In vitro effects of fluticasone propionate on IL-13 production by mitogen-stimulated lymphocytes

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Background: Corticosteroid administration produces multiple immunomodulatory effects, including down-regulation of cytokine production by CD4 T lymphocytes. Fluticasone propionate (FP) (Glaxo Smith&Kline, Greenford, UK), a highly lipophilic topical corticosteroid, has been shown to be safe and effective in the treatment of asthma and of both seasonal and perennial rhinitis.

Aims: To gain insight into the mechanisms of FP therapeutic effects, we evaluated interleukin (IL)-13 (a type 2 cytokine that seemingly plays a pivotal role in allergic mechanisms) production by mitogen-stimulated peripheral blood mononuclear cells (MNC) *in vitro*, treated or not with FP.

Methods: MNC from 10 healthy subjects and 10 asthmatic atopic patients with Parietaria allergy were stimulated v/v with phytohaemagglutinin (PHA) (50 γ/ml) or with complete medium alone as a control. Culture supernatants, *in vitro* treated or not with 10^{-7} or 10^{-8} M FP, were collected after 48 or 72 h incubation. IL-13 production was assessed by enzyme-linked immunosorbent assay. In random selected samples, after 4 or 24 h of cell cultures, RNA was extracted and IL-4 and IL-5 reverse transcriptase-polymerase chain reaction (RT-PCR) products analyzed.

Results: At 48 h, there were no differences in IL-13 concentration in PHA-stimulated cultures between healthy subjects and asthmatic patients (93.6 ± 18.9 versus 111.0 ± 25.1 pg/ml). At 72 h, similar results were obtained (63.9 ± 3.0 versus 73.3 ± 2.5 pg/ml, respectively). At this time, however, IL-13 concentrations were significantly decreased versus 48 h both in asthmatics ($p < 0.001$) and in controls ($p < 0.001$). Treatment with 10^{-7} M FP significantly reduced IL-13 production in healthy subjects and asthmatic patients both at 48 h (93.6 ± 18.9 versus 50.50 ± 10.6 pg/ml, $p < 0.001$, and 111.0 ± 25.1 versus 59.3 ± 13.6 pg/ml, $p < 0.001$, respectively) and at 72 h (63.9 ± 9.6 versus 35.5 ± 4.4 pg/ml, $p < 0.001$, and 73.3 ± 8.0 versus 40.7 ± 4.5 pg/ml, $p < 0.001$, respectively). Similar results were obtained with 10^{-8} M FP at 48 and 72 h. Accordingly, evaluation of RT-PCR products from selected cell samples showed a FP dosage-dependent inhibition of IL-4 and IL-5 mRNA production both for healthy subjects and asthmatic patients.

Conclusions: FP *in vitro* impairs IL-13 production by PHA-stimulated MNC from asthmatic and control subjects. This strengthens previous suggestions that IL-13 inhibition by steroids may, at least in part, account for their therapeutic effects.

Key words: Bronchial asthma, Fluticasone propionate, IL-13, Mitogens, Type 2 cytokines

Introduction

Corticosteroids are potent anti-inflammatory agents widely used in the management of asthma and other allergic diseases. Corticosteroid administration produces multiple immunomodulatory effects, including down-regulation of cytokine production by CD4 T lymphocytes.[1] Cytokines produced by lymphocytes are classified in two types depending on their pattern of immune response regulation. Type 1 cytokines include interleukin (IL)-2, interferon-γ and tumor necrosis factor-β, which mediate immune responses

against intracellular pathogens and promote delayed-type hypersensitivity.[2] Type 2 cytokines include IL-4, IL-5, IL-10, and IL-13, which mediate immune responses against extracellular pathogens and regulate allergic inflammation by promoting immunoglobulin E (IgE) class-switching and eosinophil activation.[2] Although IL-4 has been considered the cytokine most critical for allergic responses and for immunity against parasites, recent observations indicate that IL-13, a related cytokine, has equal or even greater importance than IL-4 in allergic inflammatory responses and host protection against parasites.[3] IL-4 and IL-13 share several functional properties; however, IL-13 can independently induce class switching and IgE secretion from human B cells. In addition, IL-13 enhances expression of CD23 and of the major histocompatibility complex class II antigens, and it may act as a monocyte chemoattractant.[4-6] Therefore, reduction of IL-13 production might be a key component of anti-inflammatory drug design.

Fluticasone propionate (FP), a highly lipophilic topical corticosteroid (Glaxo Smith&Kline, UK), has been shown to be safe and effective in the treatment of asthma and of both seasonal and perennial rhinitis. When taken by the inhaled route, FP exerts a significant beneficial effect on asthma. Moreover, FP had a higher therapeutic score than other inhaled corticosteroids. Several studies have demonstrated that FP has increased intrinsic glucocorticoid potency and high topical anti-inflammatory activity. Clinical studies showed that FP had no significant adverse effects, including effects on plasma cortisol concentrations.[7-9] To date, no studies have been performed on the *in vitro* effect of FP on the production of IL-13. Thus, to investigate the mechanisms underlying the therapeutic effects of FP, we evaluated IL-13 production by peripheral blood mononuclear cells (MNC) stimulated with phytohemagglutinin (PHA), treated or not with FP.

Materials and methods

Subjects

After informed consent was obtained, peripheral blood was withdrawn from healthy subjects (laboratory staff, $n = 10$) and from asthmatic atopic patients ($n = 10$) with Parietaria allergy. Allergic asthmatic patients had a history of more than 2 years of symptoms consistent with asthma, and a methacholine PC_{20} of 16 mg/ml or less.[10] Serum-specific IgE and skin prick tests were performed and evaluated exactly as previously described elsewhere.[10,11]

Fluticasone propionate

Pure FP was obtained from Glaxo Smith&Kline (Verona, Italy) and dissolved in anhydrous ethanol (95%) at a concentration of 10^{-3} M. It was then diluted at concentration of 10^{-7} or 10^{-8} M in complete medium (i.e. RPMI-1640) (GIBCO, Gran Island, NY, USA) supplemented with 10% heat-inactivated pool of AB sera (AB Pool; Flow Laboratories, Irvine, UK), glutamine (Flow), gentamycin, streptomycin and Hepes buffer (Boeringer Mannehim, Mannheim, Germany). Control medium was prepared using the same dilution of ethanol.[11]

Cell cultures and IL-13 assay

MNC from healthy volunteers and asthmatic atopic subjects were isolated by centrifugation on Ficoll Hipaque gradient (Nycomed As, Diagnostic, Oslo. Norway), washed twice in complete medium, and 5 $\times 10^6$/ml cells were stimulated v/v with PHA (50 γ/ml) (Sigma, Milan, Italy) or, as a control, with complete medium alone. These PHA doses were used because, in our hands, they have been demonstrated to be optimal ones.[12] Culture supernatants, with and without 10^{-7} or 10^{-8} M FP, were collected after an incubation of 48 or 72 h and stored at –70°C until assayed. IL-13 production was assessed by enzyme-linked immunosorbent assay with Quantikine™ immunoassay kits (R&D System Inc, Minneapolis, MN, USA) according to the manufacturer's instructions. All standards and samples were tested in duplicate. IL-13 results were calculated as picograms per millilitre. The detection limit in our laboratory was 32 pg/ml.

RNA extraction and mRNA synthesis evaluation

In random selected samples, after 4 or 24 h of cell cultures, RNA was extracted using the method described by Gough.[13] IL-4 and IL-5 reverse transcriptase-polymerase chain reaction (RT-PCR) products (294 pb for IL-5 and 344 pb for IL-4) were detected by electrophoresis on 2% agarose and analysed by 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM) for convenience of presentation. Because the distribution of data was not normally distributed, non-parametric tests were used for Statistical Analysis. The Wilcoxon matched pairs test was used to compare intragroup differences of IL-13, and the Mann–Whitney U-test was used to compare intergroup differences of IL-13. All analyses were performed using StatsDirect (Tidestone Technologies Inc, Cambridge, UK) on a PC. $p \leq 0.05$ was considered significant.

Results

IL-13 production by stimulated cultures treated or not treated with FP from asthmatic and healthy individuals was evaluated both at 48 and at 72 h (Fig. 1a,b).

IL-13 was detected in both healthy subjects and asthmatic patients at 48 and 72 h. At 48 h, the values of PHA-stimulated cultures did not differ between the two groups (93.6 ± 6.0 versus 111.0 ± 7.9 pg/ml, respectively). Similar results were obtained at 72 h (63.9 ± 3.0 versus 73.3 ± 2.5 pg/ml, respectively). The lack of difference in IL-13 production between healthy subjects and asthmatic patients was probably due to the small samples of subjects under study. However, 72 h IL-13 concentrations were significantly decreased when compared with 48 h concentrations in healthy subjects and in asthmatic patients ($p < 0.001$ for both).

Treatment of cultures with 10^{-7} M FP significantly reduced IL-13 production in healthy subjects and in asthmatic patients both at 48 h (93.6 ± 6.0 versus 50.50 ± 3.3 pg/ml, $p < 0.001$, and 111.0 ± 7.9 versus 59.3 ± 4.3 pg/ml, $p < 0.001$, respectively) and at 72 h (63.9 ± 3.0 versus 35.5 ± 1.6 pg/ml, $p < 0.001$, and 73.3 ± 2.5 versus 40.7 ± 1.4 pg/ml, $p < 0.001$, respectively). Similar results were obtained with 10^{-8} M FP in healthy subjects and in asthmatic patients both at 48 h (93.6 ± 6.0 versus 59.5 ± 3.8 pg/ml, $p < 0.001$, and 111.0 ± 7.9 versus 70.5 ± 5.1 pg/ml, $p < 0.001$, respectively) and at 72 h (63.9 ± 3.0 versus 40.1 ± 1.6 pg/ml, $p < 0.001$, and 73.3 ± 2.5 versus 45.5 ± 1.5 pg/ml, $p < 0.001$, respectively).

In preliminary experiments, cells from 4 h (or 24 h) stimulated cultures treated or not treated with FP from healthy subjects and asthmatic patients were collected to measure, respectively, IL-4 and IL-5 mRNA. Evaluation of RT-PCR products from cells cultured in presence of FP (10^{-7} and 10^{-8} M) showed a dosage-dependent inhibition of IL-4 and IL-5 mRNA production both for healthy subjects and asthmatic patients.
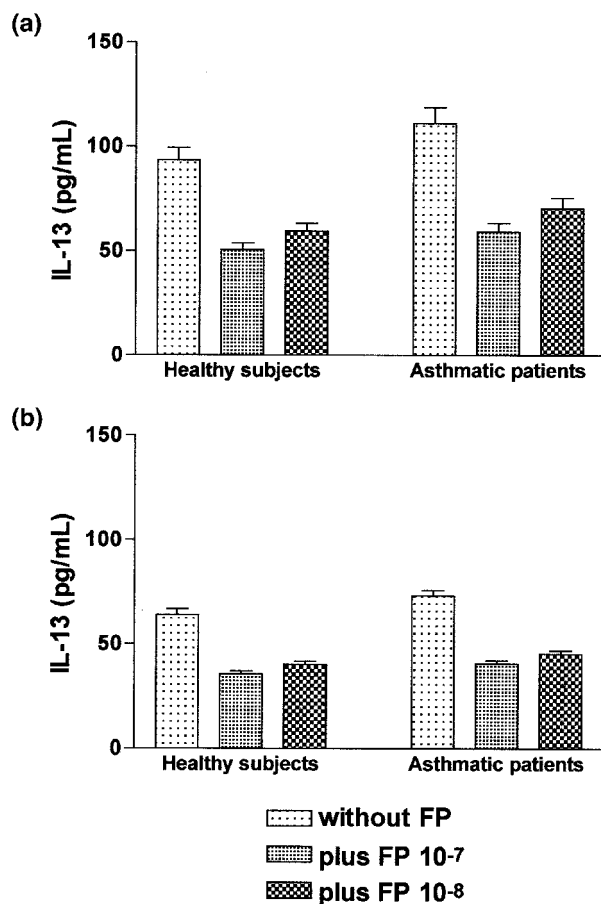

FIG. 1. (a) IL-13 levels in supernatants of PHA-stimulated 48 h cultures from healthy subjects ($n = 10$) and asthmatic patients ($n = 10$). Cultures were treated with 10^{-7} M FP, 10^{-8} M FP or control medium. Results are expressed as mean ± SEM of individual values (pg/ml). There were no significant differences between values of the PHA-stimulated cultures from the two groups of individuals. Treatment with 10^{-7} M FP significantly reduced the IL-13 production of both groups of subjects. The same results were obtained with 10^{-8} M FP. (b) IL-13 levels in supernatants of PHA-stimulated 72 h cultures from healthy subjects ($n = 10$) and asthmatic patients ($n = 10$). Cultures were treated with 10^{-7} M FP, 10^{-8} M FP or control medium. Results are expressed as mean ±SEM of individual values (pg/ml). There were no significant differences between values of the PHA-stimulated cultures from the two groups of individuals. Treatment of cultures with 10^{-7} M FP significantly reduced the IL-13 production of both groups of subjects.

Discussion

The local production and release of a number of cytokines regulate allergic upper airway inflammation. In particular, a large body of evidence indicates that IL-13 may play a crucial role in the inflammatory reaction.[3–5,14] IL-13 shares many functional similarities with IL-4, including the capacity to induce immunoglobulin isotype switching to IgE in B cells. IL-13 also causes *de novo* surface expression of the vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells of the umbilical vein and accelerates selective eosinophil migration. Interestingly, IL-13 up-regulates VCAM-1 expression in human mucosal microvascular endothelial cells, to which eosinophils adhere in bronchial and nasal mucosa. Finally, IL-13 concentrations are strongly correlated with eosinophil numbers in bronchoalveolar lavage after allergen challenge.[4,5,14] Consequently, it is not inconceivable that IL-13 is important in the pathogenesis of such eosinophil-related inflammatory disorders as nasal allergy and bronchial asthma.

Corticosteroids have proven efficacy in the treatment of allergic respiratory disorders and, regularly administered, they inhibit both the immediate and late response to allergen provocation. They exert an effect on a number of cells involved in the allergic inflammatory reaction, including down-regulation of

cytokine production, including IL-13,[1,6–9,14,15] which may explain their potent effect in clinical allergy studies. However, topical steroids control the inflammatory cell influx that increases the potential for leukotriene generation responsible for clinical symptoms.[1]

Recently, we investigated the effects of intranasal FP on subjective symptoms and on pathophysiological mechanisms in rhinitis patients monosensitive to Parietaria during the pollen season. The concentrations of eosinophils and inflammatory mediators in nasal lavages and subjective symptoms decreased significantly following FP treatment. The inhibition by FP of mast cells, eosinophils and neutrophils was clearly demonstrated by the reduced level of their mediators in nasal lavage fluid (i.e. tryptase, eosinophil cationic protein, eosinophil protein X, and neutrophil myeloperoxidase). Similarly, flow cytometric studies have shown that FP decreases the influx of total and activated eosinophils in rhinitis patients. Thus, we demonstrated that FP could influence both the cells of the early phase and those of the delayed phase of the allergic reaction.[8] In a further study, we demonstrated that *in vitro* treatment with FP impaired natural killer and lymphokine-induced killer activity in asthmatic and healthy individuals, suggesting a key role in this process for cytokine impairment by the drug.[11]

In a previous study, the expression of IL-13 mRNA and immunoreactivity in nasal biopsies from 10 normal subjects and 20 subjects with allergic rhinitis was investigated. After allergen provocation, a significant increase in IL-13 mRNA-positive and immunoreactive cells at 24 h only in subjects given placebo was observed. Thus, inhibition of the late phase after corticosteroid treatment was associated with a marked decrease in allergen-induced IL-13 mRNA-positive and immunoreactive cells. This study suggests that IL-13 expression is a prominent feature of the late phase in the nose and that inhibition of the late phase by steroid therapy might be partly attributable to inhibition of IL-13 expression.[14] In nasal polyp disease, intranasal fluticasone also reduced the number of IL-4 and IL-13 mRNA+ cells, but the expression of proinflammatory cytokines was relatively unaffected by fluticasone treatment.[6] Finally, preventive treatment of intranasal FP reduced cytokine mRNA-expressing cells before and during a single nasal allergen provocation. Preventive treatment with FP out of season resulted in a decrease in eosinophils and mRNA-positive cells for type 2 cytokines. After allergen provocation, levels of most of the measured cytokines, including IL-13, and of eosinophils were reduced using corticosteroids. Thus, preventive treatment with FP prior to contact with grass pollen was effective in reducing the increase of cytokine mRNA-positive cells in reaction to grass-pollen contact.[14]

The present results clearly show that FP *in vitro* impairs IL-13 production by PHA-stimulated lymphocytes from both asthmatic and control subjects. In accord with these data, our preliminary results seems to confirm that IL-4 and IL-5 mRNA production by PHA-stimulated lymphocytes from both asthmatic and control subjects might be inhibited by FP. On the whole, previous and present results demonstrate that type 2 cytokine inhibition by steroid may, at least in part, account for its therapeutic effects.

References

1. Barnes PJ. Molecular mechanisms of corticosteroids in allergic diseases. *Allergy* 2001; **56**: 928–936.
2. Caruso C, Candore G, Modica MA, Bonanno CT, Sireci G, Dieli F, Salerno A. Major histocompatibility complex regulation of cytokine production. *J Interferon Cytokine Res* 1996; **16**: 983–988.
3. Terada N, Hamano N, Hohki G, Ikeda T, Sai M, Yamashita T, Konno A. The potential role of interleukin-13 in eosinophilic inflammation in nasal mucosa. *Allergy* 1998; **53**: 690–697.
4. Kroegel C, Julius P, Matthys H, Virchow JC Jr, Luttmann W. Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur Respir J* 1996; **9**: 899–904.
5. Ghaffar O, Laberge S, Jacobson MR, Lowhagen O, Rak S, Durham SR, Hamid Q. IL-13 mRNA and immunoreactivity in allergen-induced rhinitis: comparison with IL-4 expression and modulation by topical glucocorticoid therapy. *Am J Respir Cell Mol Biol* 1997; **17**: 17–24.
6. Hamilos DL, Thawley SE, Kramper MA, Kamil A, Hamid QA. Effect of intranasal fluticasone on cellular infiltration, endothelial adhesion molecule expression, and proinflammatory cytokine mRNA in nasal polyp disease. *J Allergy Clin Immunol* 1999; **103**: 79–87.
7. Barnes PJ, Pedersen S, Busse WW. Efficacy and safety of inhaled corticosteroids. *Am J Respir Crit Care Med* 1998; **157 (suppl 3)**: S1–S53.
8. Di Lorenzo G, Gervasi F, Drago A, Esposito Pellitteri M, Di Salvo A, Cosentino D, Potestio M, Colombo A, Candore G, Mansueto S, Caruso C. Comparison of the effects of fluticasone propionate, aqueous nasal spray and levocabastine on inflammatory cells in nasal lavage and clinical activity during the pollen season in seasonal rhinitics. *Clin Exp Allergy* 1999; **29**: 1367–1377.
9. Ortolani C, Foresi A, Di Lorenzo G, Bagnato G, Bonifazi F, Crimi N, Emmi L, Prandini M, Senna GE, Tursi A, Mirone C, Leone C, Fina P, Testi R. A double-blind, placebo-controlled comparison of treatment with fluticasone propionate and levocabastine in patients with seasonal allergic rhinitis. FLNCO2 Italian Study Group. *Allergy* 1999; **54**: 1173–1180.
10. Di Lorenzo G, Mansueto P, Melluso M, Morici G, Norrito F, Esposito Pellitteri M, Di Salvo A, Colombo A, Candore G, Caruso C. Non specific airway hyperresponsiveness in mono-sensitive Sicilian patients with allergic rhinitis. Its relation to total serum IgE levels and blood eosinophils during and out of the pollen season. *Clin Exp Allergy* 1997; **27**: 1052–1059.
11. Di Lorenzo G, Pellitteri ME, Drago A, Di Blasi P, Candore G, Balistreri CR, Listì F, Caruso C. Effects of in vitro treatment with fluticasone propionate on natural killer and lymphokine induced killer activity in asthmatic and healthy individuals. *Allergy* 2001; **56**: 323–327.
12. Lio D, Candore G, Colombo A, Colonna Romano G, Gervasi F, Marino V, Scola L, Caruso C. A genetically determined high setting of TNF-α influences immunological parameters of HLA-B8, DR3 positive subjects: implications for autoimmunity. *Hum Immunol* 2001; **62**: 705–713.
13. Gough NM. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal Biochem* 1988; **173**: 93–95.
14. Kleinjan A, Holm AF, Dijkstra MD, Boks SS, Severijnen L, Mulder PG, Fokkens WJ. Preventive treatment of intranasal fluticasone propionate reduces cytokine mRNA expressing cells before and during a single nasal allergen provocation. *Clin Exp Allergy* 2000; **30**: 1476–1485.
15. Fushimi T, Shimura S, Suzuki S, Saitoh H, Okayama H, Shirato K. Suppression of gene expression and production of interleukin 13 by dexamethasone in human peripheral blood mononuclear cells. *Tohoku J Exp Med* 1998; **185**: 157–160