Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils

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Pan, Zhong-Zong, Lisa Parkyn, Anuradha Ray, and Prabir Ray. Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils. Am J Physiol Lung Cell Mol Physiol 279: L658–L666, 2000.—The chemokine regulated on activation normal T cells expressed and secreted (RANTES) has been implicated in eosinophil chemotaxis in asthma and allergic diseases, which are thought to be T helper (Th) type 2-dominated diseases. However, adoptive transfer of Th1 cells in mice upregulates RANTES gene expression in the lung, and increased RANTES expression has been documented in several Th1 cell-dominated conditions that are associated with neutrophilia. The in vivo role of RANTES in the pathogenesis of disease processes is not well understood. To determine the effect of RANTES expression alone in vivo, we generated transgenic mice that overexpress RANTES specifically in the lung in an inducible fashion. The airways of the transgenic mice overexpressing RANTES displayed a significant increase in neutrophil infiltration compared with that in control mice. The increased airway neutrophilia was also evident when the transgenic mice were tested in a murine model of allergic airway inflammation. RANTES expression also induced expression of the chemokine genes macrophage inflammatory protein-2, 10-kDa interferon-γ-inducible protein, and monocyte chemoattractant protein-1 in the lungs of the transgenic mice. Our studies highlight a hitherto unappreciated role for RANTES in neutrophil trafficking during inflammation. Thus increased RANTES expression, as observed during respiratory viral infections, may play an important role in the associated neutrophilia and exacerbations of asthma.

EXPERIMENTAL PROCEDURES

Generation of transgenic mice. To generate transgenic mice that express hRANTES specifically in the lung in an inducible fashion, we generated transgenic mice that overexpress hRANTES specifically in the lung in an inducible fashion. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
As the starting plasmid, a plasmid containing the hGH gene (described above) was used. This plasmid construct contains the human growth hormone (hGH) gene sequence beginning at the BamHI site in the first exon to the Not I site after the polyadenylation signal isolated from the plasmid p1017 (9) was cloned into pBlueScript II KS+ (Stratagene) between the same sites to provide intronic and polyadenylation sequences. Next, a 3.0-kb Hind III fragment containing the CC10 promoter was isolated (48) and inserted at the Hind III site in the construct in the appropriate orientation. The nrtTA fragment was isolated by digesting pUHD172-neo (19) with EcoR I following by filling in with Klenow polymerase and digesting with BamHI. This nrtTA fragment was inserted between the EcoR V and BamHI sites in the construct, yielding the final CC10 promoter/nrtTA construct. This transgene construct can express a nuclear localization signal containing a tetracycline-inducible transactivator in a lung-specific fashion. To make the plasmid construct tetracycline operator (TetOp)/hRANTES, the pBlueScript II KS+ plasmid containing the hGH gene (described above) was used as the starting plasmid. An Xho I-Cla I fragment containing the TetOp/human cytomegalovirus minimal promoter (39) was inserted between the same sites in the pBlueScript II KS+ plasmid containing the hGH gene. Next, an Xba I-Hind III fragment containing the full-length hRANTES cDNA, including its own stop codon, was inserted between the same sites in the above plasmid to generate the TetOp/hRANTES construct. The constructs were confirmed by sequencing. Transgenic mice were produced by coinjection of the two constructs into fertilized eggs from C57BL/6-SJL F1 mice.

The transgenic founder mice were identified by Southern blot analysis. The probes used were a Hind III-BamHI fragment containing the nrtTA sequence from the CC10 promoter/nrtTA construct and a BamHI I fragment containing the hRANTES region from the TetOp/hRANTES construct. These founder mice were crossed with either BALB/c or C57BL/6 mice, and the transgenic mice were identified by PCR. The upper primer for detecting nrtTA was 5'-GTCGCTTAAAGAAAAAGAAAATAAC-3', and the lower primer was 5'-TTCCAGGGCGATCGTAAACATCTG-3'. One of the primers for detecting the hRANTES transgene (upper primer) was derived from the hRANTES cDNA and the sequence was 5'-CGGCACCGCCCTCGGTGCTACCT-3', whereas the lower primer was derived from the hGH sequences and its sequence was 5'-GGGCTTATGCGGATCTCAG-3'. Five transgenic lines that were positive for both transgenes were obtained.

**Induction of hRANTES expression in transgenic mice.** All animals were 8–10 wk old and were maintained on normal water until used. For the induction of hRANTES expression, transgenic mice and nontransgenic littermates were given water containing doxycycline (Dox) hydrochloride (1 mg/ml Dox in 5% sucrose) in aluminum-wrapped bottles for 4–9 days or throughout the course of the experiment in the ovalbumin (Ova) antigen model. The water was changed every 3 days. Transgenic mice that did not receive Dox were supplied with sucrose (5%) water.

**Northern blotting and RNase protection assay.** Total cellular RNA was obtained from different tissues of transgenic mice by homogenization in TRIzol reagent (GIBCO BRL, Life Technologies, Grand Island, NY) and purification (55). Fifteen micrograms of RNA were analyzed from each tissue. Northern blotting was carried out with QuikHyb (Stratagene). The probe was made with the random labeling kit (Boehringer Mannheim, Indianapolis, IN), and the template was a BamHI fragment containing the hRANTES cDNA isolated from the TetOp/hRANTES construct.

RNA isolated from lungs was analyzed for chemokine or cytokine gene expression by RNase protection assay (RPA) with the RPA III kit (Ambion) in accordance with the manufacturer's instructions (55). The probes were made with the murine mCK-5 set for chemokine expression and the murine mCK-1 set for cytokine expression (both from PharMingen, San Diego, CA). Ten micrograms of RNA were used in each case.

**Bronchoalveolar lavage and lung histology.** Bronchoalveolar lavage (BAL) was performed as previously described (55). Briefly, lungs were lavaged with 1 ml of Dulbecco's phosphate-buffered saline (DPBS), cells were pelleted, and the supernatant was divided into aliquots and stored at −70°C. The pelleted cells were resuspended in DPBS, counted, cyto- spun, and stained with Diff-Quik (Baxter HealthCare, Miami, FL). Cell differentials were enumerated based on morphology and staining profiles. For histology, the lungs were fixed in Streck tissue fixative (Streck Laboratories) after lavage and stained with hematoxylin and eosin.

**ELISA.** The BAL fluids were analyzed for RANTES protein levels by ELISA (Endogen).

**Antigen sensitization and challenge of mice.** Mice were sensitized and challenged as previously described (55). Briefly, mice were sensitized by intraperitoneal injection of 10 μg of Ova (Sigma, St. Louis, MO) and 1 mg of alum (Resorptar, Intergen, New York, NY) on days 0 and 5. The transgenic mice were given either 5% sucrose water without Dox (−Dox) or 5% sucrose water with 1 mg/ml of Dox (+Dox) 24 h before the first intraperitoneal injection. On day 12, mice were aerosol challenged by nebulization with 1% Ova (0.01% Tween 20 and DPBS) twice, for 1 h each time, with an interval period of 4 h between the challenges. Mice were anesthetized and analyzed 24 h after the second aerosol challenge.

**RESULTS**

**Inducible lung-specific expression of RANTES.** To determine the specific role of RANTES in leukocyte chemotaxis during lung inflammation, we generated transgenic mice that overexpress RANTES in the lung in an inducible fashion with the use of a lung-specific tetracycline-inducible system previously developed in our laboratory (39) (Fig. 1A). In this system, the nrtTA driven by the lung-specific CC10 promoter is constitutively expressed in lung epithelial cells. When Dox is introduced through the drinking water, it induces the binding of the transactivator nrtTA to Tet Op/promoter sequences, resulting in induction of expression of the linked transgene (hRANTES) from a second construct (39). In this study, five transgenic lines were obtained. In the presence of Dox, RANTES expression was detected only in the lung but not in the other tissues examined (Fig. 1B). Because of the high degree of homology between human and murine RANTES, the antibodies used in the ELISA to detect RANTES protein expression detected both endogenous and transgene expression. As shown in Fig. 2, nontransgenic mice displayed a low level of expression (mean = 16.8 pg/ml). In the absence of Dox, the transgenic lines 1–3 showed slightly higher levels of RANTES expression, possibly resulting from a low level of leaky transgene expression. Transgenic mice in line 4 showed low
transgene expression in the presence and absence of Dox. Mice in line 2 showed the highest level of Dox-induced RANTES expression (average = 181.5 pg/ml). The basal expression of RANTES protein in transgenic mice in line 5 was comparable to that in their nontransgenic littermates, and there was a fourfold increase in expression upon induction with Dox (average = 69.9 pg/ml; Fig. 2). Therefore, animals from both lines 2 and 5 were used for further analysis.

Lung-specific expression of hRANTES preferentially recruits neutrophils into the lung. To determine the effect of RANTES overexpression on leukocyte chemotaxis in the lung, mice were supplied with Dox-containing water, the lungs of the transgenic mice and their nontransgenic littermates were examined histologically, and cell recruitment in the airways was assessed by BAL. As expected, in nontransgenic mice, the majority of BAL fluid cells were macrophages (Fig. 3A). When RANTES expression was induced in the transgenic mice, a significant increase in the number of neutrophils was observed in the BAL fluid (10–30% of total cells; Fig. 3, B and D). The increase in neutrophils was observed within 24 h of the induction of transgene expression and could still be observed after 12 days, the extent to which it was studied (Table 1).

Consistent with the described role of RANTES in T-cell chemotaxis (44), the lymphocyte population in the BAL fluid of the transgenic mice was also found to increase, from ~5% after 24 h to ~20% of total cells after 7 days, and was maintained at 7–10% of the total population at 12 days after induction of RANTES expression (Fig. 3E). Surprisingly, no appreciable eosinophil influx could be observed in the airways of the RANTES-overexpressing mice at any of the time points tested. Although neutrophils were increased in the airways of the mice, the lung parenchyma did not show increased neutrophil numbers or any inflammation (Fig. 3, C and D); therefore, the chemotactic gradient setup in these mice favored neutrophil migration into the airways. Similarly, in a study by Gunn et al. (21), monocyte chemoattractant protein (MCP)-1 overexpression in the lung did not cause any lung inflammation but resulted in increased monocyte and lymphocyte infiltration into the airways. Curiously, the increased neutrophil chemotaxis induced by RANTES was much more prominent in transgenic mice on a BALB/c background than in those on a C57BL/6 background (data not shown). Therefore, we estimated the peripheral blood neutrophil and eosinophil numbers in the BALB/c and C57BL/6 mice. As shown in Table 2, neutrophils account for ~25% of the circulating leukocytes in BALB/c mice but only 10% of the total cells in C57BL/6 mice. Thus under basal conditions, BALB/c mice have more circulating neutrophils, which may explain the difference in neutrophil recruitment seen in the two strains of mice.

Lung-specific expression of hRANTES can activate the expression of macrophage inflammatory protein-2, 10-kDa interferon-γ-inducible protein, and MCP-1 in the lung. Because an association between RANTES and neutrophils is not well described in the literature, we were interested in determining whether RANTES overexpression caused the induction of other cytokines and/or chemokines that might explain the increased neutrophil recruitment to the lungs of the mice. To determine this, RPAs were performed. As observed by us (55) and by others (29), a low level of RANTES is
constitutively expressed in the lungs of mice. As shown in Fig. 4, the message for macrophage inflammatory protein (MIP)-2, a potent neutrophil chemoattractant, was detectable in the lungs of the transgenic mice. In addition to MIP-2 mRNA, a prominent increase in expression of 10-kDa interferon-γ-inducible protein (IP-10) and MCP-1 mRNAs was also evident within 24 h of Dox induction in the lungs of the transgenic mice.
mice (Fig. 4, A and B). The expression of eotaxin, lymphotactin, MIP-1α, MIP-1β, or T-cell activation gene mRNA was not increased by RANTES overexpression (Fig. 4). RPA did not detect increased expression of any of the cytokines tested, including interleukin (IL)-4, IL-5, IL-10, IL-9, IL-15, IL-2, IL-6, and interferon-γ (data not shown).

Increased neutrophilia in RANTES-overexpressing mice in a murine model of airway inflammation. To determine the effect of RANTES overexpression on leukocyte chemotaxis during inflammation, transgenic mice and nontransgenic littermate controls were treated with Dox, sensitized with Ova, and then challenged with Ova by inhalation after 12 days. Twenty-four hours after challenge with Ova, mice were anesthetized, BAL fluid was recovered from the animals, and the cell differential in the BAL fluid was determined. The total cell count in the BAL fluid recovered from the transgenic mice on antigen challenge was three- to fourfold greater than that in the BAL fluid derived from the nontransgenic mice (Fig. 5). After antigen provocation, although neutrophils comprised

Table 1. RANTES levels and neutrophil and eosinophil counts in BAL fluid after induction of transgene expression

<table>
<thead>
<tr>
<th>Dox Treatment</th>
<th>RANTES, pg/ml</th>
<th>Neutrophils, %</th>
<th>Eosinophils, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>44.6 ± 3.8</td>
<td>0.02 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>Day 1</td>
<td>169.0 ± 1.5</td>
<td>5.26 ± 1.29</td>
<td>0</td>
</tr>
<tr>
<td>Day 5</td>
<td>174.8 ± 4.1</td>
<td>8.67 ± 4.5</td>
<td>0.17 ± 0.29</td>
</tr>
<tr>
<td>Day 12</td>
<td>145.3 ± 6.7</td>
<td>8.17 ± 4.07</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SD, representative of 2 experiments with both lines 2 and 5; n = 3 animals/time point. Neutrophils and eosinophils are percentages of total number of cells in the bronchoalveolar (BAL) fluid. RANTES, regulated on activation normal T cells expressed and secreted; Dox, doxycycline.

Table 2. Peripheral blood cell differential

<table>
<thead>
<tr>
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<th>BALB/c</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils, %</td>
<td>24.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>1.4</td>
<td>3.9</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>67.7</td>
<td>77.0</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>6.1</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Values are averages; n = 3 mice/strain. Peripheral blood cells were obtained from BALB/c and C57BL/6 mouse strains by cardiac puncture. Red blood cells were lysed in 1× lysis buffer for 5 min at room temperature. After 2 washes in Dulbecco’s PBS, the cells were prepared by cytoospin, and the cells on the slide were stained with Diff-Quik. All animals were bred at the Yale Animal Facility.

Fig. 4. Induction of macrophage inflammatory protein (MIP)-2, 10-kDa interferon-γ-inducible protein (IP-10), and monocyte chemoattractant protein (MCP)-1 mRNA expression by RANTES. A: lung RNA from NTg, Tg (−Dox), and Tg (+Dox) mice (24 h posttreatment) was analyzed by RNase protection assay (RPA). Because of the high degree of homology between mouse (mRANTES) and hRANTES, the transgene mRNA was also partially protected by the RANTES probe, yielding a smaller fragment. Longer exposure of the same gel revealed low expression of the transgene message in Tg (−Dox) mice. Results shown are representative of 2 experiments with 3 animals each from lines 5 and 2 and from the NTg group. B: kinetics of chemokine expression in the lungs of mice. Results are representative of 2 experiments with 2 animals in each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
DISCUSSION

Studies of chemokine function in vivo with the use of constitutive overexpression transgenic systems have been hampered by problems associated with receptor downmodulation that results from chronic stimulation by the ligand. In the present studies, we used an inducible lung-specific transgenic system to study the function of the chemokine RANTES in vivo. This system can be adapted to many cell types, including lymphocytes, with appropriate cell-specific promoters. RANTES, a member of the C-C chemokine family, is best known as a chemotactic factor for eosinophils (26, 30, 31, 33, 40) and memory T cells (43). However, recent studies (1, 3) suggest that RANTES may have biological functions that are distinct from its chemotactic effects on eosinophils. Our results show that RANTES overexpression causes increased neutrophil infiltration into the airways of mice in both the presence and absence of antigen-induced airway inflammation. Furthermore, the RANTES-overexpressing mice displayed increased expression of MIP-2, IP-10, and MCP-1 mRNA in their lungs.

Previously, RANTES was implicated in eosinophil chemotaxis based on blocking studies with antibodies (28, 54) as well as on studies that used the RANTES receptor antagonist Met-RANTES (15, 18). A surprising finding in our study is the presence of lung neutrophilia instead of eosinophilia in the RANTES-expressing mice. The principal RANTES-binding receptors are CCR1 (17, 35, 36), CCR3 (12, 36), and CCR5 (11, 37, 42). A recent study (20) highlighted an important role for CCR3 in the recruitment of eosinophils in the lung and airways of mice during eosinophilic inflammation. Although human and murine RANTES have a high degree of homology, in vitro studies (20, 36) have shown that, compared with murine CCR3, hRANTES has a low affinity for murine CCR3, the principal chemokine receptor expressed by eosinophils. Because we have expressed hRANTES in the transgenic mice, this may explain the lack of a substantial increase in eosinophils in the airways of the transgenic mice during antigen-induced inflammation. Thus our studies confirm that hRANTES is unable to cause increased chemotaxis of murine eosinophils, probably because of a low affinity for CCR3. On the other hand, because increased lymphocyte recruitment was observed in these mice, hRANTES may not discriminate between human or murine CCR1 and CCR5 in vivo. Indeed, human and murine RANTES have been shown to have similar affinities for CCR1 (36).

The increased lung neutrophilia observed in our study may be explained as follows. First, the effect on neutrophil chemotaxis may not be direct but may be mediated by other chemokines or mediators because chemokines have been shown to regulate the expression of other chemokines (18). Of the chemokines tested, RANTES increased the expression of the chemokine genes MIP-2, IP-10, and MCP-1 in vivo. Of these chemokines, MIP-2 is a potent neutrophil chemotactant (2, 14, 16). In a model of bacterial meningitis (14), a condition that involves excessive neutrophilia in the meninges, coordinate expression of MIP-2, RANTES, MCP-1, and MIP-1α was noted, with the kinetics of expression paralleling disease severity. Also, in mice in which Th1 cells were transferred or in mice infected with influenza virus, the expression of RANTES, MCP-1, and IP-10 mRNAs was increased.

Fig. 5. RANTES overexpression induced increased neutrophilia in antigen-challenged mice. All mice were sensitized and aerosol-challenged with ovalbumin (Ova). Total and differential cell counts were obtained. Values are means ± SE from a representative experiment of 2 performed with line 2 Tg mice; n = 3 mice/group. A similar profile was seen in line 5. Because line 2 displayed a low level of leaky transgene expression, a small increase in neutrophil infiltration was also evident in the absence of Dox induction. Note that the recruitment of eosinophils, lymphocytes, and monocytes was slightly enhanced by the expression of the transgene hRANTES. However, the most impressive difference between the NTg and Tg mice was the number of neutrophils in the airways of the latter. The differences in BAL fluid neutrophil counts between NTg and Tg (+Dox) mice were significant (P < 0.05).
(29); the effect on MIP-2 expression was not analyzed in this study. Interestingly, both IP-10 and MCP-1 have been implicated in neutrophil trafficking. Subcutaneous injection of IP-10 in BALB/c mice was associated with infiltration of both mononuclear cells and neutrophils in the subcutaneous tissue within 4 h of injection (49). MCP-1 was shown to behave as an efficient neutrophil chemoattractant in mice in the context of chronic inflammation (25). Similar to the potent effect of low doses of MCP-1 on neutrophils in the presence of inflammation, the local expression of multiple chemokines, RANTES, IP-10, and MCP-1 may have the ability to set up an efficient chemotactic gradient for neutrophils as observed in the RANTES transgenic mice. When viewed in combination, in a Th1 cell-dominated inflammation, RANTES may be the primary chemokine activated by Th1 cells, which, in turn, sets up a network of other chemokines such as MIP-2, IP-10, and MCP-1. To the best of our knowledge, this is the first report that shows that RANTES can induce the expression of other chemokine genes. It will be interesting to determine which chemokine receptor is responsible for the increased neutrophil chemotaxis by breeding the RANTES transgenic mice to specific chemokine receptor-deficient mice.

Second, several biological effects of RANTES have been suggested to occur in an aggregation-dependent manner. For example, in a recently described study (1), aggregated but not disaggregated hRANTES was shown to activate human neutrophils with a substantial increase in CD11b expression. Thus aggregation of RANTES in vivo may also be responsible for its neutrophil chemoattractant properties. Because resting neutrophils were shown to express CCR1 (6), it is possible that this basal level of CCR1 expression is sufficient for responsiveness to aggregated RANTES.

In the lung, Th1 cell-dominated inflammation induces neutrophilia, whereas Th2 cell-dominated inflammation promotes eosinophilia, as has been clearly demonstrated by adoptive transfer experiments (10). RANTES expression has been demonstrated in several Th1 cell-dominated conditions such as rheumatoid arthritis (38), delayed-type hypersensitivity (13), and sarcoidosis (23). In other studies, Schrum et al. (45) showed a strong expression of RANTES in a Th1 cell-type response to a gram-negative bacterium but a low expression in the Th2-type response to a nematode infection. Recently, in a murine model of rheumatoid arthritis (3), neutralization of RANTES, but not of other chemokines, greatly attenuated symptoms of the disease. In adoptive transfer experiments, Th1, but not Th2, cells were found to upregulate RANTES mRNA expression in the lung, suggesting an association between RANTES and Th1 cell-mediated effects in the lung (18; Cohn et al., unpublished observations). Also, RANTES causes chemotaxis of Th1 cells (46) and, reciprocally, interferon-γ was shown to increase RANTES expression, whereas IL-4 and IL-13 decreased expression (5, 24, 32). Although several studies show a close association between RANTES expression and a Th1 cell-type response, the specific role of RANTES in these conditions is unclear. Our studies suggest that directly or indirectly RANTES may be involved in the neutrophilia associated with Th1 cell-dominated diseases. Thus increased RANTES expression, as observed during respiratory viral infections, may play an important role in the associated neutrophilia and exacerbation of asthma (4, 7, 8, 22, 41, 47, 52). The detailed mechanism of RANTES-induced neutrophilia will be carried out in the next phase of this study. With antibody neutralization experiments and appropriate receptor-deficient mice, the direct or indirect effect of RANTES on neutrophil biology will be addressed. The use of receptor knockout mice will be particularly useful in determining the differential role of RANTES in vivo.

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