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The Role of the CC Chemokine, RANTES, in Acute Lung Allograft Rejection

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Lung transplantation is a therapeutic option for patients with end-stage lung disease. Acute allograft rejection is a major complication of lung transplantation and is characterized by the infiltration of activated mononuclear cells. The specific mechanisms that recruit these leukocytes have not been fully elucidated. The CC chemokine, RANTES, is a potent mononuclear cell chemoattractant. In this study we investigated RANTES involvement during acute lung allograft rejection in humans and in a rat model system. Patients with allograft rejection had a 2.3-fold increase in RANTES in their bronchoalveolar lavages compared with healthy allograft recipients. Rat lung allografts demonstrated a marked time-dependent increase in levels of RANTES compared with syngeneic control lungs. RANTES levels correlated with the temporal recruitment of mononuclear cells and the expression of RANTES receptors CCR1 and CCR5. To determine RANTES involvement in lung allograft rejection, lung allograft recipients were passively immunized with either anti-RANTES or control Abs. In vivo neutralization of RANTES attenuated acute lung allograft rejection and reduced allospecific responsiveness by markedly decreasing mononuclear cell recruitment. These experiments support the idea that RANTES, and the expression of its receptors have an important role in the pathogenesis of acute lung allograft rejection. The Journal of Immunology, 2000, 165: 461–472.

Materials and Methods

Patient population
Twenty-four patients with single lung transplantation at the University of Michigan were involved in this study. They were all placed on a standard pre- and post-transplantation immunosuppression protocol. Cyclosporine was begun preoperatively and continued postoperatively to maintain levels at ~300 ± 50 ng/ml throughout the first 6 wk, followed by a reduction to maintain levels at ~200 ± 50 ng/ml. Methylprednisone was begun intraoperatively and was tapered to 20 mg/day by the third postoperative month. Attempts were made to further taper the dose between 5–10 mg/day by 12 mo. Azathioprine was begun at 2 mg/kg/day 8 h pretransplantation, then adjusted to maintain a white blood cell count of ~4000/mm³. All episodes of acute rejection were treated with methylprednisone (1 g/day) for 3 days.

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All patients had routine surveillance bronchoscopy performed at 6 wk and 3, 6, 9, and 12 mo. Patients also had bronchoscopy performed at times when infection or rejection was suspected. Twelve bronchoalveolar lavages (BALs) came from healthy single lung transplant patients having routine surveillance bronchoscopy and 12 BALs came from patients with biopsy proven acute lung allograft rejection. The diagnosis and grading of acute lung allograft rejection were based on histology from transbronchial biopsies as previously described (26). There was no evidence of infection (bacterial, fungal, *Pneumocystis carinii* pneumonia, or virus) seen on BAL cytology, or transbronchial biopsy. BAL fluid was obtained from lung transplant recipients with acute lung allograft rejection and from healthy lung allografts by methods previously described (27). Briefly, the patients were premedicated with meperidine and glycopyrrolate and nebulized with 2% xylocaine. Midazolam was used to obtain an appropriate degree of sedation. We performed flexible optic bronchoscopy via a transnasal approach and wedged the scope into a subsegmental bronchus of the middle lobe or lingula of the transplanted lung. BAL was then performed with 240 ml of normal saline in 60-ml aliquots; each retrieved by low suction. After discarding the initial aliquot, an average return of 100–125 ml was obtained. The BAL solution was split into two aliquots. One aliquot was sent to clinical laboratories for evaluation of cytology, bacterial, viral, and fungal cultures and CMV shell vial. The other was stored on ice and transported to the research laboratory. The recovered BAL fluid was filtered through sterile gauze, then centrifuged for 10 min at 2000 rpm. The cell-free solution was aliquoted and frozen immediately at 70°C until batched and thawed for cytokine ELISA (27).

**Orthotopic single lung transplantation model**

To assure immunogenic standardization and histoincompatibility, these studies employed inbred, unmodified, specific pathogen-free rats, Brown Norway (BN; RT1n) and Lewis (LEW; RT1 + ). Orthotopic rat left lung transplantation was performed using a modification of the procedure reported by March et al. and Prop et al., with >85% postoperative survival (21, 28). The 15% mortality associated with this procedure was directly attributable to surgical mortality and usually occurred within 8 h posttransplantation. Neutralization of RANTES in vivo did not impact on postoperative survival. A minimum of six rats at each time point or for each manipulation were used. Animals weighing 200 and 300 g were anesthetized with a combination of 4% halothane in a mixture of 40% O2/60% N2O. The anesthetic was maintained throughout the operation using 0.5–1% halothane in 40% O2/60% N2O mixture through a Harvard rodent ventilator (Natnick, MA). The body temperature of the animal was maintained using anesthetist mattress. Intravascular volume was assured by i.v. infusion of normal saline (3 mL/h) via tail vein. The operation on the donor animal was performed by removing the anterior chest wall and infusing 1000 U (USP) of heparin into the right atrial appendage of the heart and allowing time to circulate. The right ventricle was then flushed with 50 ml of normal saline after transsection of the pulmonary vein, followed by extraction of the left lung. The operation on the recipient animal was performed via a left posterior-lateral thoracotomy in the fourth or fifth intercostal space. The left lung hilum was identified, dissected, and cross-clamped. The recipient’s left native lung was removed. The reimplantation of the donor’s lung entailed the successful anastomosis of the following structures: 1) pulmonary vein and artery were anastomosed with continuous 10-0 Ethilon, followed by removal of the clamp and restoration of perfusion; and 2) the bronchus was anastomosed with interrupted 9–0 Ethilon, followed by reinfusion at a maximal pressure of 30 cm H2O. The allograft/isograft ischemic time was determined as the time from discarding the initial aliquot until each BAL was performed.

**Histopathology**

Ten random 5-μm paraffin-embedded tissue sections for eight different lung allografts were stained with hematoxylin and eosin, and blindly reviewed and graded as previously described (26). The histopathology grading of allograft rejection was: A0 = normal, no significant abnormality; A1 = minimal, infrequent perivascular infiltrates; A2 = mild, frequent perivascular infiltrates around arteries and veins; A3 = moderate, with extension of the perivascular infiltrate into the interstitium; A4 = severe, diffuse perivascular, interstitial, and alveolar infiltrates with alveolar pneumocyte damage, potential parenchymal necrosis, infarction, or necrotizing vasculitis.

**RANTES, MIP-1α, and MIP-1β ELISAs**

RANTES, MIP-1α, and MIP-1β protein were quantitated using a modification of a double-ligand method as previously described (30). Briefly, flat-bottom 96-well microtiter plates (ImmuNo-Plate I 96-F, Nunc, Naper- ville, IL) were coated with 50 μl/well of the goat polyclonal anti-rat RANTES, rabbit anti-murine MIP-1α, or rabbit anti-murine MIP-1β (R&D Systems, Minneapolis, MN; 1 ng/ml in 0.6 M NaCl, 0.26 M H3BO3, and 0.08 N NaOH, pH 9.6) for 24 h at 4°C and then washed with PBS (pH 7.5) and 0.05% Tween 20 (wash buffer). The specificity of the polyclonal anti-RANTES Ab (R&D Systems) to rat RANTES was tested using Western blot analysis. One hundred nanograms of recombinant rat RANTES (PeproTech) showed a strong band at the appropriate m.w. for RANTES using a 1/1000 dilution of goat polyclonal anti-rat RANTES. Twenty milligrams of this anti-rat RANTES has a specific neutralizing capacity of 10 μg of rat RANTES (PeproTech) by blocking mononuclear cell infiltration in the peritoneum in response to (i.p.) administration of rat RANTES. In contrast, 20 μg of this anti-rat RANTES did not block mononuclear cell infiltration in the peritoneum in response to 10 μg (i.p.) administration of rat MCP-1 (PeproTech) in naive rats. The specificity of the polyclonal Ab against rat RANTES was achieved by inhibiting the biological effect of RANTES by passive immunization (i.p.). Animals received 20 μg of goat polyclonal IgG anti-rat RANTES or an equivalent quantity of control goat polyclonal IgG Abs on days 0, 2, and 4 and were sacrificed on day 6, 8, or 10 post-transplantation.

**FACS analysis for CCR1, CCR5, and leukocyte surface markers**

Before removal, transplanted lungs were perfused with normal saline and dissected free of the thoracic cavity. The transplanted lung was minced and

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3 Abbreviations used in this paper: BAL, bronchoalveolar lavage; LEW, Lewis rat; BN, Brown Norway; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein.
incubated for 1 h in Dispase (Collaborative Biomedical Products, Two-Oak Park, MA). The cell suspension and undigested fragments were further dispersed by drawing them up and down through the bore of a 10-ml syringe. Cells were then pelleted at 600 × g for 10 min, resuspended in sterile water for 30 s to lyse remaining RBCs, washed in 1× PBS, and resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) with 5% FCS. Cells were counted with a hemocytometer, transferred at a concentration of 5 × 10^6 cells/ml to fluorescent Ab buffer (Difco, Detroit, MI; 1% FCS, and 0.1% azide), and maintained at 4°C for the remainder of the staining process. The cell suspension was analyzed with a flow cytometer (Becton Dickinson, Bed- Francisco, CA) followed by FITC-conjugated mouse anti-rat CD3 (T cells), CD4 (T cells), CD8 (T cells), CD45RA (naïve T cells/B cells), NKR-P1A (NK cells; Pharmingen, San Diego, CA), ED1 (mononuclear phagocytes; Seratec, Kirklington, U.K.), rabbit anti-rat PMN (Accurate, Westbury, NY), or goat anti-CCR1 or CCR5 (Santa Cruz Biotechnology, Santa Cruz, CA). Double staining allowed for live gating on CD45-positive cells (leukocytes), thus permitting further analysis of subset populations. FITC-conjugated mouse, rabbit, and goat IgG were used as control Abs. Unbound Ab was washed with 1% FCS, and 0.1% azide, and the cell suspension was analyzed with a flow cytometer (Becton Dickinson, Bed- ford, MA). The data were expressed as the percentage of cells staining positively, using a modification previously described (31).

**Total RNA isolation and RT-PCR amplification**

Total cellular RNA from lung tissue was isolated by lysis of cells in a Trizol solution according to the manufacturer’s instructions (Roche, Indianapolis, IN). The RNA was alcohol precipitated, and the pellet was dissolved in diethylpyrocarbonate-water. Total RNA was determined by spectrophotometric analysis at 260 nm. Five micrograms of total RNA was reversetranscribed into cDNA and amplification was performed using the Ace- RT-PCR kit (Promega, Madison, WI) and specific primers for rodent CCR1, CCR5, or RANTES. The specific primers for the housekeeping gene, β-actin, was used as an internal control. The primers used were 5′-TTG TAA CCA ACT GGC AGG ATA TGG-3′ (sense) and 5′-GAT CCT GAT CTT CAT GGT GCT AAG-3′ (antisense) for β-actin, 5′-GAG CAG CAG CAT CTA CCT GTT CA-3′ (sense) and 5′-GCA GAA ACA AAT CTC CAC ACA-3′ (antisense) for CCR-5, 5′-CCC TCT GAA GAG TTA GCA AAT-3′ (sense) and 5′-GCC TAT GCC TCG GAC AACC-3′ (antisense) for CCR-5, and 5′-TAT GCC CCA ATA GTT GGA-3′ (antisense) for rat RANTES, giving amplified products of 764, 587, 364, and 221 bp, respectively (32). The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 45 min at 48°C and for 2 min at 95°C, followed by cycling 30 times at 95°C for 30 s and 60°C for 1 min, and then elongating at 68°C for 2 min. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA, as determined by the absence of bands when RT was replaced. After amplification, the sample (20 μl) was separated on a 2% agarose gel containing 0.3 μg/ml (0.003%) of ethidium bromide, and bands were visualized and photographed using a UV transilluminator (33–35).

**Western blot analysis of RANTES, CCR1, and CCR5**

Total protein extracts were made by homogenizing lungs in TNE lysis buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, and 2.5 mM EDTA) supplemented with 2 mg/ml aprotinin and 35 mg/ml PMSF. Cell extracts were incubated on ice for 30 min, followed by centrifugation at 4°C for 30 min. Supernatants were then removed and assayed for total protein content. Protein concentration was determined using the Bio-Rad protein assay reagents (Pierce, Rockford, IL) and comparison to known amounts of BSA. One hundred nanograms of total protein was loaded in each lane for a 12% polyacrylamide gel and subjected to SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membrane (Pierce) by electrophoretic transfer overnight in Tris-glycine buffer (20 mM Tris, 150 mM glycine [pH 8.0], and methanol added to a final concentration of 20% [v/v]). Blots were blocked in 5% skim milk in TBST buffer for 2 h at room temperature, followed by incubation in either primary goat anti-CCR1 (Santa Cruz Biotechnology; 1:100), or goat anti-CCR5 (Santa Cruz Biotechnology; 1:50) in blocking solution for 2 h at room temperature. Blots were washed for three 10-min washes in TBST and were incubated at room temperature in rabbit anti-goat HRP-conjugated secondary Ab (Dako; 1:1000). Blots were then washed for four 10-min washes in TBST, and proteins were visualized following incubation of the blots in SuperSignal chemiluminescent substrate solution according to the manufacturer’s protocol (Pierce) and exposure to X-AR-5 film (Kodak).

**Mixed lymphocyte reaction (MLR)**

Rat spleens were harvested and placed on ice in RPMI 1640. Spleens were passed through wire mesh, resulting in a single-cell suspension. Red cells were lysed using ammonium chloride lysis buffer. The recovered cells were washed three times with RPMI 1640, counted, and assessed for purity and viability. MLR was set up in 96-well flat-bottom tissue culture plates for proliferative studies and in six-well plates for cytokine analysis. Cells were mixed 1/1 with either irradiated (2000 rad) stimulator cells (one-way MLR) or nonirradiated stimulator cells (two-way MLR) in a total volume of 200 μl for 96-well plates and 1 ml for six-well plates. RPMI 1640 supplemented with 1 mM L-glutamine, 10 mM HEPES, antibiotics, and 10% FCS was used in the assay. Dilution studies of both the responder and stimulator cells were performed to determine an optimal ratio of responder to stimulator cells. The ratio of responder to stimulator cells was 1/1. Plates were then incubated at 37°C for 4 days. On day 4, cells were harvested, and [3H]thymidine incorporation was determined using a MicroBeta LSC (PerkinElmer Life Sciences, Waltham, MA). The mixture was first incubated for 45 min at 48°C and for 2 min at 95°C, followed by centrifugation at 95°C for 2 min. The mixture was then incubated for 18 h before harvest on day 6. Cells were harvested, and [3H]thymidine incorporation was determined. For cytokine determination, culture supernatants were harvested from 35-mm plates, centrifuged, and stored at −20°C until cytokine cytokine analysis was performed. Both the culture supernatants and supernatants from cells stimulated with their own irradiated cells failed to mount a proliferative response or a significant peak in cytokine production (36). In vitro neutralization of RANTES during the two-way allogeneic MLRs were performed by administering either anti-rat RANTES Abs at a dilution of 1/1000 or an equivalent quantity of control goat polyclonal Abs on days 0 through 6. In separate ex vivo MLR experiments we determined whether in vitro neutralization of RANTES could reduce allospecific responsiveness to a rechallenge with either second-party irradiated BN splenocytes or third-party irradiated Fischer splenocytes. Responder cells were splenocytes harvested from LEW rats transplanted with a BN lung and then treated in vivo with either anti-rat RANTES or control Abs. Both anti-rat RANTES and control Abs were given on day 0 and every 48 h until animals were sacrificed on day 6, and spleens were harvested. Responder cells were mixed 1/1 with either irradiated first-party self splenocytes, irradiated second-party BN splenocytes, or irradiated third-party Fischer rat splenocytes. Ex vivo MLR proliferation was determined as described above.

**Statistical analysis**

Data were analyzed on a Macintosh Power PC computer using the StatView 4.5 statistical package (Abacus Concepts, Berkeley, CA). Comparisons were evaluated by the unpaired t test and nonparametric (Mann-Whitney) analyses for statistical significance. Data were considered statistically significant at p < 0.05.

**Results**

**Elevated levels of RANTES are associated with human acute lung allograft rejection**

We determined whether RANTES was present in the lungs of patients undergoing acute lung allograft rejection. We examined the BALs from both healthy single lung transplant patients (n = 12) and single lung transplant patients with clinical, chest roentgenographic and histologic evidence of acute lung allograft rejection (n = 12). All patients were on standard protocol post-transplantation immunosuppressive regimes. Histologic grading from transbronchial biopsies ranged from A0 to A1 in the healthy group and
from A2 to A3 in the group with clinical acute lung allograft rejection. There was no evidence of P. carinii, viral, bacterial, or fungal infections in these patients. RANTES protein levels in the unconcentrated BALs were 2.3-fold greater in the human acute lung allograft rejection group than in healthy lung allograft recipients (Fig. 1).

**RANTES mRNA and protein are elevated in a temporal manner during rat acute lung allograft rejection**

To determine whether RANTES involvement in human lung allograft rejection was translational to an animal model we evaluated a unique model system of orthotopic rat lung transplantation. This rat model system has been previously studied and determined to be a reproducible, immunogenetically standardized model of acute lung allograft rejection with many characteristics of human acute lung allograft rejection. We determined the full kinetics of RANTES in our rat model of acute allograft rejection. LEW rats were subjected to lung transplantation with allografts from BN rats or from syngeneic donors as previously described (21–25). Animals were sacrificed on days 1, 4, and 6 post-transplantation, and lungs were harvested for isolation of RANTES mRNA by RT-PCR and protein measurement by specific

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**FIGURE 1.** RANTES protein levels in unconcentrated BALs from human lung allografts undergoing acute allograft rejection compared with those in healthy lung allograft recipients. *, p < 0.05.

**FIGURE 2.** RANTES mRNA and protein levels are temporally elevated in rat allografts undergoing acute lung allograft rejection. I, RT-PCR determination of RANTES mRNA from allografts and syngeneic control lungs compared with β-actin on days 1, 4, and 6. II, A, ELISA measurements of RANTES protein levels from allografts and syngeneic control lungs on days 1, 4, and 6 (n = 8 at each time point); B, ELISA measurements of MIP-1α protein levels from allografts and syngeneic control lungs on days 1, 4, and 6 (n = 8 at each time point); C, ELISA measurements of MIP-1β protein levels from allografts and syngeneic control lungs on days 1, 4, and 6 (n = 8 at each time point). *, p < 0.05.
The CC chemokine receptors CCR1 and CCR5 are expressed in the lung allograft in a temporal manner during acute lung allograft rejection

To determine whether mononuclear cell infiltration was associated with cells expressing the appropriate receptors for RANTES, we assessed the expression of CCR1 and CCR5 during acute lung allograft rejection. LEW rats were subjected to lung transplantation with allografts from BN rats or from LEW syngeneic donors as previously described (21–25). Animals were sacrificed on days 1, 4, and 6 post-transplantation, and lungs were harvested for analysis of CCR1 and CCR5 expression by RT-PCR, FACS, and Western blot analysis. In lung allografts, CCR1 and CCR5 mRNA were barely detectable on day 1; however, both CCR1 and CCR5 mRNA expression were detectable by day 4, with maximal expression occurring on day 6, the time coincidental with maximal mononuclear cell infiltration and acute lung allograft rejection (Fig. 5). In contrast, CCR1 mRNA and CCR5 mRNA expression were essentially undetectable from syngeneic lungs throughout the full 6-day time course (Fig. 5). FACS analysis of CCR1 in the lung allografts showed no significant changes on days 1, 4, and 6, respectively (Fig. 5IIIB). In contrast, these receptors showed minimal to moderate amounts of protein in lung allografts on days 1, 4, and 6 post-transplantation, and lungs were harvested for analysis of CCR1 and CCR5 expression by RT-PCR, FACS, and Western blot analysis. In lung allografts, CCR1 and CCR5 mRNA were barely detectable on day 1; however, both CCR1 and CCR5 mRNA expression were detectable by day 4, with maximal expression occurring on day 6, the time coincidental with maximal mononuclear cell infiltration and acute lung allograft rejection (Fig. 5I). In contrast, CCR1 mRNA and CCR5 mRNA expression were essentially undetectable from syngeneic lungs throughout the full 6-day time course (Fig. 5I). FACS analysis of CCR1 in the lung allografts showed no significant changes on days 1 and 4, but had a significant increase of 100% on day 6 (Fig. 5IIIA). FACS analysis of CCR5 in the lung allografts showed 170, 350, and 160% increases compared with syngeneic controls on days 1, 4, and 6, respectively (Fig. 5IIIB). Western blot analysis of CCR1 and CCR5 protein in the lung allograft homogenates showed a temporal increase on days 1, 4, and 6 (Fig. 5IIIA, A and B). In contrast, these receptors showed minimal to moderate amounts of protein on day 1 and then were virtually undetectable on days 4 and 6 in syngeneic controls (Fig. 5IIIA, A and B).

In vivo neutralization of RANTES markedly attenuates acute lung allograft rejection and the presence of CCR1- and CCR5-expressing cells in the lung allografts

Because the above studies demonstrated that the production of RANTES correlated with mononuclear cell infiltration and cells expressing CCR1 and CCR5, we next assessed whether neutralization of RANTES would inhibit extravasation of mononuclear cells expressing these receptors during acute lung allograft rejection. The specificity of the polyclonal anti-rat RANTES Abs generated in our laboratories were first assessed and compared with those of commercially available polyclonal anti-murine RANTES Abs using a Western blot analysis. Both polyclonal Abs specifically detected 100 ng of recombinant rat RANTES and murine RANTES (Fig. 6) and did not cross-react with other murine or rat recombinant chemokines MIP-1α, MIP-1β, MCP-1, C10, IP-10, Day 6 demonstrated enormous perivascular and peribronchial mononuclear cell infiltration with architectural destruction, compatible with grade A4 acute allograft rejection (Fig. 3C) (2, 26). The syngeneic controls on day 1 showed the same histology as the allografts: minimal interstitial and alveolar edema, hemorrhage, and leukocyte infiltration compatible with ischemic-reperfusion injury (37). However, day 4 and 6 samples showed essentially normal lung histology (2, 26). The amount of mononuclear cell recruitment in both lung allografts and syngeneic controls seen on histology was quantitated by FACS analysis using double staining to permit leukocyte subset analysis. The specific cell markers CD4 (T cells), CD3 (T cells), CD8 (T cells), CD45RA (naive T cell/B cells), ED1 (mononuclear phagocytes), and NKR-P1A (NK cells) showed a temporal increase in the number of cells on days 1, 4, and 6 (Fig. 4). In contrast the syngeneic controls started out with comparable numbers on day 1, then were markedly reduced throughout the rest of the time course (Fig. 4).

Expression of RANTES parallels mononuclear cell infiltration during acute lung allograft rejection

The temporal expression of RANTES mRNA and protein in the lung allografts paralleled mononuclear cell infiltration into the lung allograft. This was seen qualitatively by histology assessment of the lung allografts on days 1, 4, and 6 post-transplantation. Day 1 demonstrated only minimal interstitial and alveolar edema, hemorrhage, and leukocyte infiltration compatible with ischemic-reperfusion injury (Fig. 3A) (37). Day 4 demonstrated a significant perivascular and peribronchial mononuclear cellular infiltration, compatible with grades A1–A2 allograft rejection (Fig. 3B) (2, 26).

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MIG, KC, and MIP-2. In addition, i.p. injection of 20 mg of purified anti-rat RANTES was found to specifically neutralize rat RANTES (10 µg i.p., concomitant with neutralizing Abs)-mediated mononuclear cell infiltration in the peritoneum (Fig. 7). This effect was specific, as these Abs did not inhibit 10 µg of recombinant rat MCP-1-induced mononuclear infiltration in the peritoneum (Fig. 7).

Once the specificity of the neutralizing anti-rat RANTES Abs had been characterized, lung allograft recipients were passively immunized with either neutralizing anti-rat RANTES Abs or an equivalent quantity of control Abs on days 0, 2, and 4. The animals were sacrificed on days 6, 8, and 10. Lungs were harvested for histology (Fig. 8I) as well as FACS analysis of specific leukocyte surface markers (Fig. 8II) and of CCR1 and CCR5 (Fig. 8III). Treatment with anti-rat RANTES Abs demonstrated a significant reduction in acute lung allograft rejection compared with control Ab-treated animals (Fig. 8I, A, C, and E). The lung allografts on days 6, 8, and 10 treated with control Ab all demonstrated a histological rejection grade of A4 (Fig. 8I, B, D, and F). There was an obvious temporal increase in perivascular, interstitial, and alveolar infiltrates throughout this time course in both treated groups. However, the lung allografts on day 6 treated with neutralizing anti-rat RANTES Abs showed a dramatic decrease in rejection, with a histological grade of A1–A3, mild perivascular infiltration, and occasional extension into the alveolar interstitium (Fig. 8IA). Day 8 and 10 lung allografts treated with neutralizing anti-rat RANTES Abs also showed markedly reduced histological evidence of rejection compared with their appropriate controls (Fig. 8, C and E vs D and F, respectively). We determined the presence of leukocyte subset infiltration during acute lung allograft rejection by FACS analysis. Recipient animals treated with neutralizing anti-rat RANTES Abs demonstrated a significant reduction of infiltrating mononuclear cells expressing CD3, CD4, CD8, ED1, CD45RA, and NK cell markers at all three time points (Fig. 8II). Interestingly, FACS analysis of the lung allografts from anti-rat RANTES-treated recipients showed no significant change in neutrophil accumulation compared with control Ab-treated animals at all three time points (Fig. 8II). This reduction of infiltrating mononuclear cells correlated with the significant reductions of cells expressing CCR1 and CCR5 on days 6, 8, and 10 post-transplantation (Fig. 8III).

To further confirm that RANTES has a major role in the recruitment of mononuclear cells rather than inducing a proliferative
response during an allogeneic response, we performed in vitro studies using two-way allogeneic and syngeneic MLRs. In the two-way allogeneic MLRs RANTES protein levels were elevated in a temporal manner, with maximal levels seen on day 6 (Fig. 9). In contrast, RANTES levels in the syngeneic two-way MLRs did not increase on day 4 and only increased minimally on day 6 (Fig. 9).

FIGURE 5. CCR1 and CCR5 mRNA and protein levels are significantly elevated in rat allografts undergoing acute lung allograft rejection compared with those in syngeneic controls. I, RT-PCR determination of CCR1 and CCR5 mRNA from lung allografts and syngeneic control lungs compared with β-actin on days 1, 4, and 6 post-transplantation. II, Western blot analysis of CCR1 and CCR5 protein from lung allografts and syngeneic control lungs on days 1, 4, and 6 post-transplantation (n = 6). III, FACS analysis of CCR1 and CCR5 from lung allografts compared with syngeneic control lungs (n = 6). *, p < 0.05.

FIGURE 6. Western blot analysis showing the specificity of the polyclonal anti-rat RANTES Ab used for in vivo neutralization and polyclonal anti-murine RANTES Ab used for RANTES protein ELISA to 100 ng of recombinant rat RANTES or recombinant murine RANTES (n = 3).

FIGURE 7. Total and absolute cell counts from rat peritoneal lavage 6 h after concomitant i.p. administration of 10 μg of rat RANTES or rat MCP-1 in the presence of either normal goat serum or anti-RANTES polyclonal Abs. *, p < 0.05.
Depletion of endogenous RANTES attenuates acute lung allograft rejection and prolongs graft survival on days 6, 8, and 10 post-transplantation. I, A, C, and E, Representative photomicrographs (×100) of lung allograft sections on days 6, 8, and 10, respectively, from animals treated with anti-rat RANTES Abs (n = 8 at each time point). I, B, D, and F, Representative photomicrographs (×100) of lung allograft sections on days 6, 8, and 10, respectively, from animals treated with control Abs (n = 8 at each time point). II, A–C, Anti-rat RANTES Abs, compared with control Abs, attenuate and delay the infiltration of mononuclear cells during acute lung allograft rejection (n = 6 at each time point). III, A–C, Anti-rat RANTES Abs, compared with control Abs, reduce and delay the infiltration of mononuclear cells expressing CCR1 and CCR5 during acute lung allograft rejection (n = 6 at each time point). *p < 0.05.
Previous work has shown a decreased secretion of RANTES coinciding with Ag-specific anergy and superantigen-induced anergy (38). We therefore performed ex vivo experiments to determine whether the reduction of allograft rejection under conditions of endogenous RANTES depletion was related to altered allospecific responsiveness. LEW rats transplanted with BN lungs were treated with either anti-rat RANTES or control Abs in a manner similar to that described above. On day 6 post-transplantation, splenocytes from LEW rat spleens were harvested and subjected to MLR with irradiated first-party (self) splenocytes, irradiated second-party (BN) splenocytes, or irradiated third-party (Fisher) splenocytes. Splenocytes isolated from transplanted LEW rats treated in vivo with anti-rat RANTES Abs showed similar proliferative responses when stimulated with either irradiated first-party or irradiated second-party splenocytes (Fig. 10). In contrast, splenocytes isolated from transplanted LEW rats treated in vivo with control Abs demonstrated a significant proliferative response when challenged with irradiated second-party splenocytes (Fig. 4). The proliferative response was normalized to the proliferative response of the syngeneic control (LEW:LEW splenocytes). Furthermore, splenocytes isolated from transplanted LEW rats treated with either anti-rat RANTES or control Abs showed no significant difference in proliferative responses when stimulated with irradiated third-party splenocytes (data not shown).

Discussion

Acute lung allograft rejection is characterized by an infiltration of mononuclear cells that ultimately contribute to loss of lung architecture and function. However, the mediators that orchestrate the extravasation of leukocytes during acute lung allograft rejection have not been elucidated. Because RANTES is a potent chemoattractant for mononuclear cells (8, 12), we tested the hypothesis that RANTES and the expression of its two major mononuclear receptors, CCR1 and CCR5, contribute to acute lung allograft rejection via the recruitment of mononuclear cells to the lung.

In this study we first assessed whether RANTES was associated with human acute lung allograft rejection. Similar to previous investigators, we found a 2.3-fold increase in RANTES protein levels in BALs from lung transplant recipients with acute rejection compared with healthy lung allograft recipients (39). To determine proof of the principle that RANTES was contributing to acute allograft rejection, we employed a well-characterized model of orthotopic rat lung transplantation (21–25). In our rat lung transplantation model we measured levels of three CC chemokines; RANTES, MIP-1α, and MIP-1β. RANTES and MIP-1α were both significantly elevated in lung allografts compared with syngeneic controls, with RANTES protein levels at least 170-fold greater than MIP-1α levels. This correlates with the LEW rat adjuvant-induced arthritis model in which RANTES levels were greater than MIP-1α levels in both blood and synovial joints, and neutralization of MIP-1α did not ameliorate symptoms (29). Interestingly, MIP-1β protein levels, in contrast to either RANTES or MIP-1α levels, were lower in the lung allografts compared with the syngeneic controls. In light of the markedly elevated levels of RANTES during lung allograft rejection, and previous studies showing that RANTES is 10 times more potent than MIP-1α for inducing T cell migration (8), we studied the full kinetics of RANTES during allograft rejection.

Table 1. Proliferative response of two-way allogeneic MLR after 6 days in culture in the presence or absence of neutralizing Abs to rat RANTES

<table>
<thead>
<tr>
<th>Conditions</th>
<th>cpm</th>
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<tbody>
<tr>
<td>MLR + normal goat serum (1:1000)</td>
<td>3168 ± 350</td>
</tr>
<tr>
<td>MLR + anti-rat RANTES (1:1000)</td>
<td>3237 ± 412</td>
</tr>
</tbody>
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FIGURE 9. RANTES protein levels are significantly elevated in two-way MLRs compared with two-way syngeneic MLRs. *, p < 0.05.
We found that rat lung allografts expressed RANTES mRNA and protein in a time-dependent manner, with maximal expression and levels occurring on day 6 coincidental with maximal lung allograft rejection. We then wanted to investigate whether there was a correlation between mononuclear cell recruitment and expression of the RANTES receptors. RANTES has the ability to bind with multiple receptors (CCR1, CCR3, CCR5, CCR9, and DARC). The amount of expression of CCR3 on mononuclear cells remains controversial, although CCR3 is known to be predominately expressed on eosinophils and basophils. Although RANTES has been shown to bind to CCR9, it does not initiate a Ca\(^{2+}\) flux, and it remains unclear whether RANTES interaction with CCR9 leads to biologically relevant chemotaxis (17, 19, 20, 40). CCR4 was initially thought to bind RANTES, but latter studies have shown that its major ligands are macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) (13, 18, 41). Therefore, we focused our studies on the kinetics of CCR1 and CCR5 expression, because they have previously been shown to be the two major RANTES receptors for mononuclear cell chemotaxis (11, 14, 15, 17, 19, 20). The temporal expression of RANTES mRNA and protein in the rat lung allografts correlated with the mononuclear cell recruitment and expression of RANTES receptors CCR1 and CCR5. In contrast, the mRNA and protein levels of RANTES and its receptors, expressed by the syngeneic controls, were undetectable or decreased throughout the 6-day time course. The early elevation of CCR1 and CCR5 mRNA and protein by Western blot analysis in the syngeneic controls most likely represents ischemic-reperfusion injury, in which there is recruitment, activation, and emigration of neutrophils, which are known to express CCR1 and CCR5 (37, 42). The increase in the amount of CCR1 protein determined by Western blot analysis on days 1 and 4 compared with the amount of CCR1 protein in lung allografts determined by FACS analysis may be due to different strategies used for analyzing these receptors and different pools of receptors (i.e., cell surface vs internalization) (43). We speculate that the discrepancies of the results using different strategies for analysis of CCR expression is related to the fact that Western blot analysis is detecting both cell surface-expressed and internalized receptor protein, whereas FACS analysis is detecting predominating cell surface expression. For example, our results for the kinetics of CCR1 by Western blot and FACS analyses suggest that CCR1 protein expression during allograft rejection may be undergoing significant dynamic changes related to both internalization and cell surface expression. Alternatively, we cannot completely exclude that cells other than mononuclear cells and neutrophils (i.e., stromal or parenchymal cells) during acute lung allograft rejection may express CCR1 that was detected by Western blot analysis and not by FACS analysis.

The association between RANTES and acute lung allograft rejection described here is supported by other studies of solid organ allograft rejection (32, 44–47). RANTES mRNA was detected in infiltrating mononuclear cells and renal tubular epithelium during acute renal allograft rejection, suggesting that RANTES has a role in human allograft rejection (47). In addition, Kondo examined RANTES mRNA expression using three histoimmunocompatible models of allogeneic skin grafts in mice (44). RANTES mRNA was found to coincide with maximal allograft rejection (infiltration of mononuclear cells) in all three skin allograft models on day 7 (44). These results are similar to ours and demonstrate an important relationship between augmented expression of RANTES and mononuclear cell infiltration. In a rat acute renal allograft rejection model, RANTES mRNA expression displayed a bimodal pattern (46). The first peak occurred at 6 h after engraftment in association with ischemia-reperfusion of the allograft, and the second peak in expression occurred between 3 and 6 days postimplantation in conjunction with acute renal allograft rejection (46). Infiltrating macrophages and CD4 and CD8 cells were found to peak in the renal allografts by day 5 in parallel with maximal RANTES expression (46). These results paralleled our findings of increased RANTES expression during acute allograft rejection coinciding with increased mononuclear infiltration and allograft rejection.

To prove that RANTES and its receptors are important in the mediation of acute lung allograft rejection, we determined whether depletion of endogenous RANTES would attenuate acute lung allograft rejection and the expression of RANTES receptors on infiltrating mononuclear cells. Treatment with anti-rat RANTES Abs delayed the recruitment of mononuclear cells involved in acute rejection. This was demonstrated by histopathology and FACS analysis demonstrating a significant reduction in mononuclear cells on days 6, 8, and 10. Similar results were obtained with F(ab')\(_2\) neutralizing anti-RANTES Abs (data not shown). Concomitant with the marked reduction in mononuclear cell recruitment was a significant reduction in cells expressing CCR1 and CCR5. The specificity of RANTES for mononuclear cell recruitment was exemplified by minimal changes in the presence of neutrophils in rats treated with neutralizing Abs. Although we did not perform physiological function testing on the lungs during allograft rejection in the presence or the absence of neutralization of RANTES, it has been established that physiological measurements (i.e., forced expiratory volume at 1 s and forced vital capacity) decline in direct correlation to the magnitude of acute lung allograft rejection (i.e., mononuclear cell infiltration) (48). Based on this information, one can assume that with evidence of attenuated lung allograft rejection in the context of neutralization of RANTES, lung physiological function would be less impaired. Interestingly, although neutralization of RANTES resulted in significant attenuation of acute allograft rejection, there was still a presence of perivascular, alveolar, and interstitial leukocytes at later time points. This suggests that other CC chemokines, CXC chemokines, or other factors may be either synergistically or independently involved in acute lung allograft rejection. The attenuation of mononuclear cell recruitment by in vivo neutralization of RANTES described here is supported by other studies of disease models of inflammation that have responded to anti-RANTES Abs (29, 49). In vivo neutralization of RANTES was shown to significantly decrease the pulmonary influx of macrophages without an alteration in the number of neutrophils in a murine model of endotoxemia (49). These findings suggest the involvement of RANTES in facilitating the accumulation of macrophages in the lung during allograft rejection. Furthermore, in a rat model of arthritis, RANTES levels were increased in blood and synovial joints, and in vivo neutralization of RANTES markedly reduced arthritis scores, joint radiographic scores, and synovial joint leukocyte infiltration (29). This emphasizes the pivotal role of RANTES in mononuclear cell recruitment in two distinct diseases. Moreover, treatment with a RANTES antagonist reduced mononuclear cell infiltration as well tubular and vascular damage in a rat renal transplantation model (50). Also, low dose RANTES antagonist in conjunction with low dose cyclosporin reduced inflammatory events compared with the effect of low dose cyclosporin alone (50). This supports our findings that in vivo neutralization of RANTES significantly attenuates lung allograft rejection. Lastly, in vivo neutralization of TNF-\(\alpha\) in a rat model of acute lung allograft rejection was shown to attenuate leukocyte infiltration during allograft rejection (23). This is consistent with our findings, because TNF-\(\alpha\) is known to stimulate the expression of RANTES from a number of cell types (23, 51–53).
We further confirmed that RANTES was a CC chemokine ligand produced in response to alloantigen by one- and two-way allogeneic MLRs. RANTES protein levels increased in a temporal relationship to chronic lung allograft rejection. The findings in this study of RANTES and future studies of other CC and CXC chemokines will lead to the development of new paradigms to understand the pathogenesis of acute lung allograft rejection and its relationship to chronic lung allograft rejection. The findings in this study may ultimately result in novel therapies designed for intervention and possible prevention of both acute and chronic lung allograft rejections.

References


