dsRNA enhances eotaxin-3 production through interleukin-4 receptor upregulation in airway epithelial cells

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ABSTRACT: The exacerbation of asthma during viral infections is mainly explained by neutrophils infiltrating into the airways. However, enhanced functions of eosinophils are also observed. The aim of this study was to reveal the mechanism of how eosinophils are activated during and after viral infection of the airways, using a model of viral infection.

A synthetic double-stranded RNA, poly inosinic-cytidyric acid (poly(IC)), was transfected to a human airway epithelial cell line (BEAS-2B) and the primary bronchial epithelial cells, to mimic a viral infection. The production of chemokines from the cells was investigated.

The transfection of poly(IC), alone, marginally affected the eotaxin-3 production of the cells. However, the transfection of poly(IC) prior to interleukin (IL)-4 stimulation enhanced eotaxin-3 production. Poly(IC) transfection increased mRNA and protein expressions of IL-4 receptor (R)α and IL-2Rγ, components of the IL-4R. In BEAS-2B cells, IL-4-mediated phosphorylation of signal transducer and activator of transcription six was enhanced in poly(IC) transfected cells. This was reversed by the addition of anti-IL-4Rα antibody, suggesting the role of an increased number of IL-4 receptors in enhanced IL-4-induced eotaxin-3 production. Poly(IC)-induced upregulation of IL-4Rα was inhibited by treatment with cycloheximide or dexamethasone.

In conclusion, these results suggest that viral airway infection may enhance interleukin-4-induced eotaxin-3 production through upregulation of the interleukin-4 receptor in airway epithelial cells.

KEYWORDS: Airway epithelial cells, double-stranded RNA, eotaxin-3, interleukin-4 receptor

Bronchial epithelial cells were previously considered to function merely as a barrier to the external environment. However, accumulating evidence has revealed that the cells participate in airway inflammation through the production of a wide range of mediators, including chemokines [1]. Eotaxins are a class of these chemokines. Three eotaxins have been demonstrated: eotaxin-1, -2 and -3, which are also referred to as chemokine ligand (CCL)11, CCL24 and CCL26, respectively [2–4]. These eotaxins show potent chemotaxogenic activity towards eosinophils and are, thus, believed to play an important role in the pathogenesis of bronchial asthma [5]. The combined stimulation of tumour necrosis factor-α and interleukin (IL)-4 has been observed to induce eotaxin-1 in cultured bronchial epithelial cells [6]. The cells also produce eotaxin-3 when stimulated with IL-4 or IL-13 [7]. A study by Berkman et al. [8] demonstrated an increased mRNA expression of eotaxin-3, but not eotaxin-1, in the airway of asthmatic subjects after an allergen challenge. This suggested the important role of eotaxin-3 in late-phase asthmatic response.

The exacerbation of bronchial asthma is often associated with viral airway infections, including rhinovirus (RV) and respiratory syncytial virus (RSV) [9, 10]. Bronchial epithelial cells have been reported to produce IL-8 and RANTES in response to RV infection [11, 12]. Thus, bronchial epithelial cells play an important role in recruiting neutrophils and T-lymphocytes into the airways during viral infection. Viral associated wheeze has been demonstrated to be characterised by neutrophilic inflammation in both the upper and lower respiratory tracts without eosinophilia [13]. However, there are several pieces of evidence which also demonstrate the involvement of other inflammatory cells, e.g. eosinophils in the airways during viral infection. The increased production of eotaxin-1 after viral infection has previously been demonstrated [14].
RV infection upregulates eotaxin-1 and eotaxin-2 expression in the bronchial epithelial cells [15]. Leukotriene $C_4$ within upper airway secretions in infants with RSV bronchiolitis is likely to originate from the eosinophils [16]. It has been suggested that eotaxin-3 is specifically involved in late-phase asthmatic response, facts which may not fully explain the exacerbation of asthma during or after viral airway infection. Currently, no information is available on whether eotaxin-3 is upregulated or not during viral infection. Thus, it was hypothesised by the current authors that viral airway infection may increase the eotaxin-3 production of the bronchial epithelial cells, and possibly result in the exacerbation of bronchial asthma in ongoing asthmatic patients.

As a direct result of this hypothesis the effects of double-stranded (ds) RNA on IL-4-induced eotaxin-3 production in cultured human bronchial epithelial cells, including primary cells, was investigated in vitro. The current work, presented in this paper, investigates the possibility that viral airway infections may trigger subsequent allergen-induced airway eosinophilia, in which the upregulation of the IL-4 receptor (IL-4R) by dsRNA plays an important role in the production of eotaxin-3 in airway epithelial cells.

**MATERIALS AND METHODS**

**Cells**
The human bronchial epithelial cell line BEAS-2B, was obtained from the American Type Culture Collection (Manassas, VA, USA). Primary cultured normal human bronchial epithelial (NHBE) cells were obtained from Clonetics (Walkersville, MD, USA). Both cells were cultured as previously described [17].

**Transfection of dsRNA to the cells**
BEAS-2B and NHBE cells ($4 \times 10^5$ well$^{-1}$) were seeded onto six-well plates 24 h before transfection. Transfection was carried out using Effectene reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instruction. In brief,graded

**TABLE 1**

| Direction | Primer sequence | Base pair (nt) |
|-----------|----------------|---------------|
| **Eotaxin-3** | F 5'-GGA ACT GCC ACA CGT GGG AGT GAC-3' | 354 |
|          | Re 5'-CTG TAG GAG GAA ACA CCC TCT CC-3' | 314 |
| **β-actin** | F 5'-TCC TGT GGC ATC CAG GAA ACT-3' | 327 |
|          | Re 5'-GATT TCT GCA GCT TGT GAA-3' | 258 |
| **RANTES** | F 5'-ATT TCT GCA GCT TGT GAA-3' | 348 |
|          | Re 5'-CTC TGT GGC ATC CAG GAA ACT-3' | 510 |
| **IL-8** | F 5'-ACA CCA ATG TCT CCG ACA CTC-3' | 687 |
|          | Re 5'-AGG TAT CAG GAC GAA CAC CAG G-3' | 105 |

**Stimulation of the cells**

To induce eotaxin-3 production, cells were stimulated with 50 ng·mL$^{-1}$ of either IL-4 or IL-13 (Peprotec, London, UK) for 24 h. At the time of transfection, 20 μg·mL$^{-1}$ of polyinosinic-polycytidylic acid (poly(I); Sigma-Aldrich), a synthetic dsDNA, or 100 ng·mL$^{-1}$ of polyinosinic acid (poly(I); Sigma-Aldrich), a synthetic single-stranded RNA, was used. Cells were incubated at 37°C for 24, 48 or 72 h depending on the experiment. After 24 h of transfection, the transfection reagent was washed by the cells with PBS.

**RT-PCR**
Total RNA was extracted from the cells as previously described [19]. RT-PCR was performed by the conventional method. Sequences of the primers are shown in table 1. PCR settings were as follows for eotaxin-3, β-actin, IL-4Rα, IL-2Rγ and IL-13Rα1: first denaturing at 94°C for 2 min, then denaturing at 94°C for 30 s, annealing at 58°C for 30 s, extending at 72°C for 30 s, and finally extension at 72°C for 2 min. RANTES and IL-8 were annealed at 56°C and 50°C, respectively. For eotaxin-3, 28 cycles of amplification were performed for semiquantitative comparison because the amount of PCR products reached a plateau after 32 cycles (data not shown). For RANTES, IL-8, β-actin, IL-4Rα, IL-2Rγ and IL-13Rα1, 32, 32, 22, 36, 38 and 30 cycles were performed, respectively. The PCR cycles were determined by densitometric analyses, as previously described [20].

**Measurement of protein release in culture medium**
RANTES, IL-8 and eotaxin-3 released into culture medium were measured by ELISA (BioSource, Camarillo, CA, USA, for RANTES and IL-8; R&D systems, Minneapolis, MN, USA, for eotaxin-3). The minimum detectable doses of RANTES, IL-8 and eotaxin-3 were 3.0 pg·mL$^{-1}$, 5.0 pg·mL$^{-1}$ and 2.3 pg·mL$^{-1}$, respectively.

**Flow cytometry**
Flow cytometry analyses for the detection of IL-4Rα and IL-2Rγ was performed as previously described [20].

**Immunoblotting**
Total cellular protein was prepared 24 h post transfection, as previously described [20]. Immunoblotting for signal transducer and activator of transcription (STAT6) and phosphorylated STAT6 was carried out using anti-STAT6 (#sc-621; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-p-STAT6 (#sc-11762-R; Santa Cruz), respectively, as previously described [20].
intotheculturemediumweredeterminedbyELISA. Resultsshownaremeans
for 24 h. The concentrations of a) RANTES, b) interleukin (IL)-8 and c) eotaxin-3 released

RESULTS
Effects of poly(IC) on the productions of chemokines
The effects of poly(IC) on the productions of chemokines from BEAS-2B cells were initially investigated. Poly(IC), but not poly(I), induced RANTES and IL-8 production, as previously described (fig. 1a and b) [21, 22]. A small, yet significant increase in eotaxin-3 production was observed when transfected with 1 ng·well\(^{-1}\) or 10 ng·well\(^{-1}\) of poly(IC) (fig. 1c). Dose-dependent enhancement of the mRNA expressions of RANTES and IL-8 was observed, although no apparent induction of eotaxin-3 mRNA expression was detected when amplified to 28 PCR cycles (fig. 1d; gel shown is representative of results from three experiments). Eotaxin-3 mRNA was detected when amplified to 32 PCR cycles. Although the abundance of eotaxin-3 mRNA in poly(IC) transfected cells (10 ng·well\(^{-1}\)) was greater than that observed in nontransfected cells, the difference was not statistically significant (p=0.07; data not shown).

Transfection of poly(IC) enhanced IL-4-induced eotaxin-3 production
Stimulation with IL-4 or IL-13 induces eotaxin-3 production in BEAS-2B cells [7, 17]. The effects of poly(IC) transfection on IL-4- or IL-13-induced eotaxin-3 production were also investigated. BEAS-2B cells were transfected with increasing doses of poly(IC), IL-4 (50 ng·mL\(^{-1}\)) stimulation was started simultaneously and incubation was for 24 h. No significant enhanced IL-4-induced eotaxin-3 production was observed (fig. 2a, white column). However, 48 h post-incubation with poly(IC) transfection, prior to IL-4 stimulation (50 ng·mL\(^{-1}\) for 24 h), allowed significant enhanced eotaxin-3 production in a dose-dependent manner (fig. 2a, grey column). Figure 2b shows the effects of 100 ng·well\(^{-1}\) of poly(IC) transfection on IL-4- or IL-13-induced eotaxin-3 production. Cells were incubated for 48 h, then stimulated with or without 50 ng·mL\(^{-1}\) of either IL-13 or -14 for 24 h. Small, yet significant enhanced IL-4-induced eotaxin-3 production was observed in the cells transfected with poly(I) when compared with those in the cells without transfection. The IL-4-induced eotaxin-3 production was dramatically enhanced in poly(IC) transfected cells. IL-13-induced eotaxin-3 production was also significantly increased in poly(IC) transfected cells; however, the increase was smaller than observed in IL-4-induced production (a 3.3-fold increase in IL-4-induced production versus a 1.2-fold increase in IL-13-induced production). The effect of poly(I) and poly(IC) transfection on IL-4-induced eotaxin-3 production in NHBE cells was also investigated (fig. 2c). In NHBE cells, poly(IC) transfection enhanced eotaxin-3 production (1.5-fold increase in poly(IC) transfected cells versus 1.1-fold increase in poly(I) transfected cells, p=0.07; data not shown).

Statistical analysis
Data are presented as means±SD. The significance of the differences was evaluated by ANOVA, and multiple pairwise comparisons were carried out with the Fischer test in the analysis of protein productions of RANTES, IL-8 and eotaxin-3, and in the analysis of mean fluorescence intensity values in flow cytometry experiments. In the experiments investigating IL-4Rx mRNA expression, data were analysed by ANOVA on ranks. A p-value of <0.05 was considered statistically significant.
production in NHBE cells, as previously described [18]. IL-4-induced eotaxin-3 production was significantly higher in poly(IC) transfected cells. In contrast, transfection of poly(I) had no significant effect (fig. 2c).

**Poly(IC) enhanced expression of IL-4R complex**

Receptor regulation is one of the most important mechanisms for determining the functions of the ligands, including cytokines. Therefore, the effect of dsRNA on the expression of components of IL-4R complexes was investigated. It was observed that the transfection of poly(IC) upregulated IL-4Rα mRNA expression, in a dose-dependent manner (fig. 3a). mRNA expression of IL-2Rγ (not observed before transfection) was induced by transfection of poly(IC) (fig. 3a). However, the transfection of poly(IC) had no apparent effect on IL-13Rα1 mRNA expression (fig. 3a). IL-4Rα and IL-2Rγ protein expressions at the surface of BEAS-2B cells were further investigated by flow cytometry. Transfection of poly(IC), followed by a 2-day incubation period was found to enhance both IL-4Rα and IL-2Rγ protein expressions at the cell surface when compared with those in the cells transfected with poly(I) (figs. 3b and c). Table 2 shows dose-dependent induction of IL-4Rα and IL-2Rγ protein expressions in BEAS-2B cells. Although transfection with poly(I) or poly(dIdC) also induced the increase in IL-4Rα protein expressions, the current authors postulated that it was the effect of the transfection reagent because the transfection reagent itself induced a significant increase in IL-4Rα protein expression. A time-dependent increase in IL-4Rα expression at the cell surface was also observed (fig. 3d).

A significant upregulation of protein expression of both IL-4Rα and IL-2Rγ at the cell surface by the transfection of poly(IC) was also observed in NHBE cells. The histograms in figures 3e and f show that NHBE cells include at least two cell populations. IL-4Rα and IL-2Rγ proteins were upregulated in both these populations by transfection with poly(IC). Upregulation of IL-4Rα and IL-2Rγ mRNA expression was also observed (data not shown).

**Cycloheximide inhibited poly(IC)-induced enhancement of IL-4R**

The effect of CHX, an inhibitor of protein synthesis, on IL-4Rα protein expression at the cell surface in BEAS-2B cells was investigated. When the cells were cultured with CHX, the constitutive expression of IL-4Rα at the cell surface was significantly inhibited. Poly(IC) transfection-induced IL-4Rα protein expression was also inhibited by CHX to a similar degree (fig. 4). These results indicate the role of de novo protein synthesis in IL-4Rα production by the transfection of poly(IC).

**Transfection of poly(IC) induced increased IL-4-mediated cellular signal**

To investigate whether increased expression of IL-4R results in increased IL-4-mediated signal, the effect of poly(IC) transfection on IL-4-induced activation of STAT6 in BEAS-2B cells was investigated. Both IL-4- and IL-13-induced phosphorylation of STAT6 were enhanced in poly(IC) transfected cells when compared with those in nontransfected cells (figures 5a and b). The addition of anti-IL-4Rα antibody inhibited IL-4-induced phosphorylation of STAT6 in poly(IC) transfected cells as well as in poly(I) transfected cells (fig. 5c). It was also observed that
anti-IL-4Rα completely inhibited IL-4-induced eotaxin-3 production in poly(IC) transfected cells (data not shown). These results suggest that IL-4-induced eotaxin-3 production was totally IL-4R dependent, and that poly(IC) increased the number of IL-4R at the cell surface, thus enhancing the IL-4-mediated signal in BEAS-2B cells.
The effects of DEX on the poly(IC)-induced increase of IL-4R expression

The effects of DEX on the poly(IC)-induced increase of IL-4R expression by DEX was observed (figs 6a and b). Flow cytometry analysis, which was performed 48 h after transfection, showed significant attenuation of poly(IC)-induced IL-4R expression by DEX in a dose-dependent manner (table 3). However, DEX had no effect on the constitutive or poly(I)-induced expression of IL-4Rα (table 3). Figure 6c shows the effect of DEX plus poly(IC) on IL-4-induced eotaxin-3 production. In spite of the removal of DEX by washing the cells 24 h before IL-4 stimulation, DEX treatment attenuated IL-4-induced eotaxin-3 production, both in the cells transfected with poly(I) and in the cells transfected with poly(C). However, there was no significant difference in eotaxin-3 production between the cells treated with DEX plus poly(I) and the cells treated with DEX plus poly(IC). These results suggest that DEX treatment attenuated poly(IC)-induced IL-4R expression, thus causing the attenuation of IL-4-induced eotaxin-3 production.

**DISCUSSION**

There has been increasing interest in the relationship between bronchial asthma and various viral infections, particularly RV and RSV [10, 23]. These viruses are often associated with the exacerbation of asthma, both in children and in adults [24–26]. RV RNA or transfection of synthetic dsRNA into an airway epithelium has been shown to induce the production of several chemokines, including IL-8 and RANTES, which contribute to the recruitment of neutrophils and activated lymphocytes into the airways [21, 27]. The current study also confirmed the induction of these chemokines by a synthetic dsRNA, poly(IC), in the bronchial epithelial cell line BEAS-2B. Thus, chemokines such as IL-8 and RANTES secreted from the airway epithelium are believed to play an important role in the exacerbation of bronchial asthma.

Data are presented as mean±s.d from three sets of data. Poly(I): polyinosinic-cytidylic acid; Poly(dIdC): poly deoxyinosinic-deoxycytidylic acid; Poly(IC): poly inosinic-cytidylic acid. #: control immunoglobulin (Ig)G; *: anti-IL-4Rα; #: anti-IL-2Rγ; #: p<0.01 compares the value in nontransfected cells labelled with anti-IL-4Rα or anti-IL-2Rγ; #: p<0.01 compares the value in poly(I) transfected cells.

**TABLE 2**

| Transfection                  | Mean fluorescence intensity | Cell number |
|-------------------------------|-----------------------------|-------------|
|                               | IL-4Rα                     | IL-2Rγ      |
| None                          | 3.21±0.20*                 | 8.01±0.19*  |
| None                          | 4.79±0.20*                 | 14.80±0.11+ |
| Effectene reagent             | 6.34±0.11*                 | 14.47±0.09  |
| Poly(I) 100 ng well-1         | 6.35±0.24*                 | 14.56±0.22  |
| Poly(dIdC) 100 ng well-1      | 6.21±0.64*                 | 13.89±0.46  |
| Poly(IC) 1 ng well-1          | 7.03±0.19+                 | 17.45±0.15+ |
| Poly(IC) 10 ng well-1         | 9.42±0.15+                 | 19.88±0.14+ |
| Poly(IC) 100 ng well-1        | 11.20±0.23+                | 24.47±0.48+ |

**Effects of glucocorticoid on poly(IC) induced IL-4Rα mRNA expression**

The effects of DEX on the poly(IC)-induced increase of IL-4Rα mRNA expression in BEAS-2B cells was investigated. An attenuation of poly(IC)-induced IL-4Rα mRNA expression by DEX was observed (figs 6a and b). Flow cytometry analysis, which was performed 48 h after transfection, showed significant attenuation of poly(IC)-induced IL-4Rα expression by DEX in a dose-dependent manner (table 3). However, DEX had no effect on the constitutive or poly(I)-induced expression of IL-4Rα (table 3). Figure 6c shows the effect of DEX plus poly(IC) on IL-4-induced eotaxin-3 production. In spite of the removal of DEX by washing the cells 24 h before IL-4 stimulation, DEX treatment attenuated IL-4-induced eotaxin-3 production, both in the cells transfected with poly(I) and in the cells transfected with poly(C). However, there was no significant difference in eotaxin-3 production between the cells treated with DEX plus poly(I) and the cells treated with DEX plus poly(IC). These results suggest that DEX treatment attenuated poly(IC)-induced IL-4R expression, thus causing the attenuation of IL-4-induced eotaxin-3 production.

**FIGURE 4.** Effect of cycloheximide (CHX) on poly inosinic-cytidylic acid (poly(IC))-induced interleukin (IL)-4 receptor (R)α expression at the cell surface in BEAS-2B. At the time of transfection of poly(IC), 20 μg·mL−1 of CHX was added (100 ng-well−1). After 48 h of culture, those cells were subjected to fluorescence-activated cell sorter analysis. □: constitutive IL-4Rα expression; ——: IL-4Rα expression in the cells transfected with poly(IC); ---: cells cultured with CHX after transfection of poly(IC); ———: cells cultured with CHX without transfection. The results presented are from one of two experiments which produced similar results.

In the current study, the question was postulated whether dsRNA treatment could induce eotaxin-3 production in bronchial epithelial cells. Eotaxins-1 and -2 were demonstrated to be upregulated in viral airway infection [15]. In BEAS-2B cells, a small amount of eotaxin-3 was induced by the transfection of poly(IC). However, eotaxin-3 was not induced in NHBE cells. Although it is still obscure, the viral infection itself might not have a potent effect on eotaxin-3 production in airway epithelial cells.

The current authors raised the question of whether an airway viral infection might enhance subsequent allergic response. Allergen challenge performed after RV inoculation showed a significantly greater increase in the nasal output of eosinophil peroxidase when compared with that performed before inoculation [28]. In the present study, dsRNA transfection prior to IL-4 stimulation enhanced eotaxin-3 production in both BEAS-2B and NHBE cells. Recently, it has been demonstrated that pre-treatment with interferon (IFN)-γ enhanced IL-4-induced eotaxin-3 production, though co-stimulation with IFN-γ and IL-4 suppressed eotaxin-3 production in BEAS-2B cells [20]. Since IFN-γ is abundantly produced in a virus-infected airway, the current authors speculated that viral airway infection might sensitise the airway epithelial cells and enhance allergen-induced response, which might result in airway eosinophilia [20]. These combined observations suggest that viral infections of the airway might trigger subsequent allergen-induced airway eosinophilia in ongoing asthmatic patients.

Airway epithelial cells express two types of IL-4 receptors, type 1 and type 2 IL-4Rs [17, 20, 29]. To the current authors' knowledge, this is the first study to report that the transfection of dsRNA enhances the expression of IL-4Rα and IL-2Rγ, components of type 1 IL-4R, in airway epithelial cells. IL-4Rα

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| Poly(IC) 100 ng well-1        | 11.20±0.23+                | 24.47±0.48+ |
protein has been demonstrated to be synthesised de novo. The results indicate that the enhanced IL-4-induced eotaxin-3 production in poly(IC) transfected cells was due to the upregulation of IL-4R expression at the cell surface. The results that poly(IC) enhanced IL-4R-mediated p-STAT6 generation, and that anti-IL-4R antibody inhibited p-STAT6 generation in the poly(IC) transfected cells as well as in poly(I) transfected cells, also support the increased number of IL-4R expression at the cell surface.

The current study demonstrates that the translation of poly(IC)-induced IL-4R expression of both mRNA and protein levels was attenuated by DEX. This is the first study
that demonstrates the regulation of IL-4Rα expression by glucocorticoid in airway epithelial cells, although DEX was demonstrated to inhibit IL-4-induced IL-4Rα upregulation by translational or post-translational mechanisms in isolated T- and B-lymphocytes [30]. Since the constitutive expression of IL-4Rα protein was observed not to be affected by DEX, DEX may interfere with transcription factors induced only by dsRNA, and not with those required for constitutive IL-4R gene expression. IL-4-induced eotaxin-3 production was attenuated by DEX treatment even in the cells transfected with poly(I). This may be inconsistent with the current observation that the constitutive expression of IL-4Rα protein was not affected by DEX. Since DEX itself strongly inhibits IL-4-induced eotaxin-3 production [17], the effect of DEX may still have remained at the time of IL-4 stimulation in this experiment. These results suggest that topical glucocorticoid therapy may influence the course of allergic and inflammatory processes during viral infection by downregulating the type 1 interleukin-4 receptor involved in the mechanism of increasing eotaxin-3 production. Topical steroid therapy might decrease virus-induced interleukin-4 receptor expression, and, thus, be effective for asthmatic patients. These results might explain the relationship between viral airway infections and the exacerbation of bronchial asthma. Since the current study is only a model of viral infection, which may not perfectly reflect in vivo interactions, further study is needed to confirm the results by using real viruses or an in vivo model of a viral infection.

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