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Effects of Complement Inhibition with Soluble Complement Receptor-1 on Vascular Injury and Inflammation during Renal Allograft Rejection in the Rat

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Complement is both an effector of the humoral immune response and a stimulator of leukocyte activation. To examine the influence of complement on the allograft response, we inhibited complement using recombinant human soluble complement receptor-1 (sCR1; TP10), in an unsensitized model of rat renal allograft rejection. Lewis to DA renal transplant recipients were treated daily with 25 mg/kg sCR1 or saline and sacrificed on days 1 to 5 after transplant. Transplanted organs were examined histologically and immunohistochemically for leukocyte subset markers and for the third component of complement, C3, and membrane attack complex deposition. A second set of recipients was followed from day 5 to day 9 to assess graft survival. sCR1-treated recipients displayed >90% inhibition of plasma complement activity and a marked reduction in tissue C3 and membrane attack complex deposition. Inactivation of complement reduced the vascular injury such that there was almost complete sparing of vascular damage in day 5 sCR1-treated rats. There was a significant reduction in infiltrating leukocytes by day 5 after transplant, and complement inhibition delayed the time to reach a histologically defined end point of graft survival from 5 days in controls to 9 days in the sCR1-treated group. These results imply that the vascular and cell-mediated injury arises, in part, from complement activation. The partial inhibition of these injuries by sCR1 may have functional implications for strategies to inhibit allograft rejection. (Am J Pathol 1996, 149:2055–2066)

Acute allograft rejection is characterized by infiltrating leukocytes, which are, in part, responsible for tissue damage.[1] However, their presence alone does not necessarily indicate ongoing rejection; leukocytes are often found in well functioning grafts,[2–4] and no single cytolytic T-cell factor has been identified as a prerequisite for graft injury. This implies that humoral factors, such as complement (C), are also involved in unsensitized acute allograft rejection.

The third component of C (C3) is central to both the classical and alternative pathways of C activation, the cleavage product C3b binding covalently to both the antibody (Ab) and antigen (Ag) within immune complexes (ICs), and noncovalently to C receptors found on leukocytes. This double reactivity implies a pivotal role for C3b not only in the nonspecific clearance of foreign Ag and in local inflammation but also in the specific immune response mediated by both B and T cells.

Despite recognition that C is activated and split products are deposited in acute cell-mediated rejection,[5,6] there is little evidence concerning the influence of C upon the host's cellular immune response. C3b is known to assist in the presentation of Ag[7,8] to responsive T cells and in the proliferation of T cells in response to interleukin (IL)-2.[9] Pepys et al[10] showed that the murine B-cell response to some Ags could be dependent on C, and other reports have confirmed that C can influence B-cell responses.[11–13]

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C receptor type 1 (CR1; CD35) is a member of the regulators of C activation family of C regulatory molecules. CR1 is expressed on the surface of erythrocytes, monocytes, macrophages, neutrophils, B cells, some T cells, kidney podocytes, and dendritic cells, and in nonprimates it is also found on platelets. CR1 acts as a cofactor for factor-I-mediated degradation of the classical and alternative C3 convertases and enables degradation of C3b to the inactive form, iC3b, which no longer participates in the C cascade.

An alternatively transcribed form of CR1, lacking a transmembrane domain and soluble in plasma, has also been identified[14] with a putative role as a naturally occurring soluble inhibitor of C. The existence of CR1, and other receptors capable of binding C split products on the surface of leukocytes, may also suggest an additional role for C in leukocyte activity. A recombinant form of human soluble CR1 (sCR1) has been produced by gene transfection to Chinese hamster ovary cells, demonstrating the same capacity to inhibit C activation as CR1.[15]

Prolonged organ survival has been obtained with administration of sCR1 to recipients of discordant xenografts and in isolated organs perfused with xenogeneic blood.[16,17] Similarly, in a presensitized hyperacute model of cardiac allograft rejection,[18] sCR1 was shown to inhibit both the classical and alternative pathways of C activation.

The purpose of this study was to examine the role of C in an unsensitized model of acute renal allograft rejection. sCR1 was used to inhibit C activation and thus to assess the influence of C on the vascular and cellular components of allograft injury.

Materials and Methods

Transplantation

Orthotopic renal transplantation from Lewis ($RT1^l$) to DA ($RT1^a$) rats was performed using modifications to the technique of Fabre et al.[19] Allograft recipients were injected intravenously, immediately before revascularization and at subsequent 24-hour intervals with 25 mg/kg sCR1 in saline. Equivalent volumes of saline alone were given to allografted control rats. At sacrifice, organs were removed and portions placed either in liquid nitrogen or fixed in formalin. The study used four sets of transplant recipients. 1) To assess the plasma clearance and functional inhibition of C, sCR1-treated allograft recipients (n = 6) were examined for the first 24 hours after transplantation. 2) To test the effect of C inhibition upon allograft rejection, sCR1- and saline-treated recipients (n = 15 per group) were sacrificed on days 1, 2, 3, 4, and 5 after transplant. 3) To establish an end point for graft survival, an additional set of sCR1- and saline-treated allograft recipients were sacrificed on days 5, 6, 7, 8, and 9 (n = 18 per group). 4) To test for the impact of ischemia, untreated DA to DA renal isografts were performed, and recipients were sacrificed on day 7 after transplant (n = 3).

sCR1

sCR1 (TP10 or BRL 55730 batch STB-004-06) was produced by Biopharmaceutical Manufacturing and Development, SmithKline Beecham Pharmaceuticals (Philadelphia, PA). The protein was >98% pure and was supplied as a sterile low-endotoxin solution at 5.3 mg/ml in phosphate-buffered normal saline. It was stored at −70°C.

sCR1 Pharmacokinetic Study

Citrated blood samples were taken from the tail tip immediately before administration of sCR1 and at 1, 10, 20, 30, 45, 60, 90, and 300 minutes and 24 hours after injection. Animals were sacrificed after the last bleed. Plasma sCR1 concentrations were determined using a two-site solid-phase enzyme immunometric assay. Test samples, diluted between 1:3 and 1:40,000, and standards up to 60 ng/ml, were incubated (2 hours at ambient temperature) simultaneously with plastic beads coated with polyclonal rabbit anti-human CR1 and horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibodies directed against the same molecule (both reagents supplied by T Cell Sciences, Cambridge, MA). Unbound HRP conjugate was removed by washing the beads under a stream of H_2O. The HRP-conjugated antibody-coated beads were incubated (30 minutes at ambient temperature) with a chromogenic substrate, *O*-phenylenediamine, and the reaction was stopped by the addition of 1 ml of 1 N sulfuric acid. sCR1 concentration was directly proportional to the absorbance at 492 nm of the reaction product.

Total Hemolytic Complement (CH_{50}) Assay

To determine the functional inhibition of C, blood samples were taken from three allograft recipients in the pharmacokinetic study, before administration of sCR1 and at 1, 30, 60, 90, and 300 minutes and at 24 hours after injection. The total hemolytic activity of C was determined as the CH_{50} titer defined as the reciprocal of the dilution of rat plasma that produced lysis of 50% of antibody-sensitized sheep erythro-

cytes. The original assay of Kabat and Mayer,[20] for measurement of classical pathway activation, was adapted for microtiter plate operation using a protocol similar to that of Pruitt and Bollinger.[18]

Histology

Using standard techniques, formalin-fixed kidneys were embedded in paraffin before sectioning, and 2-μm sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff, and Martius scarlet blue.

Grade III acute rejection was scored according to the Banff classification,[1] as follows: grade I, significant interstitial infiltration and moderate tubulitis; grade II, significant interstitial infiltration, severe tubulitis, and moderate arteritis; grade III, severe arteritis, hemorrhage, and focal infarction.

Immunohistochemistry

Using standard procedures, 4-μm cryostat sections were cut and stained for leukocyte markers using an indirect peroxidase technique similar to that of Leventhal et al.[21] For C3 staining, a peroxidase-conjugated anti-rat C3 MAb was used, and for C5b-9 staining, the streptavidin/biotin indirect peroxidase technique, similar to that described by Hsu et al,[22] was used. Staining was visualized by incubation in 0.5% diaminobenzidine (Merck, Poole, UK) containing 0.01% hydrogen peroxide (Merck).

Antibodies

Tissue culture supernatant (TCS) from monoclonal hybridoma cell lines were stored at −20°C until required. Antibodies used were OX1 (CD45; leukocyte common antigen), OX19 (CD5; pan-T cells), W3/25 (CD4; T-cell subset), OX8 (CD8; T-cell subset), OX42 (CD11b; macrophage/dendritic cells), and a 1:1:1 cocktail of OX39/NDS61/NDS66 (CD25; p55 α-chain of the high affinity IL-2 receptor). Positive control staining was performed using a 1:1 cocktail of OX18/OX27 anti-rat major histocompatibility (MHC) class I. W6/32 anti-human MHC class I tissue culture supernatant was used as a negative control. Anti-rat C3 MAb was purchased from The Binding Site (Birmingham, UK) and anti-human C5b-9 neoantigen MAb, cross-reactive with the rat, from Dako (High Wycombe, U.K) as were the anti-mouse peroxidase-conjugated secondary antibody, the anti-mouse Ig $F(ab)_2$ biotinylated antibody, and the peroxidase-conjugated streptavidin complex.

Morphometric Analysis of Immunohistological Sections

Semiquantitative analysis of the percent area of positive staining for each leukocyte marker was obtained using Seescan (Cambridge, UK) image analysis software. Using a method described previously,[23] the percent area of a microscope field stained positive by immunohistochemical analysis was determined. This was performed for each leukocyte marker in five cortical fields from each allografted kidney, at days 1 to 5 after transplant.

Statistics

The means of the percent area stained positive were plotted graphically for each marker in each group of rats at each day after transplant. Statistical significance at day 5 after transplant between sCR1-treated and saline-treated control groups was determined using the unpaired Student's *t*-test. A P value of <0.05 was taken as significant.

Results

sCR1 Pharmacokinetics

Pharmacokinetic studies showed a biphasic clearance of sCR1 from the plasma of allografted rats (Figure 1). The mean peak of plasma sCR1, at 1 minute after administration of 25 mg/kg intravenous sCR1, was 782 ± 33.42 μg/ml, which was reduced by approximately 50% within 30 minutes. Plasma sCR1 then declined to trace levels (17.7 ± 7.89 μg/ml, a mean of 2.2% of peak) over 24 hours.

Systemic Complement (CH_{50}) Inhibition

The CH_{50} test measured systemic C activity in animals followed for the pharmacokinetic study. C activity (Figure 1) was inhibited to less than 10% of pretreatment (normal) activity at the peak sCR1 level (1 minute after injection). C inhibition then decayed as sCR1 cleared from the plasma over the 24-hour period. At 24 hours, hemolytic activity was still depressed to a mean of 58 ± 1.7% of normal activity, despite very low levels of detectable sCR1.

Inhibition of Tissue Complement Deposition

In control animals, with uninhibited C activity, C3 (Figure 2A) and membrane attack complex (MAC; Figure 2C) were deposited on the tubular basement membranes and in the renal vasculature. The depo-

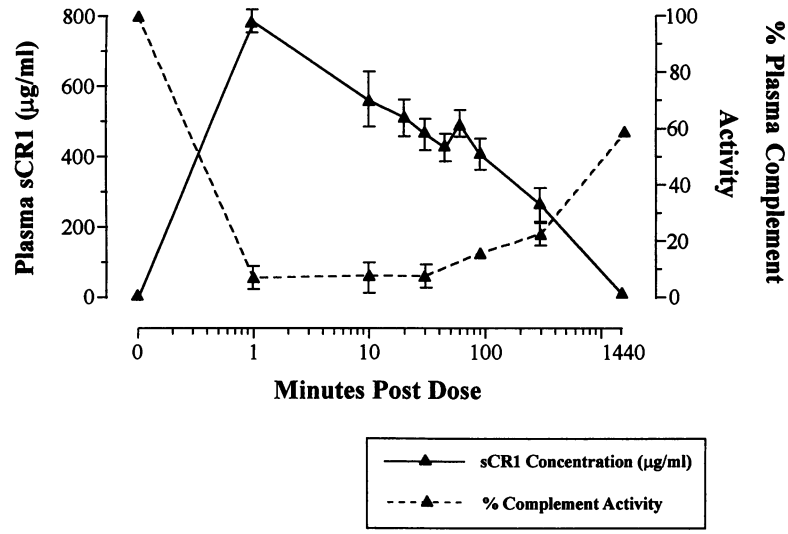

Figure 1. *The 24-hour pharmacokinetic profile of plasma sCR1 and systemic C (CH_{50}) inhibition. The means and SEM of six independent allograft recipients are shown for the sCR1 pharmacokinetic study, three of which were additionally followed for the CH_{50} profile.*

sition of C3 and MAC upon tubules between days 1 and 3 approximately coincided with areas of leukocytic infiltration. In control grafts at days 4 to 5, C deposition was more widespread throughout the rejecting kidney, particularly upon disrupted tubules. The grafts of sCR1-treated animals, in contrast, showed reduced C3 and MAC staining; the difference from control rats being most marked at day 5 (Figure 2, B and D). These results indicate that C activation through to the MAC was reduced, although not completely inhibited, in the grafts of sCR1-treated animals.

Reduction of Vascular Injury

The vascular injury in saline-treated control rats was characterized by endothelial swelling and lifting in day 4 grafts and endothelial stripping, diffuse thrombosis, interstitial hemorrhage, and focal infarction by day 5. These effects were largely prevented in the sCR1-treated rats, such that all day 5 treated animals displayed remarkably normal-looking endothelium, with typical appearances illustrated in Figure 3. Only occasional foci of interstitial hemorrhage were detected in the sCR1-treated group at this stage, with no thrombosis or tissue infarction. Thus, C inactivation did reduce the severity of vascular injury.

Reduced Cellular Infiltration

Histological analysis of control grafts showed progressive infiltration of leukocytes between days 1 and 3, focused mainly around vessels. There was an abrupt increase in infiltration of the tubulointerstitial areas at day 4, associated with disruption of the tubular basement membrane and progressive tubule damage through to day 5. At day 5, control grafts showed widespread cellular infiltration throughout the cortex and medulla with severe tubular destruction and some tissue infarction. In the grafts of sCR1-treated animals there was less dense infiltration (Figure 4) and more gradual invasion of tubulointerstitial fields through days 1 to 5, confirmed by semiquantitative analysis (see below).

Leukocyte Subset Analysis

Semiquantitative analysis, based on the area of tissue staining by immunochemical markers, confirmed that the extent of infiltration by all leukocytes was significantly reduced in sCR1-treated rats at day 5 after transplant (Figure 5; Table 1). Whereas the leukocyte population within control grafts approached plateau signal at day 5, C-inhibited rats displayed a more gradual rise in the cellular infiltrate, which was still increasing at day 5. The data show a reduction in T cells of both $CD4^+$ and $CD8^+$ phenotype and in macrophage/dendritic cells (Figure 6). The area stained for the activation marker CD25, expressed by macrophages and lymphocytes, was also reduced by approximately 50% compared with control allografts at day 5. However, as the size of cell in any particular subset will influence the data obtained by such analysis, no comparisons between cell subpopulations as a proportion of total leukocytes can be made.

Graft Survival

The initial experiment to day 5 showed that C inhibition produced a significant reduction in inflammation

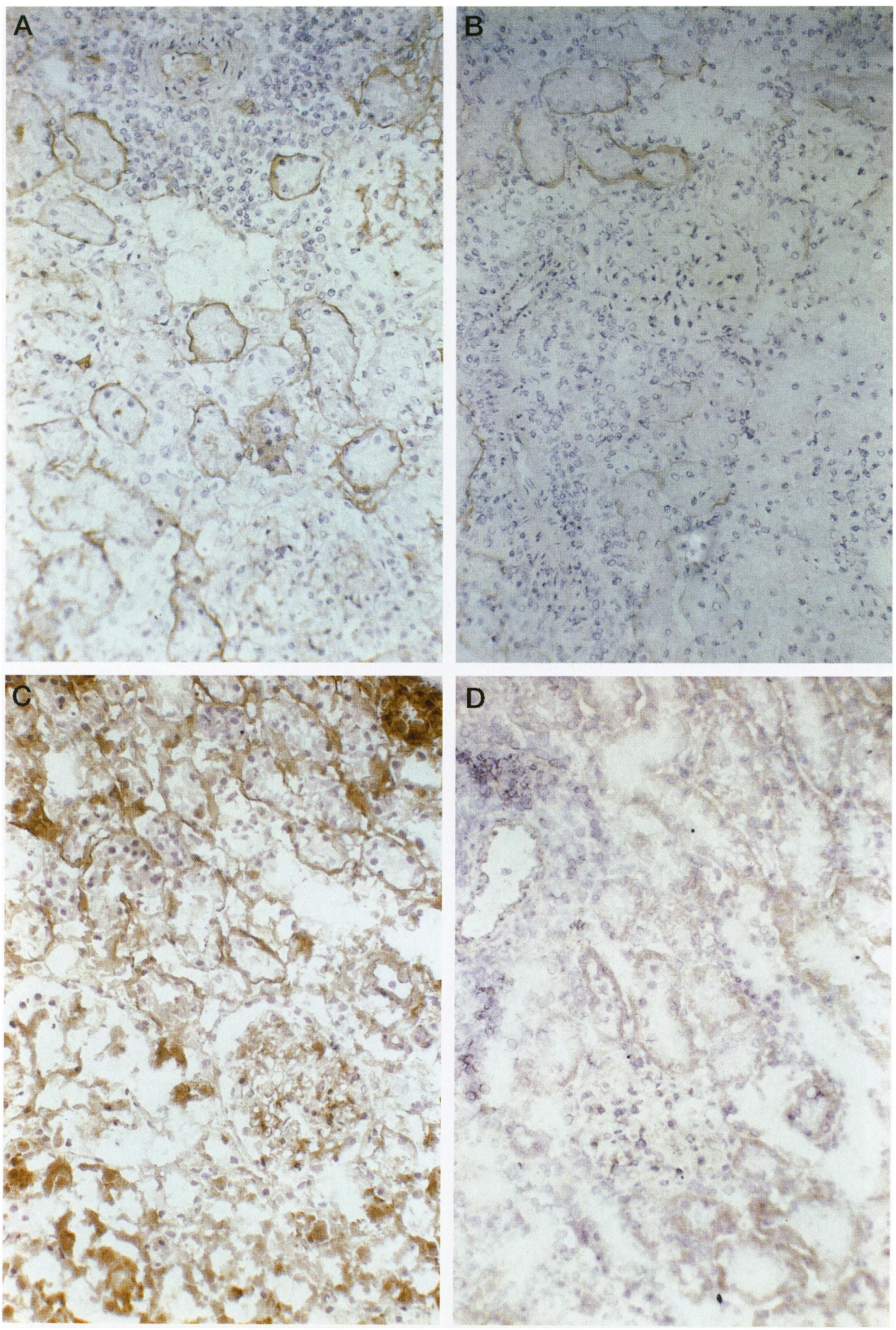

Figure 2. *Immunohistochemical staining of C activation products in renal allografts at day 5 after transplant.* A: *Saline-treated control allograft.* B: *sCR1-treated allograft, stained for C3.* C: *Saline-treated control allograft.* D: *sCR1-treated allograft, stained for MAC. Photomicrographs show markedly reduced staining of C3 and MAC on tubular basement membranes in the context of sCR1 intervention. Magnification, ×320.*

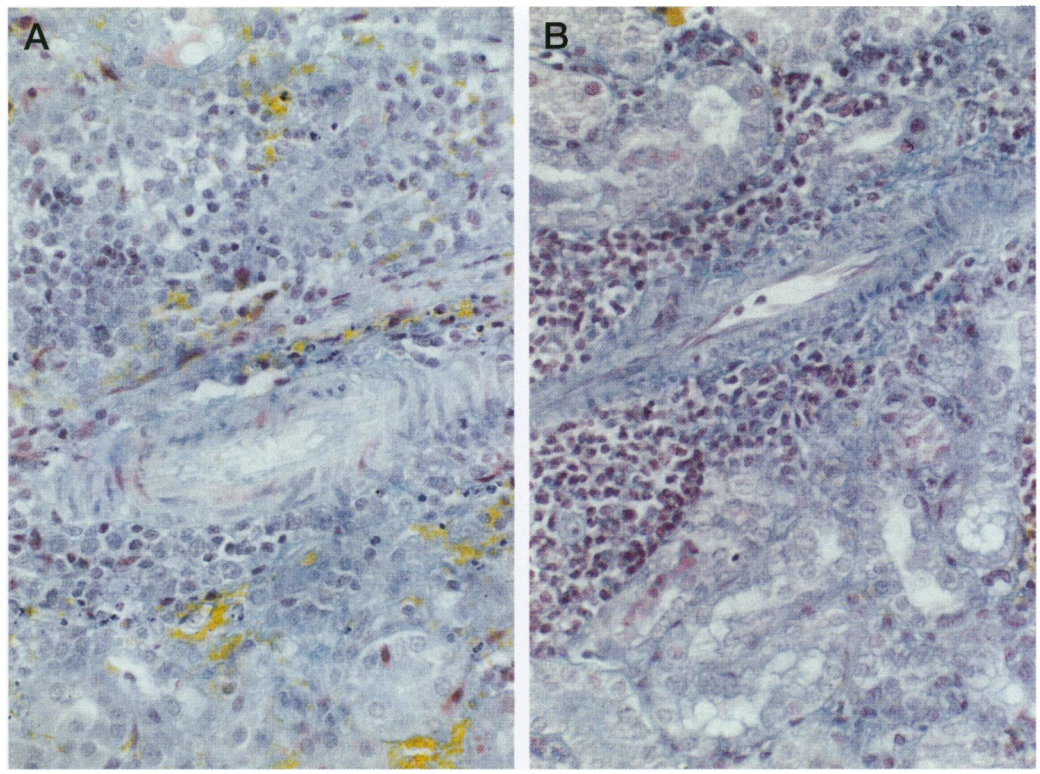

Figure 3. *MSB staining in renal allografts at day 5 after transplant.* A: *Saline-treated allograft showing stripped endothelium, disrupted tubules, and thrombosis.* B: *sCR1-treated allograft showing intact endothelium and tubules. Magnification, ×500.*

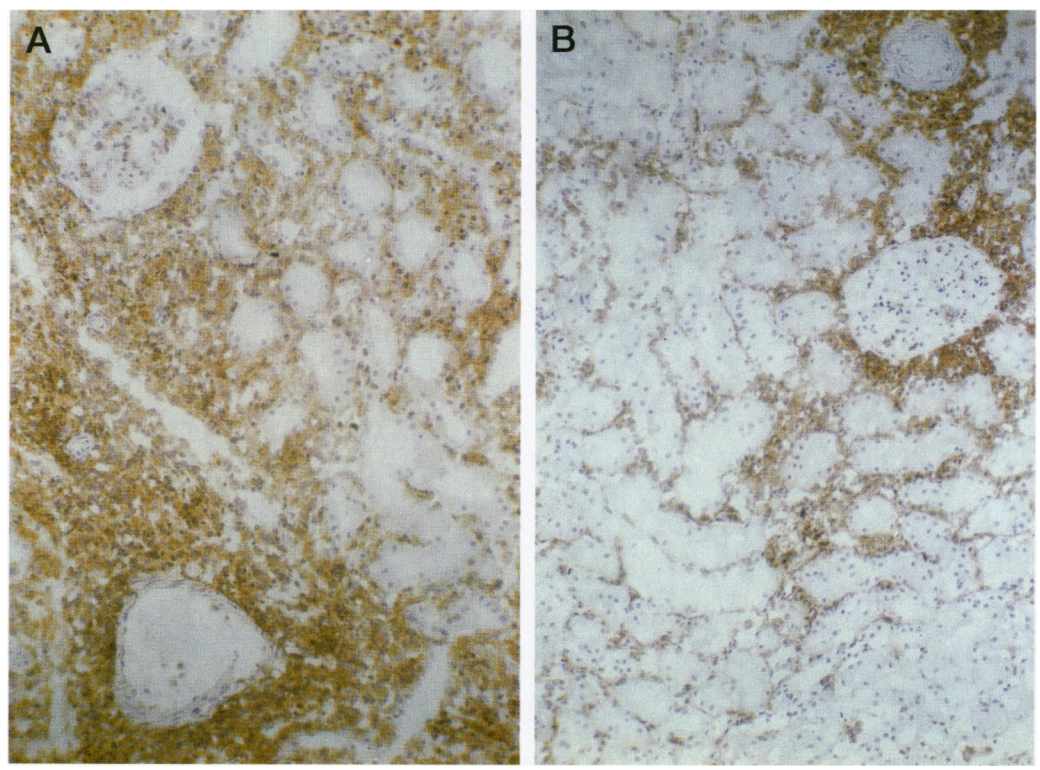

Figure 4. *Immunohistochemical staining for the leukocyte common antigen in renal allografts at day 5 after transplant.* A: *Saline-treated control showing total leukocytes within the graft.* B: *Allograft of sCR1-treated rat showing significantly fewer leukocytes present. Magnification, ×200.*

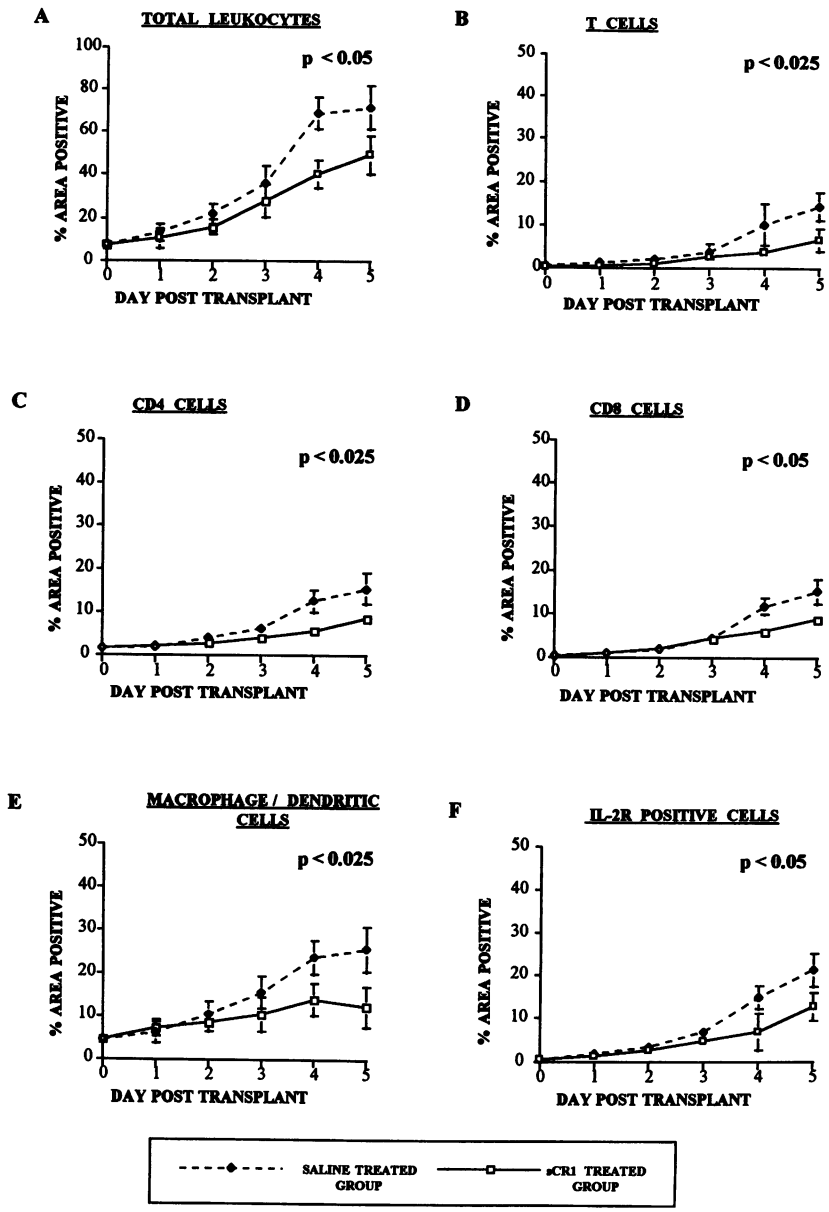

Figure 5. *Characterization of leukocyte subsets infiltrating rat renal allografts. Leukocyte infiltration of renal allografts was examined in the first 5 days after transplantation. Data points represent the means ± SEM of the percent area stained positive immunohistochemically. Markers shown are for CD45, leukocyte common antigen* (A)*; CD5, pan-T cells* (B)*; CD4, T-cell subset* (C)*; CD8, T-cell subset* (D)*; CD11b, macrophage/dendritic cells* (E)*; and CD25, IL-2 receptor* (F)*. P values are shown for the difference between treated and control groups at day 5 using the unpaired Student's t-test.*

and vascular injury. The grafts of sCR1-treated animals maintained an appearance compatible with grade I acute rejection at 5 days. In contrast, control grafts all exhibited grade II acute rejection at 4 days and grade III by 5 days.

To clarify the impact of sCR1 upon graft survival, we carried out additional transplants and analyzed the results at days 5 to 9. The findings at day 5 in this second set of recipients were consistent with those of the first set reported above. In sum, therefore, five of five control grafts exhibited grade III acute rejection by 5 days after transplant. By 7 days, control grafts displayed further tubular disruption and infarction (Figure 7A); the grafts of treated animals, although viable, showed continued infiltration and tubulitis, with some thrombosis of peritubular capillaries (grade II acute rejection; Figure 7B). At 8 days, the grafts in the treatment group still showed grade II rejection, but by 9 days, three of four of the treated rats showed evidence of grade III acute rejection (Figure 7C).

Table 1. *Mean Percent Area of Frozen Sections Stained Positive by Immunohistochemistry for Leukocyte Markers at Day 5 after Transplant*

Leukocyte antigen	Mean % area stained positive at day 5 after transplant	
	Saline-treated group	sCR1-treated group
CD45; leukocyte common antigen	71.3 ± 7.4	49.2 ± 10.7
CD5; pan-T cells	14.6 ± 3.2	6.8 ± 2.7
CD4; T-cell subset	7.4 ± 1.8	4.8 ± 1.7
CD8; T-cell subset	8.5 ± 0.9	4.8 ± 1.0
CD11b; macrophage/ dendritic cells	25.8 ± 5.3	12.3 ± 4.7
CD25; IL-2 receptor α-chain	21.3 ± 3.9	12.7 ± 3.9

Ischemic Control Studies

DA to DA renal isografts (n = 3) were examined at day 7 after transplant. The isografts all displayed near normal histology with no signs of vascular or cellular injury (Figure 7D). We therefore found no evidence that ischemic injury was directly responsible for the vascular injury observed in control allografts.

Discussion

Administration of sCR1 to inhibit C protected against renal allograft injury. Other groups have shown a similar protective effect of sCR1 on the vascular injury in presensitized hyperacute models of rejection[17,18,24,25] where endothelial injury is mediated by preformed antibodies and C. This study showed that C inhibition could also protect previously unsensitized animals against vascular rejection. In addition, C inhibition appeared to attenuate the cellular response.

A presensitized model of rat cardiac allograft rejection, using 3 mg/kg sCR1,[18] showed protection against Ab-mediated rejection. In that study, the resumption of C deposition, with subsequent rejection, was correlated to the clearance of sCR1 to a level below which inhibition was ineffective. In the present study, C inhibition was partially reversed as circulat-

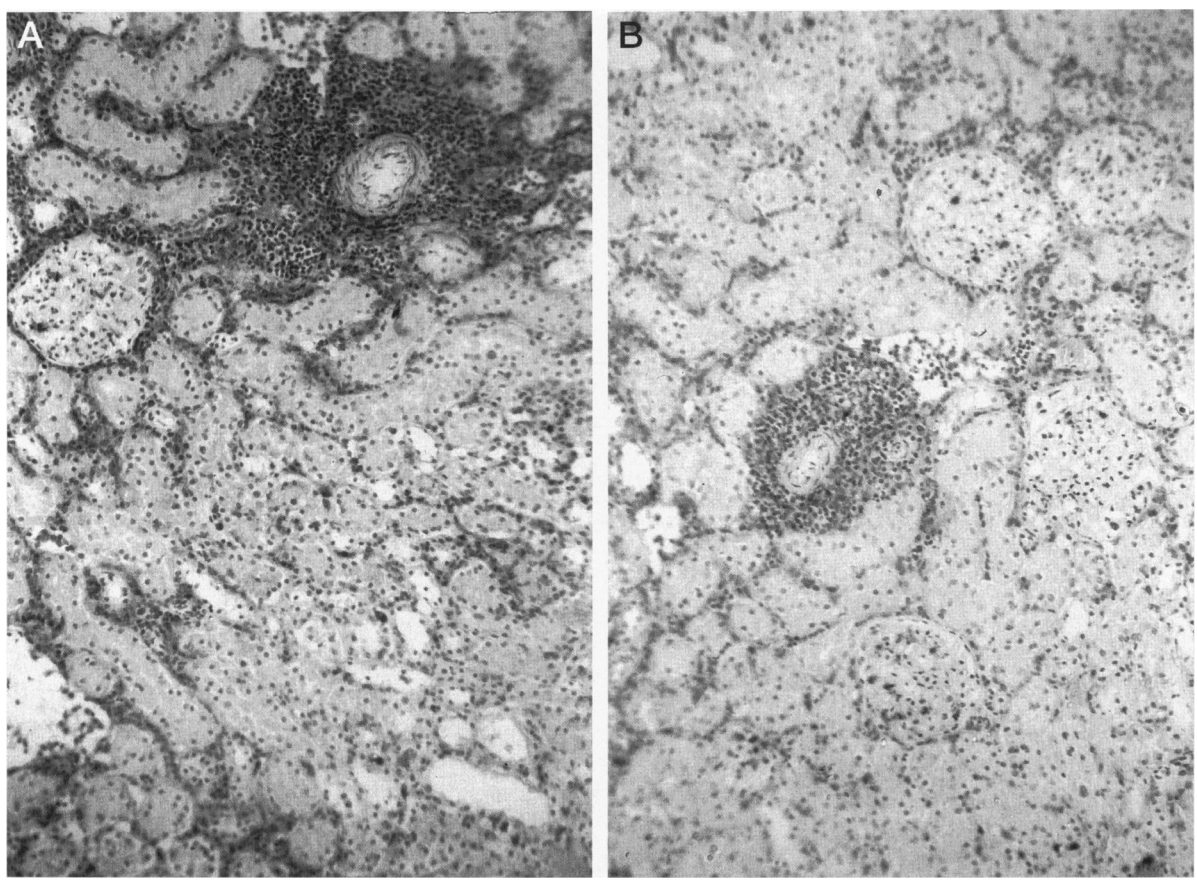

Figure 6. *Immunohistochemistry for CD25 (IL-2 receptor) in renal allografts at day 5 after transplant.* **A:** *Saline-treated control showing CD25⁺ leukocytes within the graft.* **B:** *sCR1-treated allograft with significantly fewer cells positive for CD25. Magnification, ×200.*

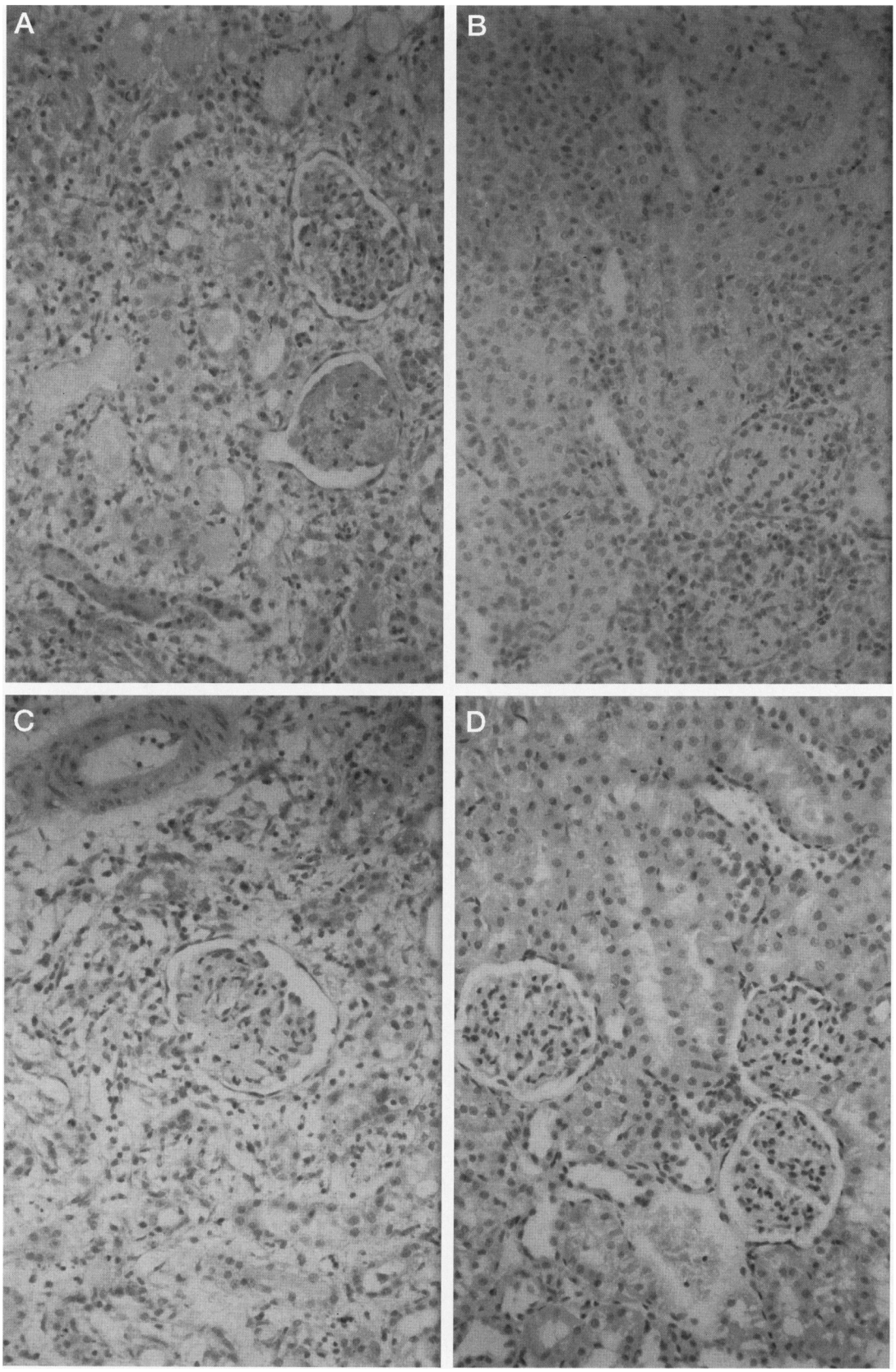

Figure 7. *H&E-stained kidney transplant tissue.* **A** *and* **B:** *Day 7 allografts from saline- and sCR1-treated recipients, respectively.* **C:** *Day 9 allograft from an sCR1-treated recipient.* **D:** *Day 7 isograft (DA to DA transplant). Magnification, ×320.*

ing sCR1 levels returned to mean baseline level at 24 hours. However, the decay of C inhibition lagged behind the clearance of sCR1 such that plasma hemolytic activity was still depressed to 58% of normal activity at 24 hours.

In pig to cynomolgus monkey, heterotopic cardiac xenotransplantation, 15 mg/kg sCR1 was used to produce a plasma concentration of 300 μg/ml,[17] shown by the same authors to be effective in preventing Ab-mediated destruction of isolated pig hearts perfused with human blood. These observations support the present data showing that, with daily administration of 25 mg/kg sCR1, achieving a mean peak plasma sCR1 level of 782 μg/ml, C was maximally inhibited by approximately 90%. Almost total plasma clearance of sCR1 within 24 hours presumably allowed continued deposition of C, although to a markedly lesser degree and without the vascular damage that had occurred by day 5 in controls.

The Lewis to DA model was chosen for the present study because it is one of aggressive allograft rejection,[26] providing a stringent test for interventional studies. The Banff criteria were used to provide an end point at which graft survival was deemed irreversible due to the presence of tissue infarction. The histopathological features at day 5 in control animals were consistent with grade III acute rejection by the Banff classification. Treatment with sCR1 appeared to prolong graft survival, as defined by this end point, until 9 days after transplant, when control grafts were almost completely necrotic.

A histopathological end point was chosen in preference to recipient survival for several reasons. First, the latter would require bilateral native nephrectomy at the time of transplantation. This would have induced increasing uremia, known to have an immunosuppressive effect. Second, prolongation of the study until terminal uremia would not seem justifiable in the face of clear-cut differences in tissue injury. Third, there is evidence that rats develop antibodies against human sCR1 after 5 days after injection (Dr. James Levin, T Cell Sciences, personal communication). This could potentially limit the efficacy of sCR1, making interpretation of the data beyond this time point more difficult. We therefore decided upon a study using a unilateral nephrectomy and more weighted toward a histological assessment during the first 5 days, allowing the recipients to maintain normal renal function.

C activation and MAC deposition do not inevitably lead to cell lysis, as nucleated cells possess protective mechanisms against this. Vesiculation of membrane fragments impregnated with sublytic quantities of MAC,[27] and increased ion efflux to counteract osmosis through MAC-derived pores, serve to protect the cell. Additionally, cell surface molecules of the regulators of C activation family, including CR1, inhibit C activation at the cell surface. It is known that cell lysis is more dependent on other factors, particularly the degree of C deposition, which was reduced in the treated allografts. These mechanisms may help to explain the protective effect on vascular injury reported here despite continued, although much reduced, C deposition in the sCR1-treated animals.

Morphometric analysis of leukocyte kinetics detected a significant reduction in the leukocyte infiltrate at day 5 after transplant with C inhibition. A number of mechanisms may help to explain this. First, the local inhibition of C may have prevented damage to the vasculature, thus maintaining the integrity of vessels and reducing the migration of leukocytes across the endothelium.

Second, proteolysis of C5 releases C5a, which is strongly chemotactic for neutrophils. Monocytes and macrophages are also known to bind the split products of C5[28] and be responsive to the C-derived chemotaxins C5a and C5a des Arg.[29] The results in this study showed a significant reduction in macrophage/dendritic cells within the grafts of treated animals. However, neutrophils were not identified in this study, so any effect of C5a chemotaxis and its potential inhibition by sCR1 is uncertain.

Third, C has been found to enhance Ag-specific presentation in a number of studies. Arvieux et al[7] showed that C3b enhanced the presentation of antigen by antigen-presenting cells to T-cell hybridomas, and Jacquier-Salin et al[8] found that C3b, covalently attached to Ag, enhanced the presentation of peptide to specific T-helper cells. These reports suggest the possibility that opsonization of Ag by C3b may enhance its uptake via surface C receptors or may influence the intracellular processing of Ag and its subsequent presentation to T cells.

Finally, follicular dendritic cells expressing C receptors, can capture ICs via C3b. These complexes are presented to responsive B cells in the germinal centers of secondary lymphoid organs. It has been suggested that C3b, bound to ICs, may function as an adhesion molecule, bridging the antigen-presenting cell and effector lymphocyte *via* their C3b receptors.[30] This IC-trapping process can be interrupted by the depletion of C.[31] Thus, C inactivation, within the graft or lymphoid system, may have led to reduced efficiency of Ag presentation and consequently to reduced activation/proliferation of leukocyte populations.

The alternative pathway of C activation is known to be important in the genesis of ischemic/reperfusion injury. However, histological analysis of isografts at day 7 after transplant did not suggest that hypoxic injury contributed significantly to the vascular injury seen in allografts. This does not exclude the possibility that, as hypoxic tissues may be rendered more susceptible to subsequent immune injury, the effects of C inhibition could have been due, in part, to reduced ischemic damage.

In conclusion, a daily dose of 25 mg/kg recombinant human sCR1 in otherwise unmodified, fully MHC-disparate, Lewis to DA rat renal transplantation produced a potent anti-inflammatory effect. Treatment with sCR1 attenuated the vascular and cellular injury seen by day 5 after transplant in saline-treated controls and delayed the time to severe irreversible rejection (defined here as Banff grade III) until day 9 in the sCR1-treated group. These data support a role for C in acute unsensitized allograft rejection, the inhibition of which may provide an additional approach to therapy.

Acknowledgments

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