Chemokines like RANTES appear to play a role in organ transplant rejection. Because RANTES is a potent agonist for the chemokine receptor CCR1, we examined whether the CCR1 receptor antagonist BX471 is efficacious in a rat heterotopic heart transplant rejection model. Treatment of animals with BX471 and a subtherapeutic dose of cyclosporin (2.5 mg/kg), which is by itself ineffective in prolonging transplant rejection, is much more efficacious in prolonging transplantation rejection than animals treated with either cyclosporin or BX471 alone. We have examined the mechanism of action of the CCR1 antagonist in in vitro flow assays over microvascular endothelium and have discovered that the antagonist blocks the firm adhesion of monocytes triggered by RANTES on inflamed endothelium. Together, these data demonstrate a significant role for CCR1 in allograft rejection.

The classic signs of acute cellular rejection during organ transplantation include the infiltration of mononuclear cells into the interstitium (1). This cellular infiltrate consists mainly of T lymphocytes, monocytes, and macrophages that are recruited from the circulation into the transplanted tissue by chemotactic molecules known as chemokines. Chemokines belong to a large family of small (8–10 kDa) inducible chemotactic cytokines, which are characterized by a distinctive pattern of four conserved cysteine residues (2). Currently over 40 chemokines have been identified and classified into two major groups, CXC and CC, dependent on the number and spacing of the first two conserved cysteine residues. The CXC class members include interleukin (IL)-8, melanoma growth stimulatory activity, and neutrophil-activating peptide-2, whereas the CC class includes RANTES, monocyte chemotactic protein-1, and MIP-1α (macrophage inflammatory protein-1). A number of studies have provided evidence for a role for RANTES in organ transplant rejection, particularly of the kidney. In a model of reperfusion injury in the rat, RANTES levels were increased over normal levels and remained high for more than a week, correlating with the peak of infiltrating macrophages (3). RANTES protein was detected in infiltrating mononuclear cells, tubular epithelium, and vascular endothelium of renal allograft biopsy specimens from patients with cyclosporin nephrotoxicity but not in normal kidney (1). A recent study suggests that RANTES may play a role in graft atherosclerosis (4). Increased levels of RANTES (both mRNA and protein) were detected in mononuclear cells, myofibroblasts, and endothelial cells of arteries undergoing accelerated atherosclerosis compared with normal coronary arteries. In another recent renal transplant study, the chemokine receptor antagonist Met-RANTES when given with low doses of cyclosporin significantly reduced renal injury including interstitial inflammation mainly by reducing the number of infiltrating monocytes (5). Mechanistically this appeared to be achieved by blocking the firm adhesion of these cells to the inflamed endothelium. In summary, these studies strongly suggest that RANTES, through activation of specific chemokine receptors on mononuclear cells, may play an important role in allograft rejection.

Based on these studies, there is strong evidence in support of the concept that the chemokine RANTES plays an important role in organ transplant rejection. Because RANTES is a ligand for the chemokine receptors CCR1 and CCR5, these receptors, located on circulating mononuclear cells, may be useful therapeutic targets in transplantation biology. These studies therefore provided the rationale for us to establish a protocol to inhibit RANTES-mediated biological activities by developing highly potent and specific nonpeptide CCR1 receptor antagonists. We have previously identified a number of specific highly potent CCR1 antagonists (6, 7) and describe here the ability of these compounds, BX471, in combination with low and high doses of cyclosporin, to significantly prolong the survival time of transplanted heart in a rat model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled chemokines were human unless otherwise indicated (Peprotech, Rocky Hill, NJ). 125I-Labeled human chemokines were obtained from PerkinElmer Life Sciences.

**Cell Lines**—The human embryonic kidney (HEK) 293 cell line was obtained from the American Type Culture Collection and was maintained in RPMI or Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin in a 5% CO2 atmosphere at 37°C. For binding assays, the cells were harvested and washed once with phosphate-buffered saline.
Cell viability was assessed by trypan blue exclusion, and cell number was determined by counting the cells in a hemocytometer.

**CCR1 Expression Vector**—Human and rat CCR1 cDNA was obtained as described (8) and inserted into a mammalian expression vector containing the SV40 replication origin, the human cytomegalovirus enhancer, the murine N-acetyl transferase gene (purine resistance) and hygromycin B gene (hygromycin resistance) similar to that described previously (9).

**CCRI-expressing Cells**—HEK 293 cells stably expressing human or rat CCR1 were grown to confluent monolayers in T225-cm² flasks as described previously (6). Cells were tested for their ability to bind 125I-MIP-1a and RANTES and biological responses by changes in intracellular Ca²⁺ or by microphysiometry.

**Chemokine Binding Studies**—The binding assays were performed either in transfected cells or in peripheral blood mononuclear cells by centrifugation methods as described previously (6). Nonspecific binding was determined in the presence of either 100 nM or 1 µM unlabeled ligand. The binding data were curve fitted with the computer program IGOR (Wavemetrics) to determine the affinity and number of sites.

**Preparation of Stock Solution of BX471**—A 25 mg/ml sterile saline solution of BX471 in 40% cyclodextrin (Aldrich) was prepared by dissolving the compound into 40% cyclodextrin in saline. The mixture was shaken followed by the addition of 230 µL of concentrated HCl. The mixture was stirred to dissolve the solute. After dissolution was complete (1 h), the pH of the solution was pH 3.3, and 1 µL KOH was added to raise the pH to 4.5. The solution was filtered through a 0.45-µm filter and stored at 4 °C.

**Determination of Pharmacokinetic Parameters in Rats**—Male Lewis rats (n = 6) were subcutaneously dosed with BX471 (50 mg/kg, three times per day) in a vehicle of 40% cyclodextrin/saline for 7 days. Blood samples were collected by cardiac puncture in EDTA-containing tubes at various times and analyzed for drug levels as described previously (7).

**Heterotopic Heart Transplant Rejection (Lewis or ACR Rats)**—Adult male, specific pathogen-free ACR (RTA1a and Lewis (RT1) rats (Charles River, Boston, MA) weighing 200–250 g were used in these studies. Vascularized cardiac allografts were heterotopically transplanted into the anterior abdominal wall of recipient rats using a modification (10) of the technique of Ono and Lindsay (11). Abdominal allografts were pulsed on a daily basis to assess graft function, and rejection was deemed complete when palpable ventricular contractions ceased.

**In Vitro Model System of Monocyte Recruitment on Microvascular Endothelium under Physiological Flow Conditions**—The interaction of monocytes with endothelium was studied in laminar flow assays performed as described (5, 12). Briefly, dermal microvascular endothelial cells grown to confluence in Petri dishes were stimulated with IL-1β (10 ng/ml) for 12 h followed by pre-incubation with RANTES (10 nM) for 30 min at 37 °C just prior to assay. The plates were assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an Olympus IMT-2 inverted microscope with ×20 and ×40 phase-contrast objectives. Isolated human blood monocytes were isolated by Nycodenz hyperonmolar gradient centrifugation as described (12) and resuspended at 5 × 10⁵ cells/ml in assay buffer (HBSS) containing 10 mM HEPES, pH 7.4 and 0.5% human serum albumin. Shortly before the assay, 1 mM MgCl₂ and 1 mM CaCl₂ was added. The cell suspensions were kept in a heating block at 37 °C during the assay and perfused into the flow chamber at a rate of 1.5 dyn/cm² for 5 min. For inhibition experiments, monocytes were preincubated with BX471 at different concentrations (0.1–10 µM) or a MeSO control for 10 min at 37 °C. The number of firmly adherent cells after 5 min was quantitated in multiple fields (at least five per experiment) by analysis of images taken with a long integration JVC 3CCD video camera and a JVC SR camera. Cell viability was assessed by trypan blue exclusion, and cell number was determined by counting the cells in a hemocytometer.

**Histology, Immunohistochemistry, and Morphometry**—The hearts were removed under deep anesthesia, quickly blotted free of blood, weighed, and then processed as needed for histology and immunohistochemistry. The organs were cut into 1-mm slices and either immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.35, or fixed in 1% HMPO, 108 mM NaPO₃, and 248 mM NaCl for 24 h or fixed in methacarn for 8 h and embedded in paraffin. Light microscopy was performed on 3-µm sections stained by periodic acid Schiff or Goldner-Elastica.

The ED1 monoclonal antibody (Serotec/Camon) was used on methacarn-fixed paraffin-embedded tissue (3 µm) to stain for rat monocytes/macrophage cells. An alkaline phosphatase anti-alkaline phosphatase detection system was used for visualization (Dako). Controls that omitted the first or second antibody for each section tested were negative.

**Histopathologic Rejection Grading System (Score: 1-3)**—Rejection in the allogeneic rat heart was graded according to Billingham (13). Mild acute rejection (score: 1) was characterized by a sparse interstitial mononuclear infiltrate often accentuated in perivascular spaces. Moderate acute rejection (score: 2) was a moderately dense perimyocytic mononuclear infiltrate with some myocyte necrosis. Severe acute rejection (score: 3) featured a dense mononuclear infiltrate with focal hemorrhage and replacement of myocytes and with occasional endothelialitis of intramural arteries. The rejection score was calculated for every tissue block, and an average score was calculated from the different blocks for every transplanted heart as rejection processes tended to be focal.

**Radioimmunounassay for Cyclosporin**—Cyclo-Trac SP whole-blood radioimmunoassay for cyclosporin kits were purchased from DaiSorin (Stillwater, MN). This method employs a specific monoclonal antibody that measures only cyclosporin. A methanol extraction step was performed for the standards, controls, and samples prior to assay. The methanol extracts were then combined with 125I-labeled cyclosporine tracer. A mixture of mouse monoclonal specific to cyclosporin A and the second antibody (donkey anti-mouse) in a single reagent was added. Following a 1-h incubation, the tubes were centrifuged, decanted, and then counted. The amount of radioactivity remaining in the pellet was inversely proportional to the concentration of cyclosporin found in each sample. The sample was assayed in duplicate, and the variation of the duplicates was <10%, otherwise the assay for that sample was repeated.

**RESULTS AND DISCUSSION**

Because RANTES appears to play an important role in organ transplant rejection, its receptor, CCR1, is a prime therapeutic target. Empirical screening of our available compound libraries to discover potential CCR1 antagonists yielded a number of compounds that were potent human CCR1 antagonists (6). One of these molecules BX471 ((R)-N-(5-chloro-2-[4-[[4-fluorophenylmethyl]-2-methyl-1-piperaziny]-2-oxoethoxy][phenyl]urea hydrochloric acid salt) had a Kᵣ of 1 nM for the human receptor (4).

The poor affinity of our CCR1 antagonists for mouse CCR1 (data not shown) precluded mouse models of disease. Thus, we tested for receptor binding of BX471 to rat CCR1 receptors by carrying out displacement binding assays with 125I-MIP-1a added. The results shown are from a typical experiment (n = 3). Inset shows the Scatchard plot of the displacement data.
rat CCR1 as it was for human CCR1, it could nevertheless compete effectively for binding to the rat receptor at higher concentrations. Additional studies with rat peripheral blood mononuclear cells revealed that BX471 was able to displace radiolabeled MIP-1α but not MIP-1β, demonstrating no cross-reactivity for rat CCR5 (data not shown).

We showed that BX471 is a functional antagonist of rat CCR1 by measuring its ability to inhibit the MIP-1α-induced transient rise in intracellular Ca²⁺ concentration in cells expressing rat CCR1. We measured the change in intracellular Ca²⁺ concentration in response to various concentrations of MIP-1α by fluorimetry using the indicator Fura-2. In these experiments, increasing concentrations of MIP-1α produced a transient rise in intracellular Ca²⁺ that was inhibited by pre-incubating the cells briefly with a 10-fold higher concentration of BX471 (data not shown). Thus, BX471 is a potent functional antagonist of the rat CCR1 receptor, capable of inhibiting calcium transients that are mediators of intracellular cell activation.

Based on the studies summarized above, pharmacokinetic studies with BX471 in rats were carried out. Male Lewis rats were subcutaneously dosed with BX471 (50 mg/kg t.i.d.) in a vehicle of 40% cyclodextrin/saline, and blood samples were withdrawn at various times and analyzed for drug levels as described in the legend to Fig. 2. As shown in Fig. 2, peak plasma levels following the subcutaneous administration of BX471 varied between 12 and 27 μM. Absorption was relatively rapid, with significant plasma levels observed at 15 min post-drug. After 8 h (trough time point), plasma drug levels were ~1–2 μM. The plasma half-life ranged between 2–3 h. Though there did not appear to be any pattern of either enhanced clearance or accumulation of the drug on repeated subcutaneous t.i.d.-dosing regimen (50 mg/kg), a considerable amount of variability was observed in the rate and extent of drug absorption on all of the days measured. These studies showed that subcutaneous dosing of BX471 at 50 mg/kg t.i.d. seemed to give adequate drug levels (steady-state BX471 levels observed between 200 and 500 min of each 8-h dosing regimen were 1 to 2 μM) over a 24-h period and allowed us to test the compound in a rat heterotopic heart transplant model.

RANTES is a ligand for CCR1 and appears to play a role in directing mononuclear cells into transplanted tissue leading to inflammation and rejection (1, 3–5). Thus, we rationalized that a CCR1 antagonist should have some utility in prolonging the survival of organ transplants. We tested this hypothesis directly by asking whether the CCR1 antagonist BX471 was efficacious in a rat heterotopic heart transplant rejection model. The mean allograft survival of animals given BX471 alone was 8.8 ± 1.2 days compared with 6.8 ± 0.8 days for control animals with ACI grafts in LEW recipients (Fig. 3).
Role of the CCR1 antagonist BX471 in Transplant Rejection

TABLE I
Rejection score for transplanted rat hearts

|                | N  | n  | Rejection Grade |
|----------------|----|----|-----------------|
| Control-oil    | 2  | 9  | 1.72 ± 0.24     |
| Control-cyclosporin | 3  | 15 | 1.45 ± 0.25     |
| BX471-oil      | 2  | 9  | 1.44 ± 0.29     |
| BX471-cyclosporin | 3  | 12 | 0.65 ± 0.14*    |

* p value relative to control oil < 0.0005.

FIG. 4. Light microscopy of allogeneic heart transplants 3 days after transplantation without (A, B) and with (C, D) BX471. A, in nonimmunosuppressed transplants a dense mononuclear infiltrate was observed. Many cardiomyocytes were vacuolated or necrotic. Interstitial edema was pronounced. B, in the cyclosporin-treated rats the inflammatory cell infiltrate was reduced, though still clearly evident, specifically around venules with focal destruction of cardiomyocytes. C, BX471-treated rats showed a focal mononuclear cell infiltrates that were pronounced with similar morphology as to be observed in nonimmunosuppressed transplants. D, BX471- and cyclosporin-treated animals showed well preserved cardiac morphology with sparse mononuclear cell infiltrates. (A–D, HE-stains; objective × 40).

FIG. 5. Immunohistologic stain for ED1-positive monocytes/macrophages in allogeneic heart transplants 3 days after transplantation without (A, B) and with (C, D) BX471. A, in nonimmunosuppressed transplants, many cells of the dense mononuclear cell infiltrate consisted of monocytes/macrophages, which were closely juxtaposed to the cardiomyocytes. B, in the cyclosporin-treated rats, the inflammatory cell infiltrate was focal and was composed primarily of ED1-positive cells. C, in the BX471-treated animals the mononuclear cell infiltrate varied significantly, aside from the dense inflammatory cell infiltrates as shown in Fig. 6, areas with moderately dense monocytic infiltrate around venules were seen. D, combined treatment of BX471 and cyclosporin resulted in a dramatic reduction in monocyte/macrophage infiltration into the allogeneic rat hearts. (A–D, APAAP reaction in Methacarn-fixed tissue; objective × 20).

combined therapy of BX471 and cyclosporin A (Figs. 4 and 5).

Based on the data from these studies, BX471 given in combination with cyclosporin resulted in a clear increase in efficacy in heart transplantation compared with cyclosporin alone.

Although we have shown that treatment with the CCR1 antagonist BX471 in combination with cyclosporin synergistically increases transplantation survival in a rat heart model compared with treatment with either drug alone, it remains a formal possibility that these data are because of drug/drug interactions that stabilize the blood plasma cyclosporin levels rather than to the true synergism of the drug combination. To determine whether BX471 had any effect on cyclosporin plasma levels, we designed a pharmacokinetic study to assess this. In this study we examined the effect of BX471 on the blood concentration of cyclosporin in the rat after a single oral dose (2.5 mg/kg) of cyclosporin followed by t.i.d. doses of BX471 at 50 mg/kg administered subcutaneously. We used a radioimmunoassay to measure whole blood cyclosporin levels in animals treated with cyclosporin in the presence and absence of BX471. Visual inspection of the time-concentration curves suggested a slight prolongation of elimination half-life of rats treated with BX471 (Fig. 6). However, statistical analysis of the paired groups indicated that there was no significant difference between the two parameters calculated (p-value for AUC was 0.224 and for the t1/2 was 0.317) and thus we can rule out drug/drug interactions as the basis for the extended survival of the transplanted rat hearts.

To gain insight into the potential mechanisms of action by which the CCR1 antagonist BX471 prolonged the rejection of transplanted rat hearts, we asked whether it could inhibit the firm arrest and diapedesis of monocytes on microvascular endothelium. It has been previously shown that perfused monocytic cells show increased attachment to IL-1β-activated DMVEC following preincubation with exogenous RANTES for 30 min (5). Treatment of isolated blood monocytes with increasing concentrations of BX471 (0.1–10 μM) showed a dose-dependent inhibition of RANTES-mediated and shear-resistant adhesion on IL-1β-activated microvascular endothelium in shear flow (Fig. 7A). Consistently, the percentage of monocytes that were found to undergo or maintain rolling interactions was dose dependently increased by pretreatment with BX471, thus serving as an inverse measure for arrest (Fig. 7B). BX471 also inhibited the RANTES-mediated adhesion of T lymphocytes to activated endothelium (data not shown). These data strongly suggest that the CCR1 antagonist is a potent antagonist that can specifically inhibit mononuclear cell adhesion to activated endothelium. It is unlikely that the CCR1 antagonist is simply inactivating the cells because e.g. activated T lymphocytes and monocytes treated with the CCR1 antagonist are still able to respond to MIP-1β in calcium flux experiments (data not shown) presumably by binding and activation of CCR5.

The dose-responsive inhibition of monocyte arrest on acti-
quantitated and expressed as cells/mm². Data represent the mean field. After 5 min of accumulation, the number of adherent cells was period and expressed as the percentage of total interactions within the rolling at low shear was assessed in the last 30-s interval of the 5-min

37 °C.

activated endothelium by BX471. DMVEC activated with IL-1

b,

ments.

monocytes were perfused at a constant shear of 1.5 dyn/cm² as de -

preincubated with RANTES were inserted in a flow apparatus, and

as anticoagulant at 0, 1, 2, 4, 6, 8, 12, and 24 h post-dosing. The
cyclosporin concentration in these samples were analyzed by the Cyclo-

Trac radioimmunoassay in rat whole blood as described under “Exper-

imental Procedures.”

FIG. 6. Effect of BX471 on cyclosporin levels in rat whole blood. Cyclosporin levels were measured in two groups of six Lewis cannulated rats. The first group received a single oral dose of 2.5 mg/kg cyclosporin diluted in olive oil. The second group received a single oral dose of the same cyclosporin solution followed by subcutaneous injection of BX471 t.i.d. at 50 mg/kg. Whole blood was collected using EDTA

FIG. 7. Inhibition of in vitro adhesion of monocytes to acti-

vated endothelium by BX471. DMVEC activated with IL-1β and preincubated with RANTES were inserted in a flow apparatus, and monocytes were perfused at a constant shear of 1.5 dyn/cm² as de-

scribed under “Experimental Procedures” and in a previous communi-
cation (5). For inhibition assays, monocytes were preincubated with BX471 at indicated concentrations or a Me₂SO control for 10 min at 37 °C. A, after 5 min of accumulation, the number of adherent cells was analyzed. Data represent the mean ± S.D. of three individual experiments. B, as an inverse measure of adhesion, the number of monocytes rolling at low shear was assessed in the last 30-s interval of the 5-min period and expressed as the percentage of total interactions within the field. After 5 min of accumulation, the number of adherent cells was quantitated and expressed as cells/mm². Data represent the mean ± S.D. of three experiments.

dose responsively inhibit the RANTES- or MIP-1α-induced up-

regulation of the β-2 integrin, CD11b (7). A number of studies

have revealed that RANTES treatment of monocytes results in an increased expression of CD11b and an up-regulation of integrin avidity for their endothelial immunoglobulin family ligands (12, 20, 21). This appears to enhance the ability of monocytes to bind to endothelial cells, which can be partially blocked with antibodies to integrins (20). These studies suggest that modulation of integrin expression and avidity by chemokines may facilitate the tissue trafficking of monocytes during inflammation.

Two recent studies have demonstrated the important role of CCR1 in organ transplantation. In the first, the efficacy of Met-RANTES, a RANTES receptor antagonist that is thought to work in part through a blockade of CCR1 (5) was examined in a renal transplantation study. Following the transplantation of Fisher RT1(ª) rat kidneys into Lewis RT1 rats, the animals treated with Met-RANTES showed a significant reduction in vascular and tubular damage relative to untreated animals. In a more severe rejection model, where Brown-Norway RT1 rat kidneys were transplanted into Lewis RT1 rats, Met-RANTES was found to augment treatment with low dose cyclosporin A to significantly reduce all aspects of renal injury. In a second study, the importance of CCR1 was examined in heart transplantation models in mice carrying a targeted deletion of CCR1(22). In four separate models of allograft survival, a signif-

significant prolongation was seen in CCR1(−/−) recipients. In one model, levels of cyclosporin that had marginal effects in CCR1(+/+) mice resulted in permanent allograft acceptance in CCR1(−/−) recipients.

In this study we have shown that inhibition of CCR1 by the receptor-specific antagonist BX471 led to a significant prolongation of cardiac allograft survival (Fig. 3). Furthermore, when the CCR1 antagonist was given in combination with a subnephrotoxic dose of cyclosporin, it markedly enhanced the survival of the transplanted organs (Fig. 3). It appears that the mech-

anism of action of the CCR1 receptor antagonist stems at least in part from its ability to inhibit the adhesion of activated immune cells to inflamed endothelium (Fig. 7). This may be because of blocking the up-regulation of integrin expression and adhesiveness that leads to imminent monocyte arrest. The ability of BX471 to enhance the effects of low dose cyclosporin treatment has clinical relevance given the dose-dependent nephrotoxicity associated with cyclosporin therapy. A reduc-

tion in the amount of cyclosporin required to achieve sufficient immunsuppression could be of considerable benefit not only in transplantation but also in the treatment of other chronic dis-

eases such as psoriasis, allergy, arthritis, and other autoim-

mune diseases.

A significant clinical advantage of BX471 may lie in its effects directly following transplantation. Early injury to the endothelium, even when it is not lethal, can have serious con-

sequences for survival of the graft. Even moderate damage can lead to the loss of the endothelial function required for ade-

quate perfusion of the graft and result in the enhanced produc-

tion of proinflammatory cytokines. By limiting immune effector cell emigration and the ensuing endothelial damage during transplantation BX471 may lower the inclination toward the development of more chronic transplant dysfunction and re-

duce the requirement for immune suppressants such as cyclos-

porin. These results strongly suggest that therapies directed toward the blockade of chemokine receptors will have a positive effect on solid allograft survival.

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A Non-peptide Functional Antagonist of the CCR1 Chemokine Receptor Is Effective in Rat Heart Transplant Rejection

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