0041-1337/93/5503-0610$03.00/0
TRANSPLANTATION

Vol. 55, 610-615, No. 3, March 1993
Printed in U.S.A.

DISTRIBUTION OF CELL ADHESION MOLECULES (ICAM-1, VCAM-1, ELAM-1) IN RENAL TISSUE DURING ALLOGRAFT REJECTION[1]

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The tissue distribution of cellular adhesion molecules (CAMs) was studied in specimens from 10 normal human kidneys and in 52 biopsies from kidney allografts with cell-mediated rejection. In addition to the vascular presence of ICAM-1, a common finding in normal kidneys, expression of ICAM-1 on tubular cells was observed in 22 graft biopsies. Compared with normal kidneys, where VCAM-1 was present on Bowman's capsules and few proximal tubular cells, a markedly enhanced expression of VCAM-1 in numerous tubuli (including distal tubular segments) was observed in 51 graft biopsies. In 41 graft specimens VCAM-1 appeared also in variable numbers of peritubular capillaries. Infiltrating leukocytes carrying VCAM-1 were observed in 7 grafts. ELAM-1 could not be found in normal kidneys but was restricted to some peritubular capillaries in 29 grafts. Comparable results were obtained with cultured renal tubular cells when stimulated by TNF-α. That the induced appearance of adhesion molecules was in fact related to actual cellular synthesis was demonstrated by Northern blot analysis. Thus, little ICAM-1 specific mRNA of 3.4-kb length could be detected in unstimulated cultured renal tubular cells, but hybridization was markedly increased after stimulation with TNF-α. A substantial amount of VCAM-1 specific mRNA of 3.2-kb length was present already in unstimulated renal tubular cells. Likewise, synthesis of VCAM-1 mRNA was enhanced by stimulation with TNF-α. TNF-stimulated endothelial cells also showed weak synthesis of VCAM-1 mRNA. The results provide further evidence that constitutive and inducible expression of cell adhesion molecules contributes to the process of allograft rejection.

The interaction of circulating leukocytes with vascular endothelial cells is of essential importance in cell-mediated inflammatory reactions (*1–3*). Among the various cell adhesion molecules (CAMs)* that are operative during this interaction, endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1, CD54), and vascular cell adhesion molecule-1 (VCAM-1, also named INCAM-110) have attracted special interest.

Recent in vitro studies have recognized a two-step mechanism in the process of adherence and transendothelial migration of leukocytes. In this model, the endothelial selectins ELAM-1, LECAM-1, and CD62 were shown to mediate initial, but reversible leukocyte rolling along endothelium, whereas ICAM-1 appears to mediate firm attachment and transendothelial migration (*4*). VCAM-1, in addition to immediate actions comparable to ELAM-1 and ICAM-1, was ascribed a functional role also in chronic inflammatory reactions (*5*). Different ligands on leukocytes are used during this process, with ELAM-1 and CD62 binding to the same carbohydrate ligand, sialyl-Lewis x, ICAM-1 binding both leukocyte β_2 integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), and VCAM-1 interacting with integrin VLA-4 (*6–8*).

Along with the different functional kinetics of vascular adhesion molecules, also differences in the expression in normal and inflamed tissues have been recognized. ICAM-1 is constitutively present on endothelial cells, on lymphoid cells, and malignant tumors (*9–12*). When stimulated by lymphokines, e.g., in the course of allograft rejection, de novo expression of ICAM-1 has been reported on renal tubular cells (*10, 13*) and hepatocytes (*14*). Likewise, expression of VCAM-1, originally thought to be endothelial specific and cytokine dependent, has recently been reported in lymphoid tissues, on Kupffer cells and on renal epithelial cells, including Bowman's capsule and tubules (*5*). Induced vascular VCAM-1 expression was observed in human cardiac allografts (*15*). In contrast, ELAM-1 does not appear in normal tissues but is restricted to inflamed endothelium, e.g., in the skin (*16*), lung (*17*), or also in cardiac allografts (*15*).

Only limited information is available with respect to the appearance of such adhesion molecules during rejection of renal allografts, therefore the tissue distribution of ICAM-1, ELAM-

[1] This work was supported by the Deutsche Forschungsgemeinschaft (SF 217:C8).

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* Abbreviations: CAMs, cell adhesion molecules; EC, endothelial cells; ELAM-1, endothelial leukocyte adhesion molecule-1; FB, fibroblasts; ICAM-1, intercellular adhesion molecule-1; kb, kilobases; LFA-1, lymphocyte function-associated antigen-1; PTC, peritubular capillaries; RTC, renal tubular epithelial cells; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

1, and VCAM-1 in normal and transplanted kidneys is investigated in this study. In addition, nonvascular expression and synthesis of these molecules are examined in more detail with cultured tubular epithelial cells.

MATERIALS AND METHODS

Renal tissues. Biopsy specimens from 52 human cadaver kidney transplants with a clear diagnosis of acute cell-mediated rejection according to clinical and pathological records were investigated. Normal renal tissue was obtained from 10 tumor nephrectomies.

The tissue distribution of cell adhesion molecules was assessed in parallel in these specimens.

Cell culture. Primary cultures of normal renal tubular epithelial cells (RTC) were derived from nephrectomy specimens according to the method of Ebert et al. (*18*) and were cultured in medium RPMI 1640 supplemented with 10% fetal calf serum, epidermal growth factor (10 ng/ml) (Boehringer Mannheim, Mannheim, Germany), and cholera toxin (10 ng/ml) (Sigma, Deisenhofen, Germany).

Human fibroblasts, transformed by SV40 were cultured in medium RPMI 1640 supplemented with 10% calf serum.

Endothelial cells (EC) were harvested from human umbilical cord vein by collagenase digestion according to Jaffe et al. (*19*). Cells were seeded in tissue-culture flasks and grown in medium M199 supplemented with 20% pooled human AB serum, nystatine (50 U/ml), insulin (10 μg/ml), endothelial growth supplement (30 μg/ml), and heparin (90 U/ml) (Sigma, Deisenhofen, Germany). All cultured EC carried specific markers such as factor VIII (V. Willebrand factor) and were stained by the EC-specific mAbs EN4 and BMA120.

All cell lines were subcultured by exposure to 0.02% trypsin and 0.05% EDTA in PBS.

For stimulation experiments, cell cultures were grown to confluence in 250-ml tissue culture flasks and were incubated in the absence or presence of recombinant TNF-α (200 U/ml) (Boehringer Mannheim, Mannheim, Germany) for 48 hr.

Monoclonal antibodies. The following mouse monoclonal antibodies were used: V9 against vimentin (M725, DAKOPATTS, Hamburg, Germany); CK2 against cytokeratin-18 (Boehringer Mannheim, Mannheim, Germany); 4F9 against human factor VIII (V. Willebrand factor) (Immunotech S.A., Marseille, France); EN4 (Sanbio, Uden, The Netherlands); and BMA120 (Behringwerke, Marburg, Germany) reactive against endothelial cells; H18/7 directed against ELAM-1 (*20*); BBA 1 against ELAM-1 (British Biotechnology Ltd., Oxford, UK); P3.58BA-14 directed against ICAM-1 (*21*); 4B9 (IgG1) directed against VCAM-1 (*22*); BBA 5 against VCAM-1 (British Biotechnology); T29/33 against common leukocyte antigen (CD45) (Hybritech Inc., San Diego, CA); mouse IgG1, IgG2a, and IgG2b antibodies against irrelevant antigens were used as controls.

Indirect immunoperoxidase staining. Indirect immunoperoxidase staining was performed as described in detail previously (*23*). In brief, 4-μm cryostat sections were prepared from normal kidney tissues and renal graft biopsies, stored at −80°C. Specimens were air-dried, fixed with cold acetone (10 min), washed in phosphate-buffered saline at pH 7.4, and were reacted serially with appropriately diluted primary monoclonal antibodies and with peroxidase-conjugated second antibody (rabbit anti-mouse immunoglobulins; Dakopatts, Hamburg, Germany). Specimens were finally stained with 3-amino-9-ethylcarbazole (Sigma), DMSO, H_2O_2 (Merck, Darmstadt, Germany) and were counterstained with hemalaun (Merck). For microscopic evaluation, slides were mounted with glycerine-gelatine (Merck). Suspended cells were cytocentrifuged on glass slides (3×10^4 cells/slide) and were processed exactly as described above.

Immunofluorescence analysis. For flow cytometric analysis, cells were suspended with 0.02% trypsin and 0.05% EDTA in PBS and reacted with the appropriate monoclonal antibody for 45 min at 4°C. After washing three times in serum-free culture medium, cells were incubated for 45 min at 4°C with FITC-conjugated rabbit anti-mouse Ig (Dakopatts). After washing in serum-free culture medium, cells were fixed in paraformaldehyde and analyzed in a FACScan (Becton Dickinson, Mountain View, CA).

Northern blot analysis. Total RNA from cultured cells was prepared by the procedure of Chirgwin (*24*). RNA (15 μg) was separated on a formaldehyde agarose gel and was transferred to nylon membranes (Hybond-N$^+$; Amersham, Brauschweig, Germany). The membranes were hybridized with a mixture of 6 single-stranded DNA oligonucleotides specific for VCAM-1 (British Bio-technology Ltd.) and with a cDNA probe of 690 bp specific for ICAM-1 (*12*, *21*). The oligonucleotides were labeled in a single reaction with terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and [α-^{32}P] dATP (Amersham) (*25*). Hybridization at 65°C was performed as described (*26*). Washings were done stepwise at 65°C with 3× saline sodium citrate buffer, 10 mM NaH_2PO_4/Na_2HPO_4, 10× Denhardt's solution, 5% sodium dodecyl sulfate for 2× 20 min and with 1 SSC, 1% SDS for 10 min. The cDNA probe was labeled by the random primer method (*27*) with [α-^{32}P]dATP (Amersham), and hybridization and washing were performed according to the method of Church and Gilbert (*28*).

RESULTS

Tissue distribution of cell adhesion molecules in normal kidneys and during cell-mediated rejection was as follows.

Intercellular adhesion molecule-1. Comparable immunohistological results with mAb P3.58-BA14, reactive against ICAM-1 were obtained with 10 normal kidney specimens. Thus, ICAM-1 was present on glomerular endothelial cells, in peritubular capillaries, in large vessels, and on some parietal epithelial cells of Bowman's capsule (Fig. 1A). During acute cell-

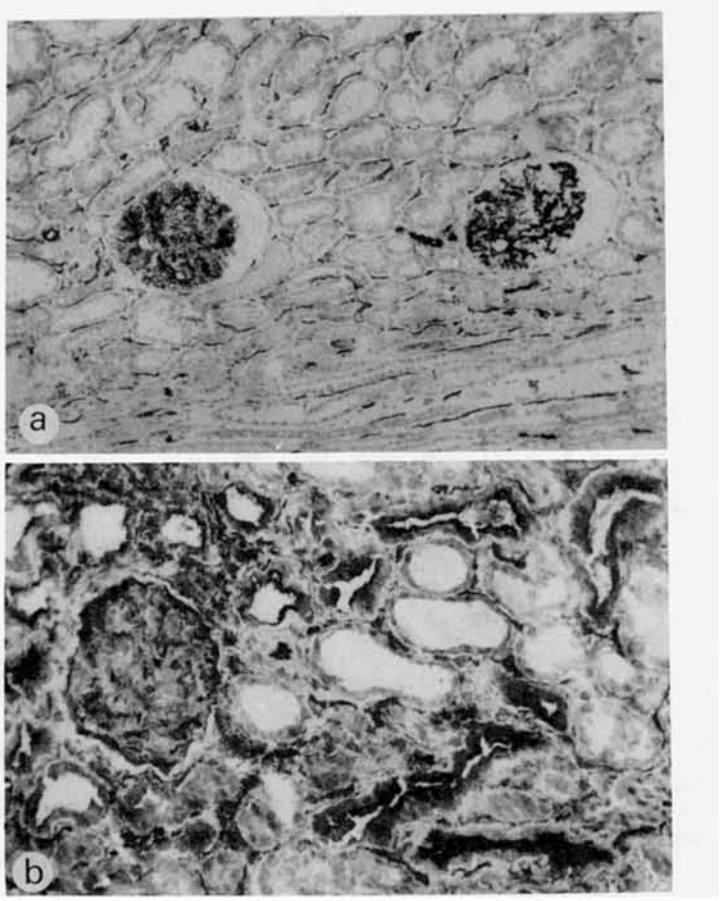

FIGURE 1. Distribution of ICAM-1. (A) Normal kidney: endothelial cells in glomerulus, peritubular capillaries, and some epithelial cells of Bowman's capsule (original magnification ×100). (B) Expression of ICAM-1 on tubular cells during rejection (original magnification ×150).

mediated rejection, focally distributed expression of ICAM-1 predominantly in proximal tubules was observed in 22 out of 52 graft biopsies (Fig. 1B). In 19 biopsies, ICAM-1 was also detectable on graft-infiltrating leukocytes.

Vascular cell adhesion molecule-1. In 10 normal kidneys, mAbs 4B9 and BBA 5 against VCAM-1 reacted with all parietal epithelial cells of Bowman's capsule and with few cells in several proximal tubules, whereas vessels were devoid of VCAM-1 (Fig. 2A). During rejection, increased tubular staining (referring to both the number of tubules and the intensity of staining) with VCAM-1 specific monoclonal reagents was observed in 51 out of 52 grafted kidneys (Fig. 2B). VCAM-1 staining was focally distributed and included distal tubular segments as well. In 41 graft biopsies, VCAM-1 appeared also in variable numbers of peritubular capillaries, ranging from single vessels to approximately 30% of all vessels present in some peritubular capillaries during rejection (original magnification ×250). (D) VCAM-1 expression on graft-infiltrating leukocytes (original magnification ×380).

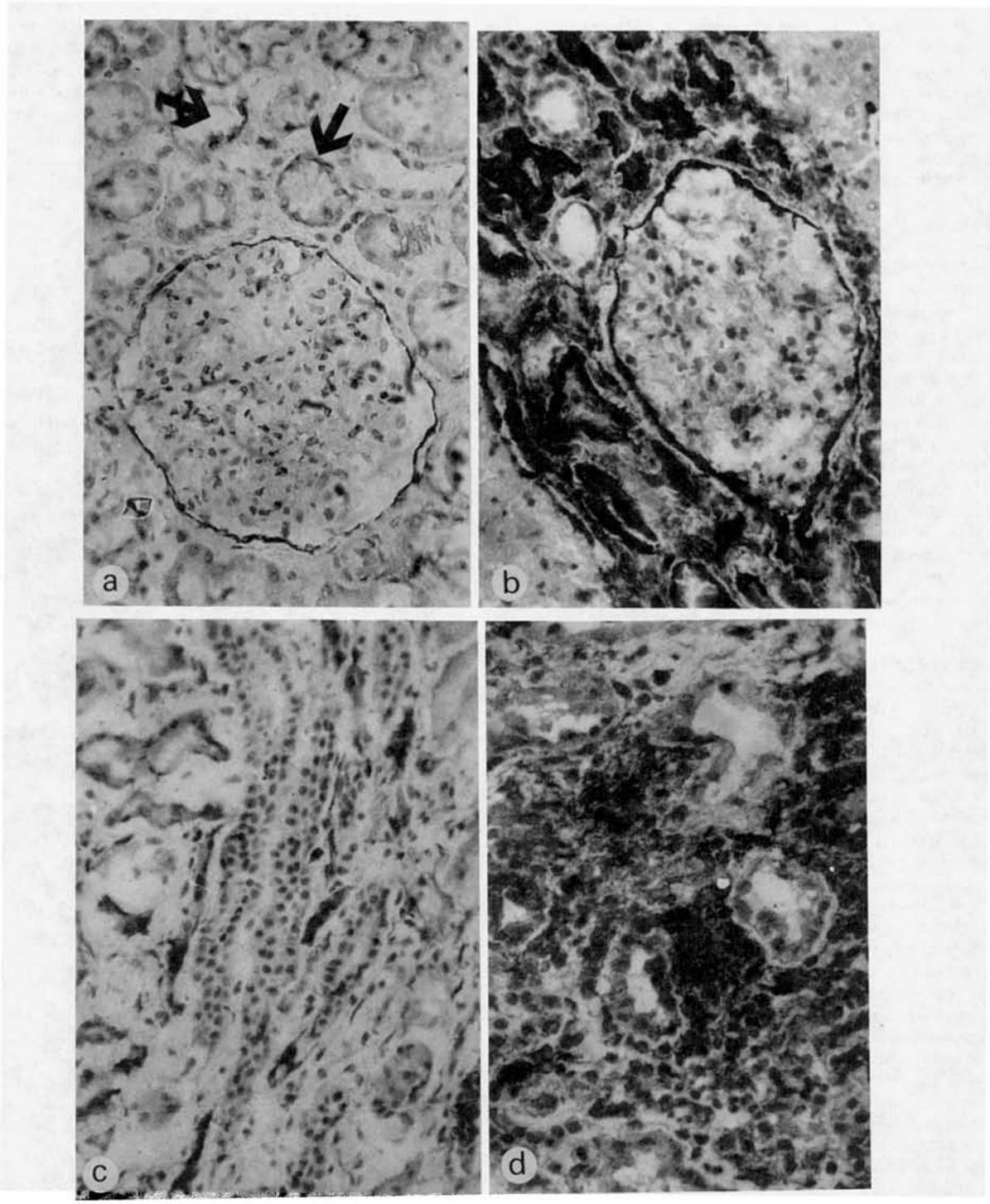

FIGURE 2. Distribution of VCAM-1. (A) Normal kidney: epithelial cells of Bowman's capsule, few proximal tubular cells (original magnification ×290). (B) Enhanced tubular expression of VCAM-1 during rejection (original magnification ×250). (C) Expression of VCAM-1 in

one specimen (Fig. 2C). However, glomerular capillaries were consistently devoid of VCAM-1. In 7 biopsies, also infiltrating leukocytes carried VCAM-1 (Fig. 2D).

Endothelial leukocyte adhesion molecule-1. No reactivity of mAbs H18/7 and BBA 1 against ELAM-1 was observed in normal kidneys (not shown). During rejection, ELAM-1 could be detected only in few peritubular capillaries in 29 out of 52 biopsies (Fig. 3). ELAM-1 could not be detected in glomerular capillaries, in tubules, or in cellular infiltrates. Immunohistological results in normal kidneys and in graft biopsies are summarized in Fig. 4 A and B.

Expression of cell adhesion molecules on cultured renal tubular cells. When various cell markers such as cytokeratin-18 (CK-18), vimentin, PAL-E antigen, factor VIII antigen, or VCAM-1 were studied, the phenotypes of cultured tubular epithelial cells and of tubular cells within renal sections were comparable (Table 1). However, ICAM-1 could be observed already on unstimulated RTC. Treatment of RTC with TNF-α resulted in enhanced expression of both ICAM-1 and VCAM-1. ELAM-1 was not present on RTC and could not be induced by TNF-α.

Presence of ICAM-1 and VCAM-1 on RTC obtained from two individuals was confirmed by flow cytometry analysis (Table 2). Increased fluorescence was consistently measured after treatment of RTC with 200 U/ml TNF-α for 48 hr.

Detection of ICAM-1 mRNA and VCAM-1 mRNA by Northern blot analysis. ICAM-1 specific mRNA of approximately 3.4-kb length was clearly detectable in a fibroblast cell line after induction by TNF-α. Prolonged exposure of the Northern blot revealed a faint hybridization signal also in unstimulated fibroblasts (not shown). In RTC, ICAM-1 specific mRNA could be detected in unstimulated cells, and increased biosynthesis of ICAM-1 mRNA was induced by TNF-α (Fig. 5A).

VCAM-1 specific mRNA of 3.2-kb length was detected in cultured endothelial cells after TNF-α treatment. No hybridization signal was obtained with fibroblasts. VCAM-1 transcripts were clearly present in unstimulated RTC; enhanced hybridization was seen following treatment with TNF-α (Fig. 5B). That roughly equal amounts of total RNA were tested in this study can be deduced from ribosomal 28s RNA.

Association of cellular infiltrates with CAMs. Cellular infiltrates in kidney grafts with rejection were readily visualized by mAbs to common leukocyte antigens such as CD45. Both patchy and diffuse interstitial infiltration were common (not shown). No clear-cut association of cell infiltrates with non-vascular expression of ICAM-1 could be determined. Likewise, the tubular appearance of VCAM-1 had no strict correlation with infiltrating cells. However, numerous capillaries bearing VCAM-1 were found to contain intraluminal leukocytes. Occasionally, intraluminal leukocytes were also present in ELAM-1 positive vessels.

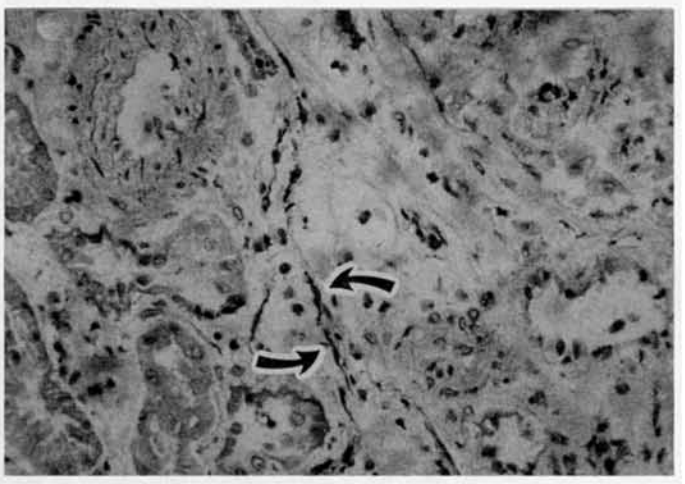

FIGURE 3. Appearance of ELAM-1 in few peritubular capillaries during rejection (original magnification ×250).

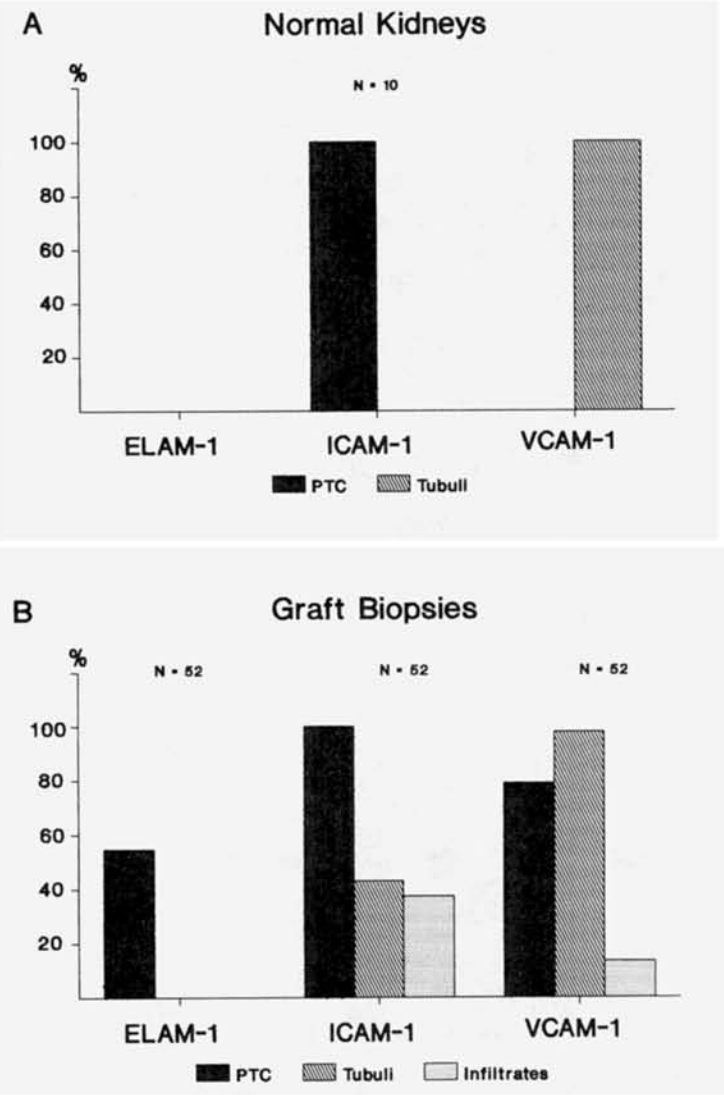

FIGURE 4. Differential distribution of ELAM-1, ICAM-1, and VCAM-1 in 10 normal kidneys (A); and during rejection in 52 graft biopsies (B).

TABLE 1. Immunochemistry of renal tubular epithelial cells

Antigen[a]	Normal kidney	Kidney allograft	RTC
CK-18	+	ND	+
Vimentin	−	ND	−
Pal-E	−	−	−
F-VIII	−	−	−
ICAM-1	−	+	+
VCAM-1	(+)	+	+
ELAM-1	−	−	−

[a] CK-18, cytokeratin 18; PAL-E, endothelial specific marker; F-VIII, factor VIII related antigen.

TABLE 2. Expression of adhesion molecules on RTC cultured in the absence or presence of TNF analyzed by flow cytometry[a,b]

RTC	ICAM-1		VCAM-1	
	−TNF	+TNF[c]	−TNF	+TNF[c]
No. 17	366 (85%)	388 (85%)	200 (5%)	238 (18%)
No. 18	305 (60%)	368 (70%)	153 (9%)	244 (22%)

[a] Figures represent mean fluorescence intensity of positive cells.
[b] Percentages in parentheses refer to the proportion of positive cells above threshold (<1% of negative control preparations).
[c] 200 U/ml TNF for 48 hr.

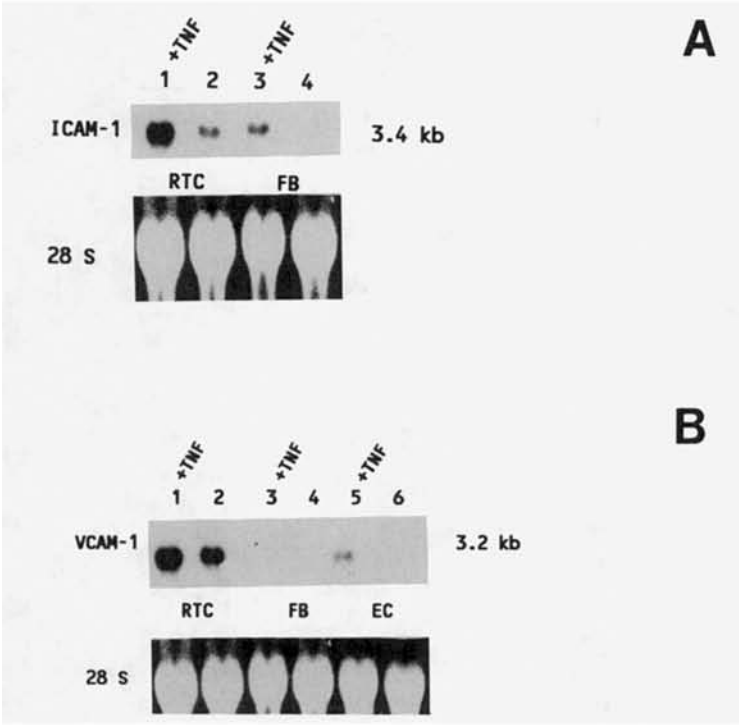

FIGURE 5. (A) Detection of ICAM-1 specific mRNA of 3.4 kb in cultured cells. Lane 1: Strong hybridization signal with renal tubular cells stimulated with TNF-α. Lane 2: Weak signal in renal tubular cells without TNF. Lane 3: Weak signal in human fibroblasts with TNF. Lane 4: Absence of ICAM-1 mRNA in fibroblasts cultured without TNF (insert: comparable intensity of 28 s ribosomal RNA). (B) VCAM-1 specific mRNA of 3.2 kb in cultured cells. Lane 1: Enhanced hybridization signal in renal tubular cells stimulated with TNF-α. Lane 2: Hybridization signal in unstimulated tubular cells. Lanes 3 and 4: Negative hybridization with stimulated and unstimulated human fibroblasts. Lane 5: Weak signal with TNF-stimulated cultured endothelial cells. Lane 6: Absence of VCAM-1 mRNA in unstimulated epithelial cells (insert: amounts of 28 s ribosomal RNA).

DISCUSSION

The vascular endothelium of renal grafts is the primary target in acute rejection (*14, 29–31*). Here, donor alloantigens, i.e., HLA class I and II molecules are recognized by the T-cell-receptor complex on recipient lymphocytes and initiate specific immune reactions. However, as is demonstrated in this study, also the lymphokine-induced expression of cell adhesion molecules, such as ELAM-1 and VCAM-1, in grafted capillaries might contribute to the cell-mediated rejection process. These molecules, in addition to ICAM-1, may serve as adhesion receptors not only for lymphocytes but also for neutrophils and macrophages, thus amplifying the immune response. Moreover, these interactions are not restricted to vessels but seem to include, in the case of ICAM-1 and VCAM-1, also the tubular system.

Several studies have already shown de novo tubular expression of ICAM-1, a member of the immunoglobulin gene superfamily, in renal allografts (*10, 13*). In addition, the number of leukocytes bearing the ICAM-1 receptors lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) and Mac-1 (CD11b/CD18) has been reported to be clearly increased during rejection (*10*). In vitro, appearance of ICAM-1 on renal cells also enhanced their susceptibility to T-cell-mediated lysis (*3*). It has been concluded that ICAM-1/LFA-1 (Mac-1) pathways are essentially involved not only in kidney (*10, 13, 29*) but also in liver (*14*) and heart allograft rejection (*15*).

It is surprising that VCAM-1, another member of the immunoglobulin gene superfamily, is constitutively expressed on Bowman's capsule and in certain proximal tubuli in normal renal tissue. During rejection all graft biopsies, with the exception of one specimen, showed increased tubular staining with VCAM-1-specific mAbs. And it is of note that tubular ICAM-1 was apparent in only 45% of rejections. However, from immunohistological studies, it could not be definitely determined whether VCAM-1 was indeed produced by tubular cells or was subject to glomerular filtration and tubular reabsorption. Now the demonstration of VCAM-1-specific mRNA in cultured RTC provides unequivocal evidence for actual tubular synthesis. It could also be shown by this method that TNF-α is able to increase biosynthesis of ICAM-1 and VCAM-1 in tubular cells.

Presence of VCAM-1 in graft capillaries during rejection was observed in about 80% of biopsies. Demonstration of VCAM-1-specific mRNA in TNF-treated cultured endothelial cells also indicates lymphokine-induced biosynthesis. It is further likely that lymphokines are involved in the expression of CAMs on leukocytes, since about 35% of biopsies showed ICAM-1 positive cellular infiltrates, compared with 15% VCAM-1 positive infiltrates. No attempts have been made in this study to reveal such an induction pathway in isolated leukocytes. That, however, TNF-α is operative locally during kidney allograft rejection has recently been demonstrated by in situ hybridization (*32*).

After all, it is interesting to note that in normal and grafted kidneys ICAM-1 and VCAM-1 apparently show some form of complementary distribution: ICAM-1 represents a basic (and inducible) constituent in capillaries that can be induced in tubules; vice versa, VCAM-1 is constitutively (and inducibly) present in tubules and can be induced in capillaries.

In contrast, ELAM-1 was not detected in extravascular sites but was confined to blood vessels in half of rejection episodes. It is difficult to draw any conclusions from immunohistological studies with respect to the supposedly early and transient expression of ELAM-1.

Considering the widespread tissue distribution of ICAM-1 and VCAM-1, and given the dynamics of leukocyte rolling, adhesion, and transendothelial migration, it is also difficult with immunohistological means to establish a firm anatomical association of leukocyte infiltration with expression of cell adhesion molecules. However, the appearance of numerous VCAM-1 positive capillaries that contain leukocytes is conspicuous. Further studies are needed to determine the pathophysiological relevance of each of these molecules, especially of ELAM-1 and VCAM-1, in kidney rejection.

In summary, the results further indicate that during allograft rejection not only specific, alloantigen-directed immune reactions are operative. Evidence is emerging that also constitutive and inducible adhesion molecules at vascular and nonvascular sites may promote rather "unspecific" adhesion of leukocytes, thus contributing to the process of cellular allograft infiltration. Future therapeutic strategies should consider these pathways.

Acknowledgments. We appreciate the expert technical assistance of Ms. E. Jeschke, Mrs. G. Schmid, and Ms. B. Maget. The following materials used in this study were provided: human skin fibroblasts by Dr. H. Wolf, Munich, Germany; mAb H18/7 by Dr. M. A. Gimbrone, Boston, MA; mAb P3.58BA-14 and a cDNA probe specific for ICAM-1 by Dr. J. P. Johnson, Munich, Germany; mAb 4B9 by Dr. T. M. Carlos, Seattle, WA.

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Received 2 April 1992.
Accepted 25 June 1992.