T Lymphocyte Adhesion Mechanisms within Inflamed Human Kidney

Studies with a Stamper-Woodruff Assay

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Renal inflammatory conditions are characterized by mononuclear cell recruitment to sites of inflammation. We have developed a modified Stamper-Woodruff assay system to analyze mechanisms of functional T cell adhesion to cryostat sections of renal biopsy material from patients with vasculitic glomerulonephritis (GN) and acute allograft rejection. Peripheral blood T cells adhered to intraglomerular, periglomerular, and tubulointerstitial regions of the cortex. Blocking monoclonal antibodies against tissue expressed ICAM-1, VCAM-1, and the CS-1 domain of fibronectin (CS-1Fn) differentially attenuated T cell adhesion. Glomerular adhesion in vasculitic GN and tubulointerstitial adhesion in acute rejection were particularly sensitive to both anti-ICAM-1 and anti-VCAM-1 antibodies, indicating a prominent role for ICAM-1 and VCAM-1 at glomerular sites in vasculitis and at tubulointerstitial sites in rejection. Furthermore, using KL/4 cells (LFA-1 expressing) and Jurkat cells (VLA-4 expressing), we demonstrated specific LFA-1/ICAM-1- and VLA-4/VCAM-1-mediated interactions within glomerular and tubulointerstitial compartments. Jurkat cells also adhered to VCAM-1-free sites, and binding was inhibitable by anti-CS-1Fn antibody, thereby demonstrating a role for VLA-4/fibronectin interactions especially at intraglomerular sites in acute rejection where VCAM-1 is notably absent. We therefore propose a prominent functional role for ICAM-1, VCAM-1, and CS-1 domain fibronectin in T cell recruitment to the inflamed kidney. *(Am J Pathol 1999, 154:503–514)*

Leukocyte infiltration is a key event in the pathogenesis of renal inflammatory states such as vasculitic glomerulonephritis (GN) and acute allograft rejection.[1,2] The migration of T lymphocytes to inflammatory sites is dependent on coordinated leukocyte-endothelial cell interactions.[3] In vasculitic GN, glomerular capillary thrombosis with characteristic focal segmental necrotizing lesions progresses to a proliferative crescentic glomerulonephritis. There is associated T lymphocyte infiltration in intraglomerular, periglomerular, and tubulointerstitial regions.[4,5] In contrast, in acute allograft rejection, T cell infiltrates are localized to the interstitium and peritubular areas and typically spare the glomerulus.[6,7]

Leukocyte adhesion is an early event for the subsequent migration into extravascular tissues.[8] T lymphocyte interactions are mediated by the β_1 integrin VLA-4 ($\alpha 4\beta 1$) and the β_2 integrin LFA-1 (CD11a/CD18). Integrins can convert between low- and high-affinity states, depending on the local environment (ie, presence of cytokines and chemokines), to mediate leukocyte adhesion.[9] LFA-1 interacts with tissue-expressed ICAM-1, whereas VLA-4 can interact with both VCAM-1 and an alternatively spliced 25-mer CS-1-peptide-containing isoform of the extracellular matrix glycoprotein fibronectin (CS-1Fn).[10]

VCAM-1 and ICAM-1 are constitutively present in the normal kidney. There is distinct VCAM-1 expression on the glomerular parietal epithelium and also on occasional tubular epithelial cells, whereas ICAM-1 expression is prominent in the glomeruli and sparse in peritubular regions. In vasculitic GN and acute allograft rejection, both ICAM-1 and VCAM-1 are heavily expressed in peritubular regions and on tubular epithelial cells[11–14]; however, glomerular expression of VCAM-1 associated with areas of segmental necrosis is a discerning feature of vasculitic GN.[15,16] Increased expression of fibronectin has been reported in experimental immune complex glomerulonephritis and rejecting renal allografts.[17–19]

Here, we describe the first study using peripheral blood T lymphocytes in a modified Stamper-Woodruff adhesion assay[20] to investigate the functional roles of ICAM-1, VCAM-1, and CS-1Fn in human renal inflammatory states. In previous studies, this technique has been applied to demonstrate adhesion molecule interactions in a variety of inflammatory conditions, including ICAM-1-

dependent T cell adhesion in a rat model of acute renal allograft rejection,[21] VCAM-1- and ICAM-1-mediated adhesion of T cell and macrophage-derived cell lines in murine lupus nephritis,[22] CS-1Fn-mediated T cell adhesion in rheumatoid arthritis,[23] ICAM-1- and VCAM-1-dependent T cell adhesion in cervical intraepithelial neoplasia,[24] VCAM-1-mediated monocyte adhesion in simian encephalitis,[25] and P-selectin-dependent eosinophil adhesion in asthma and nasal polyposis,[26] and a role for multiple adhesion molecules in mediating monocyte adhesion to human atherosclerotic plaques.[27]

We have studied the adhesive properties of peripheral blood T lymphocytes to renal biopsies taken from patients with vasculitic GN and acute allograft rejection. Furthermore, we have used an LFA-1-expressing KL/4 cell line[28] and a VLA-4-expressing Jurkat cell line in this assay system to demonstrate differential roles of ICAM-1/LFA-1-, VCAM-1/VLA-4-, and CS-1Fn/VLA-4-mediated interactions within the kidney in these disease states.

Materials and Methods

Tissue Collection and Preparation

Renal biopsy specimens from 18 patients with anti-neutrophil cytoplasmic autoantibody (ANCA)-positive vasculitis (Wegener's granulomatosis or microscopic polyangiitis with appearance of segmental glomerular lesions) and 18 patients with acute cellular allograft rejection (scoring 4(IA) to 4(IIA) on the 1997 Banff diagnostic categorization system) were used in these studies. Studies with leukocytic cell lines were performed on biopsies from 8 patients with vasculitis and 7 patients with acute rejection, whereas studies with peripheral blood T lymphocytes were performed on an additional 10 vasculitis and 11 acute rejection patient biopsies. Each biopsy was embedded in Brights cryo-m-bed (Merck, Dorset, UK), snap-frozen in liquid nitrogen, and stored at −70°C. Control tissue comprised cortical fragments from the unaffected pole of five kidneys removed for renal carcinoma. Serial 7 to 10-μm cryostat sections collected onto Superfrost Plus slides (Merck), air dried and fixed in acetone (Merck) for 10 minutes, were used in all subsequent studies. Approval for the study of human material was obtained from the local research ethics committee.

Cell Culture

A Jurkat human T lymphoblastoid cell line (a gift from the Department of Cancer Studies, University of Birmingham) and the K562 human erythroleukemic cell line transfected with LFA-1, KL/4 (a gift from Dr. M. Robinson, Celltech, Slough, UK) were used in these studies. Both cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L glutamine. For the KL/4 cells the medium was additionally supplemented with 0.2 mg/ml G418 (GIBCO, Paisley, UK).

Antibodies

The following mouse monoclonal antibodies were used: anti-human ICAM-1, RR1 (a gift from Dr. R. Rothlein;

Boehringer-Ingelheim R&D, Ridgefield, CT); anti-human VCAM-1 cell culture supernatant, Ig11 (a gift from Professor D. Haskard; Imperial College, London, UK); anti-human VCAM-1, BBIG-VI (R&D Sytems, Oxon, UK); anti-human CD11a, BCA1 (R&D Systems); anti-human VLA-4, Max68, and anti-human LFA-1, KIM185 (gifts from Dr. M. Robinson); anti-human CS-1Fn cell culture supernatant, 90.45 (gift from Dr. M. Elices; Cytel Corp. San Diego, CA); and anti-human CD3 (DAKO, High Wycombe, UK) conjugated to horseradish peroxidase (HRP; conjugate synthesized within the laboratory). All monoclonal antibodies to adhesion molecules were blocking except KIM185 (β_2 integrin activating antibody). RR1 and BBIG-VI were suitable for immunohistochemistry. We also used biotinylated rabbit anti-mouse IgG (DAKO) and HRP-conjugated streptavidin ABC complex (DAKO). Irrelevant mouse IgG (Serotec, Oxford, UK) and mouse IgM (Binding Site, Birmingham, UK) were used as controls.

Tissue Preparation

All incubations were performed at room temperature in a humidified box. Tissue endogenous peroxidase activity was blocked by treating the sections with Tris-buffered saline (TBS; pH 7.4) containing 0.3% H_2O_2 and 0.1% NaN_3, for 10 minutes.

Immunohistochemistry

The sections were treated sequentially with 0.1% avidin and 0.01% biotin to block endogenous biotin, followed by a 10-minute blocking stage with 10% rabbit serum. Three-stage indirect immunohistochemistry was performed with primary anti-human monoclonal antibodies (ICAM-1 (RR1) or VCAM-1 (BBIG-VI)), a biotin-labeled rabbit anti-mouse secondary antibody and streptavidin ABC/HRP complex. Binding of the tertiary complex was visualized by the addition of 3,3′-diaminobenzidine (DAB; Sigma, Dorset, UK). The sections were counterstained with Mayer's hematoxylin (Merck), mounted in dibutyl polystyrene xylene (DPX) (Merck), and coverslipped.

Adhesion Studies

Before the adhesion assay, cells in suspension were stimulated (peripheral blood T lymphocytes and Jurkat cells with 1 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and KL/4 cells with 25 μg/ml KIM185 for 30 minutes at 37°C. Acetone-fixed serial sections and preactivated cell suspensions were co-incubated in an agitated system in the presence of irrelevant control antibody or blocking monoclonal antibody. The binding of peripheral blood T lymphocytes isolated from patients showing evidence of acute rejection to their own biopsies was assessed. Due to the lower incidence of vasculitis as compared with acute rejection and the limited availability of blood before initiation of therapy in patients with vasculitis, T lymphocytes obtained from control volunteers were used to address peripheral blood T cell adhesion processes in vasculitic GN. We also investigated specific LFA-1- and VLA-4-mediated interactions using an LFA-1-

transfected KL/4 cell line and a VLA-4-expressing Jurkat cell line, respectively. The cell numbers (see below) used in the studies were carefully optimized to delineate specific localization of adherent cells while obtaining an appreciable level of adhesion to determine the effect of blocking antibodies.

Peripheral Blood T Lymphocyte Adhesion

Peripheral blood from patients with acute cellular allograft rejection and control volunteers was collected and heparinized. Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll/Hypaque. Mononuclear cells were collected from the interface, washed, and incubated in RPMI-1640 with 10% FCS in a petri dish (20×10^6 cells in 10 ml per dish) for 1 hour at 37°C in 5% CO_2. T lymphocytes (nonadherent cells) were collected by gentle washing with assay medium.

Preactivated T lymphocytes suspended at 5×10^6 cells per ml were overlaid on the tissue sections in a volume of 100 μl and incubated on a rotating platform at 70 revolutions per min (rpm) for 30 minutes at room temperature. Nonadherent cells were washed off with cold TBS, and sections were fixed in acetone for 10 minutes. Bound cells were labeled with the anti-CD3 HRP conjugate, and the cells were visualized by developing their peroxidase activity with DAB for 30 minutes. The sections were counterstained with Mayer's hematoxylin, mounted in DPX, and coverslipped.

KL/4 Cell Adhesion

Preactivated KL/4 cells, suspended at 5×10^6 cells per ml in RPMI-1640 containing 10% FCS, were overlaid on tissue sections in a volume of 100 μl and incubated on a rotating platform at 70 rpm for 30 minutes at 37°C. The slides were then washed in cold TBS to remove any nonadherent cells. Adherent cells were fixed in acetone for 10 minutes. Bound KL/4 cells were visualized by incubation with DAB for 30 minutes, using the endogenous peroxidase activity of K562 cells. The sections were counterstained with Mayer's hematoxylin, mounted in DPX, and coverslipped.

Jurkat Cell Adhesion

Preactivated Jurkat cells suspended at 5×10^6 cells per ml in RPMI-1640 containing 10% FCS were overlaid on the tissue sections in a volume of 100 μl and incubated on a rotating platform at 70 rpm for 30 minutes at room temperature. Nonadherent cells were washed off with cold TBS, and sections were fixed in acetone for 10 minutes. Bound cells were labeled with the anti-CD3 HRP conjugate, and the cells were visualized by developing their peroxidase activity with DAB for 30 minutes. The sections were counterstained with Mayer's hematoxylin, mounted in DPX, and coverslipped.

Blocking Studies

Before the co-incubation, serial sections and the cell suspension were incubated for 30 minutes with neutral-

izing antibodies directed against VCAM-1 and ICAM-1 at room temperature and LFA-1 or VLA-4 at 37°C. In preliminary studies, a concentration of 20 μg/ml consistently provided maximal blocking for all antibodies tested.

Combined Tissue Adhesion Assay and Immunohistochemistry

To co-localize leukocyte adhesion and tissue adhesion molecule expression, after the adhesion assay, sections were fixed in acetone for 10 minutes and stained for VCAM-1 and ICAM-1 using the immunoperoxidase staining technique described above. This order of performing the combined assay precluded any intervention by the antibodies in the initial adhesion assay.

Data Analysis

We analyzed glomerular and tubulointerstitial adhesion in turn. For each section the number of adherent cells in 5×625-μm^2 fields of tubulointerstitium were counted using a graticule and expressed as number of adherent cells per field. The total number of glomeruli in each section was counted, and the total number of adherent cells on the glomerular tuft and the parietal epithelium was expressed as number of adherent cells per glomerulus.

Data are quoted as mean and standard error of mean (mean ± SEM). Significance of differences in adhesion between control and neutralizing antibodies was determined using a one-tailed two-sample t-test. Nonparametric statistical analyses using the Mann-Whitney two-sample test confirmed the results of the t-test. Statistical advice was supplied by Dr. Roger L. Holder from the Department of Mathematics and Statistics at the University of Birmingham.

Results

Immunohistochemical Studies

Immunohistochemical (IHC) analysis confirmed the presence and up-regulation of the tissue ligands VCAM-1 and ICAM-1 in both disease states. In both vasculitic GN and acute rejection there was marked VCAM-1 expression in the parietal epithelium and tubular epithelium and at peritubular regions of the interstitium. In addition, there was distinct intraglomerular expression of VCAM-1 in vasculitic GN, which was notably absent in acute allograft rejection (Figure 1, A and B).

ICAM-1 was up-regulated in both disease states with heavy glomerular, peritubular, and tubular positivity (Figure 2, A and B).

Adhesion Studies

Peripheral Blood T Lymphocyte Adhesion in Vasculitic Glomerulonephritis

Adhesion of resting T lymphocytes isolated from peripheral blood to cryostat sections of renal biopsy material was sparse at both glomerular and tubulointerstitial
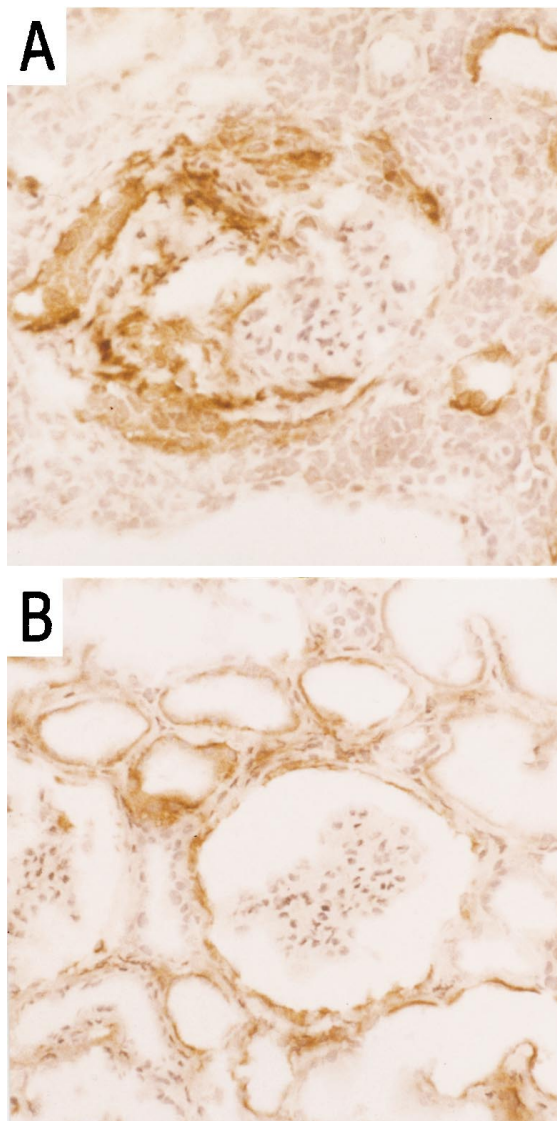
Figure 1. Immunohistochemical localization of VCAM-1 in vasculitic GN (**A**) and acute rejection (**B**) in sections of renal biopsies. Magnification, ×50.
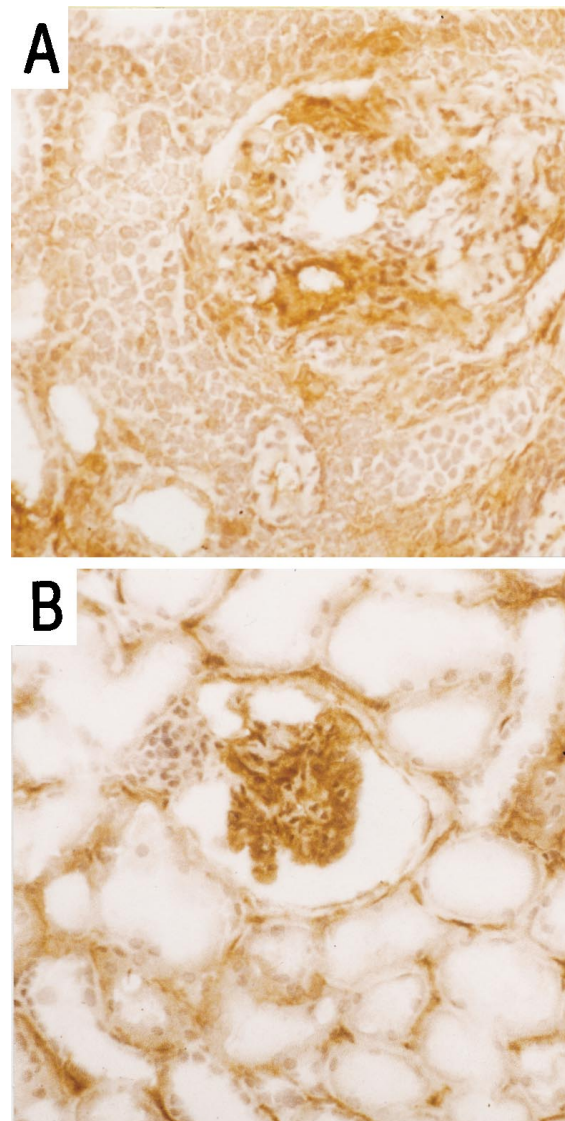

Figure 2. Immunohistochemical localization of ICAM-1 in vasculitic GN (**A**) and acute rejection (**B**) in sections of renal biopsies. Magnification, ×50.

sites. Hence, T cell integrin molecule activation with PMA was necessary to achieve optimal levels of adhesion and evaluate the inhibitory effects of blocking monoclonal antibodies. T lymphocytes did not adhere to normal tissue sections irrespective of their activation state.

T lymphocyte adhesion at glomerular and tubulointerstitial sites was differentially attenuated using the anti-ICAM-1 monoclonal antibody (Figure 3). Inhibition at glomerular sites was greater than at tubulointerstitial sites, reducing T cell adhesion by 58% (from 14.55 ± 2.4 to 6 ± 0.79) compared with 37% (from 22.1 ± 2.67 to 13.76 ± 1.4), respectively (Figure 4). In additional studies, the combination of anti-LFA-1 with anti-ICAM-1 had no effect over and above those seen with anti-ICAM-1 alone. Inhibitory effects of anti-VCAM-1 monoclonal antibody were also more prominent within the glomerular compartment. Glomerular T cell adhesion was reduced by 56% (from 14.55 ± 2.4 to 6.31 ± 2.29) as compared with 47% (from 22.1 ± 2.67 to 11.7 ± 1.56) at tubulointerstitial sites

(Figures 4 and 5). In additional studies using biopsies from an additional five patients, anti-VCAM-1 combined with anti-VLA-4 produced a small increase in inhibition of adhesion at both glomerular and tubulointerstitial sites. Thus, in these studies anti-VCAM-1 showed 63% and 46% inhibition at glomerular and tubulointerstitial sites, respectively, anti-VLA-4 showed 50% and 30% inhibition at each site, whereas the antibodies in combination inhibited by 73% and 54% at each site.

In contrast, T lymphocyte adhesion inhibition by anti-CS-1Fn monoclonal antibody was more pronounced at tubulointerstitial sites producing 62% inhibition (from 22.1 ± 2.67 to 8.2 ± 2.86) as compared with 39% (from 14.55 ± 2.4 to 8.78 ± 1.88) at glomerular sites (Figures 4 and 5). T cell adhesion was further reduced at both glomerular and tubulointerstitial regions when anti-CS-1Fn and anti-VCAM-1 monoclonal antibodies were used in combination, producing maximal inhibition of up to 72% (Figures 4 and 5). This level of inhibition was similar
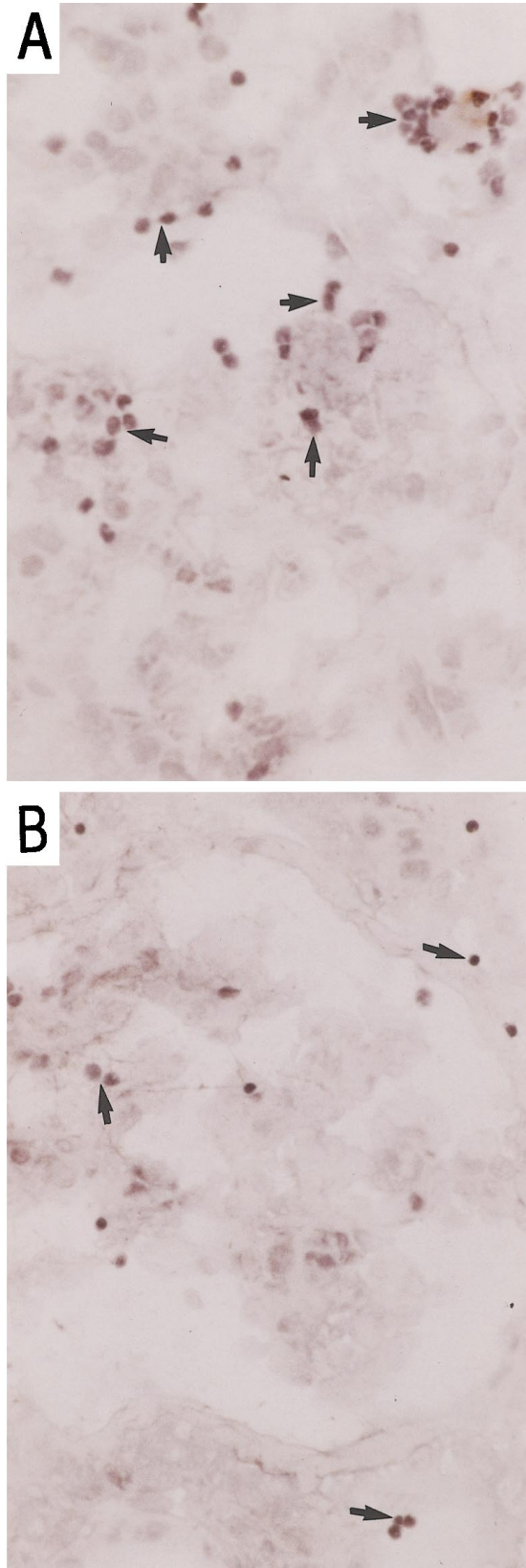
to that observed with combined anti-VLA-4 and anti-VCAM-1.

KL/4 Cell Adhesion in Vasculitic Glomerulonephritis

We used an LFA-1-transfected K562 cell line (KL/4) that was 97.1% LFA-1 positive (determined by FACS analysis), to isolate LFA-1-mediated adhesion processes. Adhesion of KL/4 cells was β_2 integrin activation dependent as no adhesion was observed by resting KL/4 cells in the absence of integrin activation by KIM185. Although normal tissue sections were able to support activated KL/4 cell adhesion (7.2 ± 3.7 and 4.7 ± 2 at glomerular and tubulointerstitial sites, respectively), this was significantly greater in the inflamed tissue (Table 1).

Anti-ICAM-1 significantly attenuated KL/4 cell adhesion at both glomerular and tubulointerstitial sites by 48% (from 14.61 ± 3.9 to 7.5 ± 2.2) and by 31% (from 13.7 ± 2.14 to 9.48 ± 2.2), respectively. This pattern of inhibition reflected that seen with anti-ICAM monoclonal antibody and peripheral blood T lymphocytes (58% and 37% inhibition in glomerular and tubulointerstitial compartments). Anti-LFA-1 produced variable levels of inhibition of KL/4 cell adhesion ranging between 25% and 60%, which was not statistically significant (Table 1).

Jurkat Cell Adhesion in Vasculitic Glomerulonephritis

We used a functional VLA-4-expressing Jurkat cell line, which was 97.66% VLA-4 positive (determined by FACS analysis) to isolate VLA-4- from LFA-1-mediated interactions. As with KL/4 cells, resting Jurkat cells were unable to adhere to either normal or inflamed tissue sections. PMA-activated Jurkat cell adhesion to normal tissue was negligible, but there was substantial adhesion in vasculitic GN. There was strong adhesion at intraglomerular, periglomerular, and tubulointerstitial sites. Anti-VCAM-1 significantly inhibited glomerular (from 8.19 ± 3.2 to 0.89 ± 0.89) and tubulointerstitial (from 23.34 ± 4.37 to 3.54 ± 2.38) Jurkat cell adhesion by up to 89% in both compartments (Figure 6). Thus, using this naturally VLA-4-expressing cell line to isolate VLA-4-mediated adhesion, effects of the anti-VCAM-1 blocking monoclonal antibody were more marked than with peripheral blood T lymphocytes where 56% and 47% inhibition of adhesion was achieved in glomerular and tubulointerstitial compartments, increasing to 73% and 54%, respectively, with combined anti-VCAM-1 and anti-VLA-4.

Anti-VLA-4 and anti-CS-1Fn monoclonal antibodies alone produced variable degrees of inhibition in glomerular and tubulointerstitial compartments (Figure 6). However, when anti-VLA-4 and anti-CS-1Fn were used in combination, Jurkat cell adhesion was significantly reduced by 88% (from 8.19 ± 3.2 to 1 ± 0.8) and by 65% (from 23.34 ± 4.37 to 8.15 ± 3.12) at glomerular and tubulointerstitial sites, respectively (Figure 6).

Peripheral Blood T Lymphocyte Adhesion in Acute Rejection

Adhesion of autologous resting T lymphocytes to cryostat sections of acute allograft rejection renal biopsies

Figure 3. Peripheral blood T cell adhesion to serial sections of renal biopsies in vasculitic GN in the presence of irrelevant control antibody (**A**) and blocking anti-human ICAM-1 monoclonal antibody; RR1 (**B**). Magnification, ×100. Adherent cells were visualized using HRP-conjugated anti-CD3 and DAB, and the section was counterstained with Mayer's hematoxylin as described in Materials and Methods.

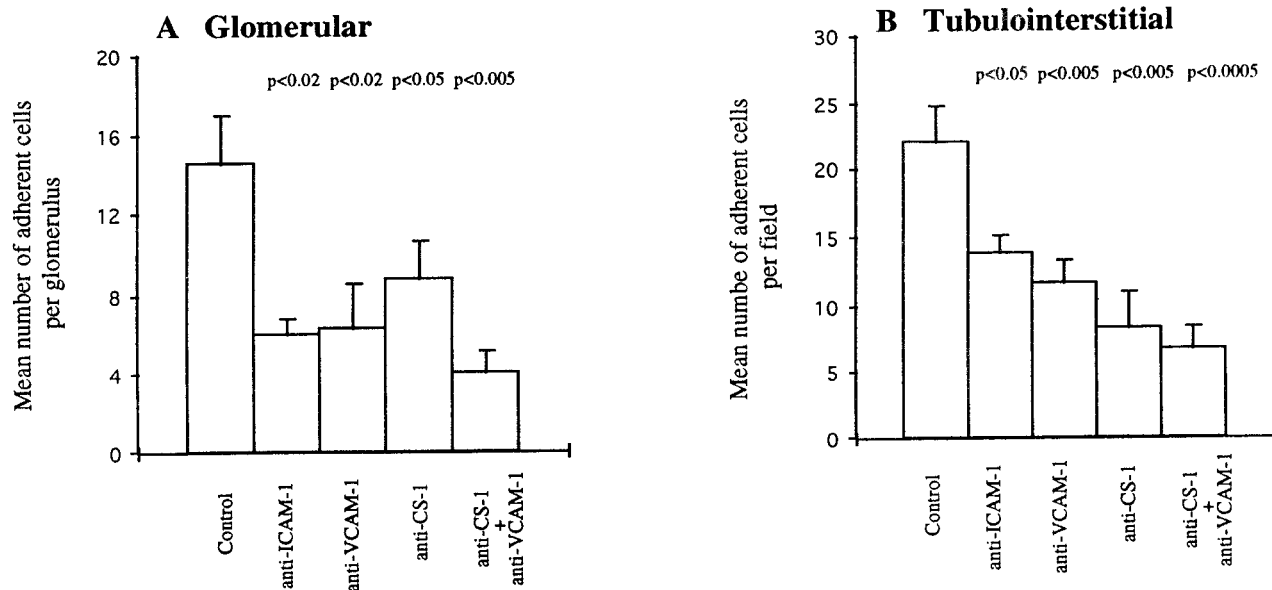## Inhibition of peripheral blood T cell adhesion in Vasculitic GN

Figure 4. Inhibition of peripheral blood T cell adhesion to glomerular (A) and tubulointerstitial (B) regions of cryostat sections of renal biopsy material from five patients with vasculitic GN using monoclonal antibodies against ICAM-1, VCAM-1, and CS-1Fn as described in Materials and Methods. Data are presented as mean number ± SEM of adherent cells per glomerulus or per field of tubulointerstitium. *P* values indicate statistical significance of inhibition.

was sparse at both glomerular and tubulointerstitial sites, necessitating previous treatment with PMA for the adhesion studies. Activated T cells bound to glomerular and tubulointerstitial sites. Compared with vasculitic GN, there was equivalent T cell adhesion at tubulointerstitial sites but less adhesion at glomerular sites (Figures 4 and 7).

In allograft rejection, 57% inhibition of T lymphocyte adhesion was achieved at tubulointerstitial sites (from 20.02 ± 3.3 to 8.58 ± 2.04) by anti-ICAM-1 monoclonal antibody compared with 49% (from 9.08 ± 1.22 to 4.59 ± 1.25) at glomerular sites (Figure 7). The inhibition by anti-ICAM-1 at tubulointerstitial sites was greater than the 37% seen in vasculitic GN, but inhibition at glomerular sites was less than the 58% seen in vasculitic GN (Figure 4). Combining anti-ICAM-1 and anti-LFA-1 did not further increase inhibition over that seen with anti-ICAM-1 alone.

Inhibition of T cell adhesion using the anti-VCAM-1 monoclonal antibody was 68% (from 20.02 ± 3.3 to 6.4 ± 1.12) at tubulointerstitial sites and 42% (from 9.08 ± 1.22 to 5.27 ± 0.69) at glomerular sites (Figure 7), similar to the pattern seen with the anti-ICAM-1 monoclonal antibody. Tubulointerstitial inhibition was greater and glomerular inhibition less than seen in vasculitis (Figure 4), reflecting the differences in the glomerular and tubulointerstitial distribution of VCAM-1 in the two inflammatory conditions. Combining anti-VCAM-1 and anti-VLA-4 reduced T cell adhesion by a further 7% at glomerular sites but had no further inhibitory effect at tubulointerstitial sites.

T cell adhesion was significantly reduced in the presence of anti-CS-1Fn by 43% (9.08 ± 1.22 to 5.19 ± 2.3) in glomerular compartments and by 67% (20.02 ± 3.3 to

6.5 ± 1.6) in tubulointerstitial compartments (Figure 7). These levels of inhibition were comparable to the 39% and 62% seen in the glomerular and tubulointerstitial compartments in vasculitis (Figure 4). Anti-CS-1Fn and anti-VCAM-1 used in combination further reduced adhesion in tubulointerstitial regions to 81% (from 20.02 ± 3.3 to 3.72 ± 0.72). In the glomerulus, however, there was less marked enhancement of inhibition of T cell adhesion using the two antibodies; anti-CS-1Fn and anti-VCAM-1 inhibited by 56% (9.08 ± 1.22 to 3.98 ± 1.6) compared with 43% (9.08 ± 1.22 to 5.19 ± 2.3) with anti-CS-1Fn used alone (Figure 7), indicating a lesser role for VCAM-1 at glomerular sites in acute rejection where VCAM-1 is mainly expressed along the glomerular parietal epithelium.

KL/4 Cell Adhesion in Acute Rejection

Adhesion of activated KL/4 cells at glomerular sites was comparable between acute rejection and vasculitic GN. However, there was enhanced adhesion of KL/4 cells to the tubulointerstitium in acute rejection (Table 1). Only modest inhibition of adhesion was achieved at both glomerular and tubulointerstitial sites by blocking antibodies against ICAM-1 and its leukocyte-expressed ligand LFA-1 (Table 1); only anti-ICAM-1 achieved marginally significant inhibition of 34% at tubulointerstitial sites.

Jurkat Cell Adhesion in Acute Rejection

Adhesion of Jurkat cells was of similar magnitude to vasculitic GN at glomerular sites (Figures 6, A and 8, A),
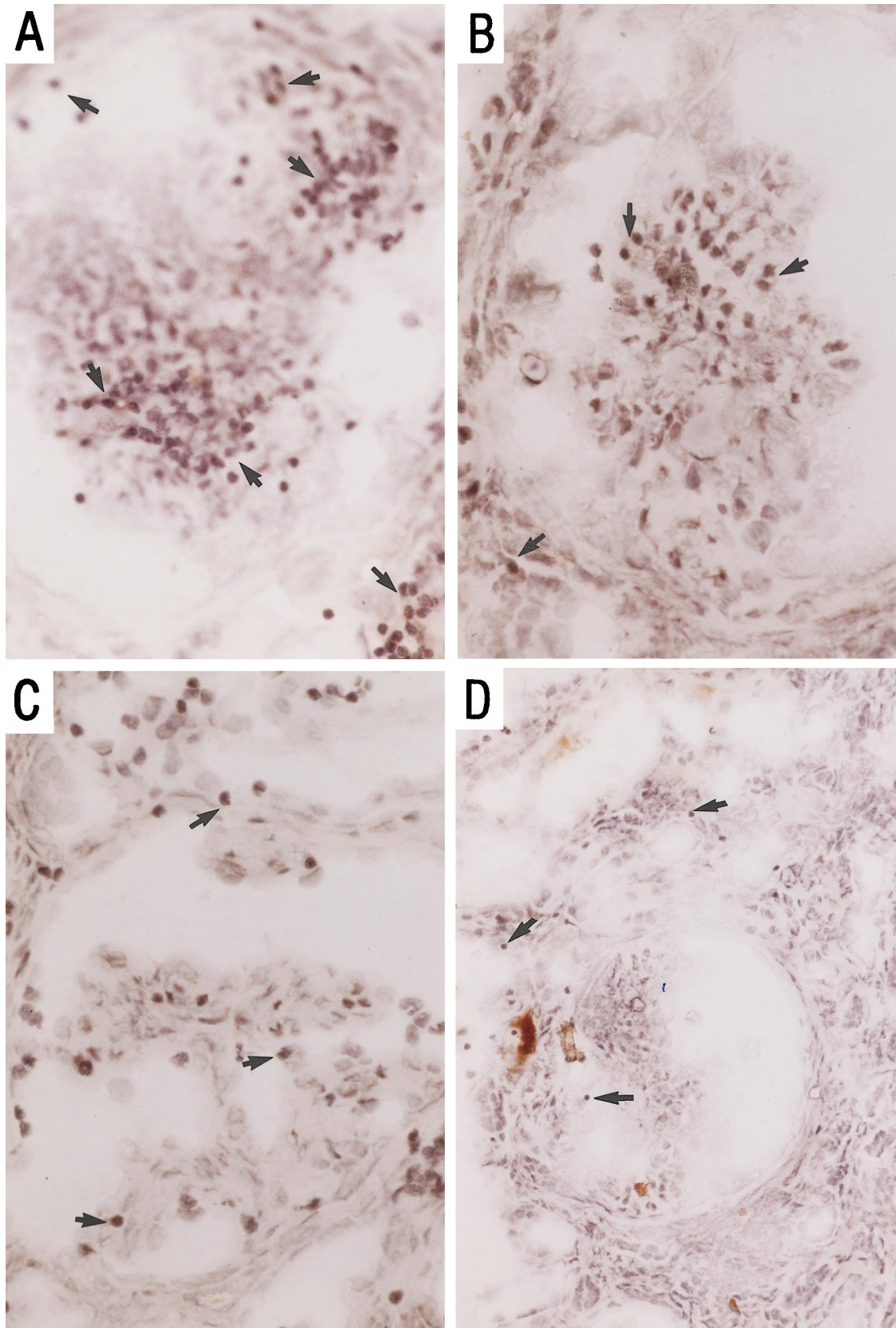
Figure 5. Peripheral blood T cell adhesion to serial sections of renal biopsies in vasculitic GN in the presence of irrelevant control antibody (**A**) blocking anti-human VCAM-1 monoclonal antibody, Ig11 (**B**), anti-CS-1Fn, 90.45 (**C**), and both antibodies (**D**). Tissue illustrated here was from a different patient from that in Figure 3. Adherent cells were visualized using HRP-conjugated anti-CD3 and DAB, and the section was counterstained with Mayer's hematoxylin as described in Materials and Methods. Magnification, ×100 (**A** to **C**) and ×50 (**D**).

Table 1. Inhibition of KL/4 Cell Adhesion

Blocking antibody	Glomerular		Tubulointerstitial	
	Vasculitic GN	Acute rejection	Vasculitic GN	Acute rejection
Control	14.61 ± 3.9	13.26 ± 4.7	13.7 ± 2.14	23.71 ± 12.4
anti-LFA-1	8.6 ± 1.8	6.4 ± 3.7	10.2 ± 2.2	9 ± 2.27
anti-ICAM-1	7.5 ± 2.2 ($p<0.1$)	9.9 ± 4.5	9.48 ± 2.2 ($p<0.1$)	15.56 ± 8.37 ($p<0.1$)

Inhibition of KL/4 all adhesion to glomeruler and tubulointerstitial regions of cryostat sections of renal biopsy material from seven patients with vasculitic GN and five patients with acute rejection using monoclonal antibodies against LFA-1 and ICam-1 as described in Methods. Inhibition is expressed as mean number of adherent cell ± SEM; *p* values indicate statistical significance of inhibition.

whereas at tubulointerstitial sites adhesion was nearly twice as great as in vasculitic GN (Figures 6, B and 8, B). Tubulointerstitial adhesion was significantly inhibited by anti-VCAM-1 monoclonal antibody by 70% (from 38.4 ± 6.9 to 11.4 ± 2.8; Figure 8). Inhibition of glomerular adhesion with anti-VCAM-1 was 49% (13.71 ± 1.5 to 6.5 ± 1.8), which was considerably less pronounced than at tubulointerstitial regions (Figure 8); this represented inhibition of VCAM-1-dependent adhesion to the parietal epithelium as there was little or no VCAM-1 expression at intraglomerular sites. (Figure 8). Anti-VLA-4 and anti-CS-1Fn monoclonal antibodies alone produced significant inhibition of similar magnitudes in glomerular and tubulointerstitial compartments. Additional increases in inhibition were observed when the two antibodies were used together, reaching maximal inhibitions of 64% (13.71 ± 1.5 to 4.9 ± 3.3) and 72% (38.4 ± 6.9 to 10.8 ± 2.5) at glomerular and tubulointerstitial sites, respectively (Figure 8).

Combined Adhesion and Immunohistochemical Studies

We further analyzed the co-localization of VLA-4- and LFA-1-mediated adhesion to their respective tissue ligands, VCAM-1 and ICAM-1. LFA-1-transfected KL/4 cells specifically adhered to ICAM-1-positive regions only (Figure 9A). Jurkat cells, however, adhered to both VCAM-1-positive and -negative sites (Figure 9B). Co-localization of Jurkat cells and VCAM-1 confirmed VLA-4/VCAM-1 interactions, whereas adhesion at VCAM-1-free sites supports the involvement of other VLA-4-mediated interactions, such as VLA-4 binding to CS-1-containing fibronectin.

Discussion

To our knowledge this is the first report of the use of the Stamper-Woodruff assay to demonstrate that the adhe-

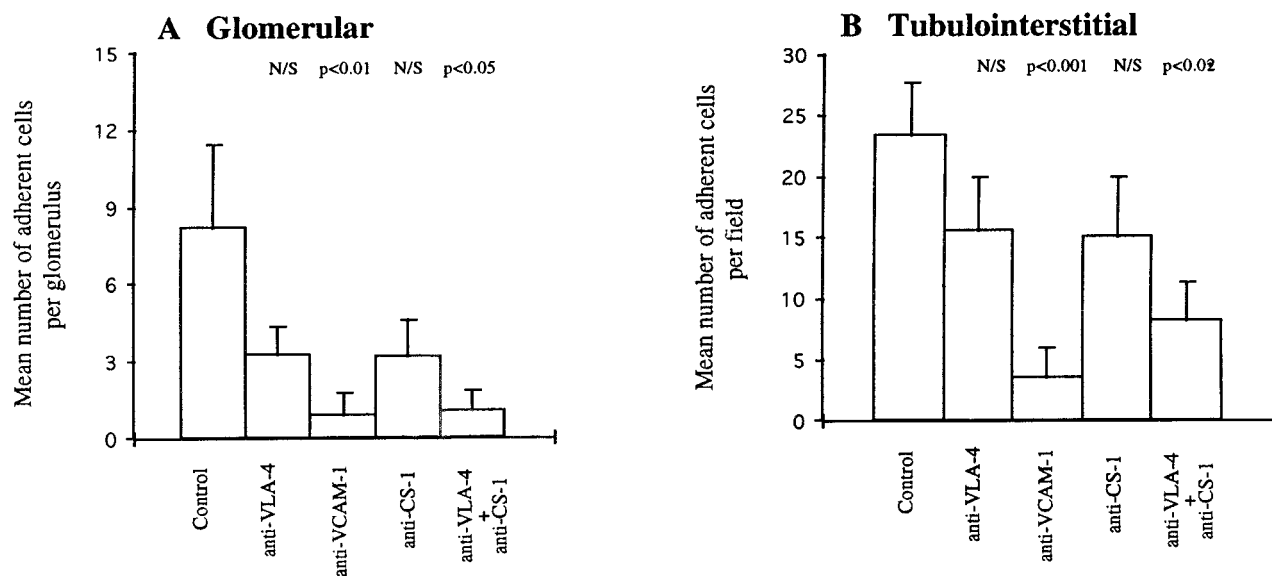## Inhibition of Jurkat cell adhesion in Vasculitic GN

Figure 6. Inhibition of Jurkat cell adhesion to glomerular (A) and tubulointerstitial (B) regions of cryostat sections of renal biopsy material from eight patients with vasculitic GN using monoclonal antibodies against VLA-4, VCAM-1, and CS-1Fn as described in Materials and Methods. Data are presented as mean number ± SEM of adherent cells per glomerulus or per field of tubulointerstitium. *P* values indicate statistical significance of inhibition.

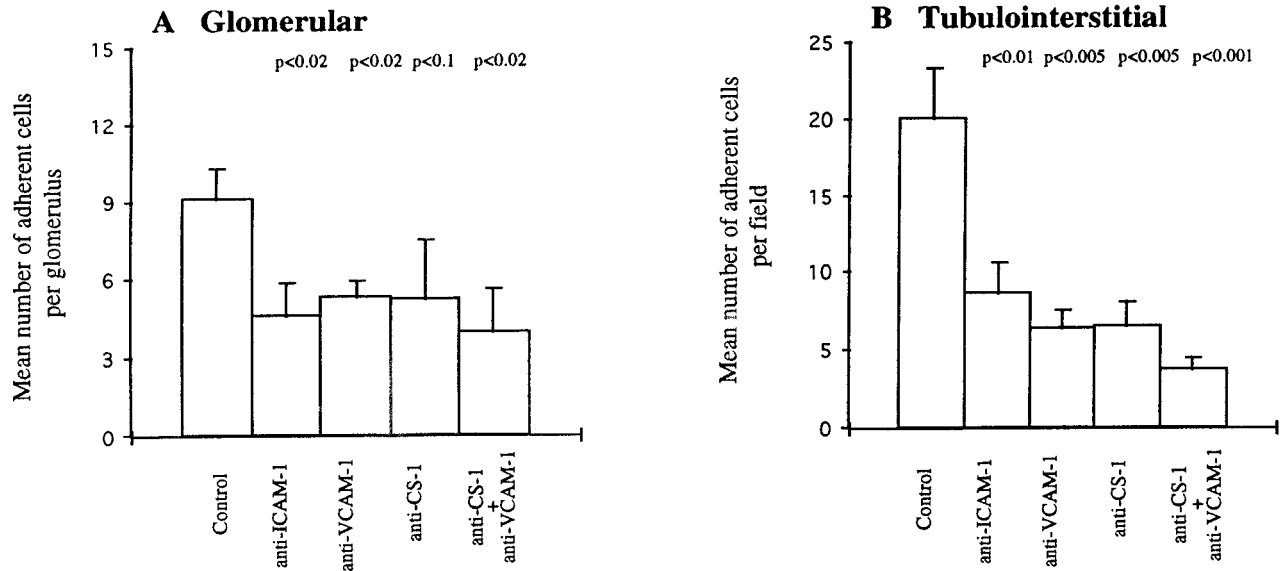## Inhibition of peipheral blood T cell adhesion in Acute rejection

Figure 7. Inhibition of peripheral blood T cell adhesion to glomerular (A) and tubulointerstitial (B) regions of cryostat sections of renal biopsy material from five patients with acute rejection using monoclonal antibodies against ICAM-1, VCAM-1, and CS-1Fn as described in Materials and Methods. Data are presented as mean number $\pm$ SEM of adherent cells per glomerulus or per field of tubulointerstitium. P values indicate statistical significance of inhibition.

sion molecules ICAM-1 and VCAM-1 have a functional role in mediating adhesion of human peripheral blood T lymphocytes and certain integrin-expressing leukocytic cell lines to cryostat sections of renal biopsies taken from patients with vasculitic GN and acute allograft rejection.

Immunohistochemical studies on serial sections confirmed previously reported differential patterns of VCAM-1 and ICAM-1 staining in the two disease states.[9–14] The T-lymphocyte-expressed integrins LFA-1 and VLA-4 mediate T cell adhesion to tissue-expressed

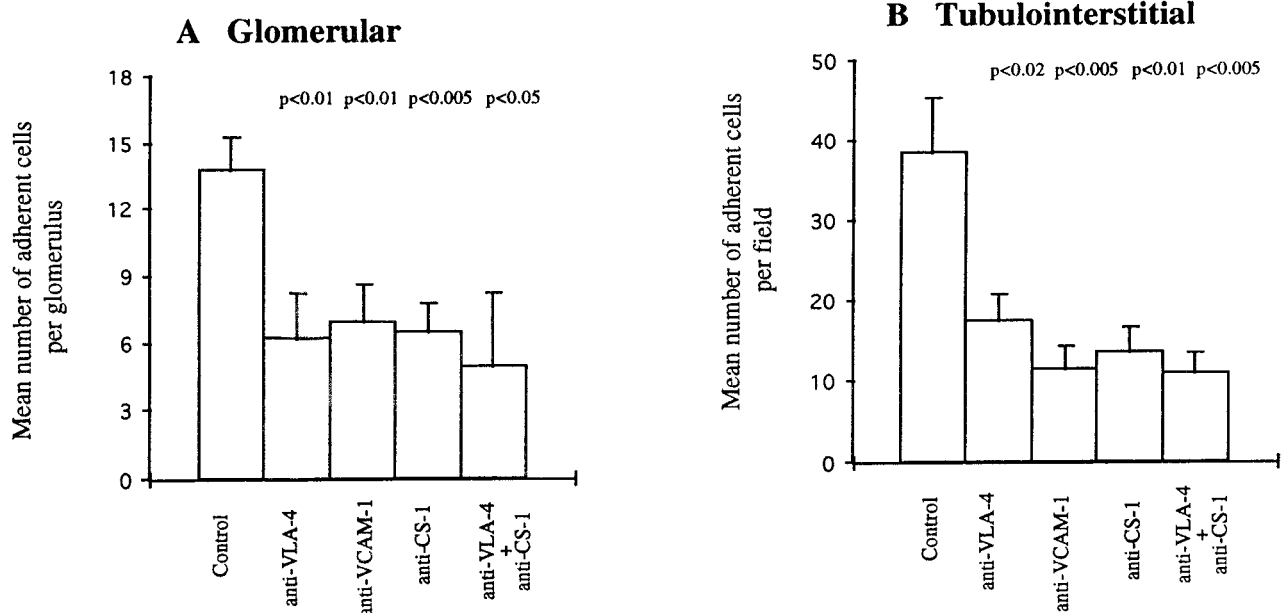## Inhibition of Jurkat cell adhesion in Acute Rejection

Figure 8. Inhibition of Jurkat cell adhesion to glomerular (A) and tubulointerstitial (B) regions of cryostat sections of renal biopsy material from seven patients with acute rejection using monoclonal antibodies against VLA-4, VCAM-1, and CS-1Fn as described in Materials and Methods. Data are presented as mean number $\pm$ SEM of adherent cells per glomerulus or per field of tubulointerstitium. P values indicate statistical significance of inhibition.
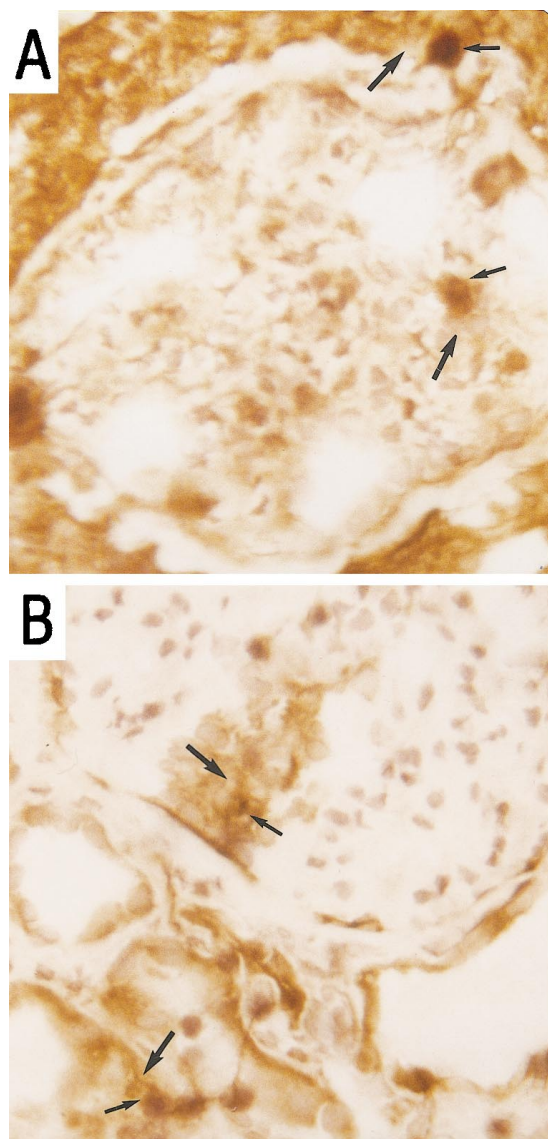
Figure 9. Co-localization of KL/4 cell adhesion (**thin arrows**) and ICAM-1 expression (**thick arrows**) (**A**) and Jurkat cell adhesion (**thin arrows**) and VCAM-1 expression (**thick arrows**) (**B**), using a combined adhesion and immunohistochemical technique. Magnification, ×100 and focused in plane of the leukocyte.

terferon (IFN)-γ, stimulate expression and up-regulation of adhesion molecules such as ICAM-1 and VCAM-1.[32,33] Expression of TNF-α and IL-1 has been demonstrated in vasculitic GN,[34] and cytokines are up-regulated to varying extents in allograft rejection.[35,36] In addition, locally produced chemotactic mediators or chemokines, such as MCP-1, RANTES, and MIP-1α, regulate the rapid switching of integrins from a low-affinity to a high-affinity state.[37,38] We have shown expression of these chemokines as well as MIP-1β in vasculitic glomerulonephritis.[39] *In vivo*, these chemokines may be the true physiological trigger for integrin activation, and in future studies our system could be adapted to test this by replacing PMA or the KIM185 antibody with a relevant chemokine.

Activated peripheral blood T lymphocytes did not adhere to normal tissue sections, but adhesion was seen in both disease states to capillary tuft and parietal regions of the glomerulus as well as to tubular and interstitial regions. Adhesion to glomerular sites was greater in vasculitic GN compared with acute rejection, whereas comparable levels of adhesion were seen to tubulointerstitial sites in both diseases. Adhesion was inhibitable by blocking tissue-expressed adhesion molecules ICAM-1, VCAM-1, and CS-1 domain fibronectin. The pattern and degree of inhibition was variable between disease states and between glomerular and tubulointerstitial compartments within the kidney. T cell adhesion at glomerular sites in vasculitic GN and at tubulointerstitial sites in acute rejection was most sensitive to inhibition by both anti-ICAM-1 and anti-VCAM-1. This observation suggests a major role for ICAM-1 and VCAM-1 in mediating T cell adhesion at glomerular sites in vasculitis and at tubulointerstitial sites in acute rejection. In both diseases, CS-1Fn appeared to play a greater adhesive role within the tubulointerstitium than within glomeruli.

Using VLA-4-expressing (Jurkat) and LFA-1-expressing (KL/4) cell lines the differential roles of β1 and β2 integrins were investigated in isolation. KL/4 cells, but not Jurkat cells, were able to adhere to normal kidney sections at glomerular and peritubular sites corresponding to the markedly higher levels of ICAM-1 in normal tissue as compared with VCAM-1. This suggests the potential for constitutively expressed ICAM-1 to mediate T cell trafficking in the normal kidney; however, this would be conditional on the presence of other permissive factors such as appropriate chemoattractant factors, which may not be available in the normal kidney. Adhesion of both cell types was substantial in both diseases, although there were some differences in the patterns of localization. Thus, adhesion of Jurkat cells and KL/4 cells was greater at tubulointersitial sites in acute rejection compared with vasculitic GN, which is consistent with the strong ability of antibodies to ICAM-1, VCAM-1, and CS-1Fn to block binding of peripheral blood T lymphocytes from patients with acute rejection at this site. This may reflect a greater density of VLA-4 adhesion ligands within the tubulointerstitial regions in acute rejection. There was little difference in adhesion of Jurkat cells or KL/4 cells at glomerular sites in the two diseases.

Inhibition of adhesion of both the KL/4 and Jurkat cell lines was achieved using blocking antibodies against

ICAM-1 and VCAM-1, respectively. The activation state of the integrins is a critical determinant of leukocyte adhesion processes.[29–31] In our system, low levels of peripheral blood T cell adhesion was possible to inflamed tissues in the absence of any previous activation, but to achieve optimal adhesion for subsequent evaluation of inhibition, the cells had to be pretreated with PMA. In contrast, activation of both the Jurkat and KL/4 cell lines was an absolute prerequisite for any leukocyte adhesion.

Up-regulation of tissue-expressed adhesion molecules and activation of their leukocyte-expressed integrin ligands promotes T lymphocyte recruitment to sites of inflammation. This process is closely regulated by local stimuli such as cytokines and chemokines. *In vitro* studies have shown that pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and in-

LFA-1 and VLA-4 and their tissue ligands ICAM-1, VCAM-1, and CS-1-containing fibronectin. The Jurkat cell line proved the most susceptible to adhesion inhibition using anti-VCAM-1 monoclonal antibody or a combination of anti-VLA-4 and anti-CS-1Fn monoclonal antibodies. Inhibition of KL/4 cell adhesion was less satisfactory, but nonetheless patterns of inhibition seen with anti-ICAM-1 were similar to those seen with peripheral blood T lymphocytes. The expression of ICAM-1 on KL/4 cells[40] may contribute to KL/4 cell adhesion to LFA-1 expressed on tissue leukocyte infiltrates. This would not be inhibited by anti-LFA-1 treatment of KL/4 cells in suspension and may explain the low levels of inhibition observed with this antibody.

Furthermore, we have demonstrated adhesion-molecule-specific localization of KL/4 and Jurkat cells in our modified Stamper-Woodruff assay system. Curiously, unlike KL/4 cells, which bound solely to ICAM-1-rich areas, Jurkat cell binding was also noted at VCAM-1-free sites. This observation and the inhibitory effect of anti-CS-1Fn suggest an important role for VLA-4/CS-1Fn interactions. Jurkat cells express a second β_1 integrin, VLA-5,[41] which is also a receptor for the fibronectin receptor. Another $\alpha4$ integrin ($\alpha4\beta7$) has also been reported on leukocytes. Like VLA-4, this molecule binds to both VCAM-1 and the CS-1 domain of fibronectin as well as a mucosal addressin cell adhesion molecule-1, MAdCAM-1.[42,43] It is possible that adhesion molecules such as MAdCAM-1 and VLA-5 are responsible for additional VCAM-1-independent adhesion processes, although we have not observed MAdCAM-1 expression in the kidney (unpublished).

Binding of peripheral blood T cells and Jurkat cells to the glomerular tuft in acute rejection was of particular interest for two reasons. First, the lack of glomerular VCAM-1 expression in acute rejection suggests a role for VLA-4/CS-1Fn or possibly VLA-5/fibronectin interactions. This is supported by the inhibitory effects of anti-CS-1Fn. Second, despite this *in vitro* evidence showing ability of peripheral blood T cells and specific integrin-molecule-expressing cell lines to bind to glomeruli in acute allograft rejection, little T cell infiltration is observed *in vivo*.[2] This highlights the necessity for other cofactors in the microenvironment. We have found little chemokine expression at this location (unpublished), so the ability to activate T cell integrin ligands may be limited in allograft glomeruli. The reasons for this are unclear but suggest a degree of protection of glomerular sites from allogeneic T cell attack.

To date, a number of studies on animal models have demonstrated that monoclonal antibodies against adhesion molecules can suppress T-lymphocyte-mediated immunological reactions. Anti-ICAM-1 and anti-LFA-1 monoclonal antibodies have been used to protect against the onset of acute cellular rejection and restore deteriorating renal function in experimental models of renal transplant with variable success.[44,45] Furthermore, an anti-β_3-integrin antibody for the prevention of arterial restenosis is available, and several other integrin-based drugs are under development.[46] This study provides further justification for the development of targeted therapeutic agents that will modify T cell retention and recruitment within the inflamed kidney.

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