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Influence of antigen distribution on the mediation of immunological glomerular injury

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Influence of antigen distribution on the mediation of immunological glomerular injury. To determine if the site of immune reaction could influence the mediation and morphological expression of glomerular injury in experimental anti-glomerular basement membrane (anti-GBM) nephritis and membranous nephropathy, we studied the events that followed the in situ reaction of rat antibody with antigen planted in either the GBM (especially the lamina rara interna) or in the subepithelial space (SE). Non-nephritogenic amounts of noncomplement-fixing sheep anti-GBM or anti-tubular brushborder antibody were injected into separate groups of rats to plant sheep IgG in the GBM and SE, respectively. Kidneys containing sheep IgG were then transplanted into naive recipients that were passively immunized with rat anti-sheep IgG. There was marked proteinuria after 2 days (antigen in GBM: 226 ± 50.7; antigen in SE: 69 ± 50.7 mg/24 hr) that was abrogated by prior depletion of complement in both groups (antigen in GBM: 10.2 ± 1.7; antigen in SE: 14.3 ± 8.7 mg/24 hr). When antigen was planted in SE, inflammatory-cell depletion with either anti-neutrophil (PMN) serum or lethal irradiation had no effect on proteinuria. In contrast, anti-PMN abolished proteinuria (12.0 ± 5.6 mg/24 hr) and irradiation reduced it by 60% when antigen was in GBM. Glomeruli of kidneys with antigen in GBM were significantly larger and more hypercellular than those with antigen in SE after transplantation into immunized recipients. Endothelial cell injury and adherence of inflammatory cells to denuded GBM were prominent in the former (antigen in GBM), while glomeruli with antigen in SE showed only subepithelial deposits, adjacent slit-diaphragm displacement, and epithelial cell foot-process effacement. Thus, the reaction of antigen and antibody in glomeruli produced complement-mediated injury which was cell-independent when complex formation occurred on the outer aspect of the GBM but was cell-dependent when the same reagents reacted more proximally to the circulation. We therefore conclude that antigen distribution can critically influence the mediation and morphologic expression of immune glomerular injury and may, in part, account for variations in the clinical and histological manifestations of antibody-induced glomerular disease in humans.

Influence de la distribution antigénique sur la médiation des lésions glomérulaires immunologiques. Afin de déterminer si le site de la réaction immune pourrait influencer la médiation et l'expression morphologique des lésions glomérulaires lors d'une néphrite expérimentale anti-membrane basale glomérulaire (anti-GBM) et d'une néphropathie extra-membraneuse, nous avons étudié les événements qui suivaient la réaction in situ d'anticorps de rat avec un antigène fixé soit dans la GBM (surtout dans la lamina rara interna), soit dans l'espace sous-épithélial (SE). Des quantités non nephritogènes d'anticorps anti-GBM, ou anti-bordure en brosse tubulaire de mouton ne fixant pas le complément ont été injectées à différents groupes de rats pour fixer de l'IgG de mouton dans la GBM et le SE, respectivement. Les reins contenant l'IgG de mouton étaient alors transplantés à des receveurs vierges passivement immunisés avec de l'IgG de rat anti-mouton. Il existait une protéinurie marquée après deux jours (antigène dans la GBM: 226 ± 50,7; antigène dans SE: 69 ± 50,7 mg/24 hrs) qui à été abrogé par une déplétion du complement dans les deux groupes (antigène dans la GBM: 10,2 ± 1,7; antigène dans SE: 14,3 ± 8,7 mg/24 hr). Lorsque l'antigène était fixé dans SE, une déplétion en cellules inflammatoires par du sérum anti-neutrophile (PMN) ou une irradiation léthale n'avaient pas d'effet sur la protéinurie. A l'opposé, l'anti-PMN supprimait la protéinurie (12,0 ± 5,6 mg/24 hr) et l'irradiation la réduisait de 60% lorsque l'antigène était dans la GBM. Les glomérules de reins ayant l'antigène dans la GBM étaient significativement plus gros et plus hyper-cellulaires que ceux ayant l'antigène dans SE après transplantation chez des receveurs immunisés. Les lésions cellulaires endothéliales et l'adhérence des cellules inflammatoires à des GBM nues étaient prédominantes chez les premiers (antigène dans la GBM) alors que les glomérules ayant l'antigène dans SE présentaient uniquement des dépôts sous-épithéliaux, un déplacement du slit-diaphragme adjacent et un effacement des pédicelles des cellules épithéliales. Ainsi, la réaction d'un antigène et d'un anticorps dans des glomérules a produit des lésions à médiation complémentaire indépentantes des cellules lorsque la formation de complexes survenait dans la partie extérieure de la GBM, mais dépendantes des cellules lorsque les mêmes réactifs interagissaient de façon plus proximale dans la circulation. Nous concluons donc que la distribution antigénique peut influencer de manière critique la médiation et l'expression morphologique des lésions glomérulaires immunes et qu'elle peut, en partie, rendre compte de variations dans les manifestations cliniques et histologiques de glomérulopathies à médiation par anticorps chez l'homme.

In glomerular diseases such as idiopathic membranous nephropathy and membranous lupus nephritis, immune deposits are located in the subepithelial space, and glomerular injury manifests as a relatively bland lesion without substantial cellular proliferation or exudation. In contrast, in diseases in which immune reactants are located in closer proximity to the circulation, such as lupus nephritis with subendothelial deposits or antiglomerular basement membrane (anti-GBM) nephritis, extensive proliferation and inflammatory cell infiltration are characteristic. In the experimental counterparts of these disorders similar observations have been made and different effector systems have been found to mediate the development

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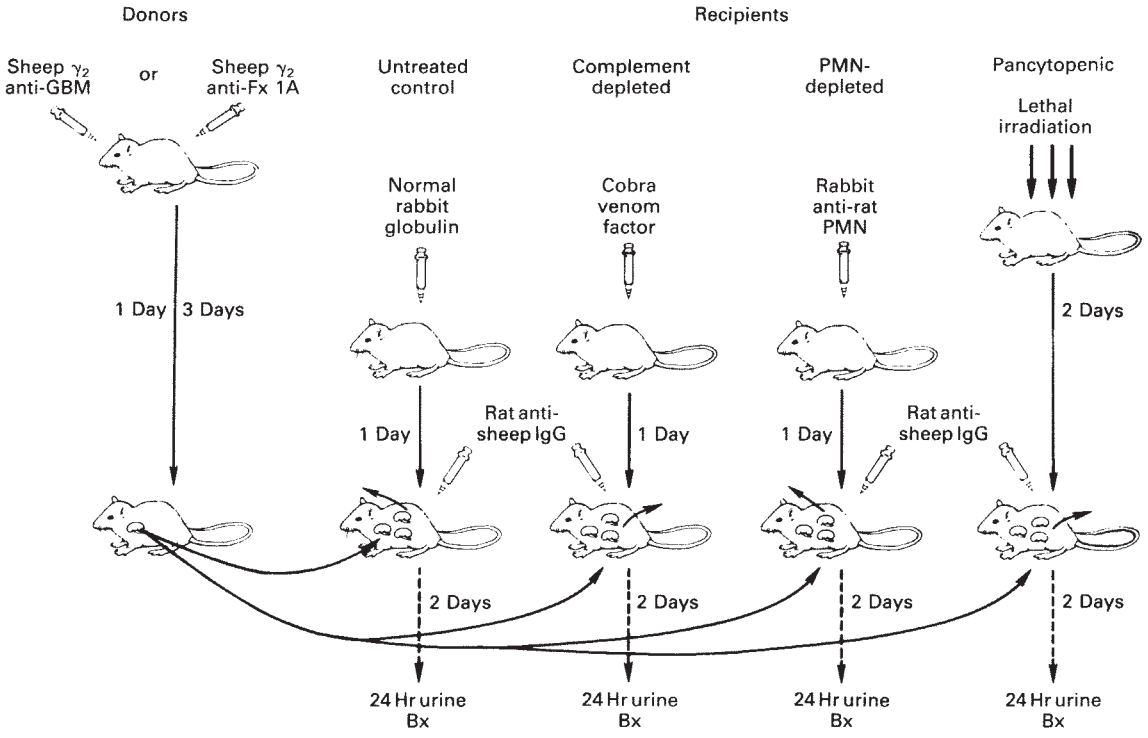

Fig. 1. *Protocol of donor and recipient immunization and recipient treatment.* Kidneys with sheep IgG planted in the GBM or subepithelial space from donors injected with a subnephritogenic dose of either γ2 sheep anti-GBM (2 mg i.v.) or γ2 anti-Fx1A (8 mg i.v.) were transplanted after 1 day or 3 days into naive recipients. Recipients were passively immunized with rat anti-sheep IgG, 1.2 ml or 1.5 ml i.v. respectively. They were treated, as shown, from the day before transplantation until termination 2 days later. Abbreviations are: PMN, polymorphonuclear leukocyte; Bx, renal biopsy.

of glomerular injury [1]. In the autologous phase of passive Heymann nephritis (PHN), host antibody reacts with heterologous IgG located in the subepithelial space; fixation and activation of complement are essential for the development of proteinuria [2]. There is an absence of infiltrating inflammatory cells and equivalent injury occurs in the face of leukocyte depletion. In contrast, the autologous phase of anti-GBM nephritis, in which host or passively administrated anti-IgG reacts with heterologous IgG located in the GBM, requires neutrophils or macrophages for glomerular injury [3–5].

Several factors could be responsible for these differences in mediation and expression of injury, including differences in heterologous antisera, schedules for active or passive immunization, and species characteristics. However, a more likely explanation is that these findings are due to differences in antigen location and the subsequent site of immune reaction. To test this hypothesis, we studied the morphology and mediation of glomerular injury induced by the same antigen and antibody reacting either proximally in the GBM or in the subepithelial space using a transplant model previously described [2]. This model is characterized by in situ reaction of antibody with a planted antigen in the absence of circulating immune complexes. Our results show that the in situ reaction of rat anti-sheep IgG with antigen (sheep IgG) in either location induces proteinuria by a complement-dependent process. However, leukocytes are required when antigen is in the GBM and not when it is in the subepithelial space. These findings are consistent with morphologic observations that demonstrate substantial hypercellularity of glomeruli only in kidneys with planted GBM antigen.

Methods

Experimental design

Two basic models, illustrated in Figure 1, were used in these studies. In both, sheep IgG was planted immunologically in the glomerular capillary wall of rat donor kidneys to serve as antigen. Donor kidneys were then transplanted into bilaterally nephrectomized recipient rats that were immunized passively with rat anti-sheep IgG. In the first model (group 1), sheep IgG was planted in the GBM by injecting subnephritogenic doses (2 mg) of the noncomplement-fixing γ2 subclass of sheep anti-rat GBM 24 hr before transplantation. In the second model (group 2), sheep IgG was planted in the subepithelial space by injecting subnephritogenic doses (8 mg) of γ2 sheep anti-rat proximal tubular brushborder antigen (Fx1A) 72 hr before transplantation. The doses and times chosen to administer sheep IgG antibody relative to transplantation were based on quantitative pilot studies (described below) and the different glomerular binding kinetics of heterologous anti-GBM [6, 7] and anti-Fx1A [8]. In both models, recipients were immunized passively with rat antiserum to sheep IgG (group 1, 1.2 ml i.v.; group 2, 1.5 ml i.v.) injected at the time of transplantation. Thus, in situ reaction of rat antibody with sheep IgG in the GBM or in the subepithelial space occurred in the transplanted kidney from the time of transplantation until sacrifice 48 hr later, in the absence of circulating immune complexes.

Table 1. Glomerular immunofluorescence, proteinuria, and serum creatinine in passively immunized recipients of transplant kidneys containing sheep IgG planted in the GBM (group 1) or in the subepithelial space (group 2)

	N	Glomerular immunofluorescence[a]			Urine protein excretion, day 2 *mg/day*	Serum creatinine, day 2 *mg/dl*
		Sheep IgG	Rat IgG	Rat C3		
Group 1			Linear			
A Unmodified	4	1–2+	3–4+	1–2+	226 ± 50.7 (163–304)[b]	1.4 ± 0.31[b]
B Complement-depleted	3	1–2+	4+	Neg-tr	10.2 ± 1.7 (8.2–11.5)[c]	1.6 ± 0.20
C Neutrophil-depleted	4	1–2+	4+	2–3+	12.0 ± 5.6 (8.7–20.0)[c]	1.2 ± 0.36
D Lethal irradiation	6	1–2+	4+	2–3+	82 ± 43.4 (48–152)[d]	1.2 ± 0.06
Group 2			Granular			
A Unmodified	5	1+	3+	1+	69 ± 50.7 (27–131)	1.3 ± 0.34
B Complement-depleted	4	1+	3+	Neg-tr	14.3 ± 8.7 (6–26)[d]	1.2 ± 0.04

[a] Immunofluorescence was graded as negative (neg) to 4+; tr represents trace.
[b] The values are mean ± SD; the range is in parenthesis.
[c] The value is significantly less than group 1A ($P < 0.028$ Mann-Whitney U test).
[d] The value is significantly less than group 1A or group 2A ($P < 0.005$ Mann-Whitney U test).

After defining the characteristics of the two models, animals in the two groups were further subdivided, as shown in Figure 1 and Table 1, to study the role of complement and inflammatory cells as mediators of the resulting proteinuria. In all recipients, serum creatinine was measured daily, 24-hr urine protein excretion was measured on days 1 to 2 post-transplantation. Renal tissue samples were then obtained for histology, immunofluorescence, and electron microscopy.

Group 1A. In the unmodified experimental group, four rats received a transplant kidney that contained γ2 sheep anti-GBM and were passively immunized with 1.2 ml rat anti-sheep IgG at the time of transplantation. To control for the possible effects of rabbit globulin given to group 1C, they also received 2.5 ml of normal rabbit globulin administered intravenously daily from the day prior to transplantation until termination. Recipients not passively immunized with rat anti-sheep IgG and transplanted with antigen-containing kidneys served as controls.

Group 1B. Four passively immunized recipients of γ2 sheep anti-GBM-containing kidneys were depleted of complement by daily injections of cobra venom factor [2, 9] beginning on the day before transplantation and continuing until termination on day 2. Serum C3 levels were monitored daily by radial immunodiffusion [10] as previously described [2, 9].

Group 1C. Four rats were depleted of neutrophils by daily administration of rabbit anti-rat neutrophil globulin, 2.5 ml i.v., from the day prior to transplantation of a γ2 sheep anti-GBM-containing kidney until termination on day 2 posttransplantation [2, 9].

Group 1D. To evaluate the role of inflammatory cells, six rats were rendered pancytopenic with lethal whole-body irradiation [2] 2 days before receiving a γ2 sheep anti-GBM-containing kidney, and were then passively immunized. Total and differential leukocyte counts were measured daily in all rats in groups 1A, 1C, and 1D [2, 9].

Group 2A. The unmodified experimental group comprised five rats. They received transplant kidneys containing sheep γ2 anti-Fx1A planted in the subepithelial space and were immunized passively with 1.5 ml rat anti-sheep IgG at the time of transplantation.

Group 2B. In four passively immunized recipients of γ2 sheep anti-Fx1A-containing kidneys, serum complement was depleted by daily injection of cobra venom factor beginning on the day before transplantation and continuing until termination. Serum C3 levels were measured daily.

Studies of the effects of leukocyte-depletion with anti-neutrophil serum (group 2C) and irradiation (group 2D) in passively immunized recipients of γ2 anti-Fx1A-containing transplant kidneys have been published previously [2].

Preparation and characterization of γ2 sheep anti-rat GBM and γ2 sheep anti-rat Fx1A

A male sheep was immunized repeatedly with lyophilized rat glomeruli emulsified in complete Freund's adjuvant (DIFCO Laboratories, Detroit, Michigan, USA). Glomeruli were prepared by differential sieving of rat renal cortices as described previously [8], and contained fewer than one tubular element per 100 glomeruli. After four monthly injections of 75 mg of glomeruli, sheep serum was collected, heat-inactivated (56°C, 30 min), and absorbed extensively with rat red cells, platelets, white blood cells, Fx1A, and rat serum absorbed to Sepharose CNBr (Pharmacia Fine Chemical, Inc., Piscataway, New Jersey, USA). The noncomplement-fixing, γ2 subclass of sheep IgG was isolated by ion-exchange chromatography as previously described [2, 9] and was then further absorbed twice (4°C, 12 hr) with lyophilized Fx1A at an IgG:Fx1A ratio of 4–6:1 (w/w). Fx1A was removed by centrifugation at ×78,680 *g* at 4°C and the absorbed IgG was applied to a size calibrated Sephacryl-200 (S-200, Pharmacia Fine Chemical) column to obtain monomeric IgG which was then stored at −70°C until used. Purity and specificity of γ2 anti-rat GBM was assessed by immunoelectrophoresis and micro-Ouchterlony immunodiffusion [11, 12] in 1% agarose gels against rat serum, rat Fx1A, and antiserum to whole sheep serum (Miles Laboratories, Inc., Elkhart, Indiana, USA), and sheep IgG (Cappel Laboratories, Inc., Cochranville, Pennsylvania, USA). The in vitro complement-fixing ability was assessed by indirect immunofluorescence as previously described [9]. Pilot studies were done in which 190 to 200 g male Sprague-Dawley rats (CD, Charles River Breeding Laboratories, Wilmington, Massachusetts,

Table 2. Quantitative studies of glomerular antigen (sheep IgG) and antibody (rat anti-sheep IgG) deposition and proteinuria, 48 hr after transplantation

Planted antigen	Glomerular binding		Urine protein excretion
	Sheep IgG	Rat IgG	
	μg/kidney		*mg/24 hr*
Sheep anti-GBM (N = 5)[a]	8.9 ± 1.26[b]	30.8 ± 5.12	21.6 ± 7.4 (16–35)[c]
Sheep anti-Fx1A (N = 5)	8.4 ± 0.73	28.8 ± 5.58	65.1 ± 49.6 (15–148)
P	NS[d]	NS[d]	0.075[e]

[a] The number of animals studied is in parentheses.
[b] The values are mean ± SD.
[c] The range is in parentheses.
[d] Student's t test was used; NS represents not significant.
[e] The Mann-Whitney U test was used.

USA) were injected with various doses of γ2 sheep anti-rat GBM to determine a subnephritogenic dose and to confirm the absence of complement fixation in vivo. The preparation and characterization of γ2 sheep anti-rat Fx1A have been described previously in detail [2, 9].

Preparation and characterization of rat anti-sheep IgG

Rat anti-sheep IgG was prepared and characterized as previously described [2]. The doses of rat antiserum used (1.2 ml i.v. in rats with sheep IgG planted on the GBM and 1.5 ml i.v. in rats with sheep IgG planted subepithelially) were predetermined from quantitative studies using a radiolabelled IgG fraction of rat anti-sheep IgG. Rat IgG was isolated from a 50% ammonium sulphate precipitate by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemical, Inc.) using 0.0175 M sodium phosphate starting buffer and a linear sodium chloride gradient to 0.3 M. Fractions enriched for IgG were pooled, concentrated in an Amicon PM-30 ultrafiltration cell (Amicon Corp.) and further purified by S-200 (Pharmacia Fine Chemical, Inc.) gel filtration chromatography. The purified IgG was concentrated to 15.2 mg/ml, shown to be pure by immunoelectrophoresis against anti-rat whole serum, and found to have a titer of 1:4 against sheep IgG.

Quantitative studies

Purified γ2 sheep anti-rat GBM and γ2 sheep anti-rat Fx1A were radiolabelled with ^{125}I and the IgG fraction of rat anti-sheep IgG was labelled with ^{131}I using the chloramine-T method [8, 13]. Specific activity was 6×10^5 to 2.3×10^6 cpm/mg IgG of which 91% was precipitable with trichloroacetic acid. The labelled reagents were diluted with the respective unlabelled antibody preparations and, in pilot studies, were injected in varying doses into two rat groups to achieve similar amounts of planted sheep IgG and rat antibody on the GBM and in the subepithelial space at the time of sacrifice. Glomerular antigen and antibody content were measured in isolated glomeruli by techniques described previously [8]. It was found that 0.64 mg γ2 sheep anti-rat GBM given i.v. 48 hr before transplantation and 6 mg γ2 sheep anti-rat Fx1A given i.v. 72 hr before transplantation produced similar quantities of planted sheep IgG in glomeruli at sacrifice 48 hr after transplantation of an antigen-containing kidney into a passively immunized recipient (Table 2). Deposits of rat anti-sheep IgG at sacrifice were similar in the two groups when 11.8 mg and 15 mg i.v. were injected at the time of transplantation of kidneys containing sheep anti-GBM and anti-Fx1A, respectively (Table 2). Urine protein excretion was measured on days 1 to 2 post-transplantation and was increased only moderately in the group given sheep anti-GBM (Table 2). For definitive studies (see *Experimental design*) the doses of anti-GBM and anti-Fx1A were therefore increased to 2 and 8 mg, respectively, while the proportionate doses of anti-sheep IgG given to the two groups of recipients remained unchanged.

Rat renal transplantation

The left kidneys of male Sprague-Dawley rats, weighing 200 to 250 g (CD, Charles River Breeding Laboratories), were transplanted orthotopically into bilaterally nephrectomized littermates using a modification of the technique of Lee [14] as we have previously described [2]. Renal function was assessed by daily measurements of serum creatinine (Worthington Diagnostics, Freehold, New Jersey, USA).

Tissue processing and immunofluorescent procedures

Renal tissue specimens were obtained from all rats 48 hr after, and some rats 4 hr after, transplantation. The unused contralateral kidneys of some donors were also examined morphologically. Tissue samples for light microscopy were fixed in 10% neutral buffered formalin, sectioned at 4 μm and stained with hematoxylin and eosin and periodic acid-Schiff reagent. In addition to qualitative assessment of glomerular and interstitial changes in all biopsy specimens, glomerular cellularity was measured and relative glomerular volume determined in untreated experimental animals from groups 1 (N = 4) and 2 (N = 5). The total number of nuclei in ten randomly selected glomerular profiles from each experimental animal was counted at a magnification of 800×. The average number of nuclei per glomerular profile was calculated. In addition, the number of nuclei per unit of surface area of glomerular cross section was also determined as the ratio between the total number of nuclei and the glomerular cross-sectional area, determined by point counting [15]. The average glomerular volume was calculated in each animal using the procedure described by Hirose et al [16].

Direct and indirect immunofluorescent (IF) procedures were performed on snap-frozen tissue as described previously [9]. All biopsy specimens were stained with the fluoresceinated IgG fractions of monospecific rabbit anti-sheep IgG, goat anti-rat IgG (Cappel Laboratories) and rabbit anti-rat third component of complement (C3), prepared as previously described [9]. Goat anti-rat IgG was not cross-reactive with sheep IgG by immunoprecipitin or IF analysis. Tissue-bound rat anti-sheep IgG did not bind the fluoresceinated rabbit antisera. IF was evaluated and photographed on a microscope equipped with EF-D epifluorescent and Microflex UFX photographic attachments (Nikon Optiphot, Nikon Inc., Garden City, New York, USA). Snap-frozen renal biopsy specimens (two each from groups 1A and 2A) were also examined histochemically for the presence of nonspecific esterase (NSE)-positive cells (phagocytes) according to the method of Yam, Li, and Crosby [17] as modified by Sterzel et al [18]. Fragments of renal cortex were immersion-

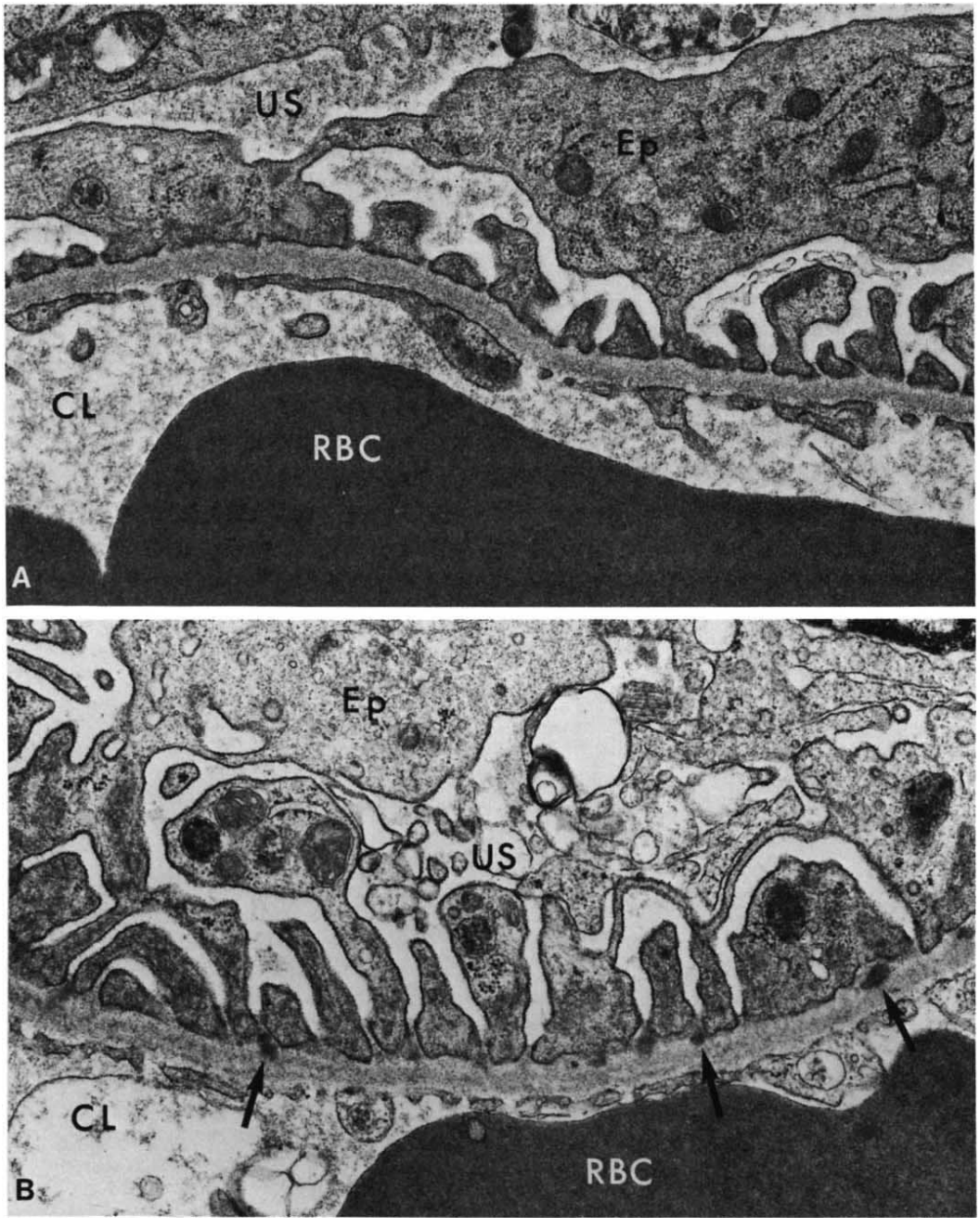

Fig. 2. *Electron micrographs of the glomerular capillary wall of donor kidneys prior to transplantation.* **A** *Planted γ2 sheep anti-GBM induces minimal alterations with occasional loss of foot-process interdigitations and slight reduction in the density of endothelial fenestrae.* (22,500×) **B** *Planted γ2 sheep anti-Fx1A induces the formation of small electron dense deposits in the lamina rara externa of the GBM* (arrows). (23,100×) Abbreviations: US, urinary space; Ep, epithelial cell; CL, capillary lumen; RBC, red blood cell.

fixed and processed for electron microscopy as previously described [2].

Statistical analysis

Results of urine protein excretion were evaluated by Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U test for the analysis of non-parametric data [19]. Parametric data, including glomerular cell counts, glomerular cross-sectional areas, and glomerular antibody binding, were analyzed by unpaired *t* test or analysis of variance [20]. Values are expressed as mean ± 1 SD or median and range unless otherwise indicated.

Results

Characterization of the experimental models

The γ2 subclass of sheep anti-rat GBM made a single cathodal arc on immunoelectrophoresis against anti-whole

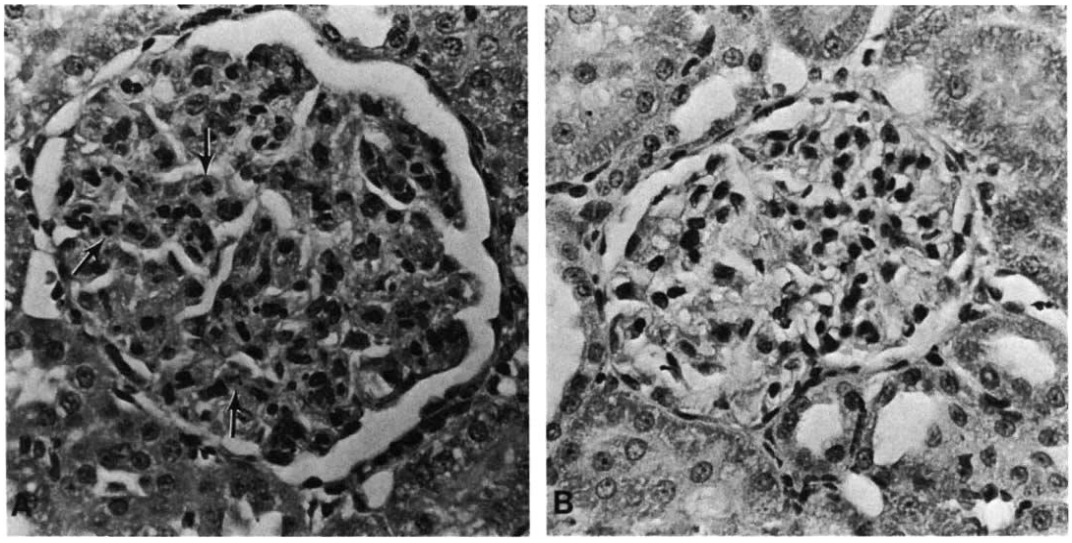

Fig. 3. *Light micrographs of representative glomeruli from animals with* **A** *passive autologous anti-GBM nephritis (group 1A) and* **B** *passive autologous PHN (group 2A).* Note the increase in glomerular diameter and infiltrating polymorphonuclear leukocytes *(arrows)* in **A.** Glomeruli in animals from group 2A **(B)** showed no histologic abnormalities. (Hematoxylin/eosin, 340×)

sheep serum and anti-sheep IgG, it was unreactive with Fx1A on immunodiffusion and, on immunofluorescence (IF), did not stain proximal tubular brush borders. It also did not fix rat C3 in vivo or human C3 in vitro. Intravenous doses of up to 4 mg produced bright, linear GBM deposits of sheep IgG but no rat IgG, C3 or proteinuria (3.6 ± 1.85 mg/24 hr, $N = 6$; upper 99% confidence limit of normal = 3.5 mg/24 hr). When kidneys from rats injected with 2 mg γ2 sheep anti-rat GBM were transplanted into naive, bilaterally nephrectomized recipients, urine protein excretion 48 hr after transplantation was less than 17 mg/24 hr. IF of these kidneys showed linear GBM deposits of sheep IgG but no rat IgG or C3. Light microscopy revealed normal glomeruli and only mild ultrastructural abnormalities as described below (Fig. 2**A**). In contrast, when identical kidneys were transplanted into recipients that were passively immunized with 1.2 ml rat anti-sheep IgG antiserum at the time of transplantation, massive proteinuria (163 to 304 mg/24 hr) was present after 48 hr together with linear deposits of sheep IgG, rat IgG, and rat C3 on the GBM by IF, glomerular hypercellularity by light microscopy (Fig. 3**A**) and extensive ultrastructural changes (Figs. 4**A** and 5) described below. This model is henceforth designated passive autologous anti-GBM nephritis.

Characteristics of γ2 sheep anti-rat Fx1A have been described previously in detail [2]. When kidneys from rats injected with 8 mg of noncomplement-fixing γ2 sheep anti-Fx1A were transplanted into naive, bilaterally nephrectomized recipients, urine protein excretion after 48 hr was less than 10 mg/24 hr. IF showed granular capillary wall deposits of sheep IgG but no rat IgG or C3. Glomerular morphology was normal except for the presence of small subepithelial electron-dense deposits (Fig. 2**B**). Identical kidneys, transplanted into recipients passively immunized with 1.5 to 2.0 ml of rat anti-sheep IgG, were markedly proteinuric after 48 hr (22 to 245 mg/24 hr) and had granular capillary wall deposits of sheep IgG, rat IgG, and rat C3 together with pronounced obliteration of epithelial cell foot processes and slit-diaphragm displacement adjacent to large, subepithelial, electron-dense deposits (Fig. 4**B**). This model is designated passive autologous PHN.

Mediation of proteinuria due to rat antibody reacting with sheep IgG on the GBM

The results reported in Table 1 and Figure 6 are those in which the renal transplant was technically successful, the serum creatinine was less than 2.0 mg/dl and not rising after 2 days post-transplantation, and no ureteric obstruction was evident at sacrifice. All recipient rats in groups 1A to 1D were immunized passively with rat anti-sheep IgG at the time of transplantation of donor kidneys containing γ2 sheep IgG on the GBM. Group 1A, treated with normal rabbit globulin, had linear capillary wall staining for sheep IgG, rat IgG, and rat C3 by IF (Table 1 and Fig. 7**A** to **C**) and severe proteinuria 48 hr after transplantation. Serum C3 levels were 91 ± 16.8% of normal. Total differential leukocyte counts at all times were no different than pretreatment values (Table 3). In recipients treated with cobra venom factor (group 1B), serum C3 levels were less than 8.5% of pretreatment values for the duration of the study. In all, IF at 48 hr post-transplantation showed linear capillary wall deposits of sheep IgG and rat IgG, but no rat C3 (Table 1 and Fig. 7**D**). Urine protein excretion at 48 hr was significantly less than group 1A ($P = 0.028$, $N = 3$) and no different than non-immunized controls (Table 1 and Fig. 6). Thus, proteinuria which results from the in situ reaction of complement-fixing rat anti-sheep IgG with sheep IgG planted on the GBM was totally prevented by depletion of complement.

Rats rendered neutropenic with anti-polymorphonuclear leukocyte (PMN) serum (group 1C, $N = 4$) were severely depleted of PMN, especially at the time of transplantation (Table 3). Moderate reduction in monocytes (Table 3) was also evident after anti-PMN. Urine protein excretion 48 hr after

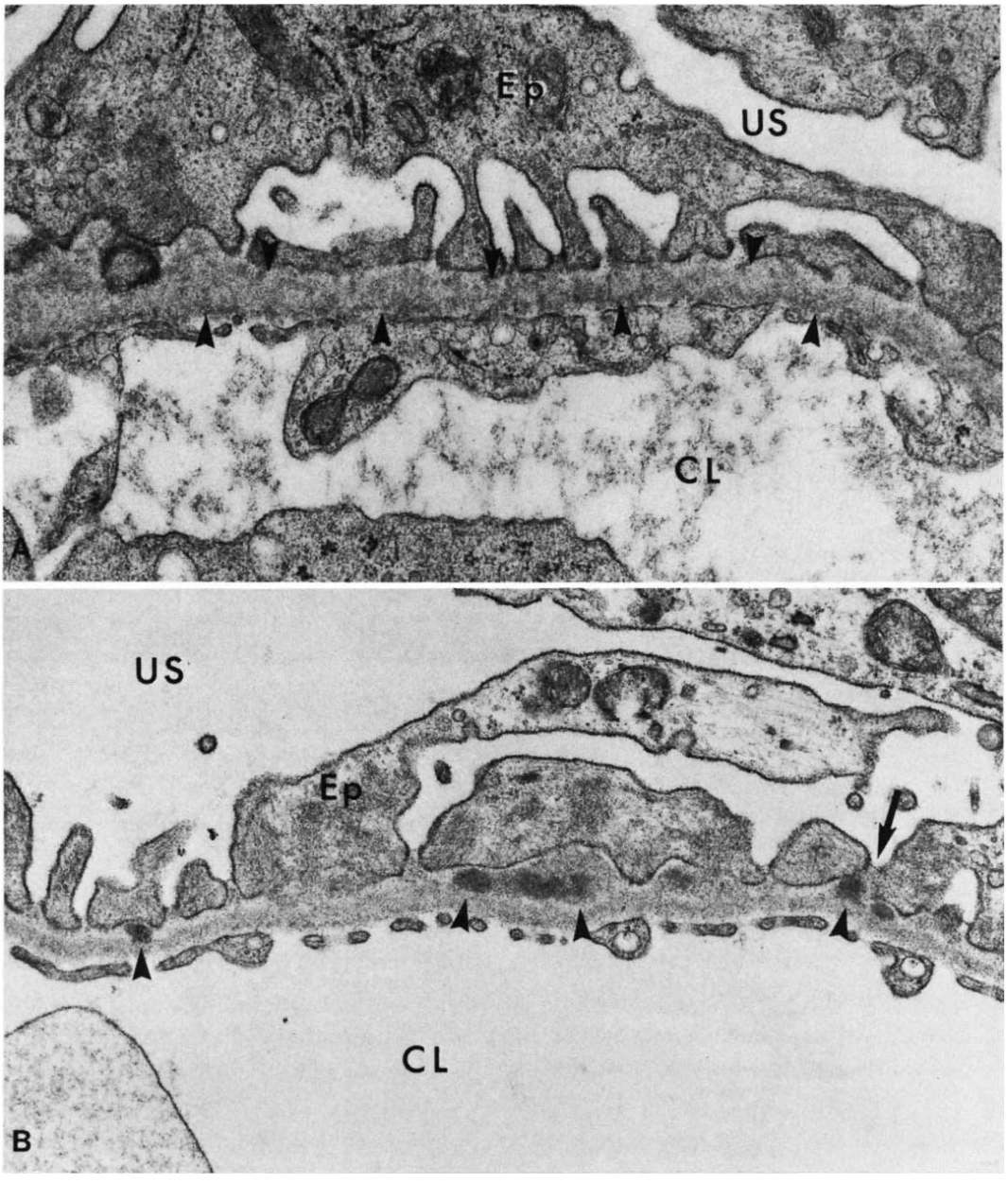

Fig. 4. *Electron micrographs of the glomerular capillary wall of donor kidneys 48 hr following transplantation and passive administration of rat anti-sheep IgG.* **A** *Group 1A: numerous small, flocculent electron densities are present in both laminae rarae* (arrowheads) *of the GBM.* The endothelial cell shows sparsity of fenestrae and epithelial cell foot processes are locally retracted and simplified. **B** *Group 2A: notice prominent subepithelial electron dense deposits* (arrowheads), *local obliteration of foot-process architecture with occasional displacement of the filtration slit-diaphragm* (arrow). The endothelial cell is unremarkable. Abbreviations are: US, urinary space; Ep, epithelial cell; CL, capillary lumen. (27,000×)

transplantation was near normal ($P = 0.014$, vs. group 1A) (Table 1 and Fig. 6). Serum C3 levels in leukopenic rats were 114 ± 15.4% ($N = 3$) of normal. Six recipients (group 1D) were lethally irradiated 48 hr prior to their receiving an antigen-containing kidney. Total leukocyte counts were reduced markedly at transplantation and for 48 hr thereafter (Table 3). Leukopenia included all cell types although mononuclear cells were proportionately more severely depleted than PMN, especially at the time of transplantation (Table 3). Urine protein excretion at 48 hr in group 1D (Table 1 and Fig. 6) was significantly less than group 1A ($P = 0.005$) though still abnormally high. Linear GBM deposits of sheep and rat IgG and rat C3 were virtually identical in groups 1C and 1D to those in group 1A by IF (Table 1). Thus, in the presence of normal serum C3 levels and abundant glomerular deposits of antibody and C3, leukocyte-depletion with anti-PMN serum completely inhibited the development of proteinuria and lethal irradiation markedly reduced it.

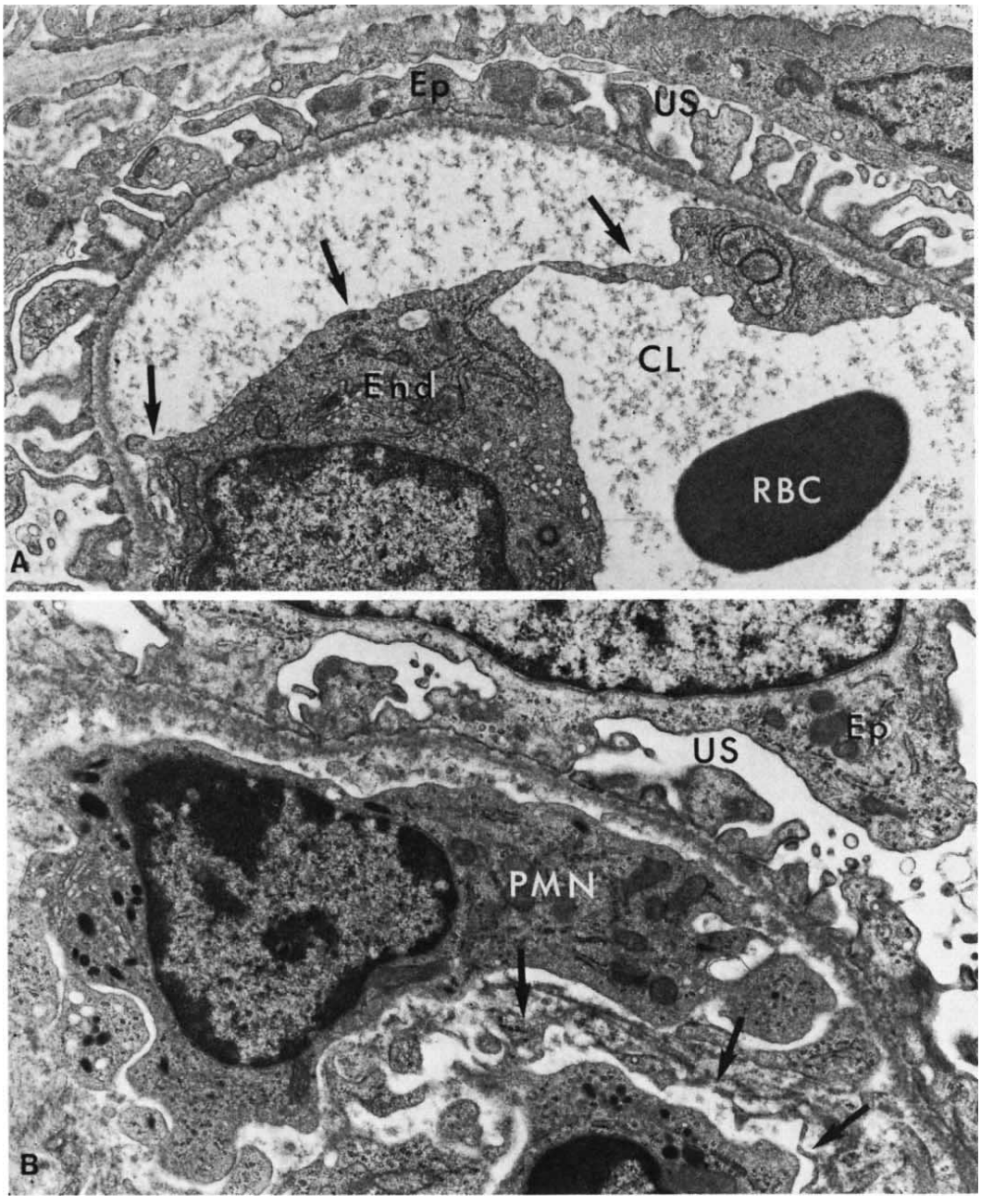

Fig. 5. *Electron micrographs showing details of the ultrastructural changes observed in passive autologous anti-GBM nephritis (group 1A).* **A** *Individual capillary loops show detachment of the endothelial cell from the glomerular basement membrane* (arrows) *and relative sparsity of endothelial fenestrae.* Notice also the irregular appearance of both laminae rarae due to small electron densities. **B** *An infiltrating PMN leukocyte is in direct contact with the denuded glomerular basement membrane.* Notice the displacement of the endothelial cell *(arrows)* by the inflammatory process. Abbreviations are: US, urinary space; Ep, epithelial cell; End, endothelial cell; CL, capillary lumina; RBC, red blood cell; PMN, polymorphonuclear leukocyte. (12,500×)

Mediation of proteinuria due to rat antibody reacting with sheep IgG in the subepithelial space

On receiving donor kidneys containing γ2 sheep IgG planted in the subepithelial space, recipient rats in groups 2A to 2D were immunized passively with the same rat anti-sheep IgG as groups 1A–1D. Rats in group 2A, either untreated (N = 3) or treated with normal rabbit globulins (N = 2), had granular capillary wall staining for sheep IgG, rat IgG, and rat C3 by IF (Fig. 7**E** to **G**), and proteinuria 48 hr after transplantation (Table 1 and Fig. 6). Serum C3 levels were 93 ± 14.8% of normal. In four recipients (group 2B) treated with the cobra venom factor, serum C3 levels were less than 4% of pretreatment values throughout the study. IF revealed granular capillary wall deposits of sheep IgG and rat IgG identical to group 2A but no rat C3 (Table 1 and Fig. 7**H**). Urine protein excretion after 48 hr was significantly less than group 2A (P = 0.002) (Table 1 and Fig. 6) and no different than nonimmunized recipients. Thus,

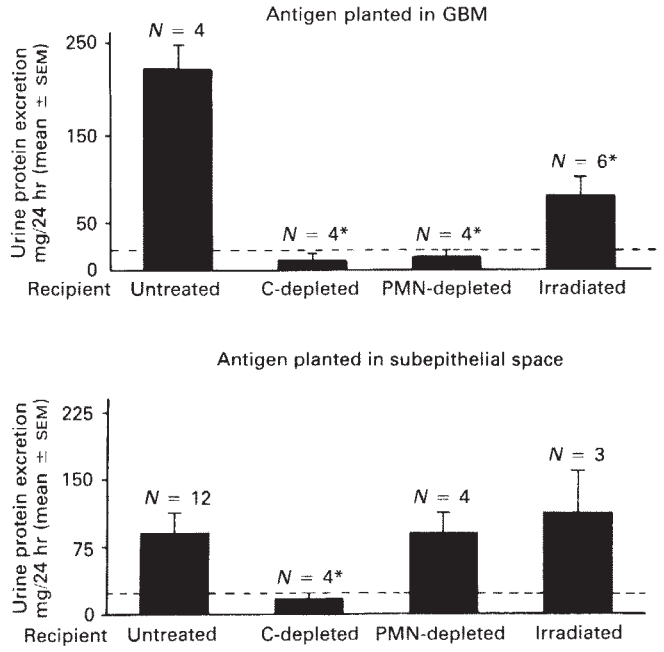

Fig. 6. *Results of 24-hr urine protein excretion 2 days after transplantation of kidneys with γ2 sheep IgG planted in the GBM or in the subepithelial space in rats passively immunized with rat anti-sheep IgG.* Recipients were either untreated, complement-depleted, PMN-depleted, or lethally irradiated. The *dashed lines* represent the upper limit of normal protein excretion by antigen-containing kidneys transplanted into nonimmunized recipients. *Asterisks* indicate a significant difference as compared to the untreated group (antigen planted in GBM, $P < 0.028$; antigen planted in subepithelial space, $P = 0.002$; Mann-Whitney U test).

proteinuria resulting from the in situ reaction of complement-fixing rat anti-sheep IgG with sheep IgG planted in the subepithelial space was abrogated by depleting rats of complement.

Results of previous studies that demonstrated the lack of effect on proteinuria of cell-depletion with anti-PMN serum or lethal irradiation in passive autologous PHN [2] are shown for comparison in Figure 6.

Morphologic studies

Donor kidneys containing planted γ2 sheep anti-GBM or anti-Fx1A, at the time of transplantation or when transplanted into nonimmunized recipients, were normal by light microscopy without an increase in glomerular cellularity. Electron microscopy of these kidneys showed good preservation of endothelial cells and minimal spreading of epithelial cell foot processes (Fig. 2**A** and **B**). No electron-dense deposits were visible in donor kidneys containing antigen in the GBM (Fig. 2**A**), but those with planted anti-Fx1A had several, small, discrete subepithelial deposits (Fig. 2**B**). Two days after immunization with rat anti-sheep IgG, light microscopy of transplanted kidneys with antigen in the GBM (group 1A) were distinctly different to those with planted subepithelial antigen (group 2A). In group 1A, glomeruli were hypercellular and swollen with occlusion of several capillary lumina and 3–5 PMN visible in most glomeruli (Fig. 3**A**). Some glomeruli contained focal areas of necrosis and loop thrombosis. Interstitial infiltrates of mononuclear cells were prominent around some glomeruli and small vessels. By comparison, glomeruli of kidneys in group 2A were less swollen and less crowded with cells, capillary lumina were widely patent and only occasional glomeruli contained PMN (Fig. 3**B**). Quantitative morphology showed significantly more cells in glomeruli of group 1A (96 ± 13.4 cells/glomerulus, $N = 4$) than in group 2A (71 ± 13.2 cells/glomerulus, $N = 5$; $P < 0.001$). In addition, the cross-sectional surface area of glomeruli in group 1A (16,081 ± 3812 μm^2, $N = 4$) was significantly greater than that in group 2A (10,745 ± 2378 μm^2, $N = 5$; $P < 0.001$) indicating a mean glomerular volume that was 1.8-fold greater in group 1A than group 2A.

On electron microscopy, similar striking differences were apparent between groups 1A and 2A. In group 1A, glomeruli showed frequent detachment of endothelial cells from the GBM (Fig. 5**A**) and loss of endothelial fenestrae (Figs. 4**A** and 5**A**). In focal areas, endothelial cells were missing completely and the GBM was in direct contact with PMN, macrophages, or red blood cells (Fig. 5**B**). In addition, several capillary lumina were collapsed or filled with PMN, mononuclear cells, or platelets. Some epithelial cells contained reabsorption droplets and showed a focal retraction of foot processes (Fig. 5). In the GBM, numerous, small, closely spaced, flocculent densities became apparent in the laminae rarae, especially the lamina rara interna in which an almost continuous line of electron-dense material was evident (Figs. 4**A** and 5). Endothelial cell detachment and GBM granularity were also seen in complement-depleted and leukocyte-depleted recipients, but infiltrating inflammatory cells and epithelial foot process abnormalities were much less prominent than in group 1A. In group 2A, the most conspicuous glomerular changes were the presence of large deposits of nonuniform density in the subepithelial space with associated displacement of overlying filtration slit-diaphragms and simplification of adjacent epithelial cell foot-processes (Fig. 4**B**). Endothelial cells were intact and normally fenestrated (Fig. 4**B**). Occasional mononuclear cells were observed in the capillary lumina, in some instances adherent to, but never displacing, endothelial cells.

Coded renal biopsy tissue specimens from two rats from groups 1A and 2A were examined histochemically for cells that stained positively for NSE. Intense, brick-red staining of all proximal tubular epithelial cells and some distal tubular epithelial cells was present in all specimens. Whereas most glomeruli in rats from group 1A contained NSE-positive cells (range 0 to 9, avg. 2.1 cells/glomerulus), most glomeruli in rats from group 2A did not (range 0 to 1, avg. 0.15 cells/glomerulus). Interstitial cellular infiltrates contained occasional NSE-positive cells in both groups particularly prominent in the periglomerular and perivascular regions in group 1A.

Discussion

These studies demonstrate an unequivocal difference in the mediation of injury in the two models used. Depleting hosts of complement completely prevented the development of proteinuria when rat antibody reacted with planted antigen both in the GBM and the subepithelial space. However, a striking difference between the two groups emerged, when hosts were depleted of leukocytes. Leukocyte-depletion with anti-PMN serum totally prevented the development of proteinuria and lethal irradiation reduced it by about 67% in the group with

Table 3. Differential peripheral blood leukocyte counts in rats with passive autologous anti-GBM nephritis; rats were either untreated (group 1A) or treated with anti-PMN serum (group 1C) or lethal irradiation (group 1D)

	PMN	Monocytes	Lymphocytes
		cells/mm³	
At transplantation			
Group 1A (N = 4)[a]	3400 (2500–4000)[b]	3550 (890–5000)	10650 (7500–19000)
Group 1C (N = 4)	60 (0–90)	590 (350–640)	10750 (6600–19000)
Group 1D (N = 6)	1850 (1200–3200)	90 (30–170)	230 (140–400)
At sacrifice			
Group 1A (N = 4)	4000 (2500–14500)	3000 (740–4300)	7000 (5300–8300)
Group 1C (N = 3)	220 (110–550)	1100 (660–1400)	6400 (5800–9100)
Group 1D (N = 6)	110 (0–370)	230 (140–770)	540 (90–1100)

Abbreviation: PMN, polymorphonuclear leukocytes.

[a] The number of animals studied is in parentheses.

[b] The values are the median; the range is in parentheses. The pretreatment values of recipients from all groups (mean ± SD, N = 14) are: total leukocytes 15300 ± 5200; PMN 2800 ± 1280; monocytes 2400 ± 1340; lymphocytes 11200 ± 2470 cells/mm³.

antigen planted in the GBM. In contrast, these maneuvers had no effect when antigen was planted subepithelially (Fig. 6) [2].

It is impossible to be certain from these studies which cells, PMN, macrophages or both, are responsible for glomerular injury in the model with antigen in the GBM. Anti-neutrophil serum was highly effective in preventing proteinuria and it virtually eliminated PMN from the circulation. However, it also moderately reduced the number of circulating mononuclear cells. Lethal irradiation, on the other hand, effectively depleted mononuclear cells but was less effective at depleting PMN and reducing proteinuria. Thus, a role for PMN is suggested but positive proof is lacking. In addition, a contributing effect of macrophages, possibly those resident in the transplanted kidney [21], has not been excluded.

Previous studies of the autologous phase of anti-GBM nephritis similarly demonstrated a critical requirement for either PMN [3] or macrophages [4, 5, 22] in the development of glomerular injury. In those studies, cell-dependent injury was shown to be complement-independent [3–5, 23] and was thought to be either Fc-receptor [24, 25] or T cell [22, 26] directed. In contrast, proteinuria was clearly complement-dependent in the present study. Although there is no simple explanation for this apparent discrepancy, the answer may relate to differences in the models especially with regard to the kinetics of antibody binding [27]. In the heterologous phase of anti-GBM nephritis, inflammatory cell-mediated injury is largely complement-dependent [28, 29] and maximum antibody binding occurs within minutes of anti-GBM administration [6, 7]. On the other hand the naturally occurring, active autologous phase develops more slowly with a greater opportunity for sensitization of host cells to the foreign antigen. In effect, our present studies are more analogous to the heterologous phase than the autologous phase of anti-GBM nephritis since recipients were not exposed to sheep IgG until transplantation of the antigen-containing kidney; immunization with anti-sheep IgG was passive and onset of injury was acute.

To attribute the differences we found in mediation and morphologic expression of injury to differences in the site of immune reaction, demands some insight into the location of the planted antigen. Several studies, using a variety of different techniques and tracers, have attempted to determine the location of the antigens to which anti-GBM antibodies bind [30–36]. Technical problems including inadequate tissue penetration of large tracers like ferritin, diffusion of peroxidase reaction product [37] and poor resolving power of immunofluorescence have made precise localization difficult. The multiplicity of putative antigens identified by heterologous anti-GBM antisera is an additional confounding factor. Nevertheless, the most convincing studies suggest that anti-GBM antibodies localize predominantly in the laminae rarae of the GBM [31, 32, 34–36]. This seems to be borne out in the studies reported here in which closely spaced electron-dense deposits became apparent in the laminae rarae after recipients of a sheep anti-GBM-containing kidney were immunized passively with anti-sheep IgG. These findings also correspond to earlier studies in which subendothelial electron densities were observed in the autologous phase of anti-GBM nephritis [38]. The deposits in our current studies were particularly abundant in the lamina rara interna, forming an almost continuous layer that was often more electron-dense than the lamina densa. While the visual amplification afforded by the second antibody may have revealed more antigen in the lamina rara interna than externa, it is more likely that a greater degree of binding occurred in the former location because it is more proximally located in the filtration pathway of antibody. In either event it is clear that a large proportion of the reaction of antibody with a planted antigen took place in the subendothelial region.

Information regarding the location of the glomerular antigen or antigens with which anti-Fx1A reacts indicates that initial binding, after in vivo injection, takes place in both endothelial and subepithelial regions but after 24 hr the binding is exclusively subepithelial [39]. More recent studies have demonstrated that at least one of the antigens identified by anti-Fx1A is located on the epithelial cell membrane, probably in coated pits [40]. In the present studies, kidneys were transplanted 72 hr after anti-Fx1A administration to ensure that all the planted sheep IgG was located in the subepithelial space. This was supported by combined phase-contrast and immunofluorescent studies. In addition, electron microscopy, before and after passive immunization with anti-sheep IgG, demonstrated exclu-

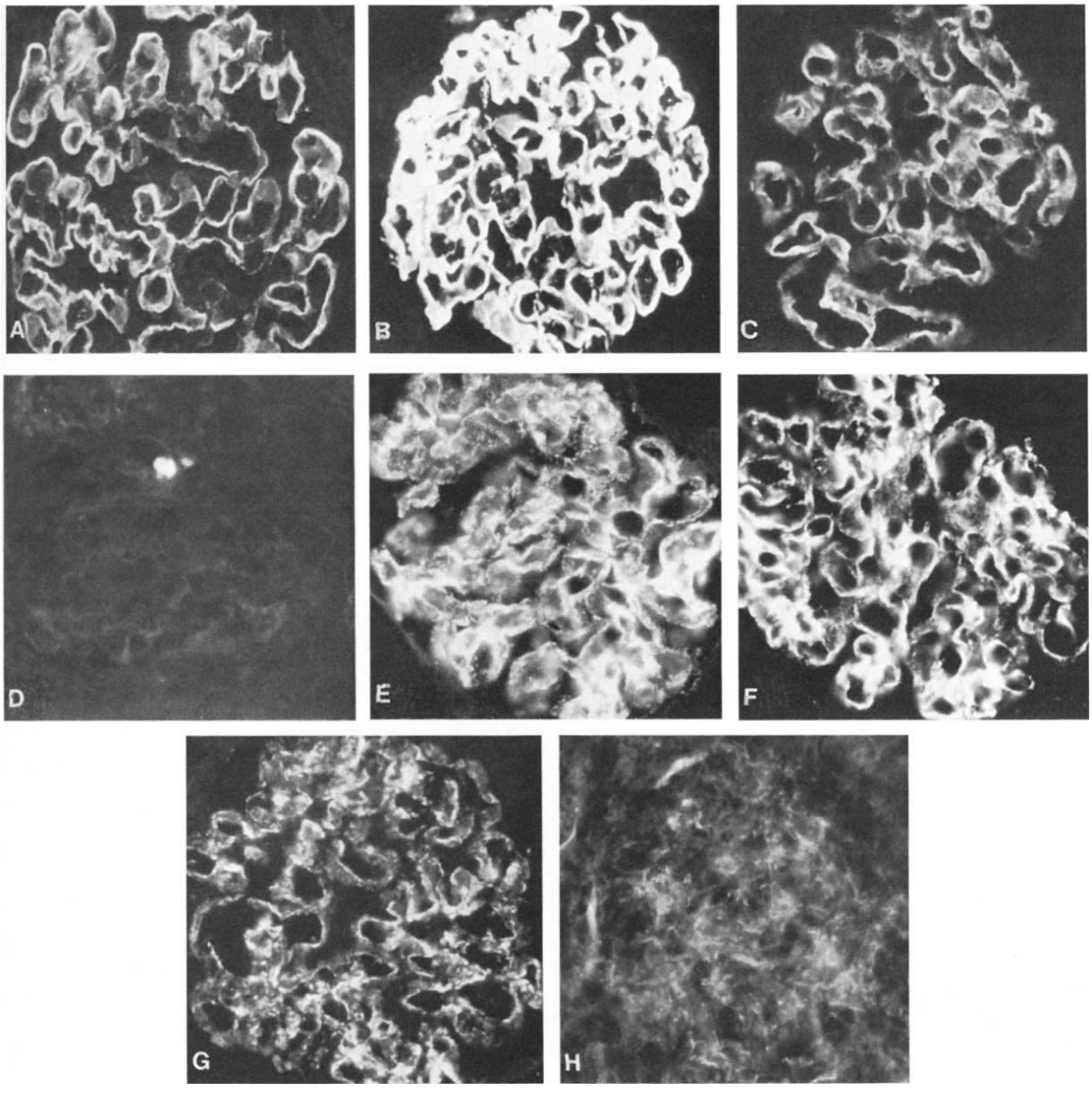

Fig. 7. *Immunofluorescent microscopy of glomeruli in renal biopsy specimens obtained 48 hr after transplantation of kidneys containing γ2 sheep IgG into rats immunized with rat anti-sheep IgG.* **A** to **C** *Group 1A—untreated.* **D** *Group 1B—complement-depleted.* **E** to **G** *Group 2A—untreated.* **H** *Group 2B—complement-depleted.* These were stained for sheep IgG (**A** and **E**), rat IgG (**B** and **F**), rat C3 (**C, D, G,** and **H**). Note the linear staining pattern in passive autologous anti-GBM nephritis (group 1); granular pattern in passive autologous PHN (group 2); and absence of staining for rat C3 in complement-depleted rats (**D** and **H**). (350×)

sively subepithelial electron-dense deposits. Thus, the location of the planted antigen and site of subsequent in situ binding of antibody was notably different in the two models.

The mechanisms by which antigen distribution influences the mediation and morphologic expression of glomerular injury have not been defined and must remain hypothetical. When complement-fixing antibody reacts with a tissue-bound antigen, complement activation results in the fixation of C3b and release of chemotaxins such as C5a. We postulate that when antigen is located in the GBM, especially in the lamina rara interna, chemotaxins gain access to the circulation producing an influx of inflammatory cells that become adherent to the immune deposits via C3b and Fc receptors. Injury is then produced largely by the action of PMN and macrophages [41, 42] and possibly by the vasoactive properties of complement [43]. By comparison, subepithelial immune deposits are sequestered from circulating PMN and macrophages which, therefore, fail to sense the release of chemotaxins and do not become adherent. In this setting, injury appears to be the direct effect of complement, possibly acting on the epithelial cell membrane via terminal complement components [44].

The morphologic changes were consistent with the studies on mediation of injury and with the above hypothesis. In rats with antigen planted in the GBM, glomeruli were larger, more

cellular, and appeared to contain more PMN than those of rats with subepithelial antigen. In the former, endothelial cell detachment, loss of endothelial cell fenestrae, and adherence of macrophages and PMN to denuded GBM were prominent findings. By comparison, in the latter, glomerular capillary walls were largely normal except for the presence of subepithelial deposits and adjacent epithelial cell and filtration slit-diaphragm abnormalities characteristic of membranous nephropathy. It is noteworthy that endothelial detachment was present in non-proteinuric kidneys of rats with planted anti-GBM that were depleted of complement or leukocytes. This suggests that endothelial integrity is not essential for the maintenance of glomerular permselectivity, at least acutely as under the conditions of these experiments.

In summary, the results of these studies show that the in situ reaction of rat anti-sheep IgG with sheep IgG planted in the GBM produces complement-mediated, cell-dependent injury associated with glomerular hypercellularity and inflammatory cell infiltration. In contrast, in situ reaction of the same rat anti-sheep IgG with sheep IgG planted in the subepithelial space produces complement-mediated, cell-independent injury with significantly less glomerular hypercellularity and no adherence of inflammatory cells. Hence, we conclude that the distribution of antigen and the site of immune reaction can critically influence the mediation and morphologic expression of glomerular injury and may, in part, account for clinical and histological differences in various forms of antibody-induced glomerular disease in humans.

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