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Infiltration patterns of macrophages and lymphocytes in chronically rejecting rat kidney allografts

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Abstract The migration of circulating leukocytes to sites of inflammation or antigen is based, at least in part, on the activities of adhesion molecules. In the context of organ transplantation, some of these have been shown to be upregulated during acute allograft rejection. As their role during chronic rejection has not been examined, we have used an established rat model to compare sequentially the presence of host cells within the grafts, as defined immunohistologically, with patterns of in vitro leukocyte binding and their dependence upon particular adhesion molecules. Various donor populations of peripheral blood lymphocytes (PBL), lymph node lymphocytes (LNL), and splenic monocytes were interacted with snap-frozen sections of allografted, isografted, and native kidneys at serial intervals up to 24 weeks after transplantation. Monocyte binding in the allografts rose at 8 weeks and peaked at 12 weeks, a period preceding the maximum numbers of macrophages noted immunohistologically in the chronically rejecting grafts at 16 weeks. Lymphocyte binding and infiltration patterns were similar, remaining stable throughout the follow-up period and consistently greater than those noted in isografts. In vitro binding of the monocytes was inhibited by mAbs against ICAM-1, LFA-1, CD18, and MAC-1; MAC-1 did not influence lymphocyte binding, although the other mAbs were effective. We conclude that adhesion molecules are responsible, at least in part, for patterns of cell populations infiltrating chronically rejecting renal allografts.

Key words Kidney, rat, chronic rejection · Rat, kidney, chronic rejection · Chronic rejection, rat kidney · Infiltrating cells, chronic rejection, rat, kidney

Introduction

The specific adhesion of cells to other cells, tissues, or tissue components is a basic feature of cell migration and recognition. It underlies various biological processes that include embryogenesis, repair, and immunity. Adhesion molecules are involved in and mediate many such cell interactions, facilitate the diapedesis of leukocytes to sites of inflammation or alloantigens, and costimulate cell activation and transformation [25]. It is known that at least some of these molecules are upregulated during acute rejection of organ allografts. For instance, ICAM-1, a member of the immunoglobulin superfamily, is expressed constitutively on the vascular endothelium and glomeruli of normal kidneys, on coronary vessels, and on bile ducts of normal liver. It increases during host immunological attack [3, 21]; in mice, mAbs directed against ICAM-1 and LFA-1 abrogate acute graft destruction [11]. Upregulation of these molecules may be triggered by cytokines elaborated after organ graft injury secondary to donor factors, surgical manipulation, ischemia, storage, or reperfusion. For instance, the pre-

sence of cytokines increases the expression of several critical molecules on human renal tubular cells, including ICAM-1 and MHC class I and class II [21].

In this study, we have compared infiltration patterns of monocytes/macrophages and lymphocytes in chronically rejecting kidney allografts in rats, as identified immunohistologically, to the differential and sequential binding of these cell populations to kidney allografts in vitro. We assessed the influence of several adhesion molecules in the process.

Materials and methods

Animals

Naive male inbred rats (Harlan Sprague-Dawley, Indianapolis, Ind.), weighing 200–250 g, were used throughout the experiments. Lewis rats (LEW, $RT1^l$) acted as graft recipients and Fisher rats (F344, $RT1^{lvl}$) as donors. LEW → LEW served as isograft controls and Wistar/Furth ($RT1^a$) as third party controls.

Kidney grafting

The left donor kidney was excised, cooled, and positioned anatomically in the host animal whose left renal vessels had been clamped and kidney removed. The donor and recipient renal artery, vein, and ureter were then anastomosed end-to-end with 10-0 prolene sutures. No ureteral stent was used. Allograft recipients ($n = 60$) were treated briefly with low-dose cyclosporin (1.5 mg/kg per day) for the first 10 days after engraftment to suppress an initial acute rejection episode [9]. The remaining native kidney was excised on day 10. Isografted hosts were either not treated ($n = 20$) or treated with cyclosporin, as described ($n = 20$).

Tissue Preparation

Grafted organs and host tissues were harvested 2, 4, 8, 12, 16, and 24 weeks after transplantation ($n = 8$ per period), snap-frozen in liquid nitrogen and stored at – 70 °C, or fixed in formalin or acid formalin for hematoxylin/eosin staining.

Antibodies

Monoclonal antibodies (mAbs) to adhesion molecules included those directed against L-selectin (HRL-3), LFA- 1 α (CD11a – WT.1), CD18 (WT.3), MAC-1 α (CD11b – WT.5), and ICAM-1 (CD54 – 1A29) [20–22, 27–29]. Antibodies against T cells (CD5 – OX- 19), monocytes/macrophages (ED-1), and MHC class II (OX-3) were obtained from Sera-Lab (Accurate Chemicals, Westbury, N. Y.).

Immunohistology

Cryostat sections of frozen tissues were stained individually with mAbs from the above panel using the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method. Stained cells were then counted with an ocular grid (600 ×, > 30 fields counted/section, 3–4 sections per kidney). The intensity of the tissue staining was evaluated on a scale of 1–4.

Preparation of cell suspensions

Peripheral blood lymphocytes (PBL) were isolated by layering heparinized blood (1 ml heparin/10 ml) over a Percoll hypaque gradient (63 %/42 %), centrifuging for 30 min at 1800 rpm, and washing with phosphate-buffered saline (PBS). Monocytes were collected from the spleen. The organs were minced and expressed through stainless steel mesh, the cells suspended, washed with PBS, layered twice over a Percoll gradient (35 %/43 %), and washed again. Lymph node lymphocytes (LNL) from cervical and axillary lymph nodes were collected after the nodes had been minced and expressed through stainless steel mesh, suspended, and washed with PBS. Cell preparations were evaluated using fluorescence staining with the appropriate antibodies and discarded if the purification was less than 95 % for PBL and LNL or less than 85 % for monocytes.

Binding assay

For binding studies, performed at 8, 12, 16, and 24 weeks after transplantation, a modified Stamper/Woodruff assay was used [26]. Aliquots of PBL and LNL ($5–10 \times 10^7$) from naive Lewis, F344, and third party Wistar/Furth rats, and monocytes ($5–10 \times 10^7$) from naive Lewis rats were incubated for 30 min at 64 rpm at 25 °C with snap-frozen sections of kidneys from naive animals and serially harvested allografts and isografts [2]. PBL and LNL from allograft and isograft recipients were also incubated with kidney graft sections from their respective hosts. The preparations were fixed in a 1.5 % glutaraldehyde solution and counterstained with thionine. Cells per field of view (c/FV) were then counted from at least 20 fields/section; four to six sections from each kidney were examined. The binding was considered specific if the percentage of cells adhering to a particular kidney component exceeded those of naive control kidneys by at least 20 %. Only sections with the tissue structure unaltered and intact at the time of evaluation were accepted.

Inhibition of binding

The method for in vitro blocking of cell binding has been described elsewhere [2]. Purified PBL, LNL, and monocytes ($5–10 \times 10^7$) were incubated with titrated concentrations (10–25 μg/ml) of the mAbs against the individual adhesion molecules for 10 min at 4 °C. PBS and an antibody to MHC class II (OX-3) were used as controls. The treated cells were then interacted at 25 °C with frozen sections of kidney grafts harvested 12 weeks after transplantation, and the extent of binding assessed was noted.

Statistics

Results were evaluated using Student's t-test.

Results

Morphological changes

Events of chronic rejection occurred in a predictable sequence in this kidney graft model. Large numbers of leukocytes infiltrated the allografts transiently during

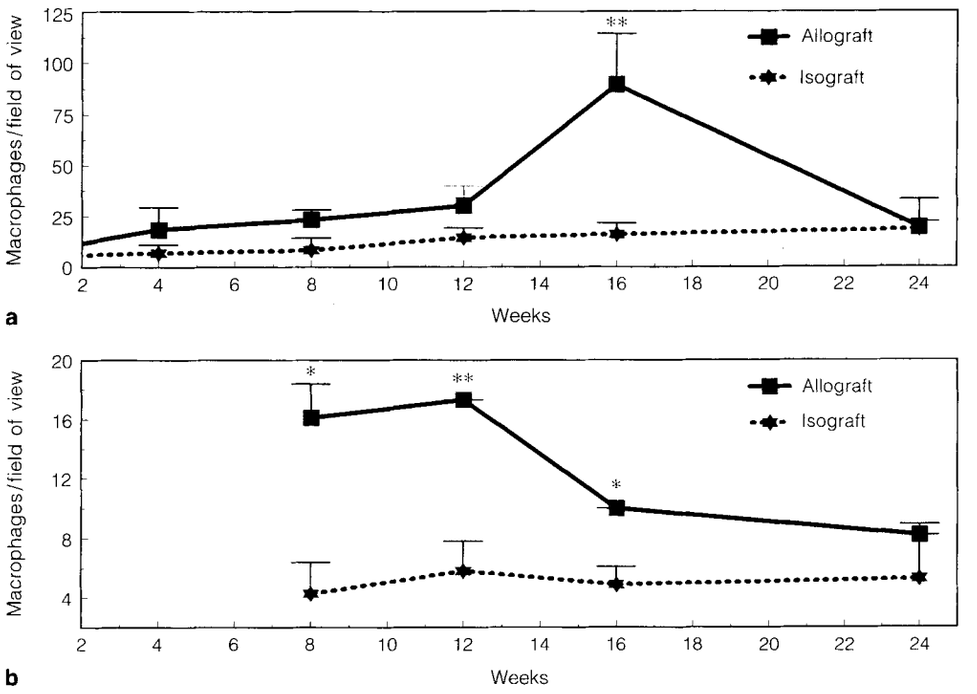

Fig. 1 a Infiltration of ED-1 + macrophages in grafted kidneys peaked between weeks 12 and 16, as assessed immunohistologically; **b** in vitro binding of monocyte/macrophages to grafted kidneys peaked between weeks 8 and 12. Negligible cellular activity occurred in isografts. Figures represent mean values from five grafts/time point. * $P < 0.01$; ** $P < 0.001$

a reversible episode of acute rejection at 1–2 weeks. Thereafter, the chronic process evolved progressively through discrete phases of cellular infiltration to eventual sclerosis. Mononuclear leukocytes appeared diffusely throughout the allograft substance by 4 weeks, localizing primarily in perivascular areas by 8 weeks and entering glomeruli in increasing numbers thereafter. Between 12 and 16 weeks, interstitial and glomerular sclerosis and intimal proliferation of arteries and arterioles were becoming evident. By 24 weeks, generalized fibrosis and sclerosis of the glomeruli had become increasingly intense. After 24 weeks, the numbers of infiltrating cells declined progressively. The recipients had begun to die of renal failure.

During the period studied, isografted kidneys showed negligible changes compared to native organs, regardless of initial treatment with cyclosporin.

Monocytes/macrophages

The migration dynamics of monocytes/macrophages were studied both immunohistologically and by in vitro binding assays. Immunohistology showed that small numbers of ED-1 + macrophages were present throughout the allografted kidneys during the first 8 weeks. The numbers increased dramatically between weeks 12 and 16, diminishing to baseline thereafter (Fig. 1 a). During this period, this population localized preferentially in glomeruli (72 % of all ED-1 + macrophages present in the tissue, 61 c/FV or 7.6 cells/glomerulus vs 0.5 cells/glomerulus in isografts at 16 weeks; Fig. 2), entering the interstitium in fewer numbers.

In vitro monocyte binding studies did not corroborate these immunohistological observations in a quantitative sense, as the total number of monocytes binding to allograft tissue remained relatively low throughout the course of chronic rejection, albeit higher than in isografts. (The number of monocytes in the allografts at 8 weeks was 16 c/FV and at 12 weeks was 17 c/FV vs binding to native and isografted kidneys, which was less than 6 c/FV at all times, $P < 0.01$; Fig. 1 b). The total numbers of binding cells diminished after this 12-week peak to levels comparable to those in the control organs (the number of monocytes in the allografts at 16 weeks was 10 c/FV and at 24 weeks was 6 c/FV). However, the specificity of monocyte binding to allograft glomeruli between 8 and 12 weeks increased to more than 60 % of cells bound versus less than 5 % on native and isografted kidneys. This correlates well with the preferential presence of macrophages in the glomeruli, as observed immunohistologically. By 24 weeks their distribution changed, with more frequent binding to areas of interstitial fibrosis and less to the fibrosing glomeruli themselves.

The inhibition of peak binding at 12 weeks using mAbs against adhesion molecules was then tested. The binding of monocytes was reduced significantly by in-

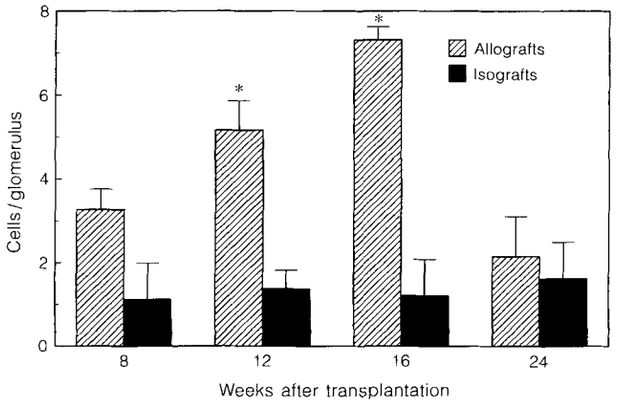

Fig. 2 Monocyte/macrophage infiltration in glomeruli of chronically rejected kidneys, as shown by immunohistology. At least 40 glomeruli were counted per graft. * $P < 0.01$

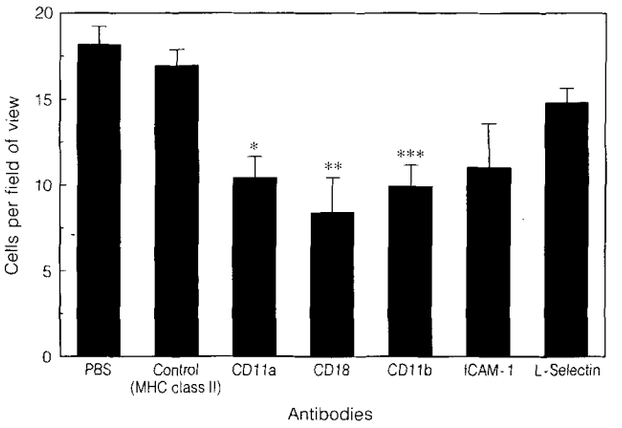

Fig. 3 Blocking of monocyte binding. At 25 °C, binding was reduced significantly by incubation with mAbs to ICAM-1 (CD54–1A29), L-selectin (HRL-3), CD11a (LFA-1 α–WT.1), CD18 (WT.3), and CD11b (MAC-1 α–WT.5). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.05$, vs controls

cubation with mAbs to ICAM-1 (– 35 %), LFA-1 α (– 39 %), CD18 (– 51 %), and MAC-1 α (– 42 %; Fig. 3).

Lymphocytes

Immunohistology showed that lymphocyte infiltration of the kidney allografts increased during the initial acute rejection episode at 1–2 weeks, then declined to relatively low but stable levels throughout the follow-up period ($P < 0.01$ vs levels of the isografts; Fig. 4 a). In contrast to macrophages, lymphocytes were almost never encountered in glomeruli, remaining distributed throughout the interstitium.

Throughout the entire follow-up period, larger numbers of lymphocytes bound in vitro to allografted kidneys than were reflected by immunohistology. The in vitro binding characteristics of PBL and LNL from grafted, native, or third party animals did not differ from one another at any time observed. In contrast, there were important differences in binding patterns depending on whether allografted, isografted, native, or third party kidneys were used as target tissues. At week 8, LNL binding to allografts localized specifically to tubules. By week 12, binding to vessels had increased slightly and preferentially (221 c/FV), but declined thereafter (Fig. 4 b). At 12 weeks, the binding cells localized to both vessels and atrophic tubules, but never to glomeruli. These changes, however, were not statistically significant. The binding of LNL or PBL to isografted and other control organs was considerably lower at all times ($P < 0.01$).

ICAM-1 staining, assessed individually as a representative adhesion molecule in this model, remained consistently elevated in the chronically rejecting allografted kidneys (3–4 +) compared to the isografts (1–2 +). At 8 and 12 weeks, ICAM-1 staining was noted primarily on tubular and vascular endothelium, shifting to atrophic tubules and infiltrating cells thereafter as the chronically rejecting grafts began to fibrose. Lymphocyte binding correlated well with its presence on various graft components (Fig. 5).

LNL binding to allografted kidneys at week 12 was significantly diminished by mAbs against specific adhesion molecules: HRL-3 (L-selectin, – 22 %), WT.1 (LFA-1 α, – 39 %), WT.3 (CD18, – 49 %), and 1A29 (ICAM-1, – 45 %). In contrast to its effect on monocytes binding, WT.5 (MAC-1 α) was ineffectual (Fig. 6).

Discussion

Macrophages and T lymphocytes comprise the predominant cell populations identified in chronically rejecting allografts in virtually all clinical and experimental settings [3, 9, 30]. In human renal allografts undergoing chronic rejection, subendothelial plaques consisting of macrophages in foamy transformation have been described [22]. Macrophages of various phenotypes have been noted in biopsies of heart allografts [18], while intimal foam cells derived mostly from macrophages, and to a lesser extent from smooth muscle cells, almost completely occupied the coronary lumina of heart grafts in cholesterol-fed and immunosuppressed rabbit recipients [23]. These results imply that not only immunological factors are involved in the reaction of the arterial wall in transplant arteriopathy but that, as in atherosclerosis, endothelial dysfunction may also be important [22].

In the immunohistological analysis, macrophages comprised the most striking cell population occurring during chronic rejection in this rat kidney model, parti-

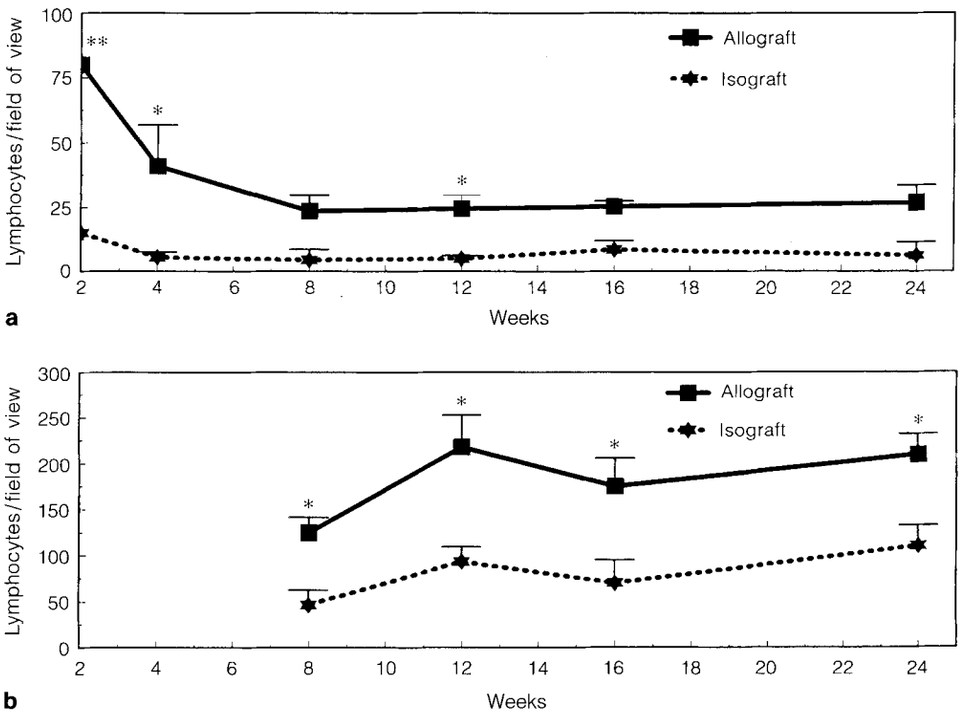

Fig. 4a Lymphocyte infiltration of organ allografts, as assessed immunohistologically, increased early during the initial reversible acute rejection episode, declining and remaining unchanged throughout the course of chronic rejection. **b** In vitro binding during the process of chronic rejection remained constantly high. Negligible cellular activity occurred in the isografts. Figures represent mean values from five grafts/time point. * $P < 0.01$; ** $P < 0.001$

cularly 12–16 weeks after engraftment. This period seems critical as progressive functional deterioration and glomerular sclerosis also develop, associated with selective macrophage infiltration of glomeruli and upregulation of the cytokines IL-1, TNF-α, and IL-6 [6, 9]. As monocyte binding to allografted kidneys in vitro is less intense than predicted by immunohistology and peaks 4 weeks earlier, the results of such binding studies suggest that either the method may be inadequate to detect all ongoing binding in the graft or that binding per se is only partially responsible for cell infiltration and does not reflect all events occurring in vivo. Alternatively, proliferation in situ may account for the larger numbers of macrophages found in the grafted kidneys by immunohistology, a point emphasized in other systems [20, 31]. In experimental focal and segmental glomerulosclerosis in rats, a model that has features similar to those observed in chronic rejection, macrophages are the dominant cells in the affected glomeruli [16]. Proliferation of mesangial macrophages is a prominent feature in the normal process of glomerular cell renewal; in hypercellular glomeruli, more vigorous local proliferation of these cells greatly amplified their potential to cause damage [5, 20]. In a rat model of acute glomerular injury induced by nephrotoxic serum, a circumstance corresponding to possible antibody involvement in chronic rejection, numbers of ED-1 + monocytes increased quickly and persisted throughout the follow-up period. Quantification of intraglomerular Ia + cells has also suggested in situ activation of monocytes within the glomeruli [7]. Conversely, the administration of antirat macrophage serum induced a profound reduction in proteinuria and a marked reduction in glomerular sclerosis [17].

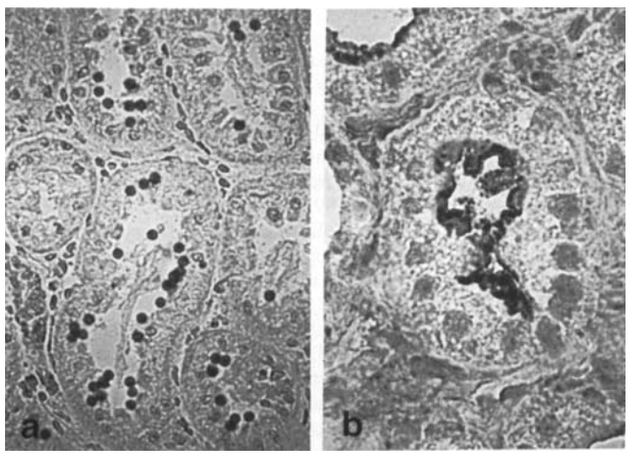

Fig. 5a, b Lymphocytes bound preferentially to areas of strong ICAM-1 staining: **a** lymphocytes bound to tubules (× 60); **b** ICAM-1 staining of tubules (× 150)

The discrepancy between in vitro lymphocyte binding and infiltration patterns of these cells as observed by immunohistology is difficult to explain. The binding tech-

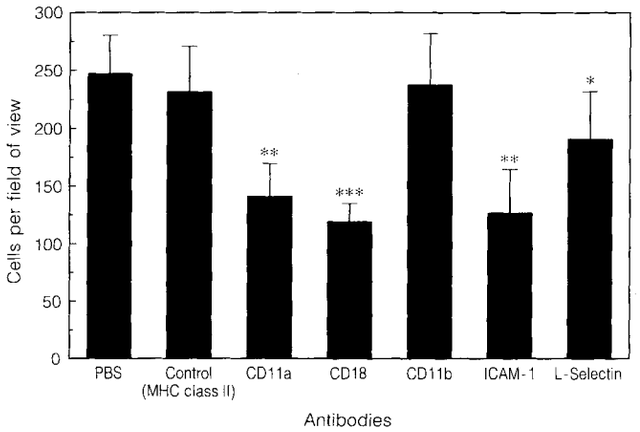

Fig. 6 Blocking of lymphocyte binding. At 25 °C, binding was reduced significantly by incubation with mAbs to ICAM-1 (CD54–1A29), L-selectin (HRL-3), CD11a (LFA-1 α–WT.1), and CD18 (WT.3). A mAb against CD11b (MAC-1 α–WT.5) was ineffectual. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs controls

nique may reflect adhesion of leukocytes to vascular endothelium based on the action of some adhesion molecules but may not reflect the action of other molecules necessary for their migration into the tissue [8, 10, 13]. That in vitro binding patterns of lymphocytes from different sources did not differ may reflect a low proportion of activated cells. More likely, it may indicate the critical nature of the endothelial cells for extravasation into the grafted organ.

A number of studies of experimental glomerulonephritis and of acute rejection of heart and kidney allografts have suggested a role for adhesion molecules in the pathophysiology of these conditions [2, 3, 21]. Indeed, in the present chronic rejection model, ICAM-1 expression on particular graft components correlated directly with lymphocyte binding patterns. Conversely, monocyte binding was inhibited by all mAbs against the adhesion molecules tested. Lymphocyte binding was also decreased by all of the mAbs used except MAC-1 α. L-selectin is probably involved in the initial adherence of monocytes to glomeruli [1, 2, 4]; VCAM-1 may also be important as its upregulation has been shown in heart and kidney allografts, and rejection can be tempered by mAbs directed against it [19].

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