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Human Airway Smooth Muscle Cells Express and Release RANTES in Response to T Helper 1 Cytokines

Regulation by T Helper 2 Cytokines and Corticosteroids[1]

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RANTES is a basic 8-kDa polypeptide of the C-C chemokine subfamily with strong chemotactic activity for eosinophils, lymphocytes, and monocytes. We determined the regulation of RANTES production by human airway smooth muscle cells in culture. While TNF-α, but not IFN-γ, increased RANTES mRNA expression and protein release, the combination of TNF-α and IFN-γ caused a greater degree of expression and release in a time- and dose-dependent manner. Sequential treatment of airway smooth muscle cells with TNF-α and IFN-γ showed that IFN-γ sensitized the cells to the stimulatory effect of TNF-α. Using a modified Boyden chamber technique, RANTES separated by reverse-phase liquid chromatography from cell culture supernatants of airway smooth muscle cells stimulated by TNF-α and IFN-γ showed a strong chemoattractant effect on human eosinophils, an effect inhibited by an anti-RANTES Ab. RANTES production induced by TNF-α and IFN-γ was inhibited partly by the Th2-derived cytokines, IL-4, IL-10, and IL-13, as well as by dexamethasone. Our studies indicate that, in addition to contractile responses and mitogenesis, airway smooth muscle cells have synthetic and secretory potential with the release of RANTES. They may participate in chronic airway inflammation by interacting with both Th1- and Th2-derived cytokines to modulate chemoattractant activity for eosinophils, activated T lymphocytes, and monocytes/macrophages. *The Journal of Immunology,* **1997, 158: 1841–1847.**

Asthma is characterized by reversible airway narrowing and inflammation of the airway wall, leading to epithelial cell damage, mucus plugging, stimulation of neural reflexes, and infiltration of eosinophils, macrophages, and lymphocytes (1–4). Structural changes to the airway wall, such as increased smooth muscle content and basement membrane thickening, associated with matrix deposition, are also present (5, 6) and may contribute to persistent airway obstruction and bronchial hyper-responsiveness (7). Until recently, airway smooth muscle was regarded to be solely contractile, because its ability to shorten in response to many inflammatory mediators leads to a reduction in airway calibre. Airway smooth muscle is also known to be capable of responding to cytokines and growth factors released from resident and/or infiltrating proinflammatory cells by undergoing mitogenesis (8). However, the possibility that airway smooth muscle may act as an effector cell in perpetuating airway inflammation by expressing and secreting inflammatory products, in particular chemotactic cytokines, has not been investigated.

RANTES[3] is a member of the C-C family of chemokines originally cloned from a subtracted cDNA library made from isolated RNA of a functional, nontransformed, Ag-stimulated T cell line (9). RANTES is a potent chemoattractant for monocytes, memory T lymphocytes, and eosinophils (10, 11), and is produced by cells including T lymphocytes, macrophages, synovial fibroblasts, and airway epithelial cells (12–16). We hypothesized that airway smooth muscle cells can express and release RANTES following stimulation by cytokines that may be released from inflammatory cells such as those present in asthmatic inflammation, in particular T lymphocytes. We investigated the effect of Th1-derived cytokines, TNF-α and IFN-γ, and of Th-2-derived cytokines, IL-4, IL-10, and IL-13, on the expression and release of RANTES from human airway smooth muscle cells in culture. RANTES is the first chemotactic cytokine shown to be expressed by human airway smooth muscle cells, and this indicates that airway smooth muscle cells, in addition to controlling airway calibre, may contribute directly to chronic airway inflammation by releasing RANTES to attract lymphocytes, macrophages, and eosinophils to the site of remodeling.

Materials and Methods

Human airway smooth muscle cell culture

Human bronchial smooth muscle was obtained from the lobar or main bronchus at lung resection from patients of either sex, undergoing surgery for carcinoma of the bronchus, as described previously (17, 18). Once in culture, human airway smooth muscle cells were maintained in DMEM containing FCS (10% v/v) supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), nonessential amino acid mixture (1:100), gentamicin

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[3] Abbreviations used in this paper: RANTES, regulated upon activation, normal T cell expressed and secreted; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MIP, macrophage inflammatory protein; RT-PCR, reverse-transcription polymerase chain reaction; TFA, trifluoroacetic acid.

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(50 μg/ml), and amphotericin B (1.5 μg/ml). All cultures were maintained in a humidified atmosphere at 37°C in air/CO_2 (95:5% v/v). Fresh medium was replaced every 72 h. Using immunofluorescence techniques for both smooth muscle actin and myosin, more than 95% of the cells displayed the characteristics of smooth muscle cells in culture (18).

Cell stimulation

Confluent human airway smooth muscle cells (passage 3–8) were growth arrested by FCS deprivation for 72 h in DMEM supplemented with sodium pyruvate (1 mM), nonessential amino acids (1:100), L-glutamine (2 mM), gentamicin (50 μg/ml), and amphotericin B (1.5 μg/ml) (Life Technologies, Paisley, U.K.); and insulin (1 μM), transferrin (5 μg/ml), ascorbic acid (100 μM), and BSA (0.1%) (Sigma Chemical Co., Poole, U.K.). After 72 h, cells were stimulated in fresh FCS-free medium containing TNF-α and/or IFN-γ in a concentration- and time-dependent manner, and in the presence of different concentrations of IL-4, IL-10, IL-13 (R&D Laboratories, Oxford, U.K.), and dexamethasone (Sigma Chemical Co.). In these experiments, dexamethasone and Th2 cytokines were preincubated for 2 h before addition of TNF-α and IFN-γ.

Reverse-transcription PCR

Total cellular RNA was extracted from adherent cells using a modification of the method of Chomczynski and Sacchi (19). Following two phenol-chloroform extractions and isopropanol precipitation, RNA samples were stored overnight and washed twice with 75% ethanol (BDH Chemicals, Poole, U.K.) and dissolved in RNase-free water. Reverse transcription of 1 μg of total RNA was performed using 15 U AMV-reverse transcriptase; 1 mM of dATP, dCTP, dGTP, and dTTP; oligo(dt_{15}) primer (0.4 μg); 30 U RNase inhibitor; 5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 9); and 0.1% Triton X-100, in a total volume of 40 μl (all from Promega Corp., Southampton, U.K.). Oligo(dt) and dissolved RNA were incubated at 65°C for 10 min and placed on ice for 5 min. The remaining ingredients were then added, and samples were incubated at 42°C for 60 min, followed by 10 min at 85°C. The cDNA was subsequently diluted to a final volume of 400 μl in nuclease-free water.

For PCR, 10 μl of the cDNA solutions were used. PCR was performed using 7.5 pM of forward and reverse primers; dATP, dGTP, dTTP, and dCTP at a final concentration of 0.2 mM each; 1.5 U Taq polymerase; 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 9); and 0.1% Triton X-100, in a final volume of 30 μl. Primers for RANTES were: 5′-TCATTGC TACTGCCCTCTGC, 3′-CCTAGCTCATCTCCAAAGAG, giving a product of 242 bp. Primers for β-actin were: 5′-GTGGGGCGCCCCAG GCACCA, 3′-CTCCTTAATGTCACGCACGATTTC, giving a product of 539 bp. PCR was conducted in a Techne multiwell thermocycler (Techne, Cambridge, U.K.) at 95°C for an initial 5 min, followed by 24 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Final extension was 10 min at 72°C. The number of cycles was chosen after determination of the linear phase of the product amplification curve from serial sampling with increasing cycles of amplification. Products were distinguished by electrophoresis on a 2% agarose ethidium bromide-stained gel, and then visualized and photographed using UV luminescence. Relative abundance of the product was assessed using laser densitometry measured from the photographic negative and expressed as a ratio of the RANTES band to the β-actin band.

Northern blot analysis

Total cellular RNA was isolated as for RT-PCR reaction. RNA samples were dissolved in RNase-free water. A total of 20 μg of cytoplasmic RNA was separated by electrophoresis on a 1% agarose gel containing 7.5% formaldehyde, and transferred to a nylon Hybond-N membrane (Amersham, Bucks, U.K.) and fixed by UV irradiation. Filters were then hybridized with a ^{32}P-labeled human RANTES cDNA probe using a multiprime DNA labeling kit (Amersham). The RANTES probe was a 410-bp *Eco*RI-*Apa*I fragment, a gift from Dr. T. J. Schall (DNAX, Palo Alto, CA). Filters were then washed at a final stringency of 0.1× SSC and 0.1% SDS at 55°C, and exposed at −70°C on Kodak XS 1 100 film for 3 to 5 days. Probes were stripped by incubating the blot in a 50% formamide solution at 70°C for 2 h before hybridization with a ^{32}P-labeled 1272-bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Densitometric quantification of the Northern blots was performed by laser densitometry (protein and DNA Imageware System; Discovery Series, New York, NY). Specific RNA levels are expressed as the ratio of RANTES to GAPDH mRNA.

RANTES radioimmunoassay

RANTES was measured as described previously (20). The rRANTES and murine anti-RANTES Ab were a gift from Dr. T. J. Schall (DNAX).

Reverse-phase HPLC separation of RANTES

The culture supernatant of human airway smooth muscle cells was collected after 96-h stimulation with TNF-α and IFN-γ (10 ng/ml each). Conditioned medium (22 ml) was acidified to pH 2 with trifluoroacetic acid (TFA), filtered (0.45 μm), and applied to C_{18} reverse-phase SepPaks (Waters: 2 × 840-mg cartridges in series, prewetted with 0.1% TFA/acetonitrile and equilibrated in 0.1% TFA/water). After washing the cartridges with 0.1% TFA/water, the bound material was eluted with 0.1% TFA/acetonitrile and the solvents were removed using a Savant SpeedVac. The sample was applied to a 300Å C_{18} HPLC column (Vydac: 4.6 × 250 mm, fitted with guard column, 4.6 × 20 mm) in 0.08% TFA and eluted at 1 ml/min with acetonitrile gradients in 0.08% TFA while collecting 0.5-min fractions (0–50% acetonitrile over 50 min, followed by 50 to 80% acetonitrile over 6 min). One percent of each fraction was lyophilized in the presence of 50 μg of BSA and redissolved in the cell culture medium for RIA. Ninety-nine percent of the fraction containing the peak of immunoreactive RANTES was lyophilized in the presence of 50 μg of BSA (low endotoxin <0.1 ng/mg), dissolved in 200 μl chemotaxis buffer, and stored in aliquots at −70°C.

Eosinophil chemotaxis assay

Human eosinophils were separated and purified by using an immunomagnetic cell-sorting method (MACS system; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Blood was mixed 1:1 with HBSS (Sigma Chemical Co.) and centrifuged through a two-layer Percoll (Pharmacia, Uppsala, Sweden) density gradient (1000 × *g*, 20 min, 20°C). After removing the mononuclear fraction and the lysed red cells, the remaining leukocyte fraction was resuspended in RPMI 1640 medium (Sigma Chemical Co.) containing 5 mM of EDTA. Neutrophils were extracted from this fraction using a mAb against CD16 conjugated with micromagnetic beads and separated in a magnetic separation column. The separated fraction contained >99% eosinophils.

Eosinophil chemotaxis assay was assessed by a modified Boyden method, as previously described (11), using nitrocellulose filters (150 μm thick; 8 μm pore size). Eosinophils (50 μl of 1 × 10^6 cells/ml) were pipetted into each upper well, and chemoattractants were (28 μl vol) placed in the lower well. Cellular migrational response was determined by counting the number of cells on the underside of the filter. Results are expressed as the number of cells in 10 random high power fields. The dose of anti-RANTES Ab (R&D Systems, Abingdon, U.K.) was calculated for 50% inhibition.

Data analysis

Data are reported as mean ± SEM. Comparison between groups was performed using Mann-Whitney test. A p value of <0.05 was considered to be significant.

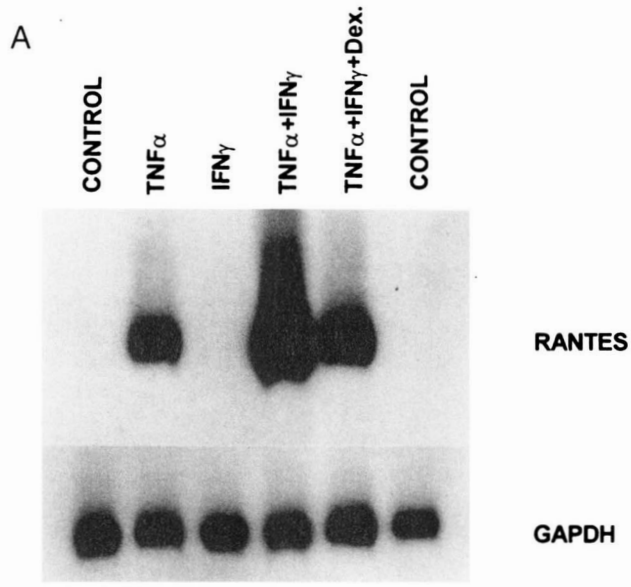
Results

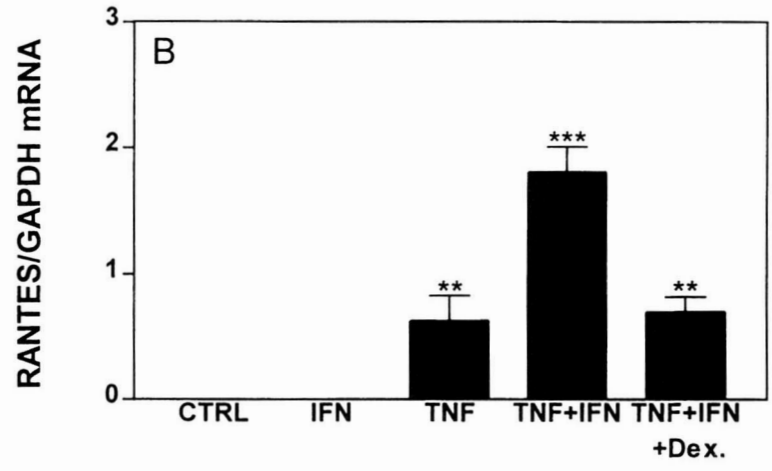
Induction of RANTES protein and mRNA expression by TNF-α and IFN-γ

Human airway smooth muscle cells were stimulated for 24 h with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml), or with both TNF-α and IFN-γ. TNF-α alone induced RANTES protein and mRNA, as measured on Northern analysis, whereas IFN-γ had no effect. Using a mixture of both cytokines, there was a large induction of RANTES protein and mRNA, which was significantly higher than in control or with single cytokine stimulation (Fig. 1). We found that IL-1β was not effective when tested alone and had no potentiating effect on the response to TNF-α, IFN-γ and TNF-α + IFN-γ (data not shown).

Dose effect

To investigate the dose response of TNF-α and IFN-γ on RANTES expression, cells were plated on 24-well plates and stimulated either with TNF-α or IFN-γ alone at concentrations of 0, 1, 3, 10, 30, and 100 ng/ml for 24 h. In addition, TNF-α (10 ng/ml)





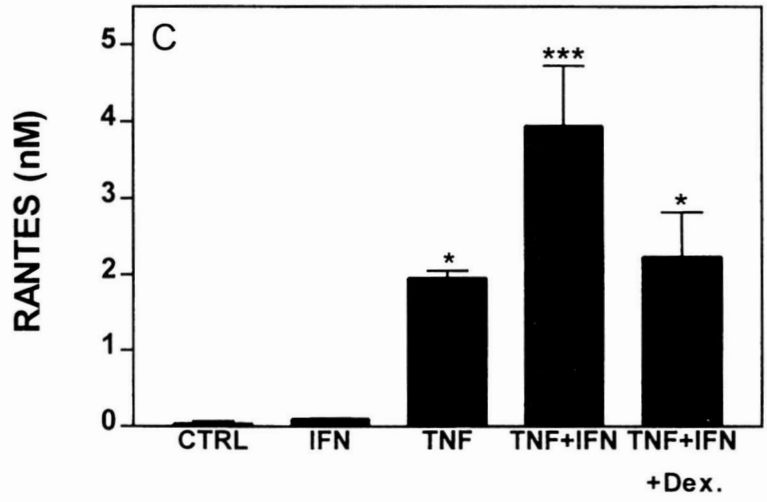


FIGURE 1. Effect of IFN-γ (10 ng/ml), TNF-α (10 ng/ml), TNF-α + IFN-γ (10 ng/ml each), and TNF-α + IFN-γ in the presence of dexamethasone (10^{-6} M) on RANTES mRNA and protein expression after 24-h stimulation. *A*, Representative Northern blot of RANTES and GAPDH mRNA expression in human airway smooth muscle cells after stimulation and the effect of dexamethasone. *B*, Mean ratio of RANTES and GAPDH mRNA, as measured by densitometric analysis. *C*, RANTES immunoreactivity in supernatants from human airway smooth muscle cells treated as above. Data are the mean ± SEM of three experiments. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with control.

or IFN-γ (10 ng/ml) was added to each concentration of the alternate cytokine to test the synergistic effect of one cytokine on the other. IFN-γ had no effect on its own up to a concentration of 100 ng/ml, while TNF-α induced the release of RANTES, the highest effect being seen at 100 ng/ml. However, in the presence of TNF-α (10 ng/ml), IFN-γ induced a marked dose-dependent

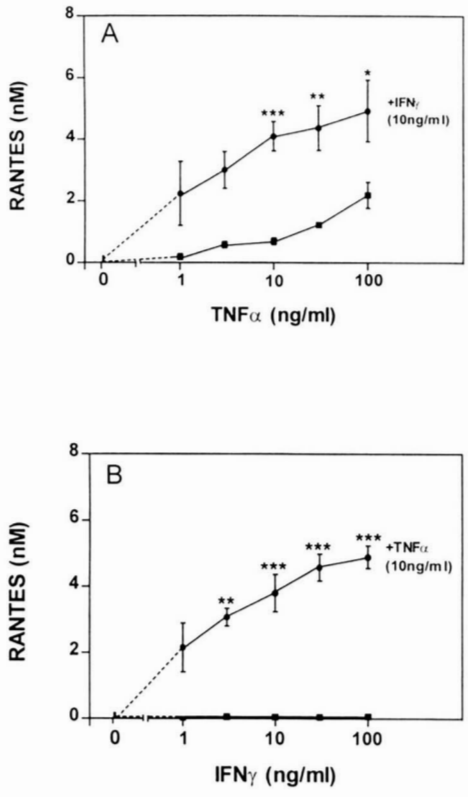


FIGURE 2. Dose-response curves of TNF-α and IFN-γ alone, and the combination of TNF-α and IFN-γ on RANTES production after 24-h incubation with human airway smooth muscle cells. *A*, TNF-α alone (■) and in the presence of IFN-γ at 10 ng/ml (●). *B*, IFN-γ alone (■) and in the presence of TNF-α at 10 ng/ml (●). Data are the mean ± SEM of three experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the response to TNF-α alone (*A*) or IFN-γ alone (*B*).

increase in RANTES production. A similar potentiating effect of IFN-γ (10 ng/ml) on the response to increasing concentrations of TNF-α was also seen (Fig. 2).

Time course

To determine the time course of RANTES production, human airway smooth muscle cells were cultured in presence of TNF-α or IFN-γ, or TNF-α + IFN-γ. Cell culture supernatants and cells were collected at various time points. There was a time-dependent increase in RANTES mRNA, expressed as a ratio of β-actin mRNA after stimulation with a combination of TNF-α and IFN-γ (Fig. 3). A time-dependent increase in RANTES protein release was also observed following stimulation with TNF-α and with the combination of TNF-α and IFN-γ (Fig. 3). A synergistic effect of TNF-α and IFN-γ on RANTES protein was observed at all time points.

Sequential effects of TNF-α and IFN-γ

To determine which of the two cytokines, TNF-α or IFN-γ, was responsible for priming RANTES release, we examined the effect of sequential stimulation with TNF-α and IFN-γ. Human airway smooth muscle cells were cultured for the first 24 h with TNF-α, IFN-γ, or a mixture of both cytokines at a concentration of 10 ng/ml each. Cells were then stimulated for an additional 24 h with or without TNF-α and IFN-γ individually and RANTES concentration measured in the culture supernatant. Incubation with IFN-γ enhanced the effect of TNF-α added 24 h later, while incubation with TNF-α did not enhance the effect of IFN-γ added 24 h later (Table I).

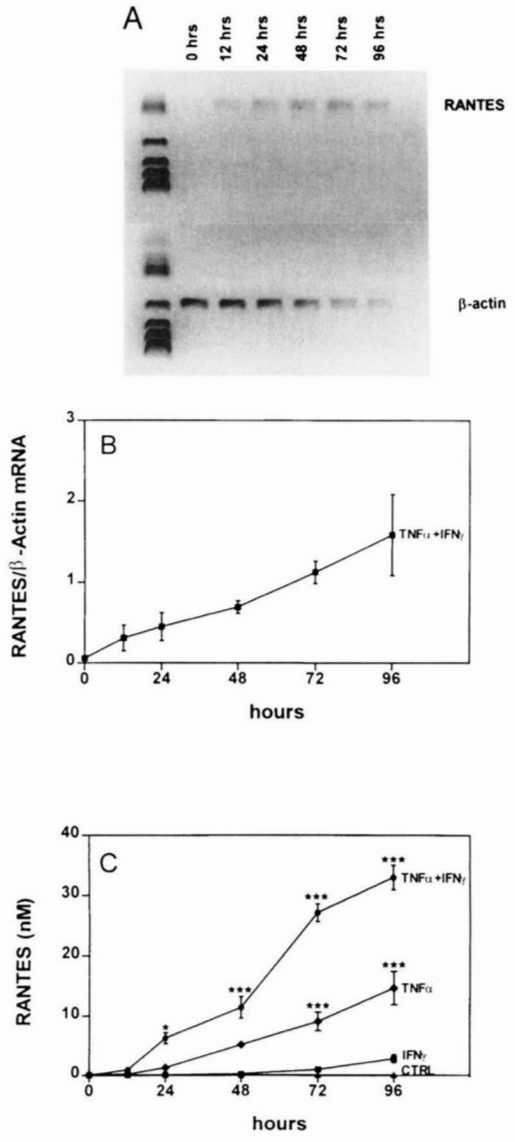


FIGURE 3. Time course of RANTES mRNA abundance and protein release in human airway smooth muscle stimulated with TNF-α and IFN-γ (10 ng/ml each). *A*, Time course of expression of RANTES and β-actin mRNA, using RT-PCR. *B*, Ratios of RANTES mRNA to β-actin mRNA measured by densitometry at various time points after stimulation with the combination of IFN-γ and TNF-α. *C*, RANTES protein release after stimulation of human airway smooth muscle cells with TNF-α (♦), IFN-γ (■), and TNF-α + IFN-γ (●) at a concentration of 10 ng/ml each and from control unstimulated cells (△). Data are the mean ± SEM of three experiments. $^*p < 0.05$ and $^{***}p < 0.001$ compared with control (unstimulated cells).

Macrophage inflammatory protein-1α (MIP-1α) release

In two sets of airway smooth muscle preparations, TNF-α (10 ng/ml), IFN-γ (10 ng/ml), and IL-1β (10 ng/ml) did not increase MIP-1α release at 24 h with values of 5 and 5.8 pM, 7 and 6 pM, and 7.5 and 5 pM, respectively, compared with 7 and 6 pM for control unstimulated samples. The combination of TNF-α, IFN-γ, and IL-1β increased MIP-1α release to 53 and 52 pM, which is much lower when compared with the amount of RANTES released.

Effects of IL-4, IL-10, IL-13, and dexamethasone on RANTES expression

To investigate the inhibitory effect of Th2 cytokines and corticosteroids on RANTES expression, human airway smooth muscle

Table I. *Effect of sequential addition of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) on RANTES production from human airway smooth muscle cells*

First Incubation (24 h)	Second Incubation (24 h)	RANTES (nM)[a]
	IFN-γ	0.1 (0.1, 0.1)
	TNF-α	1.7 (1.8, 1.7)
	TNF-α + IFN-γ	6.3 (6.4, 4.7)
IFN-γ		0.5 (0.1, 0.9)
IFN-γ	TNF-α	3.3 (3.5, 3.1)
	IFN-γ	0.1 (0.1, 0.1)
TNF-α		5.1 (5.6, 4.8, 5.0)
TNF-α	IFN-γ	5.8 (5.2, 6.3)
IFN-γ + TNF-α		12.7 (12.8, 12.6)

[a] RANTES was measured in the medium from the second incubation. Data are the mean of two or three experiments with individual results shown in brackets.

cells were stimulated with a combination of TNF-α and IFN-γ (10 ng/ml each) in the presence of IL-4, IL-10, IL-13, and dexamethasone. IL-4, IL-10, and IL-13 had no effect on the release of RANTES from unstimulated smooth muscle cells. IL-4, IL-10, and IL-13 treatment resulted in a significant reduction of RANTES production in comparison with cells without treatment with Th2 cytokines. The maximal effect was observed at a concentration of 10 ng/ml (Fig. 4). Dexamethasone inhibited RANTES release by 49.6 ± 16.5% at 10^{-9} M ($p < 0.001$), with no significantly greater inhibition at higher concentrations. The inhibition of RANTES release was accompanied by a reduction in RANTES mRNA, as assessed by Northern analysis at 10^{-6} M of dexamethasone (Fig. 1).

We also investigated the effects of IL-4, IL-10, and IL-13 (10 ng/ml each) and dexamethasone (10^{-7} M) on TNF-α-induced release of RANTES. IL-4, IL-10, and IL-13 did not induce significant inhibition of RANTES release. However, dexamethasone reduced RANTES release from 450 ± 50 pM to 192 ± 50 pM ($n = 5$; $p < 0.05$).

Reverse-phase liquid chromatography

RANTES was concentrated and substantially purified from TNF-α- and IFN-γ-stimulated human airway smooth muscle cell-conditioned medium by using C_{18} reverse-phase HPLC. The majority of immunoreactive RANTES eluted in a single fraction (37–37.5 min, ~26% acetonitrile) corresponding to a small peak of absorbance at 214 nm and clearly separated from the majority of nonimmunoreactive proteins (Fig. 5). This fraction was lyophilized with carrier protein and dissolved in buffer for the eosinophil chemotaxis assay.

Eosinophil chemotaxis

RANTES, released by IFN-γ/TNF-α-stimulated airway smooth muscle cells and substantially purified by HPLC, induced eosinophil chemotaxis at concentrations of 10^{-11} to 10^{-7} M, with significant inhibition at 10^{-9} to 10^{-7} M (Fig. 6), as previously shown for platelet-derived rRANTES (11, 12). When tested with a neutralizing anti-RANTES Ab (at a concentration predicted from the manufacturer's information to achieve a 50% inhibition of the response), the chemotactic response to 10^{-7} M of smooth muscle RANTES was inhibited by 56% (Fig. 6).

Discussion

We have shown that human airway smooth muscle cells in culture can be induced to express RANTES mRNA and release RANTES protein, particularly when incubated with a combination of Th1-derived cytokines. This effect is concentration dependent, with

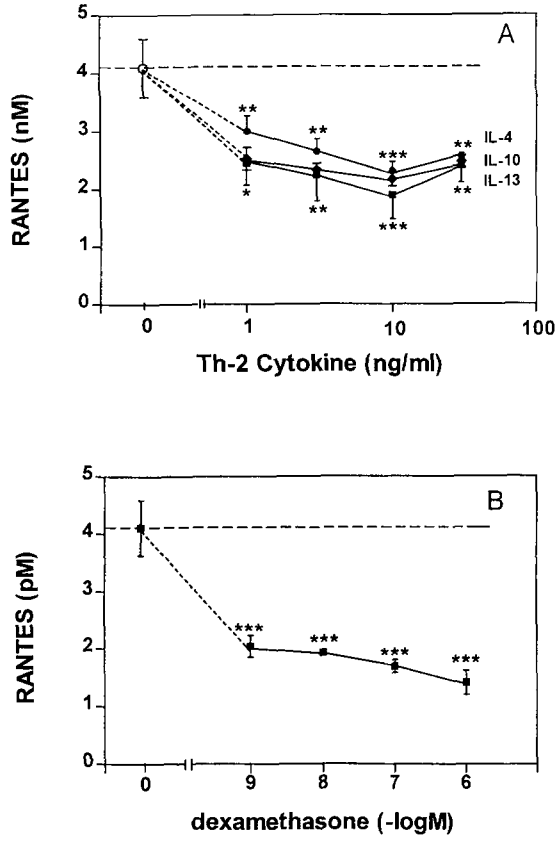


FIGURE 4. Inhibition of RANTES release from human airway smooth muscle cells, stimulated with a combination of TNF-α and IFN-γ (10 ng/ml each), by Th2 cytokines and dexamethasone. *A*, Significant inhibition of RANTES release by each of IL-4 (●), IL-10 (♦), and IL-13 (■). *B*, Significant effect of dexamethasone on RANTES release by human airway smooth muscle cells. Data are the mean ± SEM of three experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with responses in the absence of Th2 cytokine or dexamethasone (shown as dashed lines).

IFN-γ sensitizing the cells to the effect of TNF-α. The increase in RANTES mRNA expression preceded the rise in RANTES protein release, and RANTES mRNA expression increased with time. Smooth muscle-derived RANTES protein had potent chemoattractant activity for eosinophils. The Th2-type cytokines, IL-4, IL-10, and IL-13, produced concentration-dependent inhibition of the release of RANTES. A similar inhibition of RANTES release was also seen with dexamethasone. These results indicate that airway smooth muscle may contribute directly to airway inflammation by interacting with Th1- and Th-2-derived cytokines, and by attracting inflammatory cells to the airway submucosa through the release of the chemoattractant cytokine, RANTES.

RANTES gene induction by TNF-α and IFN-γ has been described in many cell types, such as airway epithelial cells, macrophages, and endothelial cells (13, 14, 16), but this is the first demonstration of chemoattractant cytokine production by airway smooth muscle cells. IFN-γ stimulates RANTES gene expression in macrophages (13), and TNF-α has similar effects in fibroblasts, mesangial cells, and renal tubular epithelial cells (15, 21, 22). A synergistic interaction between TNF-α and IFN-γ has been described in fibroblasts, endothelial cells, and airway epithelial cells for RANTES gene expression (14–16). Synergy between TNF-α and IFN-γ has also been described in macrophages for the production of oxygen radicals, nitric oxide, and the expression of adhesion molecules (23–25). In our study, IFN-γ increased the

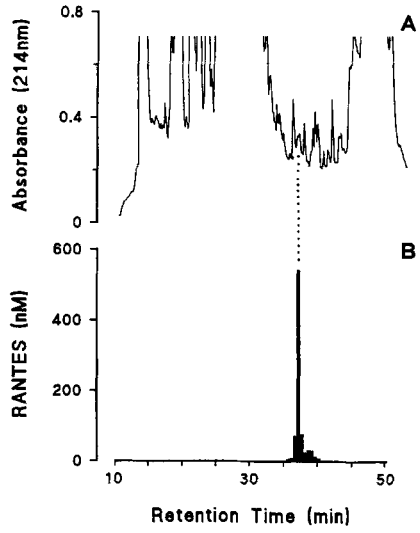


FIGURE 5. Reverse-phase HPLC separation of RANTES from supernatants of stimulated airway smooth muscle cells. RANTES in conditioned media was concentrated and substantially purified using C_{18} reverse-phase HPLC. The majority of immunoreactive RANTES was eluted in a single fraction at 32 to 32.5 min (*B*), corresponding to a small peak of absorbance at 214 nm (*A*).

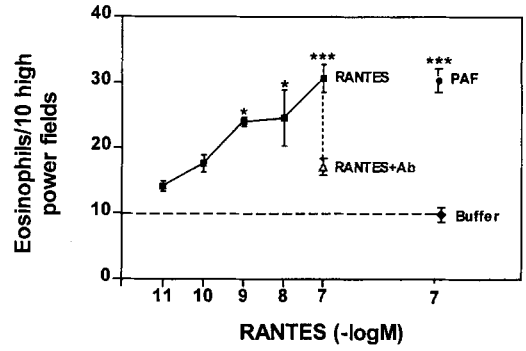


FIGURE 6. Human airway smooth muscle RANTES induces eosinophil chemotaxis. RANTES was purified substantially by reverse-phase HPLC (see Fig. 5). Eosinophil chemotaxis was measured by a modified Boyden chamber method, and results are expressed as migrated eosinophils per 10 high power fields. In the presence of a limited amount (see text) of anti-RANTES Ab (△), there was significant inhibition of the response induced by 10^{-7} M of RANTES ($p < 0.01$). Platelet-activating factor was used at 10^{-7} M (●) as a positive control. The dashed line shows the eosinophil count in wells treated with the control buffer (PBS/RPMI medium + 0.25% BSA). Data are the mean ± SEM for three experiments. $*p < 0.05$ and $***p < 0.001$ compared with buffer-treated wells.

release of RANTES induced by TNF-α, but TNF-α did not modulate the effect of IFN-γ. One potential mechanism for the enhanced effect of IFN-γ on TNF-α effects could be mediated through an increase in TNF-α receptor expression induced by IFN-γ, as has been shown on human monocytes (26, 27). Other mechanisms could involve postreceptor events, such as the activation of activator protein-1 and nuclear factor-κB, as has been demonstrated for the stimulated expression of IL-8 expression by the synergistic action of IFN-γ and TNF-α (28), as the upstream region of the RANTES gene contains both activator protein-1 and nuclear factor-κB binding sites (29).

The continuing time-dependent increase in RANTES mRNA and protein expression following TNF-α alone may indicate that this response could be related to increased mitogenesis and changes in airway smooth muscle cell phenotype. Although we did not measure any indices of proliferation in our study, a previous study using similar concentrations of TNF-α has shown that it induces modest proliferation in cultures of human airway smooth muscle (30). However, this occurred after 48 h of incubation, while significant expression of RANTES mRNA and release of RANTES were observed earlier at 12 to 24 h after exposure to TNF-α. It is not known whether mitogenesis of human airway smooth muscle stimulated with TNF-α alone or in combination with IFN-γ is modulated by the preceding expression and production by these cells of RANTES.

In addition to RANTES, other C-C chemokines may also be produced by human airway smooth muscle cells. Thus, we have shown that macrophage-inflammatory protein-1α (MIP-1α) is also released, although to a lesser extent than RANTES, after stimulation with the combination of IFN-γ and TNF-α. The newly described C-C chemokine, eotaxin (31), is also released, as detected by reverse-phase liquid chromatography (manuscript in preparation). Thus, it is likely that airway smooth muscle cells produce a wide range of C-C chemokines other than RANTES. We have also recently demonstrated release of high levels of the C-X-C chemokine, IL-8, from airway smooth muscle cells.

Subsets of $CD4^+$ T cells have been defined on the basis of distinct cytokine secretion patterns and their concomitant immunomodulatory role. Th1-derived cytokines include TNF-α and IFN-γ, while IL-4, IL-5, IL-10, and IL-13 are Th2-derived cytokines (32). IL-4 and IL-5 are involved in the development, maturation, and increased survival of eosinophils and mast cells (33–35), and have been found to be expressed in airways of patients with asthma (36). IL-10 mRNA expression is also increased in asthmatic airways (37). The Th2-derived cytokines, IL-4 and IL-13, are known to inhibit the release of RANTES from endothelial cells, but only IL-4 reduces RANTES release from airway epithelial cells, with IL-10 being inactive in both cell types (14, 16). We demonstrate that all three Th2-derived cytokines, IL-4, IL-10, and IL-13, inhibit the release of RANTES from airway smooth muscle cells stimulated by a combination of TNF-α and IFN-γ. IL-4, IL-10, and IL-13 could inhibit RANTES production in airway smooth muscle in vivo either by a direct effect on the smooth muscle cells or through the inhibition of IFN-γ and TNF-α release by T cells and macrophages (38–41). However, since IL-4, IL-10, and IL-13 did not inhibit TNF-α-induced release of RANTES, it is likely that these cytokines inhibited only IFN-γ-mediated enhancement of TNF-α effects, when cells were stimulated by a combination of IFN-γ and TNF-α. Corticosteroids inhibited RANTES mRNA and protein expression, indicating an effect at the transcriptional level. It is of interest that the maximum degree of inhibition achieved by the Th2-derived cytokines and by corticosteroids was similar and did not exceed 50%, suggesting that these agents may achieve this inhibition by similar mechanisms.

T cells are a significant feature of many airway inflammatory diseases such as asthma (4, 36), and are often found in the airway submucosa, often in close proximity to the smooth muscle. Another source of the proinflammatory cytokines, IFN-γ and TNF-α, is the monocyte/macrophage, which in asthmatic airways can release these cytokines at concentrations that stimulate release of RANTES from airway smooth muscle cells (42). Based on the findings we report in this work that RANTES production in vitro is stimulated by Th1-derived cytokines and modulated by Th2-derived cytokines and that activated T lymphocytes can adhere to airway smooth muscle cells (43), T cell-derived cytokines may be the stimulus for airway smooth muscle cell production of RANTES in vivo. The role of RANTES or other C-C chemokines

released from airway smooth muscle in the progression of the asthmatic airway pathology into a chronic state is not known. In addition to being a chemoattractant for T cells, monocytes, and eosinophils (10, 11), it is not known whether RANTES can induce airway smooth muscle proliferation.

Our results indicate that the airway smooth muscle should not be regarded solely as a specialized cell capable of contractile responses. Proinflammatory cytokines and several growth factors are capable of modulating airway smooth muscle phenotype and mitogenesis (8), and the resulting increase in airway smooth muscle mass may contribute to airway obstruction and bronchial hyper-responsiveness in asthma (7). The additional secretory potential of airway smooth muscle, particularly in terms of RANTES release, adds another dimension to the putative role of airway smooth muscle in airway inflammation. Thus, airway smooth muscle could contribute directly to the recruitment of inflammatory cells such as eosinophils, lymphocytes, and monocytes to the airways (10, 11, 44). Whether RANTES produced by airway smooth muscle cells could also in turn contribute to altered smooth muscle function and airway remodeling is not known. Our observations support the notion that airway smooth muscle could be a major contributor to the inflammatory and pathophysiologic features of the airways in asthma.

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