

Double-stranded RNA activates RANTES gene transcription through co-operation of nuclear factor- κ B and interferon regulatory factors in human airway epithelial cells

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Summary

Background Regulated on activation, normal T cells expressed and secreted (RANTES) is a member of the CC chemokine family and contributes to viral-induced airway inflammation including exacerbations of asthma. Double-stranded RNA (dsRNA) is known to be synthesized during replication of many viruses and a ligand of Toll-like receptor 3. We hypothesized that dsRNA may mimic viral infection and induce RANTES expression in airway epithelial cells.

Objective We first confirmed that dsRNA up-regulated RANTES mRNA and protein synthesis in the airway epithelial cells. We next focused our studies on the transcriptional regulation of RANTES.

Methods Airway epithelial cell line BEAS-2B and normal human bronchial epithelial cells were used *in vitro* study. Levels of RANTES mRNA and protein expression were determined with RT-PCR and ELISA. Mechanisms of transcriptional regulation were assessed by electrophoretic mobility shift assay and dual luciferase assay using RANTES promoter-luciferase reporter plasmids.

Results Activation of nuclear factor- κ B (NF- κ B) was confirmed by nuclear protein binding to a DNA probe derived from the RANTES promoter. Activity of the RANTES promoter was increased by dsRNA. The stimulation with dsRNA was partially inhibited in plasmids mutated at either of the binding sites for NF- κ B or IFN regulatory factors (IRFs). When both sites were mutated, the activation was totally abrogated.

Conclusion These results imply that dsRNA activates NF- κ B and IRFs and these transcription factors activate transcription of the RANTES promoter and its protein expression in airway epithelial cells.

Keywords asthma, double-stranded RNA, epithelial cells, IFN regulatory factor, NF- κ B, RANTES, Toll-like receptor, viral infection

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Introduction

Respiratory viral infections can trigger exacerbations of asthma and chronic obstructive pulmonary disease (COPD). Human rhinovirus (HRV), respiratory syncytial virus (RSV), influenza virus and parainfluenza virus are particularly common viruses causing asthma exacerbations [1–3]. These viruses are RNA viruses and synthesize double-stranded RNA (dsRNA) during replication in infected cells. DsRNA is known to stimulate production of IFN- α/β [4, 5] and plays an important role as a trigger of the host defence against viral infection. Investigators have reported that intracellular dsRNA binds dsRNA-dependent protein kinase (PKR),

which mediates signal transduction [6]. Moreover, recent studies identified the Toll-like receptor 3 (TLR3) as a receptor that mediates the response to dsRNA and have implicated TLR3 in the signalling pathway by which dsRNA induces IFNs [4, 5, 7].

RANTES is a member of the CC branch of the chemokine family and binds to receptors CCR1, CCR3 and CCR5. It is chemotactic for T lymphocytes, monocytes, eosinophils, and basophils [8–13]. Recent studies have suggested that RANTES contributes to the pathogenesis of many kinds of diseases including allergic diseases, such as asthma, allergic rhinitis, and dermatitis [13–18]. RANTES also contributes to virus-related disease. For example, one receptor for RANTES, CCR5, was found to be a fusion co-factor of HIV and administration of RANTES could antagonize the infection of CD4⁺T cells by HIV. RANTES is also known to induce apoptosis of cells infected with HIV [19, 20]. On the other hand, it has been reported that RANTES contributes to

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the airway inflammation caused by several viral infection in human and animal models [21–25].

RANTES expression is up-regulated in the airway of asthmatics infected with viruses and it may contribute to the exacerbations of asthma through attracting inflammatory cells into the epithelium [21, 26]. One of major cell sources expressing RANTES is the airway epithelial cell. Inflammatory cytokines and several kinds of viruses, such as HRV, RSV, and influenza virus, are known to induce RANTES in airway epithelial cells *in vitro* [14, 27–31].

Reports concerning the mechanisms for inducible RANTES gene expression following viral infection have been accumulating. Some studies have indicated that transcription factors, notably members of the nuclear factor- κ B (NF- κ B) and IFN regulatory factor (IRF) families, bind to the promoter region of RANTES and play a crucial role in gene expression [32, 33]. Although respiratory viral infections are known to activate these transcription factors [33–36], the role of dsRNA in this process has not yet been fully elucidated in airway epithelial cells.

In the present study, we investigated the up-regulation of RANTES expression by dsRNA in airway epithelial cells. We provide evidence for a role of NF- κ B and IRF in the transcriptional activation of RANTES by dsRNA. These findings provide insight into the mechanisms of epithelial activation by dsRNA and RANTES expression.

Materials and methods

Cell culture and reagents

BEAS-2B is a human airway epithelial cell line transformed with adenovirus 12-SV40 hybrid virus (a kind gift from Dr Curtis Harris) [37]. BEAS-2B cells were cultured in DMEM/F12 with 10% FBS, 100 U/mL penicillin and 100 ng/mL streptomycin (Invitrogen Corporation, Tokyo, Japan) at 37 °C with 5% CO₂ in humidified air. Normal human bronchial epithelial (NHBE) cells were purchased from BioWhittaker, Inc. (Tokyo, Japan) and cultured in bronchial epithelial growth medium. The dsRNA poly (I : C), single-stranded RNA poly (I) and poly (C) were purchased from Sigma (Tokyo, Japan) and dissolved in distilled water.

RT-PCR

Gene expression of TLR3, RANTES, and β -actin was analysed by RT-PCR as described before [29]. Total RNA was extracted from cells after the incubation with or without indicated reagents using ISOGEN reagent (Nippon-Gene, Tokyo, Japan). First-strand cDNAs were synthesized from isolated RNA templates in the presence of Moloney–Murine leukaemia virus reverse transcriptase (Amersham Pharmacia Biotech, Tokyo, Japan). The polymerase chain reaction was performed using Taq polymerase (Roche Diagnostics, Tokyo, Japan) and was performed for 30 cycles of: denaturation step, 94 °C for 45 s; annealing step, 57 °C for TLR3 and 61 °C for RANTES and β -actin for 45 s, and extension step 72 °C for 60 s; followed by a final extension of 72 °C for 10 min (Perkin-Elmer Cetus, Norwalk, CT, USA). The sequences of the primers used were: TLR3, 5'-AAATTGGGCA AGAACTCACA GG-3' (forward) and 5'-GTGTTTCCAGAGCCGTGCTAA-3' (reverse);

RANTES, 5'-ATGAAGGTCTCCGCGGCACGCCT-3' (forward) and 5'-CTAGCTCATCTCCAAAGAGTTG-3' (reverse) and β -actin, 5'-GTGGGGCGCCCCAGGCACCA-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse). PCR-amplified products were analysed by 2% agarose gel electrophoresis and ethidium bromide staining followed by visualization with an ultraviolet transilluminator.

Assay of RANTES protein release into the culture medium

Concentrations of RANTES in the collected culture medium were determined with a commercially available system for ELISA (R&D systems) as described previously [38]. The limit of detection in the assay was 8 pg/mL.

Nuclear extraction

BEAS-2B cells were treated as indicated and then harvested with a scraper and then nuclear extracts were prepared as described before [39] and was stored at –80 °C.

Electrophoretic mobility shift assays (EMSA)

DNA–protein-binding assays were based on the methods previously reported [39]. Aliquots of 5 μ g of nuclear extracts were incubated in reaction buffer containing ³²P-labelled oligonucleotide with or without unlabelled oligonucleotide probe at room temperature for 20 min. In some experiments, antibodies against p50 (NF- κ B1), p65 (Rel A), and STAT1 (Santa Cruz Biotech, Tokyo, Japan) were incubated with the mixture for 30 min after incubation with labelled probe. The reaction products were analysed by electrophoresis in a 5% polyacrylamide gel. The gels were dried and analysed by autoradiography. The sequences of the oligonucleotide probes used in EMSA for detecting NF- κ B are indicated in Table 1. This probe includes the two proximal-binding elements for NF- κ B of the RANTES promoter that were reported before [34, 35]. Oligonucleotides were synthesized by Invitrogen Corporation.

Construction of RANTES promoter-luciferase reporter plasmids (pRANTs)

A 884 bp fragment of the promoter region of the RANTES gene was cloned into a promoter-less luciferase reporter pGL2-Basic Vector (Promega, Madison, WI, USA) and was referred to as pRANT-WT (a kind gift of Dr Thomas J. Schall) [27]. Putative transcription factor-binding sites in pRANT-WT were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), using the pRANT-WT as a template. Three constructs, pRANT-m κ B, pRANT-mISRE (ISRE, IFN-stimulated response element), and pRANT-m κ B&ISRE, were synthesized by temperature cycling using site-mutated primers and PfuTurbo DNA polymerase (Stratagene). Non-mutated template plasmid was digested with *DpnI* endonuclease (Stratagene). The mutated plasmid was transformed into XL1-Blue cells (Stratagene) and purified using the Qiagen plasmid purification kit (Qiagen, Tokyo, Japan). Sequences of mutated sites are indicated in Table 1. The plasmid in which the two binding sites for NF- κ B of the promoter were mutated is referred to as pRANT-m κ B. The plasmid in which the binding site for IRF families, so called

Table 1. The sequences of oligonucleotides for EMSA or pull-down that contain two binding sites for NF- κ B and a binding site for IRF in the *RANTES* promoter

Cis-responsive elements	Position	Oligonucleotide used in EMSA/pull-down and sequence of mutated sites in the reporter plasmids
ISRE	-122 to -96	5'-GGCTATTTAGTTTTCTTTCCGTTTTGTGC-3'
		5'-GGCTATT <u>G</u> CAGTTT <u>G</u> CTTTG <u>C</u> CGTTTTGTGC-3'
NF- κ B1 and 2	-57 to -27	5'-TTTGGAAACTCCCTTTAGGGGATGCCCTCA-3'
		5'-TTTGGAAAC <u>GG</u> CCTTTAGG <u>CC</u> ATGCCCTCA-3'

The sequences of mutated sites in the *RANTES* promoter-luciferase reporter plasmids are underlined. EMSA, electrophoretic mobility shift assays; NF- κ B, nuclear factor- κ B; IRF, IFN regulatory factor; ISRE, interferon stimulated response element.

ISRE [33–35], was mutated is referred to as pRANT-mISRE. The plasmid in which the sites of both NF- κ B and ISRE were mutated is referred to as pRANT-m κ B&ISRE (see Fig. 1). The sequences of mutated sites in the pRANTs are underlined (Table 1).

The sequence of each plasmid was confirmed using a gene analysis system (Takara Syuzou, Shiga, Japan).

Transient transfections and luciferase assay

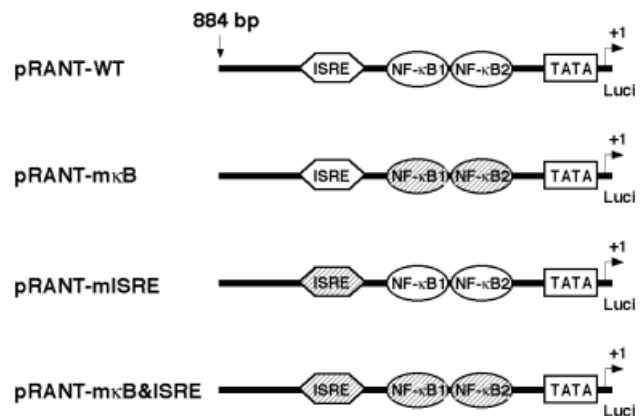
BEAS-2B cells were seeded into six-well plates and were transfected with 1 μ g of reporter plasmids and 10 ng of a control renilla-luciferase vector pRL-TK (Promega) using 3 μ L of Fugene 6 transfection reagent (Roche Diagnostics) and then incubated for 48 h in 2 mL medium. Cytokines or poly (I:C) (dsRNA) were then added and 8 h later cells were harvested. The luciferase activity was measured using the Dual-Luciferase Assay System (Promega) and a luminometer Gene-Light 55 (Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of the pRANT was normalized to renilla-luciferase activity and expressed as fold induction compared with the control value [38].

Statistical analysis

Analysis of data was performed using Stat-View IV (Abacus Concept, Inc., Berkeley, CA, USA). Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical differences were determined by analysis of variance with Fisher's PLSD.

Results

We first confirmed that airway epithelial cell lines, BEAS-2B and NCI-H292, and NHBE cells constitutively expressed mRNA of TLR3 (Fig. 2). Peripheral blood mononuclear cells were used as control. We also used TLR3 cDNA that was cloned from a human lung cDNA library (Invitrogen Corporation) as a positive control. The sequence of cloned TLR3 cDNA was confirmed using a gene analysis system (Takara Syuzou) (data not shown).

**Fig. 1.** Schema of *RANTES* promoter-luciferase reporter plasmids are depicted. Mutated binding sites in the promoter were indicated with dash.

We next analysed whether dsRNA activates the expression of *RANTES* mRNA in airway epithelial cells. A minimal level of expression of *RANTES* mRNA was detected in unstimulated BEAS-2B cells (Fig. 3a) and NHBE cells (Fig. 3b). Stimulations for 24 h with 50 μ g/mL of dsRNA increased the expression of *RANTES* mRNA in both BEAS-2B cells and NHBE cells. These data indicate that dsRNA may regulate *RANTES* expression at the level of transcription.

RANTES protein production was dose dependently increased by stimulation of BEAS-2B cells for 24 h with dsRNA (Fig. 4a). The greatest induction of *RANTES* protein was observed using dsRNA at a concentration of 50 μ g/mL (1.54 ± 0.18 ng/mL). *RANTES* protein production was increased by dsRNA in a time-dependent manner and reached a plateau by 24 h in BEAS-2B cells (Fig. 4b). Neither of two preparations of single-stranded RNA (poly I and poly C) increased *RANTES* protein production, indicating that the effect on *RANTES* induction was specific for dsRNA (data not shown). Release of *RANTES* protein into the medium of NHBE cells was profoundly increased 24 h after treatment with dsRNA suggesting that this response occurs with airway epithelial cells that have not been immortalized (2.15 ± 0.2 ng/mL) (Fig. 4c).

We next focused experiments on the mechanisms of regulation of *RANTES* transcription by dsRNA. EMSA

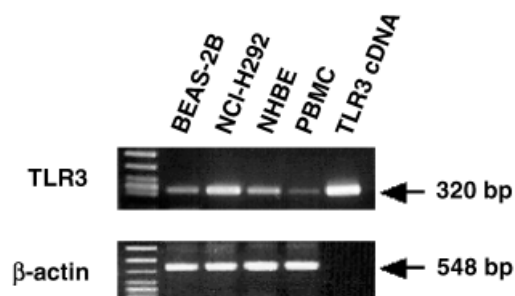


Fig. 2. Expression of mRNA of Toll-like receptor 3 (TLR3). RNA was extracted from indicated cells and was subjected to RT-PCR for detection of mRNA of TLR3 or β -actin. Representative of four independent experiments.

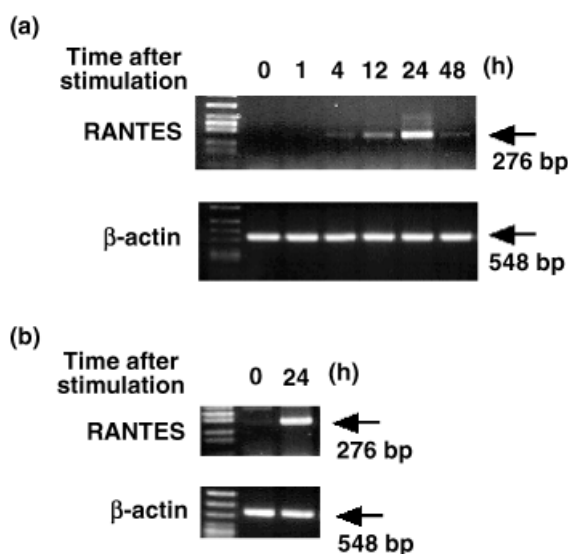


Fig. 3. Effect of double-stranded RNA (dsRNA) on the expression of RANTES mRNA. RNA was extracted from BEAS-2B cells, which were incubated with 50 μ g/mL of dsRNA for 1–48 h (a) or normal human bronchial epithelial cells incubated for 24 h (b). Extracted RNA was subjected to RT-PCR for detection of RANTES or β -actin mRNA. Representative of four independent experiments.

was performed to determine whether stimulation with dsRNA results in formation of active NF- κ B and IRF. EMSA with nuclear extracts from BEAS-2B cells stimulated with dsRNA (50 μ g/mL) resulted in formation of a binding complex (complex 1) using a DNA probe spanning the putative-binding sites for NF- κ B1 and 2 in the RANTES promoter. A strong band was observed using extracts from cells harvested at 1–2 h after stimulation (Fig. 5a). This binding complex was blocked or supershifted by antibodies to p50 and p65 NF- κ B Rel family members. Antibodies against other Rel family members (Rel B, c-Rel, and p52) and pre-immune rabbit and goat IgG did not influence complex formation (data not shown). The binding specificity of the NF- κ B complex was further assessed by competition with unlabelled oligonucleotides (Fig. 5b). Taken together, these results suggest that dsRNA induces the formation of a sequence-specific NF- κ B complex composed of p50/p65 heterodimers.

Using EMSA, an IRF–DNA-binding complex was barely detectable, presumably due to the lower efficiency of EMSA for IRF compared with EMSA for NF- κ B (data not shown).

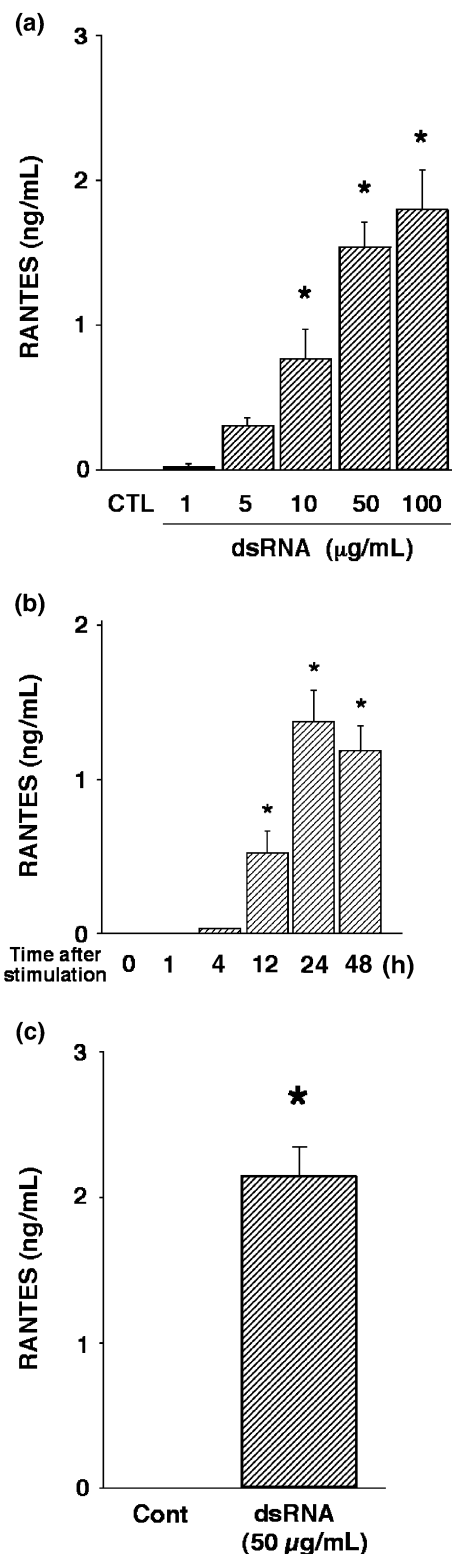


Fig. 4. RANTES protein release into the medium of BEAS-2B cells. (a) BEAS-2B cells were treated for 24 h with double-stranded RNA (dsRNA) (1–100 μ g/mL). (b) BEAS-2B cells were incubated for 1–48 h with 50 μ g/mL of dsRNA. (c) Normal human bronchial epithelial cells were incubated with 50 μ g/mL of dsRNA for 24 h. Concentrations of RANTES protein in the medium were analysed by ELISA. The data are presented as the mean $\pm$ SEM of a total of six independent experiments (* $P < 0.05$ compared with control).



Fig. 5. (a, b) Translocation of nuclear factor- κ B p50/p65 and its binding to the proximal region of the RANTES promoter was confirmed using electrophoretic mobility shift assays. Representative of four independent experiments.

To determine whether dsRNA activates IRF and induces subsequent binding to the RANTES promoter, we performed western blot analysis of nuclear protein precipitated by binding to a RANTES promoter-derived DNA probe containing the ISRE (see Table 1). IRF-3 and IRF-7 were detected in extracts of untreated cells. The level of IRFs seemed to be slightly increased after incubation with 50 μ g/mL of dsRNA for 1–2 h, however, we could not detect statistically significant change in the level of IRFs after stimulation with dsRNA (data not shown).

DsRNA (50 μ g/mL) increased firefly-luciferase activity in BEAS-2B cells transfected with the pRANT-WT reporter in a dose-dependent manner indicating that dsRNA activates RANTES transcription (Fig. 6a). To compare the ability of dsRNA to induce RANTES transcription with that of other stimuli, we analysed the effect of TNF- α and IFN- γ on the activity of the RANTES promoter (Fig. 6b). TNF- α (100 ng/mL) increased the activity of pRANT-WT, while IFN- γ (50 ng/mL) did not affect the activity of pRANT-WT at all. To test the roles of binding sites for NF- κ B and IRFs in transcription of the RANTES gene induced by these stimuli, luciferase reporter plasmids mutated at relevant sites in the proximal promoter were utilized. A reporter using the RANTES promoter mutated at the binding site for NF- κ B (referred to as pRANT-m κ B) nearly completely inhibited the response to stimulation with TNF- α and partially inhibited the response to stimulation with dsRNA (Fig. 6c). A reporter plasmid, which was mutated at the putative IRF-binding site, pRANT-mISRE, showed partial loss of dsRNA stimulation, while the stimulation with TNF- α was unaffected. Neither TNF- α nor dsRNA activated the reporter, pRANT-m κ B&ISRE, which was mutated at the binding sites for both NF- κ B and IRF.

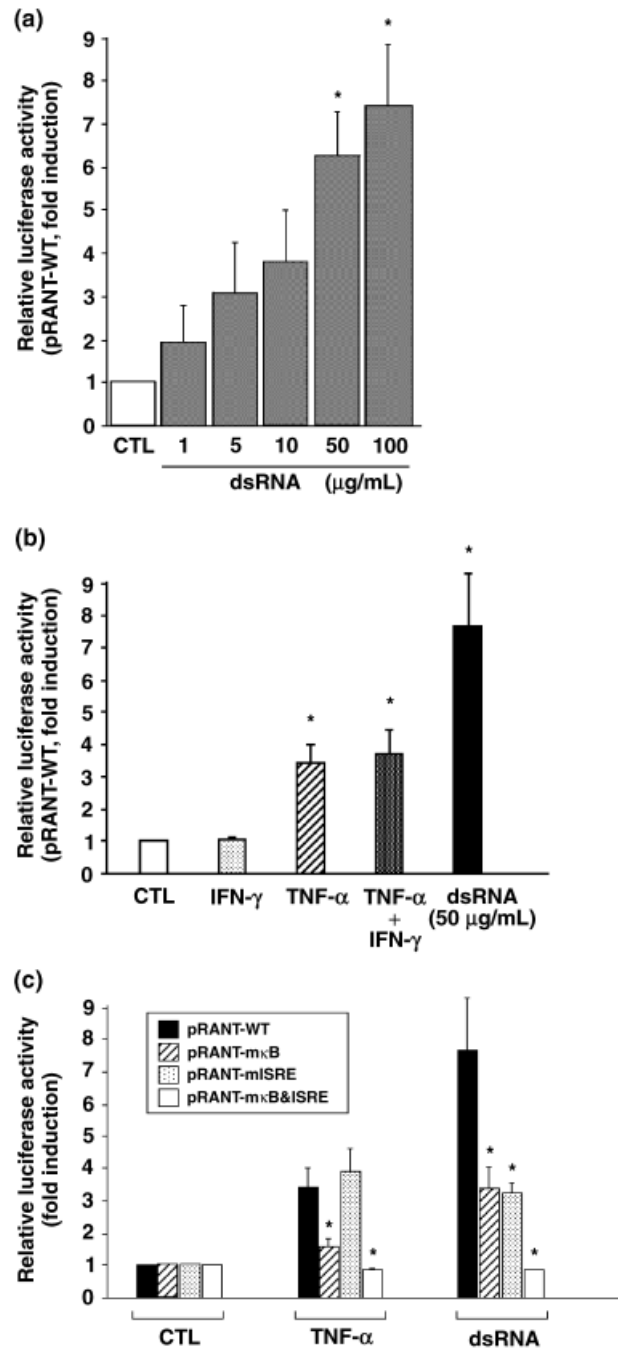


Fig. 6. (a) Double-stranded RNA dose dependently activated wild-type RANTES promoter-luciferase reporter (pRANT-WT). (b) Effect of TNF- α (100 ng/mL), IFN- γ (50 ng/mL), and double-stranded RNA (dsRNA) (50 μ g/mL) on RANTES promoter activity (* P < 0.05 compared with control) and (c) the influence of response elements for nuclear factor- κ B and IFN regulatory factor (* P < 0.05 compared with pRANT-WT). The data are presented as the mean $\pm$ SEM of a total of three independent experiments.

Discussion

We demonstrate here that dsRNA up-regulates RANTES gene transcription through co-operation of NF- κ B and IRFs in airway epithelial cells.

DsRNA seems to mimic the effects of virus and induces the expression of numerous genes involved in inflammation and

host defence. Many of these genes, of which RANTES is an example, play a protective role against viral infection or can trigger inflammation following viral infection. It has been hypothesized that dsRNA secreted from infected cells may contribute to the immune and inflammatory response of adjacent non-infected cells against viral infection as well as in infected cells. While viral infection can stimulate host cells by several mechanisms, we have focused on dsRNA as a viral product that activates epithelial responses.

NF- κ B is comprised of a homodimer or heterodimer of Rel family proteins, including NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel. It is sequestered in an inactive form in the cytoplasm by the inhibitory molecules known as I κ B [40]. TNF- α activates NF- κ B by inducing the phosphorylation and degradation of I κ B and leads to the translocation of NF- κ B to the nucleus where it can bind to specific-binding sites of relevant promoters. It has been reported that NF- κ B regulates many kinds of genes and plays a crucial role in inflammatory diseases [40–43]. It has been reported that TNF- α and several different viruses induce activation and subsequent binding of NF- κ B to sites in the RANTES promoter [32, 34, 35, 44, 45]. Our data using EMSA and reporter assays clearly show that dsRNA activated NF- κ B and increased the activity of the RANTES promoter in part through binding sites for NF- κ B.

IRF family was originally identified as regulators of the IFNs. It has been shown that IRF-1 and p48, essential components of IFN-stimulated gene factor 3, are critical for the IFN-mediated anti-viral response. Recent studies have shown that viral infection and dsRNA can also activate IRF-3 and IRF-7 [4, 43, 46]. We could not clearly demonstrate which subtype of IRF family play crucial role in the up-regulation of RANTES expression with dsRNA. We need further study to assess the quantity of the binding of IRF to DNA and analyse the role of each transfection factor using its dominant negative. However, the results of the promoter assay using the mutant reporter plasmids showed the importance of both NF- κ B and IRFs in the response to dsRNA. These findings are in agreement with the results that have been recently reported from the experiments using viral infection [34–36]. In our results, co-operation of NF- κ B and IRFs are important for activation of RANTES promoter with dsRNA. Although the mechanisms are not clear, co-activators such as CBP/p300 or Ets family members might be candidates for the factors that mediate the co-operation. CBP/p300 are known to bind NF- κ B and IRF and may contribute to the interaction of these transcription factors with transcriptional machinery [47–49]. Ets family, such as PU.1, is known to interact with NF- κ B and IRF family and activate target genes synergistically [50]. Fitzgerald et al. [46] recently reported that I κ B kinase-epsilon (IKKepsilon) and TANK-binding kinase-1 (TBK1) activate not only NF- κ B but also IRF3 suggesting that these kinases may co-ordinate the signalling pathway of TLR3-dsRNA. Further studies will be necessary to investigate the mechanisms of the co-operation between NF- κ B and IRF in the regulation of RANTES transcription.

It is not clear what pathway mediates the signal of dsRNA to NF- κ B or IRFs in our studies. While the response to TNF- α was largely dependent on NF- κ B, the response to dsRNA required response elements for both NF- κ B and IRFs. Thus

dsRNA may activate more than one pathway. Uetani et al. [51, 52] recently reported that dsRNA activates NF- κ B and IRF-1 through activation of PKR and these transcription factors up-regulate NOS2 gene expression in airway epithelial cells. Meusel et al. [53] reported the up-regulation of TNF- α via PKR activated by dsRNA in airway epithelial cells. Quite recently, Gern et al. [54] reported that dsRNA activated PKR and induced RANTES expression in bronchial epithelial cells. These reports indicate the possibility of involvement of PKR in the induction of RANTES expression by dsRNA in airway epithelial cells. While data about the role of PKR in the signal of dsRNA have been reported, recent data showed that TLR3 recognizes dsRNA [5, 7]. While the role of TLR3 in airway epithelial cells is unknown, we showed that airway epithelial cells constitutively express mRNA for TLR3 (Fig. 2). It may be reasonable to hypothesize that both of PKR and TLR3 may be associated with the signal transduction by dsRNA that activates NF- κ B and IRFs to activate the transcription of the RANTES gene. Doyle et al. [55] recently reported that agonists of TLR3 and TLR4, such as dsRNA and LPS, respectively, activate NF- κ B and IRF3 and both transcription factors are involved in the induction of IFN- β and chemokines RANTES and IP-10 in murine macrophage. Jiang et al. [56] recently reported that PKR is involved in the activation of NF- κ B through dsRNA-induced TLR3-mediated signalling pathway. These data are in agreement with the data presented in this report and we hypothesize that co-operative action of NF- κ B and IRFs might be important in our study, while Doyle et al. indicated IRF3 plays a crucial role in their murine model.

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