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Chronic renal allograft rejection: the significance of non-MHC alloantigens

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Abstract. We studied the role of polymorphic endothelial antigens other than MHC in antibody-mediated chronic renal allograft rejection in two models. In the first model, donor Lewis rat kidneys were transplanted into BN recipients that had been made tolerant for donor class I antigens at the B cell (antibody) level. In this setting Lewis kidney grafts were chronically rejected with stable renal function but increasing proteinuria (> 100 mg/24 h). Rejected graft tissue showed mononuclear cell infiltration and the presence of glomerular vasculonecrotic lesions with fibrinoid material, associated with IgG and IgM deposition, but with absent or weak C3 binding. Graft endothelium showed no expression of MHC class II antigens. Serum antibodies were not reactive with donor class I antigens, but did react with endothelial non-MHC alloantigens. In the second model, more direct information on the role of endothelial non-MHC alloantigens in renal allograft rejection was obtained by transplanting Lewis 1 N kidneys into unmodified BN recipients (MHC-matched transplants). Here, similar to the first model, the animals developed severe proteinuria with stable renal function. Histopathological examination showed mononuclear cell infiltration and deposition of IgM and IgG along the glomerular vasculature, but this time in the presence of strong C3 reactivity. However, glomerular vasculonecrotic lesions with intense fibrin deposition were not observed. The data showed that although clinically the two kidney transplantation models used gave similar chronic rejection phenomena, histopathologically some striking differences were observed in the glomeruli. The precise mechanisms effecting chronic rejection of the grafts is still a puzzle. However, immune reactivity against graft (endothelial) non-MHC antigens may play a significant role.

Key words: Renal allograft – Chronic rejection – Non-MHC – Endothelial cells

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Clinically, chronic renal allograft rejection is still a problem. Despite improved HLA matching and immune suppression strategies, kidney grafts can be subjected to rejection several months or years after transplantation [1–3]. The mechanisms that are involved in chronic rejection puzzel (transplantation) scientists from various disciplines, and have led to scientific research from fields such as immunology, pharmacology, and hemostasis and thrombosis. The vascular endothelium is a major target of immune reactivity in vascular organ transplants such as the kidney. Endothelial cells express high levels of class I major histcompatibility complex (MHC) antigens [4–7], and they can be induced to express class II antigens by cytokines released in local immune reactivity [8–10]. However, in closely matched transplants, MHC antigens cannot be considered major targets in the process of rejection A contribution to rejection in such closely-matched transplants seems to be delivered by non-MHC alloantigens expressed by graft cells, and, in particular, those expressed by endothelial cells [11–13]. If graft endothelial non-MHC antigens are indeed targets in chronic rejection, one may expect various degrees of vasculonecrotic lesions, dependent not only on the type (humoral or cellular) and intensity of the immune response, but also on the type and local constitution of the blood vessels involved (e. g., arterial or venous, small or large vessels). In this study we compared two rat models of renal transplantation with clinical phenomena of chronic rejection in which major involvement of (endothelial) non-MHC alloantigens could be expected. Histopathological and serological studies were executed to determine the immunological mechanisms related to chronic rejection of non-MHC mismatched kidney grafts. In the first model Lewis kidneys were transplanted into BN recipients that had been made tolerant to Lewis erythrocytes (Lew-E). These Lew-E tolerant BN rats were unable to make an antibody response to Lewis class I antigens [14, 15]. As a result, these BN recipients chronically rejected the Lewis allografts (survival > 40 days), clinically showing increasing proteinuria, but stable renal function (serum urea levels < 200 mg/100 ml). In the second model, kidneys from

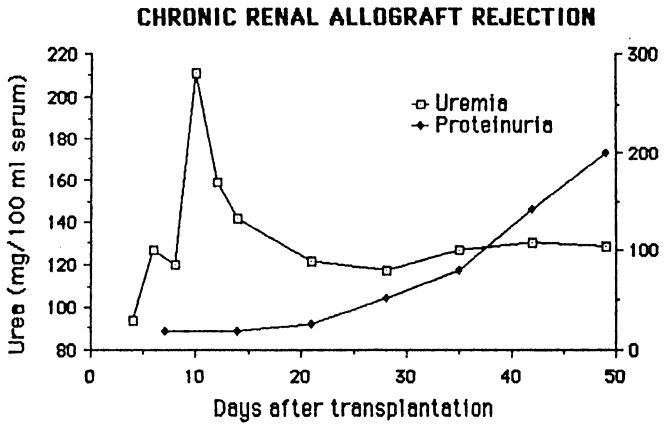

Fig. 1. Serum urea and urine protein levels in Lew-E tolerant BN recipients after grafting of Lewis kidneys (average values; $n = 7$)

Lewis IN congenic rats were transplanted into BN recipients. Here, the renal grafts that only differed from the recipient in non-MHC antigens were chronically rejected with similar clinical signs as the previous model, e.g. increasing proteinuria, stable renal function and a survival of more than 40 days. Histopathological and immunohistochemical analysis of rejected grafts, and analysis of recipient serum was carried out to study local immunological reactivity and antibody reactivity with donor endothelial cells respectively. The data showed that in both models clinical rejection of the kidney grafts was attended with histological rejection showing glomerular and interstitial lesions with mononuclear cell infiltration and antibody deposition in the renal vasculature. Serological studies demonstrated antibody reactivity with endothelial cells. Although glomerular lesions in both models were not similar, we suggest that (endothelial) non-MHC alloantigens played an important role in the development of (humoral) immune reactants against the graft, eventually leading to rejection.

Materials and methods

Experimental animals and surgery. Lewis ($RT1^l$) and Brown Norway (BN; $RT1^n$) rats were obtained from the Central Animal Facility of the University of Limburg. Only male rats were used. The animals were maintained under SPF conditions until use and had free access to food and water. Lewis IN ($RT1^n$) and BN.1L ($RT1^l$) were from the Central Institute for Laboratory Animal Breeding in Hannover, Germany. Donors and recipients of renal grafts were between 10 and 12 weeks of age. LEW erythrocyte (LEW-E) donor rats were 12–20 weeks old.

Kidneys were perfused via the abdominal aorta with 100 ml cold Collins solution, containing 2% bovine serum albumen, using 40 cm hydrostatic pressure. The kidneys were then transplanted into bilaterally nephrectomized BN recipients using standard techniques [16].

Induction of tolerance to LEW class I MHC antigens. BN recipients were, unless indicated as unmodified, intravenously infused with high doses of Lewis erythrocytes (LEW-E) to induce B cell (antibody) tolerance to Lewis class I MHC antigens [14, 17]; 0.5–1.0 10^{10} LEW-E were infused at 6, 4 and 2 weeks before transplantation. Details of erythrocyte purification and infusion have been described previously by Majoor et al. [14]. Prior to transplantation the serum of the BN recipients was measured for LEW-E hemagglutinating antibodies. Only animals in which hemagglutinating antibodies were absent were used as graft recipients.

(Immuno)histology. Animals were perfused with cold saline via the left heart ventricle. Grafts were removed and slices of the tissue were snap frozen in isopentane and stored at −70°C until use. Another piece of graft tissue was fixed in 4% formalin and processed for paraffin embedding. Paraffin sections (2–4 mm thick) were routinely stained with silvermethenamine and counterstained with hematoxylin and eosin (HE). Heart tissue used for serum staining was removed, snap frozen and used for frozen sections.

For immunohistochemistry, 4–6 µm thick frozen sections were cut, acetone-fixed for 10 min and air dried for at least 60 min using a fan. Sections were stained with antibodies using a 2 step immunoperoxidase technique as previously described by Duijvestijn et al. [18]. Briefly, sections were incubated with the first antibody for 60 min and were then washed in PBS. Next, the sections were incubated with the second step reagent, a horse radish peroxidase (HRP) conjugated rabbit-anti-mouse Ig or a HRP-conjugated goat-anti-rabbit Ig (DAKO, Denmark). To block cross-reactivity with rat Ig in the sections, 2% normal rat serum was added to the conjugates. For staining of heart sections, sera diluted 1/80 were used. The second stage antibody was a HRP-conugated rabbit-anti-rat Ig (DAKO, Denmark). After washing, the sections were incubated with a diamino-benzidine solution containing 0.02% H_2O_2 for 10 min. Subsequently, the sections were rinsed, counter-stained (not for heart sections) with hematoxylin, dehydrated, and covered with Entallan (Merck, Germany). Immunofluorescence staining with FITC-conjugated antibodies was performed on non-fixed frozen sections. Sections were covered with 50% glycerol in PBS and examined under epifluorescent light.

Antibodies used were W3/13 (pan T-cel marker), OX-6 (recognizes MHC class II antigens), and OX-18 (anti MHC class I antigens), donated by A. Williams (Oxford, England), EDI (monocyte/macrophage marker) donated by C. Dijkstra (Amsterdam, The Netherlands), MARM (anti IgM), and MARGI, 2a, 2b, 2c (anti IgG subsets) obtained from Sanbio (The Netherlands), anti C3 (recognizes complement component 3) donated by M. Daha (Leiden, The Netherlands) and RECA-1 (anti-endothelial cell antigen) from our laboratory.

Results

Clinical parameters in chronic renal allograft rejection

In both rat models used to study rejection mechanisms, and in particular the involvement of non-MHC alloantigens, recipient protein and urea levels in urine and serum were measured after kidney transplantation. When Lewis kidneys were transplanted in Lew-E tolerant recipients ($n = 7$), a transient rejection crisis was frequently observed around day 10, as measured by increasing serum urea levels. No proteinuria was measured at that time. However, from week 3 on, while urea levels remained stable, a slowly increasing proteinuria occurred in all animals, reaching avarage levels of more than 200 mg/24 h around day 50 (Fig. 1). Recipients suffering from proteinuria had severe weight loss, were in a poor clinical condition and were sacrificed between 6 and 12 weeks after transplantation. By this time they had developed severe proteinuria. The pattern of clinical data of Lewis 1N renal grafts rejected in unmodified BN recipients ($n = 4$) was similar. Proteinuria developed later, around week 5 or 6, but also reached levels of more than 200 mg/24 h and led to severe weight loss. Although in the 1st days after transplantation serum urea

Table 1. (Immuno)histology of chronic kidney rejection

	Antibody	Kidney graft of Lewis → Lew-E tolerant BN	Kidney graft of Lewis 1N → BN
Mononuclear cell in intration/ margination	na (HE)	+	+
T cells	W3/13	+	+
Monocytes/ macrophages	ED-1	+	+
Class I expression	OX-18	diffuse	diffuse
Class II expression in tubules	OX-6	some tubules	some tubules
Class II expression on endothelium	OX-6	–	–
Glomerular vasculonecrotic lesions with fibrin deposition	na (HE)	+	–
IgM deposition	MARM	glomerular capillaries (sometimes mesangial)	glomerular capillaries
IgG deposition	MARG (1, 2a, 2b, 2c)	entire renal vasculature	entire renal vasculature
C3	anti C3	– (–/+) in glomeruli	+ in glomeruli

na, not applicable
HE, hematoxylin/eosin staining

levels in some animals were rather high, levels stabilized after the 1st week, seldomly exceeding 200 mg/100 ml. In contrast to the first model, a transient rejection episode with increasing urea levels around day 10 was not observed.

Histopathology and immunohistochemistry (Table 1)

Similarities between the two models of chronic renal allograft rejection. Renal allografts were studied in a late stage of chronic rejection (about 7 to 10 weeks after transplantation), when the recipients had severe proteinuria, but stable renal function. In both models of chronic rejection, interstitial mononuclear infiltrates and marginating mononuclear leukocytes in glomeruli and peritubular capillaries were observed. Infiltrating and marginating cells consisted predominantly of monocytes/macrophages (ED-1 positive) and T cells (W3/13 positive). In the interstitial infiltrates B cells and plasma cells (IgM or IgG positive) were also present, whereas neutrophils were only occasionally observed. Class II expression (OX-6) was seen on marginating leukocytes present in glomerular and peritubular capillaries, and in interstitial infiltrates. In addition, the epithelial cells of some tubules, especially those in the vicinity of interstitial infiltrates expressed class II MHC antigens. No class II expression was seen on vascular endothelium of peritubular and glomerular capillaries or other renal blood vessels. Immunostaining for IgM and IgG (subclasses 1, 2a, 2b, 2c) in chronically rejected renal grafts showed, in addition to stained B and plasma cells, selective deposition along the renal vasculature. All IgG subclasses were present in the renal vessels, including glomerular and peritubular capillaries. Figure 2 A shows a staining pattern representative of all IgG subclasses of Lewis kidneys rejected by Lew-E tolerant BN recipients. A similar staining pattern was obtained for Lewis 1N kidneys rejected by unmodified BN recipients. In both models of chronic renal allograft rejection IgM deposition was detected selectively in the glomerular vasculature and sometimes in a few large blood vessels; no IgM was detected in the peritubular capillaries. Control syngeneic transplantations showed no graft pathology.

Discrepancies between the two models of chronic renal allograft rejection. A striking difference between the two models was the type of glomerular lesions in the individual models. Undoubtedly the glomerular lesions were related to the proteinuria developed by the recipients. In the Lewis renal grafts rejected by Lew-E modified BN recipients, the glomeruli were irregular in morphology, were frequently large with swollen endothelium and showed vasculonecrotic lesions with eosinophilic fibrinoid material deposited in variable intensities (Fig. 2 B). In the Lewis 1N renal grafts no such glomerular vasculonecrotic lesion with fibrinoid necrosis was observed, although swollen glomerular endothelium and mononuclear leukocytes were seen in most glomeruli (Fig. 3 A). Complement C3 deposition was studied in the rejected kidneys, and absent or only weak C3 staining was observed in the glomeruli of Lewis grafts in Lew-E tolerant BN recipients, whereas strong granular C3 staining was observed along the glomerular capillaries of Lewis 1N grafts in unmodified BN recipients (Fig. 3 B).

Reactivity of serum antibodies with non-MHC endothelial cell antigens

Serum was collected from renal allograft recipients in a late stage of chronic rejection when the animals had severe proteinuria, but stable renal function. Immunostaining with sera diluted 1/80 from BN recipients of both chronic rejection models clearly showed reactivity with Lewis heart endothelial cells, but not with heart muscle cells. No reactivity was seen with BN endothelial cells. Serum staining patterns in Lewis hearts were similar to immunostaining with RECA-1, a monoclonal antibody specific for rat endothelial cells [19]. Due to background problems, and perhaps endothelial low density antigen expression, kidney sections could not be used for the detection of anti-endothelial cell reactivity of serum antibodies. Because recipients of Lewis renal transplants were antibody-tolerant for Lew-E and therefore also for class I, serum reactivity with heart endothelial cells, which did not express class II antigens, was most likely directed against endothelial non-MHC antigens. Also the serum antibodies from BN recipients of Lewis 1N kidneys must have been directed against endothelial non-MHC antigens, because both graft and recipient were MHC haplotype-

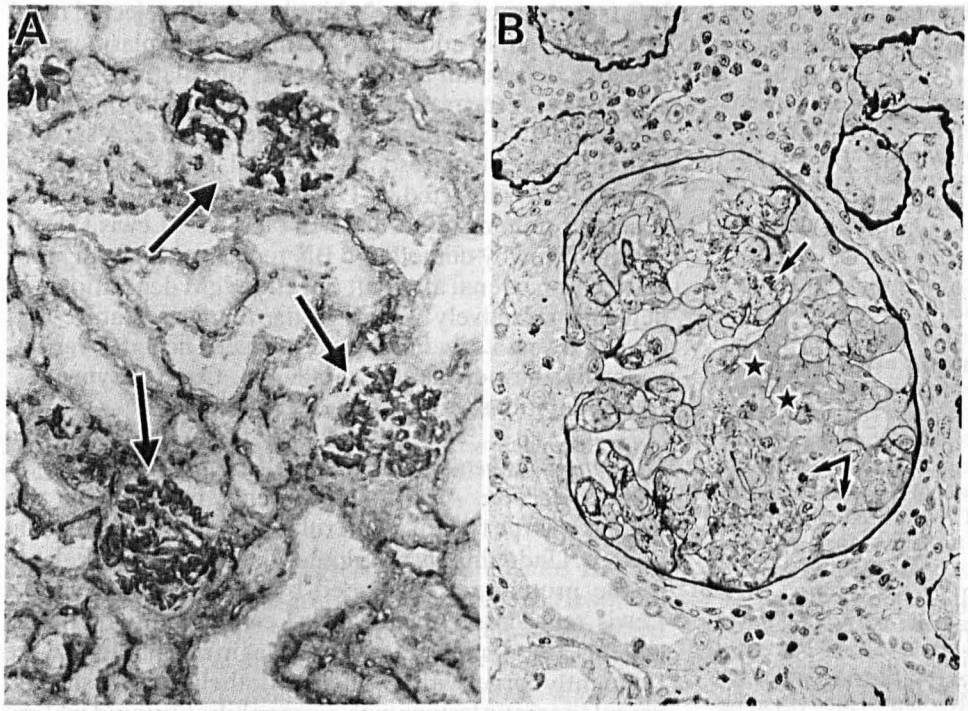

Fig. 2 A, B. Chronically rejected Lewis kidney in Lew-E tolerant BN recipient. **A** Immunoperoxidase staining for deposited IgG2c with antibody MARG2c. Note deposition along glomerular *(arrows)* and peritubular capillaries. × 200.
B HE/silver staining shows glomeruli with vasculonecrotic lesions with fibrin deposition *(asterisks)*. Note marginated mononuclear leukocytes in the glomerulus *(arrows)*, and the presence of interstitial infiltrate. × 500

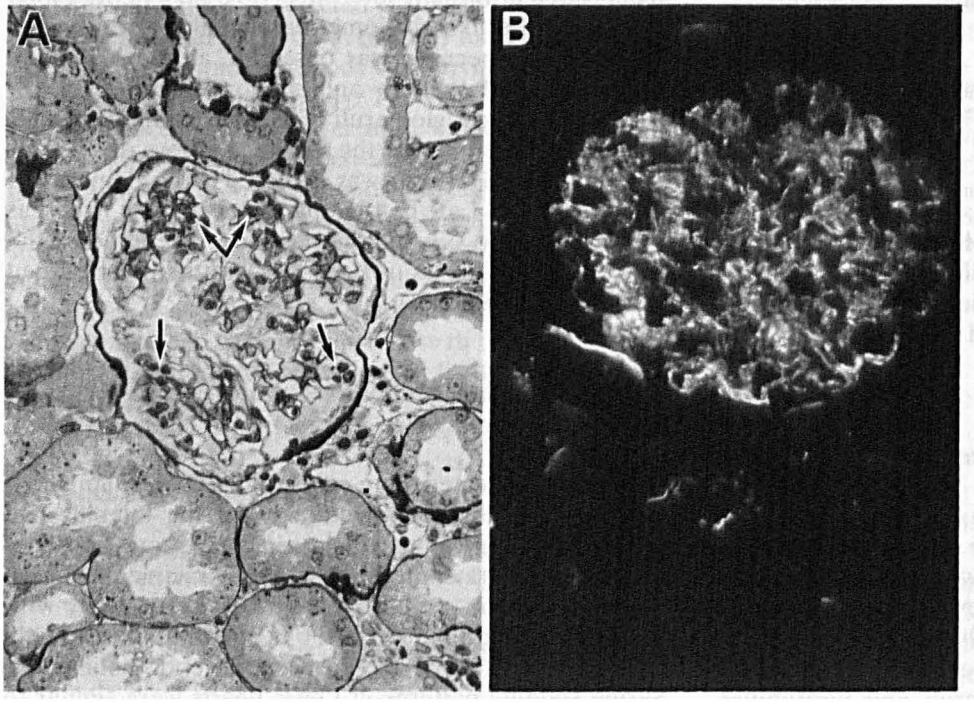

Fig. 3 A, B. Chronically rejected Lewis 1N kidney in unmodified BN recipient. **A** HE/silver staining shows absence of vasculonecrotic lesions with fibrin deposition in the glomeruli. Note glomerular mononuclear cell margination *(arrows)* and the presence of interstitial infiltrate. × 500.
B Fluorescent staining using anti-C3 antibodies shows deposited C3 in glomeruli. Note the granular staining along the glomerular capillaries. × 500

matched. Because staining for class I in heart sections also gave a staining pattern similar to RECA-1 (data not shown) the sera were tested on heart sections from Lewis 1N (carrying the BN MHC type) and BN 1L (carrying the Lewis MHC type) congenic strains, to ascertain that reactivity was with endothelial non-MHC antigens and not with MHC antigens. Sera from recipients in both transplantation models did not react with BN.1L endothelial cells (which excludes reactivity with Lewis MHC class I antigens), but did react with Lewis 1N endothelial cells, demonstrating that reactivity was indeed with non-MHC antigens on endothelial cells (Table 2). Control BN sera, or sera from control transplantations showed no reactivity with heart endothelial cells.

Discussion

We studied two models of chronic renal allograft rejection in the rat. Keeping in mind that, (1) endothelial cells in vascularized allografts can be considered a first and major target in rejection, and that, (2) endothelial cells are po-

Table 2. Reactivity of recipient serum antibodies in chronic rejection

Recipient	Graft	No. of transplantations tested	Serum staining[b] with heart endothelium of:			
			Lewis[c]	BN[d]	Lewis 1N[e]	BN.1L[f]
Lew-E tolerant BN	Lewis kidney	5	+	–	+	–
Unmodified BN	Lewis 1N	3	+[a]	–	+[a]	–

[a] One recipient, which was sacrificed 24 days after transplantation showed only weak staining of endothelium
[b] Frozen heart sections were stained with 1/80 diluted recipient serum using the immunoperoxidase technique
[c] MHC RT1[l], [d] MHC RT1[n], [e] MHC RT1[n], [f] MHC RT1[l]

tent responders to various cytokines released in local immune reactivity, we emphasized the role of vascular endothelial cells in this study. Since in mismatched grafts the MHC disparity leads to acute rejection, our models of chronic rejection were based on a partial MHC-tolerance of the recipient, or on non-MHC mismatch only. In the first model, the BN recipient had been made antibody (B cell) tolerant to donor erythrocytes, and therefore to donor class I antigens (rat erythrocytes are class I positive), leading to chronic renal graft rejection. Apparently, antibodies to graft class I antigens, which are highly expressed by endothelial cells, play a significant role in acute rejection. We observed intense interstitial mononuclear (monocytic and lymphocytic) cell infiltration and margination in peritubular and glomerular capillaries. Apparently, high cellular reactivity in these grafts is involved in the rejection process. We have suggested in a previous study, based on the lymphocyte profile and the moderate tubular damage, that this local immune reactivity is involved in regulatory (e.g. B cell help) rather than cytotoxic activity [20]. Because in the present study chronic rejection was clinically manifested by proteinuria in the presence of good renal functioning, we suspected glomerular lesions of playing a major role in the rejection process. The glomerular vasculonecrotic lesions that we observed with intravascular coagulation and deposition of IgM and the various IgG subclasses suggested that the lesions are most likely brought about by an antibody mediated mechanism. The fact that none or only weak C3 deposition in the glomeruli was detected suggested that glomerular thrombotic mechanisms leading to occlusion and glomerular lesions, may be due to glomerular endothelial cell activation (possibly with induced procoagulant activity) rather than antibody-mediated endothelial disruption. Also, other studies refer to the presence of non-cytotoxic anti-endothelial cell antibodies in chronic rejection [12]. The vasculonecrotic lesion occurred selectively in the glomeruli and not in the renal blood vessels, for example the peritubular capillaries, where IgG was also found to be deposited along the vessel wall. This suggested that in the glomeruli it may be the combination of IgM and IgG deposition, probably supported by effects of locally released cytokines, that triggered the (endothelial) thrombotic mechanisms [20, 21]. With respect to the reactivity of the deposited immunoglobulins, our date showed that they were not directed to graft endothelial MHC class II antigens, which were not expressed on the renal endothelium, nor to class I antigens because the recipient was antibody tolerant to graft class I antigens. The reactivity of the recipient sera with Lewis 1N but not BN.1L heart endothelial cells demonstrated that the serum antibodies, and thus most likely also antibodies deposited in the graft, reacted with graft endothelial non-MHC antigens. The data from our second model of chronic renal allograft rejection (Lewis 1N → unmodified BN) showed a similar clinical condition, and also similar immune reactivity in the graft, demonstrated by cellular infiltration and vascular IgM and IgG deposition, as the first model. However, major differences were found in the glomeruli. Glomerular vasculonecrotic lesions with intense fibrin deposition were absent, and strong glomerular C3 deposition was observed. This suggested that although the clinical data of chronic rejection were similar in the two models, the actual mechanism leading to glomerular protein leakage may have been different. Most likely, the C3 deposition was related to direct vascular damage and loss of the integrity of the glomerular filtration unit (viz. the layer of endothelial cells, glomerular basal membrane, and epithelial cells). Serum antibody reactivity with endothelial cell non-MHC antigens was also demonstrated in this model. The selective deposition of IgM in the glomeruli and not in other renal vessels in both models may indicate that we were dealing with locally formed or preformed and trapped immune complexes. To understand further the mechanism(s) responsible for the development of proteinuria during rejection, precise information on the localization of glomerular immunoglobulins and/or immunoglobulin-antigen complexes is essential, and, therefore, currently under investigation in our laboratory. In agreement with other authors [22–25], we concluded that, although different mechanisms may effect proteinuria in chronic kidney rejection, in this study non-MHC alloantigens on vascular endothelial cells played a significant role in chronic rejection of MHC-matched or partly (class II) mismatched renal allografts.

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