
A model of allergen-driven human airway contraction: β_2 pathway dysfunction without cytokine involvement

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Background: In our previous in vitro model, allergen incubation of passively sensitized human airways reduced the response to salbutamol. However, whether cytokines play a role in this model is still unknown.

Objective: To investigate interleukin 1 β and tumor necrosis factor α expression in allergen-challenged human airways.

Methods: Nonasthmatic airways (n = 13) were passively sensitized by overnight atopic serum incubation and then challenged with allergen for 1 hour (n = 9). After repeated washouts, airways were immersed in physiologic salt solution for 6 hours and finally in formaldehyde for immunohistochemical studies. The effect of co-incubation in anti-interleukin 1 β and anti-tumor necrosis factor α specific neutralizing antibodies on salbutamol response was also studied (n = 4).

Results: No differences were found among control, sensitized, and challenged rings in the number of inflammatory cells. The percentage of basement membrane covered by epithelium was similar in the different conditions. There was a higher percentage of degranulating to total mast cells in allergen-challenged rings than in sensitized rings ($P < .001$). A significant correlation was observed between allergen-induced contraction and mast cell degranulation ($r = 0.88$; $P < .001$). The sensitization procedure was validated by paired allergen-induced contractions. No expression of the 2 cytokines was detectable up to 6 hours after allergen challenge, and specific neutralizing antibodies did not attenuate the impaired response to salbutamol in allergen-challenged rings.

Conclusion: These data suggest that in our in vitro model of allergic inflammation, β_2 pathway dysfunction can occur without cytokine involvement, thus supporting previous results that suggest a role for leukotrienes.

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INTRODUCTION

We previously demonstrated in an in vitro model that allergen incubation of passively sensitized human airways caused a reduced response to salbutamol.¹ Further studies have shown that G_s protein impairment was responsible for allergen-induced β_2 pathway dysfunction² and that leukotrienes could be regarded as the causative agents.³

Although previous data on the expression of inflammatory cells and intercellular adhesion molecule 1 did not show any difference between allergen- and sham-challenged airways after 2 hours,⁴ there are no data on cytokine involvement in this model. Particularly, it is unknown whether allergen challenge or passive sensitization procedures can alter cytokine expression.

Among the cytokines, interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) concentrations are found to be increased after an allergen challenge in vivo in bronchoal-

veolar lavage in patients with asthma^{5,6} and in vitro in passively sensitized human airways and lung fragments.^{7,8} Furthermore, these 2 cytokines can alter β_2 pathway function,⁹ with mechanisms involving G_s ¹⁰ or G_i ^{11,12} proteins.

Incubation in a high serum IgE concentration can alter functional properties of airway smooth muscle.¹³ The low-affinity receptor Fc ϵ RII (CD23) may play a role in the development of nonspecific hyperresponsiveness. Up-regulation of Fc ϵ RII receptor expression represents an inducible phenomenon largely due to IgE immune complex-coupled activation of the receptor and associated with Fc ϵ RII-induced autologous production of IL-1 β by the atopic sensitized airway smooth muscle, as demonstrated by successful pretreatment using a monoclonal anti-CD23 blocking antibody.¹⁴ Although in our model we did not find differences in the expression of inflammatory cells and intercellular adhesion molecule 1 between passively sensitized and control airways,⁴ we did not study mast cell degranulation. However, functional studies^{1–3} show the same contractile responses to carbachol in passively sensitized and control airways. Therefore, our aim was to study IL-1 β and TNF- α expression and their contribution to allergen-induced β_2 dysfunction. The procedure of passive sensitization and the effectiveness of allergen challenge were validated by allergen-induced contractions and mast cell degranulation evidence.

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METHODS

Bronchial samples were obtained from 13 men without asthma aged 56 to 72 years undergoing surgery for lung cancer. None of the patients received β_2 -agonists, theophylline, or anticholinergic drugs in the 24 hours before or during surgery. None of the patients were sensitized to *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*, as evaluated by skin prick testing (Lofarma, Milan, Italy).

Surgical specimens were immersed in aerated (95% oxygen and 5% carbon dioxide) physiologic salt solution (PSS) of the following composition: 110.5mM sodium chloride, 3.4mM potassium chloride, 2.4mM calcium chloride, 0.8mM magnesium sulfate, 1.2mM potassium hydrogen phosphate, 25.7mM sodium bicarbonate, and 5.6mM dextrose. Rings (internal diameter, 3–5 mm; length, 4–5 mm) as far as possible from the site of malignancy were prepared by careful dissection and removal of parenchymal tissue. Care was taken to avoid epithelial damage.

Passive Sensitization

Three or 4 rings from each patient were passively sensitized by overnight (18-hour) incubation in 1 mL of serum diluted with 9 mL of PSS from different *Dermatophagoides*-sensitized donors (sensitized rings). The serum concentration of specific IgE to *Dermatophagoides* of the sensitized donors was greater than 17.5 Phadebas RAST Units/mL (fourth RAST class; Pharmacia, Uppsala, Sweden), and the mean $\pm$ SD total serum IgE concentration was 305 ± 83 IU/mL. Bronchial samples were maintained at room temperature and were continuously gassed with 95% oxygen and 5% carbon dioxide. One ring was sham incubated in 1 mL of serum from nonatopic donors (total serum IgE concentration <20 IU/mL and negative radioallergosorbent test results for *Dermatophagoides*) diluted in 9 mL of PSS (control ring).

Allergen Challenge

One sensitized ring from each patient ($n = 13$) was washed with PSS for 15 minutes and then challenged for 60 minutes with 5 mL of aerated (37°C) PSS containing a 200-AU/mL *Dermatophagoides* mix (Lofarma) (challenged rings). In 4 cases, 1 sensitized ring was also incubated, before allergen challenge, in anti-TNF- α (0.028 μ g/mL) and anti-IL-1 β (0.10 μ g/mL) (Inalco, Milan, Italy) (challenged rings with antibodies). The concentrations used for both antibodies were double the weight of those that cause 50% inhibition of biological activity of specific targets. The other sensitized ($n = 13$) and control ($n = 13$) rings underwent the same procedure and were sham challenged for 60 minutes in 5 mL of aerated (37°C) PSS. In 4 cases, 1 sensitized ring was also treated with anti-TNF- α and anti-IL-1 β antibodies (sensitized rings with antibodies). Rings were then washed with fresh PSS and aerated (37°C) in 5 mL of PSS for 6 hours or were used for functional studies. At the end of the experiment, rings were immersed in 4% formaldehyde at 4°C for 24 hours. In 3 cases, other challenged rings, at the end of the

experiment, were immediately stored in fresh PSS for Western blot analysis.

Histologic Examination

Bronchial rings ($n = 24$), previously immersed in formaldehyde, were dehydrated through graded alcohols and xylene, embedded in paraffin, cut into 2- μ m sections using a rotative microtome, and stained with hematoxylin-eosin and toluidine blue for eosinophil and mast cell quantification, respectively. Analysis was performed using a light microscope (Leica, Cambridge, England) over adjacent, nonoverlapping, high-power fields (at $\times 500$ magnification with the aid of an eyepiece graticule) until all the available area of 1 section was covered and for 1 entire section of bronchial ring. Results are expressed as the number of cells per square millimeter of submucosa. Mast cells were also examined at $\times 1,000$ magnification to detect ongoing degranulation. Mast cells were considered degranulated when the presence of 1 or more metachromatic granules around it was detected. The intraobserver mean coefficients of variation of 3 repeated measurements were 7% for eosinophils and 5% for mast cells.

Immunohistochemical Analysis of TNF- α and IL-1 β Expression

Paraffin-embedded sensitized ($n = 9$) and challenged ($n = 9$) rings were cut into 2- μ m sections onto slides (Superfrost Plus; Menzel, Braunschweig, Germany) and baked at 60°C for 30 minutes, deparaffined in xylene, rehydrated through graded alcohols, and rinsed in distilled water. All samples were treated with hydrogen peroxide to quench endogenous peroxidase. Nonspecific sites were blocked with a 1:2 goat serum. Sections were incubated for 30 minutes in an appropriate dilution of primary antibody as TNF- α (mouse monoclonal IgG1 antibody; Santa Cruz Biotechnology, Santa Cruz, CA) and IL-1 β (rabbit polyclonal antibody IgG; Santa Cruz Biotechnology), rinsed 3 times in phosphate-buffered saline solution at pH 7.6, and incubated for 20 minutes in multilink biotinylated immunoglobulin. Then the slides were rinsed 3 times in phosphate-buffered saline solution at pH 7.6 and incubated for 20 minutes in horseradish peroxidase streptavidin solution. Antibody binding was demonstrated by incubation in liquid diaminobenzidine substrate pack (BioGenex, San Ramon, CA). The negative control rings were not incubated in primary antibody. Samples were counterstained with Mayer hematoxylin (Sigma-Aldrich Corp, St Louis, MO), dehydrated through graded alcohols and xylene, and mounted in Eukitt (O. Kindler, Freiburg, Germany). Finally, the slides were examined under a light microscope (Olympus U-SPT; Olympus, Hamburg, Germany) connected to a videotape recorder and a computerized image system (Leica MC 500 Image Analysis; Leica, Cambridge, England). Cells that stained positive with each antibody are automatically counted at a magnification of $\times 100$ in all specimens, and cell counts are expressed as the number of cells per square millimeter of mucosa and submucosa.

Western Blot Analysis

Challenged rings ($n = 3$) were homogenized with tissue protein extraction reagent (T-PER; Pierce Biotechnology Inc, Rockford, IL) and a protease inhibitor and were centrifuged at 10,000 rpm for 5 minutes to pellet cellules or tissue debris. The supernatant was collected and stored at -20°C . A sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel at 12% loaded up the samples, the marker, and the positive control with an electrophoretic run. Proteins on the gel were transferred electrophoretically to a nitrocellulose membrane sheet at 12 mA, overnight, at room temperature. The nitrocellulose sheet was saturated with milk at 5% and subsequently was incubated in primary antibodies (TNF- α and IL-1 β) at room temperature for 3 hours, with shaking. After washing with wash buffer solution, the membrane was incubated in secondary antibodies for 90 minutes at room temperature, with shaking. Finally, the nitrocellulose membrane, after washing, was incubated in nitroblue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (NBT/BCIP; Pierce Biotechnology Inc) diluted in alkaline-buffered solution for 10 minutes. The reaction was stopped with distilled water.

Assessment of Sensitization

One sensitized ring from each patient ($n = 12$) was suspended by 2 stirrups in 25-mL baths containing aerated PSS (37°C). The lower stirrup was connected to a stationary hook at the bottom of the tissue bath, and the other stirrup was connected by a silk string to a force transducer (model FT03D; Grass Medical Instruments, Quincy, MA) connected to a strip chart recorder (model TA 4000; Gould, Valley View, OH). Sensitized rings were allowed to equilibrate in the baths for 2 hours and were washed every 15 minutes. During this time, the rings were progressively stretched to a resting force of 1g, which had been determined previously to correspond to the optimal length in human bronchi of this size. The length was not changed throughout the study. Next, contraction was obtained with 10^{-6}M carbachol ($n = 9$). After repeated washouts that returned the resting force to previous values, sensitized rings were exposed to a dose of 1,000 AU of *Dermatophagoides* mix (Lofarma) for 1 hour, and the contractile response was recorded. Successful passive sensitization was assumed if the allergen challenge resulted in a contractile response greater than 0.5g, and contraction was expressed as a percentage of 10^{-6}M carbachol contraction. If

a sufficient number of rings were available, 1 paired control ring ($n = 9$) underwent the same procedure to rule out the presence of natural sensitization.

Effect of Anti-TNF- α and Anti-IL-1 β Neutralizing Antibodies on the Salbutamol Response Curve

In 4 cases, rings treated with neutralizing antibodies (1 sensitized and 1 challenged) were compared with 1 challenged and 1 control ring in functional studies and underwent the same procedure described previously herein for isometric force recording. Contraction was obtained with 10^{-6}M carbachol, and relaxation was studied by adding salbutamol cumulatively from 10^{-9} to 10^{-4}M in a half-log increment manner.

Data Analysis

The active force was measured from the resting force before the carbachol-induced contraction. Data were analyzed using 1- or 2-factor repeated-measures analysis of variance with the Newman-Keuls post hoc test. Categorical data were compared using the χ^2 test. Correlations were performed using the Pearson test. Differences were considered statistically significant at $P < .05$. Data are presented as mean $\pm$ SD.

RESULTS

Microscopic Evaluation and Immunostaining

The percentage of basement membrane covered by epithelium was similar in all conditions. No differences were found among control, sensitized, and challenged rings in the numbers of eosinophils and mast cells. There was a higher percentage of degranulating to total mast cells in challenged rings than in control and sensitized rings ($P < .001$), with no difference between sensitized and control rings ($P = .61$). Data on inflammatory cells are summarized in Table 1. A measurable expression IL-1 β and TNF- α was not detected in sensitized or challenged rings. Results of Western blot analysis for IL-1 β and TNF- α proteins were negative in challenged rings.

Functional Studies

Verification of passive sensitization. The mean weight of bronchial rings used for verification of passive sensitization and exclusion of natural sensitization ($n = 18$) was 141 ± 62 mg, and the mean resting force was $1.2 \pm 0.3\text{g}$. Mean weights

Table 1. Histologic Data From Control, Sensitized, and Challenged Rings*

	Control rings ($n = 6$)	Sensitized rings ($n = 9$)	Challenged rings ($n = 9$)
Eosinophils, No./mm ² of submucosa	42.7 ± 17.9	37.4 ± 20.8	40.1 ± 19.4
Mast cells, No./mm ² of submucosa	37.2 ± 6.3	40.6 ± 13.2	43.4 ± 7.5
Degranulating to total mast cells, %	$11.1 \pm 7.4^{\dagger}$	$17.5 \pm 10.3^{\dagger}$	59.1 ± 26.2
Basal membrane covered by epithelium, %	77 ± 11	75 ± 9	70 ± 15

* Data are expressed as mean $\pm$ SD.

$^{\dagger} P < .01$ vs challenged rings.

and resting forces of rings were not significantly different within ($P = .35$) or between ($P = .22$) studies.

Response to carbachol. The response to 10^{-6} M carbachol of sensitized ($n = 13$) and control ($n = 9$) rings was 20 ± 12 g/g (force per wet tissue weight) and 19 ± 7 g/g, respectively, without any difference between the 2 groups ($P = .25$).

Response to allergen. All sensitized rings responded to *Dermatophagoides* mix and developed a contractile force equal to $52\% \pm 19\%$ of that elicited by 10^{-6} M carbachol. Control rings never responded to allergen challenge. Allergen-induced contraction, expressed as a percentage of the 10^{-6} M carbachol response, was significantly correlated to degranulating mast cells ($r = 0.88$; $P < .001$), expressed as a percentage of total mast cells (Fig 1).

Effect of incubation in specific antibodies on the salbutamol response curve. The mean weight of bronchial rings ($n = 16$) was 97 ± 49 mg, and the mean resting force was 1.2 ± 0.2 g. Mean weights and resting forces of rings were not significantly different between groups ($P = .11$ and $P = .37$, respectively). The response to 10^{-6} M carbachol of sensitized and control rings was 8.8 ± 3.4 g/g and 9.3 ± 4.4 g/g, respectively. The response to 10^{-6} M carbachol of challenged rings with or without antibodies was 6.1 ± 4.0 g/g and 9.4 ± 3.6 g/g, respectively. No difference was found among different conditions ($P = .63$).

In challenged rings, the dose-response relaxation curve to salbutamol was significantly shifted to the right compared with that in control and sensitized rings (interaction $P \leq .001$ for each comparison) (Fig 2). No difference was observed between control and sensitized rings ($P = .93$). Co-incubation in anti-IL-1 β and anti-TNF- α antibodies did not change the dose-response relaxation curve to salbutamol in challenged rings ($P = .11$).

DISCUSSION

The main findings of the present study are that (1) 1-hour allergen challenge plus up to 6 hours of ongoing reaction was

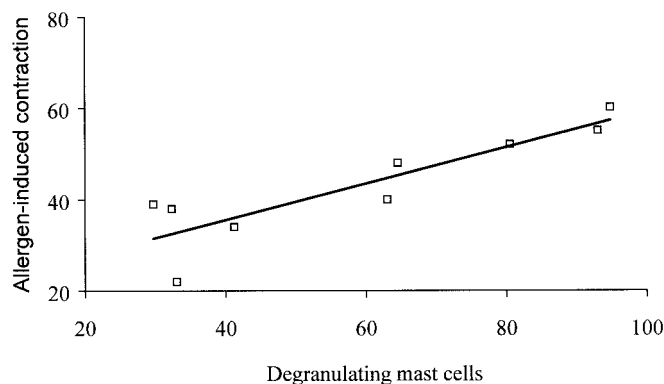


Figure 1. There was a significant correlation between allergen-induced contraction, expressed as a percentage of the response to 10^{-6} M carbachol, and degranulating mast cells, expressed as a percentage of total mast cells ($r = 0.88$; $P < .001$).

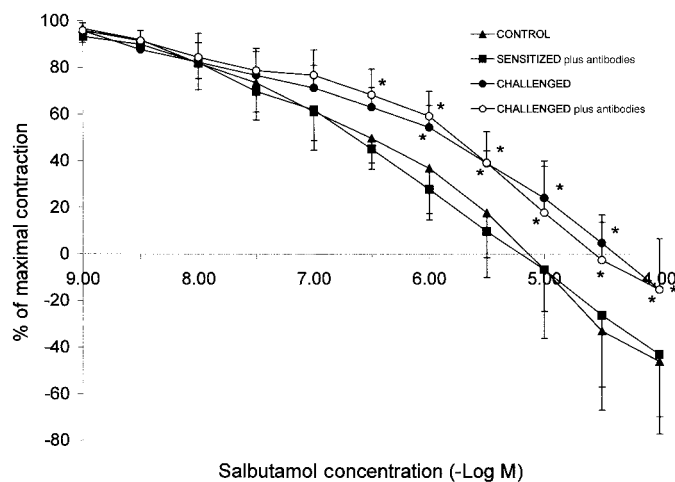


Figure 2. Mean relaxant effect of salbutamol in carbachol-contracted human bronchial rings, expressed as a percentage of maximal contraction induced by 10^{-6} M carbachol. In challenged rings, the reduced relaxation to salbutamol occurred similarly in the presence or absence of neutralizing antibodies. Asterisk indicates values of challenged rings that were significantly different ($P < .05$) from corresponding values of control and sensitized rings; horizontal line, the resting force before carbachol-induced contraction. Error bars represent SD ($n = 4$).

not followed by IL-1 β and TNF- α expression, and neutralization of these cytokines with specific antibodies did not attenuate the allergen-induced dysfunction of relaxation; and (2) passive sensitization itself did not alter inflammatory cell expression and mast cell degranulation. Therefore, in our human airway model, cytokines are not involved in allergen-induced β_2 pathway dysfunction. Data on cytokine expression are strongly supported by in vitro contraction studies.

We know that in our in vitro model only resident cells can respond to allergen challenge and that extrapolation to an in vivo model cannot be performed. However, we consider that unlike in an in vivo model, other factors may counteract allergen β_2 pathway dysfunction. Furthermore, in an in vivo study, data interpretation would be more difficult, ie, for peripheral blood interference, by cytokine production from lymphocytes or by chemotaxis of inflammatory cells. Finally, previous data on 8 patients without asthma⁴ were not used for statistical analysis, and new experiments were performed.

In this study, IL-1 β and TNF- α were undetectable in sensitized rings and remained unexpressed 6 hours after 1-hour allergen challenge. It is possible that early transient increases in cytokine levels were undetected or that cytokines secreted before measurements may not be accounted for in this study. However, results of additional experiments with neutralizing antibodies consistently exclude a role for these 2 cytokines in allergen-induced β_2 dysfunction.

In guinea pig tracheas, impairment of β_2 -receptor response is reproduced by IL-1 β and TNF- α incubation.⁹ The combination of IL-1 β and TNF- α ,¹¹ even at concentrations present in asthmatic airways, inhibits responses to isoproterenol in

human airway smooth muscle cells, probably resulting from the ability of TNF- α to augment IL-1 β -induced prostanoid formation. However, in all cases, data were from 18-hour incubation studies.^{9,14,15} To our knowledge, there are only 2 in vitro studies^{7,8} that show an increase in IL-1 β or TNF- α after an allergen challenge. The first study⁷ demonstrated a significant increase only for TNF- α 2 hours after allergen stimulation. However, cytokines served as proof of ongoing allergen reaction, and no contractile studies were performed. The authors inferred successful sensitization from increased cytokine concentrations in the supernatants after allergen challenge and from the ability of the same cytokines to increase adhesion molecule expression. Finally, the authors used a concentration of allergen that was shown by other researchers¹⁶ to be capable of producing a bronchial smooth muscle contraction as potent as that induced by 10⁻³M acetylcholine. This approach can explain some discrepancies with our results because we used an allergen concentration demonstrated to elicit a force approximately 55% $\pm$ 25% of 10⁻³M acetylcholine.⁴ Nevertheless, because the dose chosen by us was demonstrated to be sufficient to cause functional consequences, ie, alteration of the salbutamol response,¹ our results also seem to be relevant. We have functional (allergen-induced contraction) and morphologic (mast cell degranulation) data that support the effect of allergen incubation. Furthermore, these data were significantly correlated (Fig 1). The second study⁸ demonstrated an increase in TNF- α on supernatants from 2 hours after allergen challenge. However, data were from lung fragments, and, although a possible role for lymphocytes was excluded, macrophages from alveoli can be an important source of TNF- α .¹⁷

Others studies that demonstrated increased TNF- α release and expression were related to human anti-IgE incubation,^{18,19} able to trigger low- or high-affinity IgE receptors not only on mast cells but also on eosinophils and macrophages.^{20,21} The study by Ohkawara et al¹⁸ demonstrated an increase in TNF- α on supernatants 2 hours after anti-IgE incubation of passively sensitized human peripheral lung tissue. Again, results on pulmonary fragments, including alveoli with macrophages, could be different from ours. Finally, pulmonary fragments were harvested at 2 and 4 hours but during challenge and not after a 60-minute challenge. The study by Casale et al,¹⁸ in a similar model, demonstrated after a 30-minute anti-IgE challenge a weak but significant increase in TNF- α expression, particularly on eosinophils. Again, triggering with anti-IgE can directly stimulate eosinophils and cause the induction of TNF- α messenger RNA, with an amplifying effect.

In our study, passively sensitized human airways did not demonstrate morphologic differences from control airways, as evaluated by inflammatory cell numbers and mast cell degranulation. The percentage of basement membrane covered by the epithelium was similar between the 2 conditions. These results are consistent with previous functional data that show no differences in contractile responses to carbachol between sham-sensitized and sensitized human bronchial rings.¹⁻³ Therefore, in our human airway model, passive sen-

sitization cannot alter function,¹⁻³ activate cells, and express cytokines. Our results are different from those of Berger et al,²² showing that passive sensitization of human airways with an IgE concentration similar to ours (305 vs 323 IU/mL) induces mast cell degranulation and release of tryptase, resulting in an enhancing histamine response on functional studies. However, although the data from the 2 studies can be compared, mast cell identification methods were different. Furthermore, none of these studies²³ can rule out the presence of cytokines in serum from atopic subjects. The serum samples we used for the passive sensitization procedure were collected from individuals allergic to house dust mites who had rhinitis or asthma, usually during asymptomatic periods. The serum samples collected by Berger et al²² were from subjects with asthma, probably under environmental challenge, as inferred by high eosinophil blood concentrations (>500 cells/mm²). Under these conditions, it is not possible to discern whether passive sensitization-induced alterations are really due to IgE itself or to cytokines present in asthmatic serum.^{12,23} Finally, data on histamine response are conflicting. Watson et al²⁴ found an increase in the magnitude of maximal contractions to histamine in airways sensitized with high-IgE serum (>3,000 IU/mL) but not with serum containing approximately 600 IU/mL.

In conclusion, in our model, IL-1 β and TNF- α cannot be advocated as causative agents of 1-hour allergen challenge consequences on the β_2 pathway. These data support our previous findings and suggest a role for mast cell mediators, particularly leukotrienes.³

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