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Original article

Cytokine secretion in nasal mucus of normal subjects and patients with allergic rhinitis

M.C. Scavuzzo [a,*], V. Rocchi [b], B. Fattori [c], F. Ambrogi [b], A. Carpi [d], R. Ruffoli [a], S. Manganelli [b], F. Giannessi [a]

[a] *Dipartimento di Morfologia Umana e Biologia Applicata, Università di Pisa, Via Roma 67, 56126 Pisa, Italy*
[b] *Unità Operativa di Immunoallergologia, Azienda Ospedaliera Pisana di S. Chiara, Pisa, Italy*
[c] *Dipartimento di Neuroscienze, 3ª Unità di ORL, Università di Pisa, Pisa, Italy*
[d] *Dipartimento di Medicina della Procreazione e dell'Età Evolutiva, Università di Pisa, Pisa, Italy*

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Abstract

Allergic rhinitis is regulated by the local production and release of several cytokines. The levels of Th2 cytokines IL-4, IL-6, IL-10 and the Th1 cytokine IFN-γ were studied in nasal mucus from 30 subjects with allergic rhinitis and 45 non-atopic healthy controls. In this study a sampling technique for collecting nasal mucus, well tolerated by the subjects and with a minimal stimulation of the mucosa, was performed. The cytokine concentrations in nasal mucus samples were detected and quantitated by a new paramagnetic particle-based immunofluorescent assay system more sensitive than the conventional ELISA techniques. The new technique showed reliable values of the measured parameters. The nasal mucus from allergic patients contained significantly higher concentrations of IL-4 (25.5 ± 3.6 pg/ml; $P < 0.001$) and IL-10 (1300 ± 190 pg/ml; $P < 0.05$) compared to the nasal mucus from control subjects (15.2 ± 2.3 and 532 ± 28 pg/ml, respectively, for IL-4 and IL-10). No significant modification in IFN-γ levels of allergic patients was found when compared to control group (respectively, 19.9 ± 3.3 vs. 25.7 ± 5.1 pg/ml; $P > 0.05$). Moreover, the allergic patients showed lower levels of IL-6 concentrations in the nasal mucus compared to control subjects (64.8 ± 9.1 vs. 129.0 ± 18.1 pg/ml; $P = 0.0099$). These data can be interpreted by the hypothesis that in response to environmental allergens there is a preferential Th2 polarity by activated CD4+ T cells and that the cytokines IL-6 and IL-10 have, respectively, an important anti-inflammatory and counterregulatory action in the pathogenesis of allergic rhinitis.

Keywords: Allergic rhinitis; Cytokines; Nasal mucus

1. Introduction

Allergic rhinitis is characterized by the development of nasal mucosal inflammation in response to natural allergen exposure. Inflammatory allergic disorders are characterized by the production of numerous cytokines and chemokines by activated cells present in target tissues, including T cells, mast cells, macrophages and eosinophils. Moreover, allergic inflammation is associated with a shift in the balance between cytokines produced by Th1 and Th2 cells toward a Th2 predominance [25]. The allergen induces Th2 lymphocyte proliferation with the release of characteristic combination of cytokines such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and granulocyte–macrophage colony-stimulating factor (GM-CSF). Th1 cells produce a different cytokine profile characterized by IFN-γ secretion.

IL-4 cytokine appears to be an essential requirement for IgE production and IL-4 production is critical for the development of Th2 cells [12]. Inside, IFN-γ inhibits IgE production and plays a negative regulatory role in the Th2 cell development [12]. However, the mechanisms underlying the preferential activation of Th2 cells by environmental allergens in atopic individuals still remain unclear.

IL-6 is a pleiotropic cytokine with multiple effects during the course of a wide variety of infectious and inflammatory disorders [33,39]. In addition, IL-6 is released in the late phase of the allergic response [2] and an increased production in asthma of this cytokine is documented [7,33]. However, the role of IL-6 in inflammatory reaction remains unde-

* Corresponding author. V. F.lli Kennedy, 27, Casciavola 56123, Pisa, Italy.
E-mail address: m.scavuzzo@int.med.unipi.it (M.C. Scavuzzo).

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fined although this moiety has important immunoregulatory properties [16].

The Th2 IL-10 is an anti-inflammatory cytokine and its major effect is to attenuate the release of pro-inflammatory cytokines [34], allergen-induced airway inflammation and non-specific airway responsiveness [29].

A number of recent studies on nasal mucus samples suggest that the level of IL-4 increases in allergic rhinitis [4,5,23]. On the contrary, the IFN-γ production is reduced in nasal mucus of allergic rhinitis [3] and in peripheral T cells of asthmatic patients [35]. However, in literature there are discordant data concerning cytokine levels both of IL-6 and IL-10 in allergic rhinitis [13,15], in allergic and asthmatic school-age children [21], and after mucosa allergen provocation [17].

The aim of the study was to evaluate the use of a relatively new technique—the ImmunoFlow-IFA (BioErgonomics, St. Paul, MN, USA) flow cytometry-based immunofluorescent assay kits—more sensitive than currently used ELISA methods, for measurement of cytokines IL-4, IL-6, IL-10 and IFN-γ in the nasal mucus and to determine their concentration in atopic rhinitis patients.

2. Materials and methods

2.1. Subjects

Thirty subjects (18 males and 12 females) aged between 18 and 51 years (mean age 32.1 ± S.D. 8.9) suffering from allergic rhinitis were selected and involved in the study. The patients presented a personal history of allergy and a positive skin prick tests (SPT) (Lofarma®, Milan, Italy) for a panel of the following allergens: rye grass, birch, cypress, hazel-tree, composite mix, ragweed, olive-tree, *Alternaria tenuis*, house dust mite, mould mite, *Blattella germanica, Periplaneta americana*, cat and dog epithelial dander and latex. Histamine dihydrochloride solution at 1 mg/ml as control positive and physiological solution as negative was used. Reactions were read after 15 min and a test was considered positive if the diameter of the wheal exceeded the negative control by more than 3 mm. The SPTs gave the following results: five subjects positive for rye grass; three for olive-tree and rye grass; three for cypress; one for *Alternaria tenuis*; one for cypress, hazel-tree and birch; five for house dust mite; three for ragweed; nine polisensitized to more than three allergens.

Smokers, subjects exposed to professional irritative substances, subjects with infective rhino-sinusitis, or recent upper respiratory tract infections, or other systemic illness were excluded from the study.

The use of local or systemic corticosteroids or antihistamines was stopped at least 4 weeks before the collection of mucus; depot steroid therapy was stopped at least 6 weeks before the collection of mucus. Acetyl salicylic acid intolerance was absent in the history of the patients.

Forty-five subjects (20 males and 25 females) aged between 29 and 41 years (mean age 34.1 ± S.D. 4.8) were recruited as control group. These subjects were healthy, non-smokers, with no previous respiratory pathology and had never used any topic or systemic therapy in the last 4 weeks before the collection of mucus.

The purpose of the study was described to the subjects and their informed consent was obtained. The Medical Ethics Committee of the University Hospital of Pisa approved the protocol.

2.2. IgE levels

Serum total IgE levels were assessed by a nephelometric method (Behring, Marburg, Germany). Specific IgE levels were determinated by CAP-FEIA, according to the manufacturer's instructions (Pharmacia®, Uppsala, Sweden) and results of CAP class 2 (0.7–3.5 kU/l) or more were considered positive.

2.3. Collection of nasal mucus

The collection of the nasal mucus samples was carried out according to the method of Ruocco et al. [26], which is well tolerated by the subjects and induces a minimal stimulation of the mucosa. The nasal mucus was collected in patients with ongoing nasal symptoms and in control group. Each sample was obtained using a square (1 × 1 cm) of sterile pre-humidified (10 μl of PBS) gauze, which was gently placed for 10 s between the inferior turbinate and the nasal septum. Two specimens were taken from each nasal fossa in all subjects. The pieces of gauzes imbibed with nasal mucus were then placed in a plastic tip and put into a test tube in which 1000 μl of PBS at pH 7.4 were added. The mucus was eluted by centrifugation at 2000 rpm for 20 min at 4 °C. The mucus samples were then collected in plastic tubes and stored at –20 °C until tested for cytokine quantification.

2.4. Quantification of cytokine secretion

The human cytokines IL-4, IL-6, IL-10 and IFN-γ were detected and quantitated by the ImmunoFlow-IFA (BioErgonomics) flow cytometry-based immunofluorescent assay kits [9], which use captured particles with distinct light scatter characteristics and permit analysis with a sensitivity in the femtomolar range.

Cytokine capture beads (10 μl) were incubated in 500 μl of sample for 60 min at room temperature with nutritional mixing. The beads were then washed twice with 2 ml of IFA buffer and vortexed to resuspend them. The fluorescent-labeled reporter antibody (10 μl) coupled to phycoerythrin (PE) was then incubated with the capture beads for 30 min at room temperature with nutation. The beads were washed with IFA buffer and are then resuspended in 500 μl of PBS for flow cytometric analysis.

Standard curves were developed in the range 20 ng/ml–2 pg/ml for IFN-γ and 5 ng/ml–500 fg/ml for IL-4, IL-6 and IL-10. Mean channel fluorescence of the resultant beads was compared to a standard curve developed from the mean

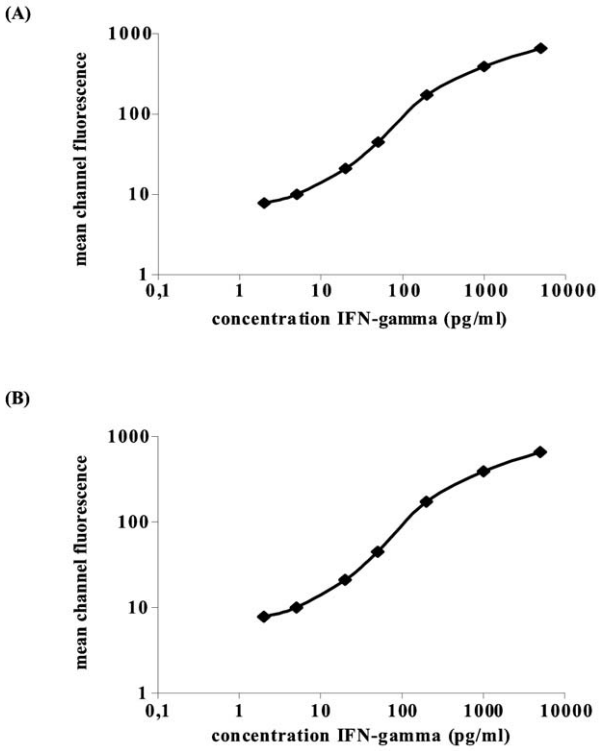

Fig. 1. Standard curve plotted as concentration vs. mean channel fluorescence (log vs. log) by ImmunoFlow-IFA (BioErgonomics) flow cytometry-based immunofluorescent assay kits. (A) Standard curves for IL-4, IL-6, IL-10; (B) standard curve for IFN-γ.

channel fluorescence of similarly treated capture beads incubated with pre-diluted standards composed of known concentrations of cytokine included with the kits. Four standard curves are thus obtainable from one set of calibrators (four results obtained from each calibration point shown in Fig. 1).

2.5. Flow cytometric analysis

Flow cytometric analysis was performed by using a FACScan flow cytometer (Becton Dickinson Labware, Bedford, MA, USA). Data were acquired and analyzed using the CellQuest software (Becton Dickinson). Polystyrene spheres were used for the calibration and the normalization of the flow cytometry each time; 10 000 events were recorded from each sample. Forward versus side scatter gating was employed to exclude doublets and irregularly shaped beads. Data were displayed as FL2 histograms using log amplification on the PE channel. The standard curve was developed by plotting the mean channel fluorescence of each standard versus cytokine calibrator concentration adopting the logarithmic scale. The concentration of cytokine secretion was determined by comparing mean channel of the fluorescence peak of the samples to the standard curve.

2.6. Statistical analyses

The statistical analysis was performed with Stat view SE + Graphics™ for Macintosh.

The Wilcoxon sign-rank test was used for a comparison between the control group and the atopic patients. A confidence level of 95% was adopted to assess the statistical significance of the results. All data are presented as mean ± S.E.M.

3. Results

3.1. Standard curves of flow cytometry-based immunofluorescent assay kits

The representative standard curves for IL-4, IL-6, IL-10 (Fig. 1A) and IFN-γ (Fig. 1B) and the same data plotted as the mean channel fluorescence of each peak versus the concentration of the cytokine standard are shown in Fig. 1. The technique used in this study to detect and to quantitate cytokine secretion in nasal mucus is different from the usual method based on the well-known enzyme-linked immunoabsorbant assay (ELISA). ImmunoFlow-IFA (BioErgonomics) flow cytometry-based immunofluorescent assay kits measured the human cytokines. The flow cytometry-based immunoassay systems use monoclonal antibody-coated paramagnetic particles, which are added directly to the nasal mucus to capture the specific cytokine, and fluorochrome-conjugated monoclonal antibodies to report the presence of the captured cytokine. The total time of the assay is 120 min. Standard curve was developed using pre-diluted cytokine standards included with the kit and the concentration of the cytokine in the nasal mucus was directly proportional to the mean channel fluorescence of the particle. The mean channel fluorescence of unknown samples was compared to the standard curve to determine concentrations.

The performance of the ImmunoFlow-IFA flow cytometry-based immunofluorescent assay kits for all four cytokines was previously evaluated by Collins [9] using cell culture supernatants, but not on nasal mucus sample. The current ELISA technology is a 4–5 h assay with a range of detection of 2000–15 pg/ml. High sensitivity ELISA assays have a narrow range of analysis of 20–0.5 pg/ml and may require overnight incubation to achieve that level of sensitivity. The typical ImmunoFlow-IFA assay requires a total time of 120 min and has a range of analysis of 5000–0.5 pg/ml and the IFN-γ assay has an upper limit of 20 000 pg/ml.

3.2. IgE levels

The total serum IgE levels ranged between 50 and 425 kU/l in allergic rhinitis patients (mean: 226.6 ± 103.5 kU/l S.D.; $P < 0.0001$) and between 17 and 110 kU/l in controls (mean: 55.5 ± 35.1 kU/l).

3.3. Nasal mucus cytokine determination

The nasal mucus levels of IL-4, IL-6, IL-10 and IFN-γ were measured in non-allergic donors and atopic patients. The concentrations of the studied cytokines were expressed as pg/ml and their average levels were shown in Table 1.

Table 1
Total IgE levels in serum, and IL-4, IFN-γ, IL-10 and IL-6 levels in nasal fluids from patients with allergic rhinitis and control subjects

Subjects	IgE (kU/l)	IL-4 (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
Allergic rhinitis patients ($n = 30$)	226.6 ± 103.5	25.5 ± 3.6	19.9 ± 3.3	1300 ± 190	64.8 ± 9.1
Controls ($n = 45$)	55.5 ± 35.1	15.2 ± 2.3	25.7 ± 5.1	532 ± 28	129.0 ± 18.1
P	***	*	>0.05	**	**

Data are expressed as mean ± S.E.M. of three independent experiments. The Wilcoxon sign-rank test was performed comparing atopic patients with controls. * $P < 0.01$; ** $P < 0.05$; *** $P < 0.0001$.

The nasal mucus from allergic rhinitis patients contained significantly higher concentrations of IL-4 (25.5 ± 3.6 pg/ml; $P < 0.01$) and IL-10 (1300 ± 190 pg/ml; $P < 0.05$) compared to the nasal mucus from control subjects (15.2 ± 2.3 and 532 ± 28 pg/ml, respectively, for IL-4 and IL-10).

Data in Table 1 show that there were no significant differences in IFN-γ concentration between the two groups of subjects. However, a slight trend towards decreased IFN-γ secretion was found in the allergic rhinitis subjects (19.9 ± 3.3 vs. 25.7 ± 5.1 pg/ml; $P > 0.05$). In addition, IL-6 levels in allergic patients resulted to be significantly lower compared to control subjects (64.8 ± 9.1 vs. 129.0 pg/ml; $P = 0.0099$).

4. Discussion and conclusions

We tested a new quantitative flow cytometry detection assay for measuring cytokine secretion in nasal mucus of normal subjects and patients with allergic rhinitis. Therefore, we have shown for the first time that it is possible to measure cytokine concentrations from human nasal mucus using multiplexing abilities of the flow cytometry. The flow cytometers are multiparametric analyzers that allow easy multiplexing of assays by isolation on the basis of differences in light-scatter or fluorescence characteristics and show a sensitivity in the femtomolar range. As reported by Collins [9], this new methodological approach for measuring cytokine secretion in nasal mucus shows to have more sensitivity, wider range of analysis and to require a shorter duration of assay when compared to the conventional ELISA techniques.

We compared the levels of IL-4, IL-6, IL-10 and IFN-γ in the nasal mucus of allergic rhinitis patients versus a control group by the collection method of Ruocco et al. [26]. We found higher levels of IL-4 in the nasal fluid of allergic rhinitis patients compared to controls, in agreement with previous studies performed in adults and children [5,22,23,30,37].

Then, in the IgE mediated response, the central role of IL-4 was sustained like an increase of B cell proliferation, IgE switching, MHC II molecule expression, Th2 lymphocyte differentiation which facilitates proliferation and activity of eosinophils and mast cells [31]. Moreover, in allergic disorders the Th2 cells predominate over Th1 cells and differentiate to produce IL-4, but not IFN-γ. Our data seem to be in agreement with this dichotomy, defined Th1/Th2 paradigm [14,25,32,36], in fact the level of IFN-γ is higher in the control subjects than in patients. However, this result is not statistically significant at the adopted confidential level.

It is particularly interesting that IL-10 concentrations in nasal mucus were significantly lower in non-atopic than in allergic subjects. This result is consistent with recent reports indicating an increase of IL-10 in plasma levels of atopic asthmatic patients after challenge with specific allergen [21,36], in broncho-alveolar lavage fluid of atopic [19] and asthmatic patients [24], in peripheral lymphocyte culture of allergic subjects [20], in nasal fluids both of subjects during seasonal rhinoconjunctivitis [5] and after challenge with specific allergen [17]. It is worth noticing, however, that some authors found a decrease of IL-10 levels in tears [10], nasal fluid [11], plasma [15] of atopic subjects compared to control group.

We also found that IL-6 levels in rhinitis allergic subjects were significantly lower than in the control group. IL-6 is considered an important pro-inflammatory cytokine, with a key role in the pathogenesis of local inflammation during viral infection of upper airways [27]. However, discordant data have been published so far; some authors did not detect any difference of IL-6 concentrations between atopic patients and healthy subjects [13,21], while others found a significant increase of this cytokine in chronic sinusitis and allergic rhinitis [5]. A possible explanation of our result could be related to double effect of IL-4, because this cytokine has pro-inflammatory effects, which down-regulate IL-6 production [1]. Moreover, IL-10 produced by both Th2 and Th1 lymphocytes, as well as mononuclear phagocytic cells, B lymphocytes and mast cells, inhibits the secretion of IL-6, IFN-γ, IL-2, IL-4, IL-5, TNF-α, IL-1β, IL-8 and IL-12. Thus, IL-10 could be considered a determinant factor for allergic inflammation [18], because it could mitigate allergic inflammation by its ability to inhibit IgE production, reduce eosinophil survival and induce allergen-specific tolerance [8]. The counterregulatory activity of IL-10 could be explained with its delayed presence (at least 8 h after allergic stimulation, with a peak between 12 and 24 h) compared to IL-1, IL-2, IL-4, IFN-γ, which are detectable 15–30 min after the stimulation. Then, IL-10 could be a regulator of the immune response, where inflammatory stimulus induces the production of pro-inflammatory cytokine, which facilitates IL-10 release to reduce the reaction course [6].

Some authors found a reduction of IL-10 concentration in the fluid of broncho-alveolar lavage of asthmatic subjects and proposed that a deficiency of anti-inflammatory activity could be attributed to the increase of pro-inflammatory cytokine secretion involving the worsening of disease [28]. Moreover, some studies documented an IL-6 anti-inflammatory activity. For instance, Xing et al. [38] recorded

a significant increase of the mortality and pro-inflammatory cytokines (TNF-α and IL-1) after a challenge with endotoxin in mice depleted of IL-6 gene. In these mice, the lack of IL-6 did not interfere on the concentrations of anti-inflammatory cytokines, particularly of IL-10. The authors concluded that IL-6 is an anti-inflammatory cytokine, necessary to control local flogistic response both acute and systemic. A recent work [35] confirms previous result, accounting specifically for Th2 response. The authors demonstrated that the lack of IL-6 gene implied an allergen phlogosis induced by Th2 in mice exposed to allergen by inhalation. This phlogosis was characterized by eosinophilia both in tissue and broncho-alveolar lavage, by production of IL-4, IL-5, IL-13 and chemokines and by bronchial hyperactivity with methacoline. Therefore, the IL-6 inhibitory action may be explained either as the result of a negative effect on production of macrophage pro-inflammatory cytokine, or as an increase of synthesis of anti-inflammatory molecules like antagonist of IL-1r, or else as a production of anti-proteinases.

Therefore, we conclude that in the pathogenesis of allergic rhinitis, IL-6 and IL-10 seem to have, respectively, important anti-inflammatory and counterregulatory actions during allergic inflammation. However, the role of IL-6 and IL-10 has not been fully elucidated in the development of allergic rhinitis and further studies are necessary to understand their role.

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