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Co-stimulation of cultured peripheral blood mononuclear cells from intrinsic asthmatics with exogenous recombinant IL-6 produce high levels of IL-4-dependent IgE

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Co-stimulation of cultured peripheral blood mononuclear cells from intrinsic asthmatics with exogenous recombinant IL-6 produce high levels of IL-4-dependent IgE. I.M. Sánchez-Guerrero, N. Herrero, M. Muro, R.P. Vegara, M. Campos, A.M. García-Alonso, M. Rocío Álvarez. ©ERS Journals Ltd 1997.

ABSTRACT: Asthma is an inflammatory airway disorder, traditionally subdivided into extrinsic, immunoglobulin E (IgE)-mediated, and intrinsic asthma of unknown aetiology. IgE synthesis requires contact between T- and B-cells and a signal provided by interleukin (IL)-4, which can be modulated by IL-6. The objective of this study was to evaluate the effects of IL-4 and IL-6 on total IgE synthesis by peripheral blood mononuclear cells from intrinsic and extrinsic asthmatics.

Peripheral blood mononuclear cells from intrinsic and extrinsic asthmatic patients and from healthy subjects were cultured and stimulated with pokeweed mitogen, recombinant IL-4 and IL-6. The IgE level in serum and supernatants was measured by an enzyme-linked immunoassay.

Serum IgE was significantly lower in intrinsic asthma than in extrinsic asthma, but significantly higher than in control subjects. IgE production by cultured mononuclear cells from extrinsic asthmatics was not modified after exogenous IL-4 and IL-6 addition. However, intrinsic asthmatics showed enhancement of IgE synthesis in response to IL-4 stimulation, reaching a threefold increase of the spontaneous IgE values, when simultaneous recombinant IL-4 plus IL-6 stimulus was used.

Our results indicate that exogenous recombinant interleukin-6 can significantly upregulate the interleukin-4-dependent immunoglobulin E synthesis in intrinsic asthma. This suggests that immunoglobulin E could also play a role in the pathogenesis of intrinsic asthma, in which an interleukin-6 threshold would be critical. *Eur Respir J 1997; 10: 2091–2096.*

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One of the most challenging problems in all developed countries is the increasing number of patients with asthma. Bronchial asthma is a chronic inflammatory disease of the airways characterized by infiltration of inflammatory cells [1–4]. Although the precise immune features of the airway inflammation in asthma have not so far been well defined, the examination of biological activities shows that various types of cells are involved. Among them, activated T-cells can regulate immunoglobulin (Ig)E production by B-cells in extrinsic asthma [5] through direct contact and by release of cytokines, thus providing the appropriate basis for immunoglobulin production and direct isotype switching. However, limited information is available on the immune mechanisms of intrinsic asthma.

Interleukin (IL)-6 is a cytokine, with a wide range of biological activities, produced by many different cell types, including tumoral cells [6–9]. It is involved in the regulation of inflammatory responses [10–13], and plays a critical role in the modulation of immune responses. The B-cell response is regulated by different factors. IL-4 acts in the early B-cell activation to stimulate proliferation and heavy chain isotype switching to IgG and IgE, while IL-6 is a late-acting differentiation factor for human activated B-cells to become plasma cells [14, 15]. IgM, IgG and IgA production by pokeweed mitogen (PWM)-stimulated peripheral blood mononuclear cells (PBMCs) is also reported to be IL-6-dependent [14, 16, 17].

In recent years, IgE synthesis by B-cells has been extensively studied and is now known to require at least two signals [18]. The first signal is provided by a physical contact between B- and T-cells and the second is delivered by IL-4, which is IgE-isotype-specific. In addition, it has been reported that IL-6 can enhance IL-4-induced IgE synthesis [19–22], and that the presence of IL-2 is also required to produce an optimal humoral response [16, 23].

In order to investigate whether intrinsic asthmatic patients are able to produce high quantities of IgE, we assessed the *in vitro* effect of human recombinant IL-4 (rhIL-4) and IL-6 (rhIL-6) on IgE production by PBMCs from such patients as compared to extrinsic asthmatics and healthy controls. The results of the experiments in this study suggest that IgE may also be involved in the immunological processes of intrinsic asthma.

Materials and methods

Study subjects

Peripheral blood samples were obtained from 57 nonsmoking asthmatics, selected according to the definition of the American Thoracic Society on the basis of clinical symptoms (history of wheezing, chest tightness, and/or shortness of breath relieved by an inhaled β-agonist), physical examination and pulmonary function tests, demonstrating normal results or reversible airway obstruction, manifested by a postbronchodilator increase in forced expiratory volume in one second (FEV_1) >15%. The asthmatic subjects were assigned either to the extrinsic or intrinsic group. Extrinsic asthmatics had a positive history of a bronchoconstrictive response after allergen exposure and one or more positive skin-prick tests with common allergen extracts, such as house-dust mite, moulds, pollens and animal danders. Intrinsic asthmatics developed symptoms following respiratory tract infections, but had no history of allergen-induced bronchospasm and negative skin-prick tests.

No patient was in acute respiratory distress at the time of the study or had required treatment for an exacerbation with oral or intravenous corticosteroids in the previous 6 months. Medication used by asthmatics included inhaled β-agonists on an as-needed basis, which was withheld at least 12 h before blood sampling. None of the extrinsic asthmatics had ever received immunotherapy. Thirty healthy individuals with no history of atopy and negative skin-prick tests were used as controls. None of them had taken any medication in the previous 3 months. The characteristics of the patients are presented in table 1.

Study design

The aim of the study was to evaluate differences in the total IgE level in serum from extrinsic and intrinsic asthmatic patients and controls. Changes in *in vitro* IgE synthesis by PBMCs upon stimulation with PWM, rhIL-4 and rhIL-6 were also studied.

Serum IgE measurement

A modified isotype-specific enzyme-linked immunosorbent assay (ELISA) was performed, as described previously [24], to measure serum IgE in asthmatic patients and controls. Therefore, microtitre plates (Microwell MaxiSorp; Nunc, Roskilde, Denmark) were coated with 100 μL·well^{-1} of mouse antihuman IgE monoclonal antibody (Dakoppats, Glostrup, Denmark) at 1/75 dilution in 0.1 M $NaHCO_3$ buffer (pH 9.6) overnight at 4°C. After washing, the wells were blocked with 100 μL·well^{-1} of bovine serum albumin (BSA) 1% in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C. After further washing, 100 μL of serum or IgE control was added in duplicate. The standard IgE used as control was purchased from Behringwerke AG (Marburg, Germany). Following a 24 h incubation at 4°C, the wells were washed another three times. Then 100 μL of 1/3,000 diluted alkaline phosphatase-labelled goat polyclonal antihuman IgE (Caltag Laboratories, Burlingame, CA, USA) was used as a second antibody and incubated by shaking at room temperature for 2 h. Subsequently, 100 μL of p-nitrophenyl phosphate (Kallestad, Austin, TX, USA) substrate solution was added to the wells after three washes with PBS-Tween 20 0.5%. Absorbance was read at 405 nm with an ELISA reader (Whittaker 2001, Salzburg, Austria). The detection limit of this ELISA was 9.6 ng·mL^{-1} and its coefficient of variation 6.7%.

Table 1. – Characteristics of the subjects studied

	Extrinsic asthma	Intrinsic asthma	Control
Subjects n	30	27	30
Sex M/F	10/20	13/14	12/18
Age yrs‡	29 (12–40)	42 (35–60)	33 (22–47)
≥1 positive skin-prick test	30	0	0
Serum IgE ng·mL^{-1}#	465±458	57±42	17±13

‡: mean, and range in parenthesis; #: mean±SEM. M: male; F: female; IgE: immunoglobulin E.

Cell preparation and culture

A randomized sample of 10 subjects from each group investigated was further selected to analyse the IgE production *in vitro*. PBMCs from patients and controls were isolated from heparinized venous blood by Ficoll density gradient centrifugation (Lymphoprep; Nicomed Pharma, Oslo, Norway) as reported previously [25]. These cells were washed three times and resuspended in medium RPMI 1640 (M.A. Bioproducts, Walkersville, MD, USA), supplemented with 10% foetal calf serum (Flow Laboratories, Irvine, UK), and cultured in flat-bottomed microtitre plates (Costar Corp., Cambridge, MA, USA) at 2×10^5 cells·well^{-1}, either alone or supplemented with PWM (Gibco Laboratories, Grant Island, NY, USA) at 1/100 (v/v), rhIL-4 (100 UI·mL^{-1}) and rhIL-6 (20 UI·mL^{-1}) (R & D System, Abingdon, Oxon, UK) or their combinations to induce IgE synthesis, as described previously [24]. The culture plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 14 days to allow IgE isotype switching. Five replicates were performed on each point in every experiment. Nonstimulated supernatants were removed on days 3 and 14 of culture to determine the spontaneous production of IgE, and the stimulated supernatants were recovered on day 14 to measure the induced IgE synthesis.

Supernatant IgE measurement

IgE levels in culture supernatants were determined by using a commercial low level IgE ELISA (The Binding Site, Birmingham, UK), following the manufacturer's technical guidelines. The coefficient of variation and the sensitivity of this IgE assay were 4.1% and 0.31 ng·mL^{-1}, respectively.

Statistical analysis

Statistical analysis of serum IgE values was performed in the log-data by using a one-way analysis of variance (ANOVA) to compare differences between the

three study groups. This analysis was completed by using a two-tailed, unpaired Student's t-test. Differences between mean values of supernatant determinations were analysed according to Student's t-test. Only p-values below 0.05 were considered significant. Data are presented as mean±SEM.

Results

As expected, serum IgE values, measured by ELISA, were higher in patients with extrinsic asthma (466±457.7 ng·mL^{-1}) than those observed in intrinsic asthma and healthy subjects. However, using this sensitive method, we observed that IgE values (mean±SEM) from intrinsic asthmatic patients (57.3±42.3 ng·mL^{-1}) were significantly higher than those detected in healthy subjects (17.1±13.1 ng·mL^{-1}) ($p<0.001$). These data are presented in figure 1.

Table 2 summarizes the measurements of basal serum IgE and spontaneous IgE release in culture supernatants from patients and controls. In cultures without mitogenic stimulus and prolonged for 14 days, the cells were not able to survive. Therefore, the supernatant IgE measurement showed values similar to those observed on day 3 of culture, which is considered to be a spontaneous IgE production. In fact, nonstimulated lymphocytes died in prolonged cultures. Consequently, IgE production did not increase. Therefore the data corresponding to spontaneous IgE production on day 3 are presented in figure 2, which seems reasonable because IgE production in culture needs mitogenic stimulus and incubation for a period of 14 days [24]. The spontaneous nonstimulated IgE production appeared to be enhanced in the atopic patient group, as compared with intrinsic asthmatics and controls. In contrast to the results observed in serum, the lowest values of spontaneous IgE production corresponded to the intrinsic asthmatic group. However, none of these results reached statistical significance.

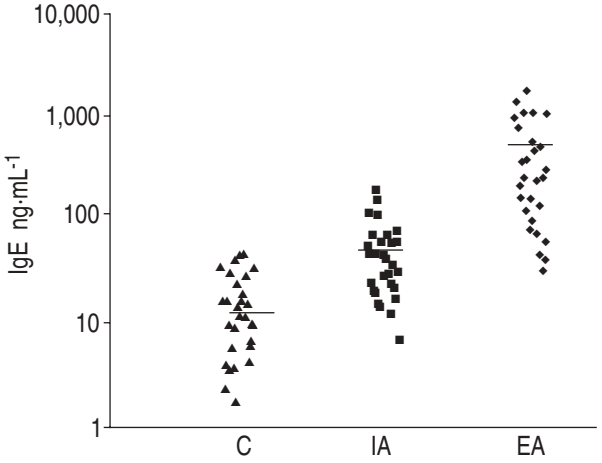

Fig. 1. – Distribution of serum IgE in the study groups. IgE was measured by ELISA in extrinsic asthmatic patients (EA), intrinsic asthmatic patients (IA) and control subjects (C). Data are presented in the log-rank. Horizontal bars represent mean values. IgE: immunoglobulin E; ELISA: enzyme-linked immunosorbent assay.

Table 2. – Serum IgE against spontaneous IgE *in vitro* production by the selected subjects from each group

Group	Subjects	Serum IgE ng·mL^{-1}	Spontaneous IgE production pg·mL^{-1}
Extrinsic asthma	10	202±94.4	836.7±564.1
Intrinsic asthma	10	44.9±23.2	434.5±298.2
Control	10	11.1±5.5	530.0±119.4

Data are presented as mean±SEM. Spontaneous IgE production was measured in supernatants recovered on day 3 of culture without stimulus. IgE: immunoglobulin E.

Measuring IgE levels in extrinsic asthmatic patients on day 14, it was found that rhIL-4 either alone or in combination with rhIL-6 produced no consistent change in IgE response as compared to the values observed in the spontaneous or PWM-induced IgE production. Interestingly, in intrinsic asthmatic patients, IgE was markedly increased after stimulation with rhIL-4 ($p<0.05$), rising to values similar to those observed in extrinsic asthmatics. The addition both of rhIL-4 and rhIL-6 had a potentiating effect that resulted in an IgE level three times that of spontaneous production, which was significantly ($p<0.02$) higher than that observed in extrinsic asthmatics (fig. 2a).

All experiments with healthy subjects performed on day 14, under similar experimental conditions, yielded a strong enhancement of IgE levels in relation to their spontaneous production, when PBMCs were stimulated

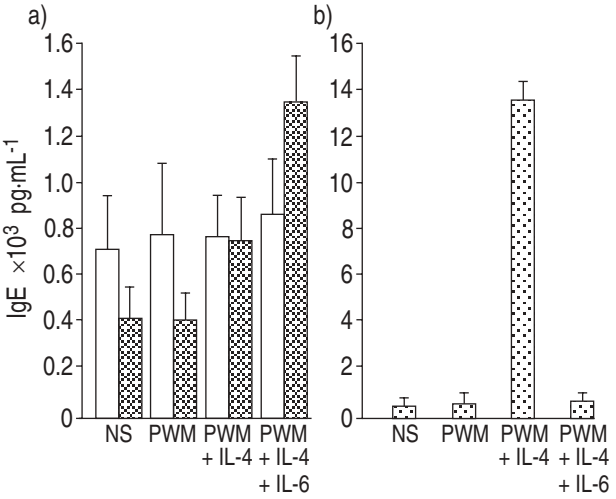

Fig. 2. – Influence of recombinant human interleukin (rhIL)-4 and rhIL-6 on the IgE response elicited on day 14 by PWM-stimulated PBMCs. The supernatants were harvested on day 14 of the culture from: a) intrinsic asthmatic (▩) and extrinsic asthmatic (□) patients; and b) healthy control subjects. The production of total IgE was tested by ELISA. Results are expressed as mean±SEM. PWM: pokeweed mitogen; PBMCs: peripheral blood mononuclear cells; NS: nonstimulated; IL: interleukin. For further definitions see legend to figure 1.

Table 3. – Supernatant IgE levels in the study groups following the use of different stimuli

	Control	Extrinsic asthma	Intrinsic asthma
Nonstimulated (day 3)	530±231	737±363	434±123
Nonstimulated (day 14)	512±249	697±323	401±203
Day 14 PWM	593±377	804±362	427±109
Day 14 PWM + IL-4	13827±437	850±229	776±234
Day 14 PWM + IL-4 + IL-6	737±203	890±314	1378±327

PWM: pokeweed mitogen; IL: interleukin; IgE: immunoglobulin E.

with rhIL-4 ($p<0.0001$). However, double stimulation with rhIL-4 and rhIL-6 in these subjects not only resulted in an enhancement of IgE synthesis but even reverted the rhIL-4-induced rise of IgE, whose values returned close to the basal values ($p<0.05$) (fig. 2b). Supernatant IgE levels for each study group following the use of different stimuli are summarized in table 3.

Discussion

Our study provides evidence that, upon simultaneous stimulation with PWM and exogenous rhIL-4 and rhIL-6, the PBMCs of intrinsic asthmatics can synthesize *in vitro* high levels of IgE, even greater than IgE production in extrinsic asthmatic patients. We were interested in these respiratory disorders because they share similar clinical manifestations, despite exhibiting different immunological characteristics. The mechanism responsible for this dichotomy remains unclear, but it would be tempting to hypothesize that an impaired helper T-cell function and a different lymphokine network could be involved in the pathogenesis of each type of asthma. A relative dominance of T-helper type-2 (Th2)-mediated responses has been reported for extrinsic asthma [5, 26], but it is unknown, so far, which pattern of mediators regulates intrinsic asthma.

The detection of a moderate increase of serum IgE levels in intrinsic asthmatic patients, as well as our previous finding that serum CD23 was enhanced in these subjects [27], supports the idea that the IgE system could also be implicated in this type of asthma, although *via* another regulatory pathway. Therefore, this work was focused on researching the *in vitro* production of IgE in both diseases, studying their behaviour under identical culture conditions.

MAGGI *et al*. [28] have reported that IL-6 potentiates the IL-4-induced IgE synthesis by B-cells in atopic patients, but our results showed that the amounts of IgE produced by PBMCs from atopic asthmatic patients either in the presence or absence of IL-6 did not differ significantly, even in the presence of exogenous rhIL-4. Since it is known that B-cells from these patients are induced to switch to IgE production, the endogenous IL-4 may be enough to activate it. In addition, IL-6 can be synthesized not only by T-cells but also by activated monocytes and alveolar macrophages [14, 29]; thus, it is probable that the amount of endogenous IL-6 produced by atopic patients could be optimal, and exogenous addition of rhIL-6 and rhIL-4 would not enhance IgE production.

When PBMCs from intrinsic asthmatic patients were analysed, we observed that IgE production appeared to be conditioned by rhIL-4 and rhIL-6. It has been reported that IL-4, which induces IgE synthesis [18, 30–32], is decreased in intrinsic asthma [33]; hence, the exogenous addition of this interleukin could explain the fact that intrinsic asthmatics produced IgE in similar quantities to those produced by extrinsic asthmatics. Nevertheless, it was surprising that, when rhIL-6 and rhIL-4 were added simultaneously, the increase in the level of IgE in this group exceeded the production of IgE by atopic patients. These findings indicate that PBMCs from intrinsic asthmatic patients are able to lead to an IgE response, at least under *in vitro* conditions.

In the control group, exogenous rhIL-4 was able to induce a strong IgE response by PBMCs, which may be due to the effect exerted on resting B-cells to induce IL-4 receptor and CD40-L expression. Moreover, exogenous IL-4 could lead to an immune deviation from uncommitted T-helper (Th0) phenotype to Th2, which increases the endogenous IL-4 production, resulting in IgE switching of the control subject lymphocytes. However, B-cells from extrinsic asthmatic patients would not be affected in this way, while intrinsic asthmatic patients, exhibiting slightly but significantly elevated serum IgE values, could represent an intermediate situation, where the degree of cell differentiation would allow an intermediate rhIL-4-induced IgE response. Although MAGGI *et al*. [28] reported that rhIL-6 had no effect on the synthesis of IgE by B-cells from nonatopic donors, we found that the supplementary rhIL-6 abrogated the IL-4-exogenous effect on IgE synthesis by PBMCs from healthy controls; but it should be taken into account that we used a different experimental system and PBMCs instead of isolated B-cells.

The impaired response to exogenous IL-6 could be explained by several mechanisms. Firstly, it might be suggested that cells from intrinsic asthmatics were unable to release appropriate amounts of IL-6; but we are not convinced of this, because when IL-6 was measured in sera and culture supernatants, we found similar amounts of this cytokine in intrinsic and extrinsic asthmatic patients (data not shown). Secondly, as has recently been reported, the amplitude of the IL-6 response seems to be determined by the ratio of two subunits (gp80 and gp130) of the IL-6 receptor on the cell surface. Thus, if gp80 membrane expression was low, IL-6 could not induce appropriate responsiveness. Indeed, IL-6 induces gp130 subunit expression, which, in turn, determines the level of IL-6/gp80 binding on the membrane to induce transduction of specific signals [34]. Moreover, the unusual fact that IL-6 shows an agonistic behaviour with its receptor [35] could suggest that, in the presence of an excess of exogenous IL-6, gp130 receptor subunit expression could be induced on the cell surface, allowing the amplifying action of IL-6. Taking these considerations into account, it is quite reasonable to conclude that endogenous IL-6 levels in intrinsic asthmatic patients were restrictive to allow an adequate gp130 expression, which would be enhanced by exogenous addition of rhIL-6.

It is now well established that the regulatory events for IgE synthesis are triggered by the activation of CD4+ T-cells, which requires physical interaction between T- and B-cells involving the T-cell receptor complex and major histocompatibility complex class II molecules [36]. Once activated, T-helper cells express other essential surface molecules, which interact as ligand-receptor pairs, such as CD28-B7, CD21-CD23, and CD40-gp39 [37–39], providing a second signal by T-cell derived interleukins, particularly IL-4 and IL-6. In a murine model, HILBERT *et al*. [40] examined the differential IL-6 requirements of primary and secondary antigen-specific B-cell response, and concluded that the antibody secretion of naive B-cells is entirely IL-6-dependent, while the antigen-primed B-cell responsiveness is essentially IL-6-independent. It has also been reported that IL-6 does not play a significant part in antibody

responses to the influenza virus by human tonsillar mononuclear cells [41]. Considering these observations, we believe that in intrinsic asthmatic patients, rhIL-6 is likely to trigger T-helper cells to induce a subsequent signal necessary for the development of primary response, while this cytokine would not act in extrinsic asthmatic patients since they could only undergo a secondary response.

At the time of this study and on the basis of the information available, little is known about the possible participation of IgE in the pathogenesis of intrinsic asthma [42]. For the first time, this report demonstrates that PBMCs from intrinsic asthmatic patients, at least under the present experimental conditions, are able to exceed the *in vitro* production of IgE by extrinsic asthmatic patients. This finding suggests that in the *in vivo* microenvironment airway cells, such as monocytes, alveolar macrophages and bronchial epithelial cells, which can secrete increased local amounts of IL-6 [43], may act on B-cells delivering a strong *in situ* IgE response that is likely to be responsible for the clinical manifestations of intrinsic asthma.

In summary, our data provide circumstantial evidence that the peripheral blood B-lymphocytes from intrinsic asthmatics may cause a high immunoglobulin E response in the presence of appropriate amounts of interleukins. This fact, together with the reports of other authors [42], supports the idea that both types of asthma could be caused, at least in part, by a common immunological feature, possibly regulated *via* different pathways. This possibility has yet to be demonstrated by further investigations to analyse the regulatory T-cell cytokine network in more detail, since a better understanding of immunoglobulin E function and production in intrinsic asthma will open new ways for designing more rational approaches to the therapy of this disease.

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