaid with the eluate or serum. The specificities of antibodies in the serum and kidney eluates of nephritic sheep were studied by indirect IF on cryostat sections of human and sheep, adult or fetal lung and kidney; on human skin, placenta, and skeletal and heart muscle; on rat kidney, ocular muscles, skin, lung, and eye; and on monkey eye. Human fetal and adult tissues were obtained at autopsy from the University of Chicago.

Absorption Studies

Sheep kidney eluates and serums that showed antibody activity were absorbed twice with various antigens as described.[10] Briefly, 1 ml of eluate (diluted

1:2) or serum (diluted 1:4) was absorbed with 50 mg (wet weight) of human or sheep GBM; 300 mg (wet weight) of sheep or human lung basement membrane (LBM); 50 mg of mouse liver powder; 20 mg of whole, heat-killed nephritogenic Group A streptococci; or 1 ml of human red blood cells (RBCs) (Group AB, Rh+). If the titer of antibodies was very high against human GBM and LBM, we also absorbed the serum at a dilution of 1:640 to avoid using excess absorbing material. Human and sheep LBM[9] and GBM[1] made as described were used for absorption; the supernatants were tested by indirect IF for antibodies.

Additional Observations on Treated and Untreated Sheep

We have studied over 250 sheep in various past experiments. Growing sheep have an increase in spontaneous deposits of IgG and C3 in their glomeruli and in the number of fixed glomerular cells.[8,10,11] Sixty-four sheep given heterologous GBM developed anti-GBM nephritis, and 62 died from it by Day 100; 13 of 17 sheep given pooled homologous GBM and CFA developed anti-GBM nephritis, but only 7 died from fulminating nephritis before Day 100. Four sheep given autologous GBM, prepared as in this report, did not develop anti-GBM nephritis.

This experience provides a basis for analysis of the functional, morphologic, and immunohistologic status of kidney glomeruli of untreated and variously treated sheep. This experiment was designed with these facts in mind.

Results

Immunization of Sheep

All sheep given heterologous GBM and CFA (Groups III and V) developed progressive anti-GBM glomerulonephritis, characterized by an abrupt onset of proteinuria, hematuria, casts, azotemia (Table 1). All kidneys had petechiae and histologic changes as described previously.[1] Hemorrhagic, exudative, necrotizing glomerular changes were rapidly followed by extensive crescent formation.

Three of 6 sheep given homologous GBM and CFA also developed the clinical and morphologic features of progressive anti-GBM glomerulonephritis (Figure 1, Table 1). The other 3 sheep given homologous GBM showed no evidence of anti-GBM antibodies or nephritis.

Table 1—Clinical Course, Histology, and Immunohistology in Sheep Given Autologous, Homologous, or Heterologous GBM

Antigen (no. of sheep)	GBM* (mg wet wt) (range)	High-speed product: Sediment (mg wet wt) (range)	High-speed product: Lyophilized supernatant (mg dry wt) (range)	Day of onset of azotemia (range)	Day of death (range)	BUN at sacrifice (mg/100 ml) (range)	Immunofluorescence patterns of IgG and C3 at sacrifice†: GBM	Immunofluorescence patterns of IgG and C3 at sacrifice†: TBM	Kidney lesions‡
Autologous									
6	219-315	120-232	15-35	None	131-190§	20-35	Mesangial 1-3+ VRP 1-3+	0	Glomerular amyloidosis (3)‖ Arterial disease (1)
Homologous									
3	180-383	140	None	32-88	38-88	41-360	Linear 3-4+	10-70% 1-4+	Acute (3)
3	195-375	180-330	23-30	None	80-190§	20-30	Mesangial TR-3+ VRP 1-3+	0	Glomerular amyloidosis (1)‖ Arterial disease (1)
Heterologous									
2	300	None	None	39-41	48	>300	Linear 3-4+	5-90% 3-4+	Acute (2)
5	180	None	None	31-34	37-70	>300	Linear 3-4+	5-90% 3-4+	Acute (5)

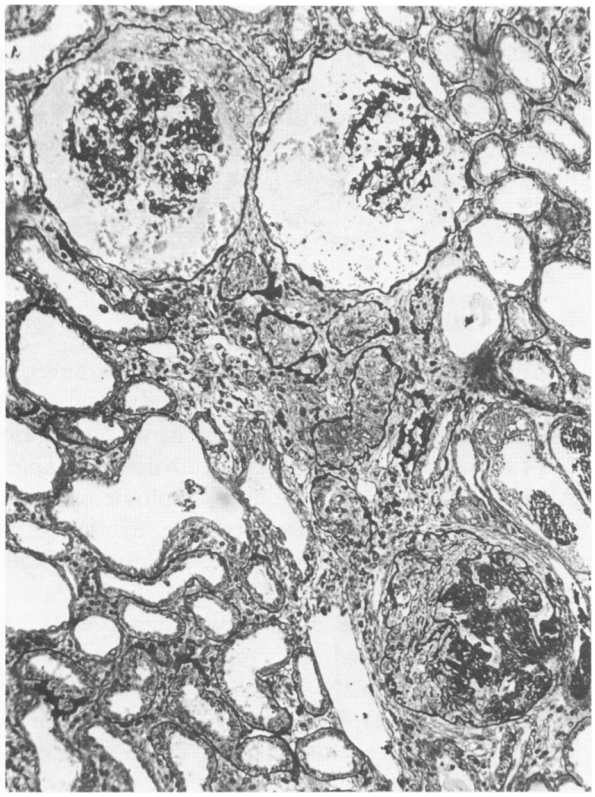
* In all 7 sheep given heterologous GBM and 2 sheep given homologous GBM nephritis developed in three injections. One homologous GBM-injected sheep got 7 injections (including 140 mg of high speed sediment). All sheep given autologous GBM and 3 sheep given homologous GBM received 6-12 injections of GBM antigens.

† The IF patterns of staining for IgG and C3 were identical in nephritic kidneys, but segments of TBM from 5-10% of tubules from nonnephritic kidneys reacted with anti-C3 serum. VRP, vascular root pattern, a characteristic, intense, granular, IF pattern in the hilar area.

‡ Acute: hemorrhagic, exudative, necrotizing, crescentic glomerulonephritis. Arterial disease: severe intimal and/or medial proliferation. The number in parenthesis is the number of animals with the indicated lesion.

§ Two sheep given homologous GBM died on Days 80 and 161, and 3 sheep given autologous GBM died on Days 48, 105, and 135 of nonrenal causes.

‖ Focal, segmental, and global masses of IgG and C3 correspond to amyloid deposits in the glomeruli.



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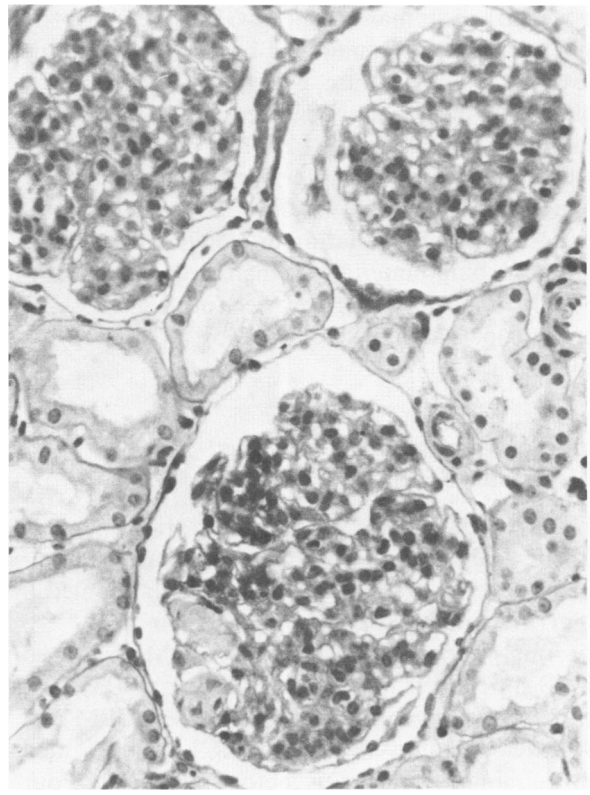

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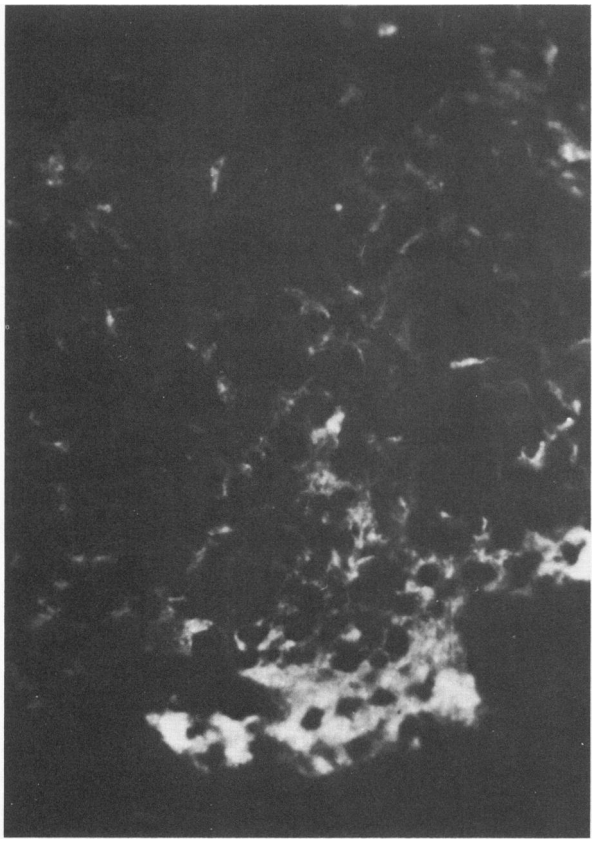

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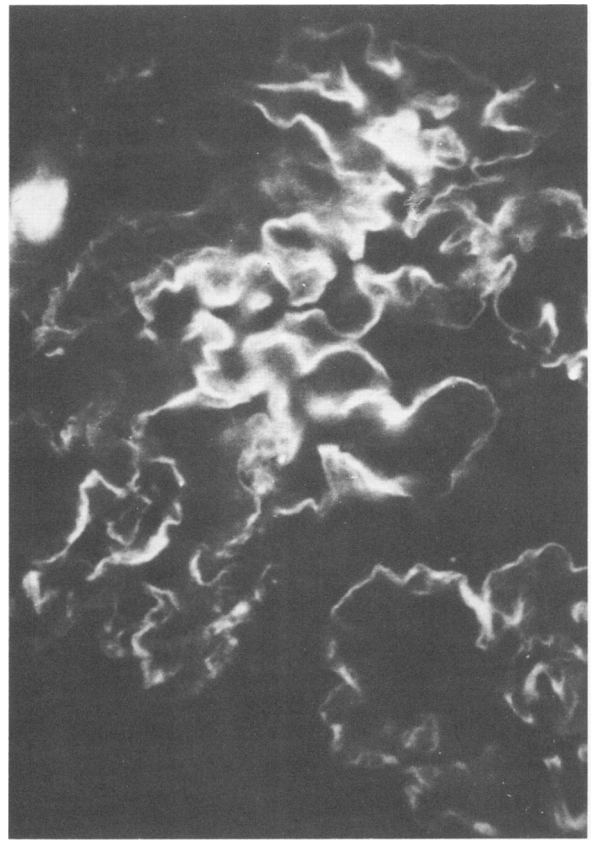

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By contrast, none of the sheep given autologous GBM and CFA or CFA alone had any evidence of anti-GBM antibodies or nephritis (Figure 2, Table 1). Three of the sheep given autologous GBM and 6 of the 13 sheep given CFA-only developed proteinuria without hematuria or azotemia on Days 90–206.

The average number of fixed glomerular cells/glomerular cross-section in animals in Groups I, II, and IV at the end of the experiment was not significantly different from the number in the preinjection kidneys nor from the number in the renal biopsy specimens of the age-matched controls in Groups VI and VII. This result suggests the number of fixed glomerular cells, which increases in growing sheep,[8,10,11] tends to level off in sheep about 18–24 months of age.

In addition to anti-GBM nephritis, the following pathologic changes in the kidneys were seen in the experimental animals. Glomerular amyloidosis was found in 3 of 6 sheep given autologous GBM, 1 of 6 sheep given homologous GBM, and 6 of 13 sheep given CFA only. Focal segmental intimal and/or medial and/or nodular proliferative arterial lesions (with obliteration of some arterial lumens) were found in 1 of 6 sheep given autologous GBM, 1 of 6 sheep given homologous GBM, and 4 of 13 sheep given CFA only. Foci of interstitial nephritis were seen in a few animals in each group, including 2 untreated control animals in Group VII. One or more of these conditions were invariably seen when proteinuria was found. No amyloidosis, arterial vasculopathy, or evidence of anti-GBM nephritis was seen in the preinjection renal biopsies or in the kidneys of the 10 untreated control sheep (Group VII).

At the end of the experiment on Day 190, 1 sheep given homologous GBM, 1 sheep given autologous GBM, and 1 sheep given CFA only—none of which had evidence of anti-GBM antibodies or nephritis—were given human GBM and CFA. The antigen and immunization schedule used in Group V was used. All 3 died of fulminating anti-GBM nephritis in 37–40 days. This outcome proved that these sheep could develop anti-GBM nephritis with the right antigenic stimulus and that treatment with autologous or homologous GBM had not rendered them tolerant or hyperresponsive.

Immunofluorescence in Sheep Kidneys

In the preinjection renal biopsies all glomeruli stained for IgG and C3 by IF in a variable, irregular mesangial pattern, as previously described.[10] Sections through the vascular pole usually showed a more intense granular or confluent staining for IgG and C3 in the hilar area, a staining that we called the vascular root pattern (VRP).[3,10] At death from nonrenal causes or at the end of the experiment, all sheep given autologous GBM and 3 sheep given homologous GBM had similar variable VRP and mesangial-type staining for IgG and C3 in the glomeruli (Table 1, Figure 3). This staining was indistinguishable from that in the age-matched, CFA-treated, and untreated control sheep.

By contrast, the kidneys of all sheep given heterologous GBM and 3 sheep given homologous GBM had a characteristic, intense (4+) linear staining for IgG and C3 along all of the peripheral GBM and the TBM of a variable number of proximal tubules (Figure 4, Table 1).

Light and Immunofluorescence Microscopic Study of Lungs

None of the sheep given heterologous or sheep GBM had lung hemorrhages during life or at autopsy. The light-microscopic findings were identical to those described for sheep given homologous and autologous LBM.[10] Several sheep had lung parasites. All sheep given CFA, with or without GBM antigens, had focal or confluent granulomas. At sacrifice, the alveolar walls from animals given injections were much thicker than the thin, largely acellular alveolar walls of untreated sheep. There were no alveolar hemorrhages or stigmata of Goodpasture's disease.[10] By IF microscopy there was no linear staining for IgG along the alveolar LBM in any lung from sheep with anti-GBM nephritis or other treated sheep.

Indirect Immunofluorescence of Serum

By indirect IF the serums in all sheep with anti-GBM nephritis induced by heterologous GBM con-

Figure 1—Kidney from sheep with anti-GBM nephritis induced by homologous GBM. Three glomeruli are in various phases of damage, from early hemorrhage into Bowman's space to extensive crescent formation. Interstitial fibrosis and cellular infiltrates, as well as tubular atrophy and dilation, are present. (Silver methenamine, ×120) **Figure 2**—Kidney from sheep given autologous GBM and CFA. There are focal segmental deposits of amyloid in one glomerulus. The cellular architecture, excluding the amyloid, is representative of glomeruli from all sheep given autologous GBM and sheep given CFA only (H&E, ×300) **Figure 3**—Cryostat kidney section from sheep injected with autologous GBM, showing typical granular and confluent mesangial staining for IgG by IF. The staining is more intense at the hilum (VRP) and decreases toward the periphery. This pattern is representative of all autologous and 3 of the nonnephritic homologous GBM-treated sheep or all age-matched controls. (×535) **Figure 4**—Typical linear staining for IgG by IF along peripheral GBM, characteristic of autoantibodies reacting with GBM of sheep with anti-GBM nephritis. In this case the nephritis was induced by homologous GBM. (×535)

tained antibodies that reacted with human, rat, or rabbit GBM and LBM. Six of the 7 serums had enough autoantibodies to react with the preinjection autologous or homologous GBM and TBM, but all 6 serums reacted equivocally (±) with the LBM from 9 sheep. The titers of antibodies in one representative serum from a sheep given human GBM were 80 against sheep GBM, 8,192 against human LBM, and 16,384 against human GBM and TBM. The nephritic serum reacted with the GBM, TBM, and Bowman's capsule from a 14-week human fetus and from both a 2-kg and a 250-g sheep fetus. Absorption of the nephritic serum (diluted 1:4) with human GBM or LBM removed all antibodies reactive with sheep GBM or LBM but left antibodies reactive with human GBM or LBM (Table 2). At a dilution of 1:640, absorption of the nephritic serum with human GBM, but not with sheep GBM, removed all antibodies reactive with human GBM, TBM, or LBM. Absorption with human LBM left a residual group of antibodies reactive only with human GBM.

A different situation was found in the serums of sheep with anti-GBM nephritis induced by homologous GBM. These serums reacted with human and rat GBM and TBM but only equivocally with human LBM. Only two reacted with autologous and homologous GBM and TBM, but these two serums reacted equivocally with the LBM from 9 sheep (Table 3). The serum antibody titers of 1 representative sheep were 8 against rat and human GBM, 32 against sheep GBM, and 0 against human and sheep LBM. The nephritic serum reacted with the GBM and TBM from a 14-week human fetus but not with fetal LBM from sheep and man. Absorption of the nephritic serum (diluted 1:4) with human GBM or LBM removed all antibodies against human or rat GBM but left antibodies reactive with fetal and adult sheep GBM, ie, sheep-specific autoantibodies (Table 3). Absorption of the serum with sheep GBM or LBM removed all antibodies reactive with sheep GBM.

No autoantibodies were found in the serums of all 6 sheep given autologous GBM or in 4 of 6 sheep given homologous GBM (1 of which had anti-GBM nephritis) or in any serum obtained from the CFA-treated or untreated control sheep on Days 60–190.

Elution Studies of Kidneys and Lungs

Autoantibodies were identified in the kidney eluates of all sheep with anti-GBM nephritis induced by heterologous GBM. By radial immunodiffusion about 420 μg of IgG/ml was present in a representative eluate from a sheep given human GBM. When this nephritic eluate was layered over sheep and human kidney and lung and tested by indirect IF, the human and sheep GBM and TBM and human LBM stained in an intense (4+) linear pattern (Table 2). However, the staining of sheep LBM was equivocal (±) on repeated tests with 8 sheep lungs. The titer of antibodies in this eluate was 16 against human or sheep GBM. The eluted autoantibodies also reacted with the GBM, TBM, and Bowman's capsule from a monkey, rabbit, rat, guinea pig, and both a 2-kg and a 250-g fetal sheep; with a 14-week fetal human kidney; and with fetal human LBM. They reacted with membranous structures in blood vessel walls and subsyncytial membranes of human placenta and with the sarcolemma and capillaries of skeletal muscle and

Table 2—Specificity of Autoantibodies in Serum and GBM Eluates of Sheep With Nephritis Induced by Human GBM

	Indirect immunofluorescence				Immunodiffusion for IgG
	Sheep		Human		
	Kidney†	Lung‡	Kidney	Lung	
GBM eluate*					
Unabsorbed (diluted 1:4)	4+	±	4+	4+	+
Absorbed with‖					
Group A streptococci, human RBC, or mouse liver powder	4+	±	4+	4+	+
Human GBM or LBM	0	0	0	0	0
Sheep GBM or LBM	0	0	0	0	0
Serum§					
Unabsorbed (diluted 1:5)	4+	±	4+	4+	Not applicable
Absorbed with human or sheep GBM or LBM	0	0	3+	2+	
Unabsorbed (diluted 1:640)	0	0	4+	4+	
Absorbed with‖					
Human GBM	0	0	0	0	
Human LBM	0	0	1+	0	

* Eluate antibody titer: 16 against sheep or human GBM.
† Linear staining along both GBM and TBM.
‡ Linear staining along LBM.
§ Serum antibody titers: 16,384, 8,192, and 80 against human GBM, human LBM, and sheep GBM, respectively.
‖ Diluted 1:640.

heart, basement membranes of the dermal–epidermal junction, hair follicles, sebaceous glands, and sweat glands and ducts of skin in man and rat. They also reacted with the sarcolemma of ocular muscles, lens capsule, Descemet's membrane, and choroid capillaries of monkey and rat eye and with rat LBM. Absorption of the nephritic eluate with sheep or human GBM or LBM abolished staining against all human, sheep, and rat tissues tested (Table 2).

Again, a marked difference was found in the kidney eluates of 2 sheep with anti-GBM nephritis induced by homologous GBM. When a representative eluate, containing about 620 μg IgG/ml, was layered over sheep and human kidney and lung and tested by indirect IF, the human and sheep GBM stained intensely, the TBM of sheep and man less so. However, both human and sheep LBM stained equivocally. The titers of this eluate were 20 and 60 against human and sheep GBM, respectively. It reacted with the GBM and less with the TBM from a 14-week fetal human kidney and both a 2-kg and a 250-g fetal sheep and with the lens capsule and Descemet's membrane of rat and monkey eye. Absorption of this eluate with human GBM or LBM removed all autoantibodies reactive with human GBM but left a residual population of autoantibodies reactive with sheep GBM, ie, sheep-specific autoantibodies (Table 3). Absorption of the eluate with either sheep GBM or LBM removed all autoantibodies against sheep or human tissues.

The eluates of kidneys from all sheep given autologous GBM, 3 of the 6 sheep given homologous GBM, all sheep given CFA only, and untreated control sheep did not contain antibodies against sheep or human GBM. Eluates of lungs from nephritic sheep did not contain autoantibodies or react with human GBM or LBM.

Discussion

All sheep given heterologous GBM and 3 of 6 sheep given homologous GBM antigens and CFA had clinical, morphologic, and immunopathologic features of progressive, crescentic anti-GBM glomerulonephritis.[1,2] By contrast, none of the 6 sheep given autologous GBM or 3 of 6 sheep given homologous GBM and CFA developed any evidence for anti-GBM nephritis. The staining by direct IF of their kidneys was indistinguishable from that in the age-matched, CFA-treated, or untreated control animals and reflects the spontaneous deposits of IgG and C3 seen in untreated sheep.[10,11]

The nephritogenic efficacy of heterologous, single-kidney homologous, and autologous GBM in this study is comparable to that in our previous experience (summarized in Materials and Methods). In the present study we did not have enough test animals to distinguish single-kidney homologous GBM (3/6; number with nephritis/total number of sheep tested), from autologous (0/6) or from heterologous GBM (5/5). However, if we add our previous result with autologous GBM (0/4) to that for autologous GBM in this study, we get 0/10, which is significantly different ($P = 0.035$, Fisher's exact test) from the result for single-kidney homologous GBM. Thus, we show clearly that sheep are very tolerant to their own GBM, but this tolerance is easily broken by heterologous or homologous GBM.

In a similar experiment,[10] homologous LBM failed to induce anti-GBM antibodies or nephritis. The explanation may be that the sheep given homologous LBM came from a semi-closed colony of closely related Suffolk sheep, and there may have been less antigenic differences between homologous LBMs in the Suffolk sheep than between homologous GBMs from

Table 3—Specificity of Autoantibodies in Serum and GBM Eluates of Sheep With Nephritis Induced by Homologous GBM

	Indirect immunofluorescence				
	Sheep		Human		Immunodiffusion for IgG
	Kidney†	Lung‡	Kidney	Lung	
GBM eluate*					
Unabsorbed (diluted 1:2)	4+	±	4+	±	+
Absorbed with					
Group A streptococci, human RBC, or mouse liver powder	4+	±	4+	±	+
Human GBM or LBM	2+	0	0	0	+
Sheep GBM or LBM	0	0	0	0	0
Serum§					
Unabsorbed (diluted 1:4)	4+	±	3+	±	Not applicable
Absorbed with human GBM or LBM	2+	±	0	0	
Absorbed with sheep GBM or LBM	0	0	0	0	

* Eluate antibody titers: 20 and 60 against human and sheep GBM, respectively.
† Linear staining along both GBM and TBM.
‡ Equivocal staining along LBM.
§ Serum antibody titers: 8 and 32 against human GBM and sheep GBM, respectively.

the mixed-breed sheep used in the present study. Rabbits immunized with autologous kidney sediment have been reported[12] to develop a little antibody but no disease. However, no confirming evidence was sought for autoantibodies in the serum or kidney eluates. If autoantibody were present, it might be due to a species difference in handling autologous GBM or to alteration of the autologous antigen during preparation, thus making it immunogenic.

The renal amyloidosis[13] and proliferative arterial lesions noted in this study were probably induced by injections of CFA, but we are unaware of any prior association of arterial vasculopathy induced by injections of CFA.

Autoantibodies to GBM and LBM were found in the serum and kidney eluates of sheep with anti-GBM nephritis induced by human, rat, or rabbit GBM and CFA but not in the autologous-GBM-treated sheep or controls. The specificity of antibodies in human-GBM-induced nephritis shows that human and sheep GBM and LBM share closely related antigenic determinants (Table 2). The equivocal staining of sheep LBM with autoantibodies by indirect IF was unexpected and may be due to less shared antigenic determinants per unit area of sheep LBM. Identical results were obtained with autoantibodies induced in sheep by human LBM.[10] Autoantibodies reactive with sheep and human GBM and LBM were absorbed out of the eluates by sheep LBM preparations, thus demonstrating that sheep LBM does contain antigenic determinants in common with sheep GBM and human GBM and LBM. Moreover, sharing by sheep and human LBM of common antigenic determinants has been shown previously.[10] The equivocal staining by IF of sheep LBM with autoantibodies *in vitro* could explain the absence both of autoantibodies along the sheep LBM *in vivo* and of alveolar hemorrhages. Perhaps human LBM has more antigenic determinants capable of reacting with autoantibodies *in vivo*.

The fact that these eluted autoantibodies reacted with basement membranes of various adult and fetal human and sheep organs and with rat, monkey, guinea pig, and rabbit organs indicates that these autoantibodies are not species- or organ-specific and are not even specific for vascular basement membranes; yet they seem to injure only the kidney. Similar autoantibodies were induced in sheep by human LBM.[10] These findings confirm that anti-GBM nephritis is an exception to two old dogmas: that autoimmune diseases are induced by organ-specific antigens and that they are preferably induced by autologous antigens.[8]

The autoantibodies were reactive with basement membranes of diverse function and embryologic origin.[14] The distribution of identical or closely related autoantigenic determinants in extrarenal structures is unexpectedly widespread and suggests some unifying structural and/or functional purpose(s).

Because serums from sheep with human-GBM-induced anti-GBM nephritis contained high titers ($\geq$8,192) of antibodies against human GBM and LBM and a low titer (80) against sheep GBM, only a small fraction of the total antibodies were autoantibodies. These were true autoantibodies, however, reacting with autologous GBM and TBM as well as with adult and fetal, sheep and human GBM and TBM and human LBM.

In contrast to the single population of autoantibodies induced by heterologous GBM, the autoantibodies induced by homologous GBM consisted of two populations. One was similar to that described above for human GBM-induced autoantibodies and reacted with antigens common to sheep and human GBM and LBM. Its equivocal reaction by indirect IF with human LBM was unexpected, but removal of all autoantibodies reacting with human GBM in serum or eluate by absorption with human LBM indicated the presence of a common antigen in human LBM. Thus the equivocal reaction with human LBM may result from a lower density of common antigenic determinants in human LBM than in GBM. Evidence of a second population of sheep-specific autoantibodies was indicated by the serum antibody titers (20 against human GBM, 80 against sheep GBM) and confirmed when absorption of the serum or eluates with human GBM or LBM left only autoantibodies reactive with sheep GBM (Table 3). Because absorption with sheep LBM removed all staining against sheep GBM, there were no sheep kidney-specific autoantibodies.

Studies in anti-GBM nephritis in man show that a few kidney eluates do not bind to heterologous GBM,[15] suggesting that a few patients do have human-specific anti-GBM autoantibodies. If human kidney eluates demonstrating species cross-reactivity were shown to also contain a second population of human-specific autoantibodies, this would also indicate an endogenous origin of immunogenic stimuli.

None of the sheep given heterologous, homologous, or autologous GBM, not even those sheep with anti-GBM nephritis, had lung hemorrhages or lung lesions like those reported in Goodpasture's disease. The sheep given CFA, with or without antigens, did develop extensive granulomas in the lung, but these changes are a well-known effect of CFA.[10]

Focal linear deposits of IgG along human LBM and autoantibodies to GBM and LBM in lung eluates have been found in Goodpasture's syndrome.[16] However, we were unable to induce alveolar hemorrhages or *in vivo* binding of autoantibodies to sheep LBM in nephritic sheep despite the production of autoantibodies to sheep LBM. Moreover, similar results were

found in sheep with anti-GBM nephritis induced by human LBM.[10] Some important questions are thus again raised about the precise role of autoantibodies in Goodpasture's syndrome.[10]

We showed that adult human and sheep GBM, LBM, and TBM antigenic determinants are present early in the fetus, as previously described.[10] GBM is thus exposed to the bloodstream during most of fetal and all of adult life, a situation conducive to lifelong tolerance to self-antigens.[17] Because there are traces of GBM-related antigens in the blood of animals,[18,19] we have proposed that sheep have a low-zone tolerance to GBM and LBM[2]; ie, T cells are tolerant, but B cells are not. In another experiment,[10] this precarious state of tolerance was easily broken by heterologous LBM, but not by homologous or autologous sheep LBM. In contrast, the tolerance in this study was broken by *homologous* sheep GBM from a single kidney as well as by pooled homologous GBM.

Because the specificity of autoantibodies induced by homologous GBM in sheep may be similar to that in human non-Goodpasture anti-GBM nephritis,[15] we propose a hypothesis for non-Goodpasture anti-GBM nephritis. Some unknown event may alter the GBM so that it becomes a cross-reacting antigen, like homologous GBM, and induces autoantibodies of a restricted specificity that do not react easily with LBM *in vivo*.

A study of the specificity of autoantibodies in human anti-GBM nephritis, as described in this report and previously,[10] might indicate whether the immunogenic stimulus in human anti-GBM disease is the GBM, LBM, or one of the other extrarenal sources of GBM-related antigens. This study clarifies a report[20] on the difference in specificity between kidney eluate and serum antibody in sheep given human GBM by injection. We show here that eluates contain only autoantibodies, whereas sheep serums contain large titers of heteroantibody as well.

Although anti-GBM autoantibodies are generally accepted as causing renal disease, a number of questions remain unanswered, such as the circumstances that induce the formation of pathogenic autoantibodies and the unknown factors that allow autoantibodies to localize in and injure the lung.[21]

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