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Eosinophils in the bronchial mucosa in relation to methacholine dose-response curves in atopic asthma

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Möller, Gertrude M., Shelley E. Overbeek, Cornelia G. Van Helden-Meeuwsen, Henk C. Hoogsteden, and Jan M. Bogaard. Eosinophils in the bronchial mucosa in relation to methacholine dose-response curves in atopic asthma. *J. Appl. Physiol.* 86(4): 1352–1356, 1999.—Asthma is characterized by both local infiltration of eosinophils in the bronchial mucosa and bronchial hyperreactivity (BHR). A detailed characterization of BHR implies analysis of a histamine or methacholine dose-response curve yielding not only the dose at 20% fall of baseline forced expiratory volume in 1 s (FEV_1), but also a plateau (P) representing the maximal narrowing response in terms of percent change in FEV_1 and reactivity as the steepest slope at 50% of P (%FEV_1/doubling dose). In the baseline condition, the specific airway conductance (sGaw) may be considered closely related to airway lumen diameter. In 20 nonsmoking asthmatic patients, methacholine dose-response curves were obtained, and a sigmoid model fit yielded the BHR indexes. Immunohistochemistry with the monoclonal antibodies (EG_1 and EG_2) was used to recognize the total number of eosinophils and activated eosinophils, respectively. The number of activated eosinophils was significantly correlated to both P ($r = 0.62$; $P < 0.05$) and sGaw ($r = -0.52$; $P < 0.05$), whereas weaker and nonsignificant correlations were found for dose at 20% fall of baseline FEV_1 and the total number of eosinophils. We conclude that the number of activated eosinophils can be considered a marker of the inflammation-induced decrease of airway lumen diameter as represented by the plateau index and sGaw.

bronchial mucosa; bronchial hyperreactivity; methacholine dose-response curve

ATOPIC ASTHMA IS CHARACTERIZED by local infiltration of several activated inflammatory cells in the bronchial mucosa, even in subjects with mild asthma (6). Airway inflammation produces hyperemia in the lamina propria (27). The process of vasodilation and plasma leakage may increase airway wall thickness, which may directly contribute to airway narrowing (24, 27). Eosinophils in particular have been associated with epithelial damage (5, 14) through their release of different basic proteins with cytotoxic properties, such as major basic protein, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin, and eosinophil peroxidase. The number of eosinophils in the bronchial mucosa and in bronchoalveolar lavage fluid has been correlated with the severity of asthma and the mechanisms that underlie airway hyperresponsiveness (4, 18, 25, 26), one of the most prominent characteristics of asthma (3).

Airway hyperresponsiveness is accompanied by a decrease in the threshold of airway narrowing in response to a variety of nonspecific stimuli (15). At present, airway responsiveness is usually measured in dose-response curves as the provocative concentration (PC) of histamine or methacholine causing a fall of 20% in the forced expiratory volume in 1 s (FEV_1), which is called the sensitivity (S; $\log_2 PC_{20}$). Previous studies (12, 22) have focused on the importance of characterizing the entire histamine or methacholine dose-response (MDR) curve not only by S, but also by reactivity, defined as the slope at 50% of the curve (R; %FEV_1/doubling dose), and the maximal airway narrowing response value [plateau (P); %change in FEV_1]. It was found that dose-response curves from asthmatic subjects show a shift to the left and have a steeper slope and higher maximal response value compared with those of normal subjects (28). To interpret the entire dose-response curve accurately, a model fit to the data is often necessary. Reaching an experimental P may be associated with a large drop in FEV_1, causing feelings of severe dyspnea in the patient and making the measurement very uncomfortable for the patient. Extrapolation of the data may, therefore, be necessary. Moreover, the model gives a smoothing of the individual data fluctuations, because of varying levels of patient cooperation or other causes. In our investigation, indexes were obtained as parameters of a sigmoid model, the cumulative Gaussian distribution (CGD) function, which was fitted to the dose-response curves (1).

Indexes of the MDR curve, which describe different aspects of bronchial hyperreactivity (22), may be correlated with inflammatory cell numbers. Another lung function index, which is an indicator of the size of the airway lumen, is the specific (volumic) airway conductance (sGaw; Ref. 29) to be obtained by body plethysmography. Inflammation, contributing to airway narrowing, may also be correlated with this variable. We, therefore, tested the hypothesis that, in mild to moderate asthma, indexes from the entire MDR curves and baseline sGaw are related to the presence and activation of eosinophils in the lamina propria.

METHODS

Patients. Patients were selected when they met the following criteria during the baseline measurements: PC_{20} histamine $\leq$ 8 mg/ml and $\geq$9% reversibility in FEV_1, relative to

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baseline, after inhalation of 1,000 μg of β_2-adrenoceptor agonists. Atopy was defined by at least one positive skin-prick test to a panel of 16 common aeroallergens in the presence of positive and negative controls.

In the month preceding the baseline measurements, patients were only allowed to take inhaled short-acting β_2-agonists on an as-needed basis. All other medication was stopped. Patients with a history suggesting respiratory infection or exacerbation of asthma in the month before the study were excluded.

Twenty nonsmoking atopic asthmatic patients [15 men; median age 26 yr (range 21–56 yr)] entered the study. Median FEV_1 was 2.98 liters (range 1.94–5.36 liters), and for percent predicted the median was 87% (range 47–108%). Approval was obtained by the Medical Ethics Committee of the hospital. All patients gave informed consent.

Design. After the first visit, baseline values were established during two morning or afternoon sessions. At one of these sessions, a flow-volume curve was constructed, bronchodilator response was measured, and intradermal skin testing was performed; at the other session, body plethysmography was carried out followed by the methacholine provocation test. Bronchoscopy was performed 1 wk after the baseline visit and was performed by the same operator (S. E. Overbeek).

Bronchoscopy. Fiber-optic bronchoscopy (model BF IT 10, Olympus, Tokyo, Japan) was performed with atropine (0.5 mg im) as premedication. Terbutaline, two puffs of 250 μg per nebuhaler, was given 30 min before the procedure. The nose, throat, and vocal cords were anesthetized with topical lidocaine spray. An Olympus fenestrated forceps (model FB19C) was used to take three to six biopsies from segmental or subsegmental divisions of the main bronchi.

Immunohistochemistry of bronchial biopsies. Each biopsy specimen was immediately placed in isotonic saline and frozen within 20 min in Tissue-Tek OCT embedding medium (Miles, Naperville, IL). Samples were stored at −80°C until use.

Sections (6 μm) were cut by using a cryostat and were collected on poly-L-lysine-coated (Sigma Chemical, St. Louis, MO) slides. The sections were air-dried for at least 1 h and stored at −20°C until use. Before immunohistological staining, frozen tissue sections were brought to room temperature and fixed in acetone for 10 min.

Immunohistochemical staining of bronchial biopsies was performed with the immuno-alkaline phosphatase anti-alkaline phosphatase method (9) by using new fuchsin (Chroma-Gesellschaft, Stuttgart, Germany) as the chromagen.

The following monoclonal antibodies were used: EG_1, recognizing ECP in resting and activated eosinophils, and EG_2, recognizing the cleaved form of ECP in activated eosinophils (Pharmacia, Uppsala, Sweden). The dilution was 1:100 for both EG_1 and EG_2.

Control slides were treated similarly excluding the primary antibody and by using an unrelated antibody of the same isotype and concentration.

The interval between the sections was at least 12 μm to avoid overlapping of cells.

Quantification of eosinophils. In the majority of cases, two biopsy specimens, out of three to six biopsies taken, were coded, and the observer (G. M. Möller) counted 5–10 sections in a blinded fashion for each antibody and each biopsy at a magnification of ×400 (Zeiss microscope 903844). Thus the total number of sections for the $n = 20$ patients per marker was ~300, and per-patient mean values were calculated. With the use of an eyepiece graticule, the number of positively stained cells was counted in a zone 100 μm deep in the lamina propria along the length of the epithelial basement membrane (BM), which had to be covered with epithelium over at least 500 μm. Most of the biopsies were of adequate size, without crush artifacts. Cells were counted if they stained red and contained a nucleus. The cell counts were expressed as the number of cells per millimeter of BM. The within-observer χ^2 test for goodness of fit for three repeated counts was <5% for EG_1 and EG_2.

Quantification of the lamina reticularis. Biopsies were coded before analysis. Subepithelial reticular collagen thickness was measured by using a 10 × 100 oil objective and a digital image-processing system (IBAS 2000 system, Kontron, Munich, Germany). The thickness of the BM was quantitated by measuring only the BM covered by at least a basal epithelial cell layer and where the lamina propria under the BM was at least 100 μm deep. The thickness of the BM was measured in five random fields in two sections for each patient.

MDR curves. The methacholine challenge test was performed by using the standardized 2-min tidal-breathing technique (8). Dose-response (%FEV_1) curves were obtained after the patients inhaled doubling concentrations of acetyl-β-methylcholine bromide (0.03–256 mg/ml) in normal saline. Dose was expressed as the $\log_2$ of the methacholine concentration in milligrams of methacholine per milliliter of saline. Thus 1 mg/ml is equivalent to 0.82 mg/ml of methylcholine-chloride solution. When chloride and bromide data are compared, a fixed conversion constant of 0.29 should, therefore, be subtracted from the $\log_2$ dose bromide values. A test was interrupted if the FEV_1 fell by more then 60% or if unpleasant side effects or dyspnea compelled the patient to stop.

Curve fitting. Although S was obtained by linear interpolation of adjacent data points of the dose-response curve according to international standards (19), other indexes of the sigmoid curve were obtained by fitting with a CGD function (1). This fit yielded the R as slope in the 50% point of the curve (%FEV_1/doubling dose) and the P value. If the last three PC values showed a variation coefficient of <5% of the mean value, this mean value was considered as the experimental P estimate; otherwise, the model fit was used from which the extrapolated P value was obtained. For R only, model estimates were used.

Pulmonary function. FEV_1 was measured with a heated pneumotachometer system (Jaeger, Würzburg, Germany), corrected to BTPS conditions and expressed as percent predicted (19).

sGaw was measured in a volume-constant body plethysmograph (Jaeger body test). The value was derived from the ratio of the inverse of the effective resistance (Reff) and the intrathoracic gas volume during the measurement. Body plethysmography was assessed with a humidified and thermostated (37°C) rebreathing bag during normal breathing, whereas panting frequency during the volume measurement was ~0.5–1 Hz.

Data analysis. Correlation coefficients between cellular and pulmonary function indexes were obtained by Spearman's rank method; $P < 0.05$ was considered significant.

RESULTS

Curve fitting and sGaw. In 12 patients, the P value had to be obtained with the model fit because experimental determination was not possible. An example of a P extrapolation by a model fit is given in Fig. 1. Median values and ranges of R and P values were 9.06% FEV_1/doubling dose (range 3.31–16.7% FEV_1/doubling

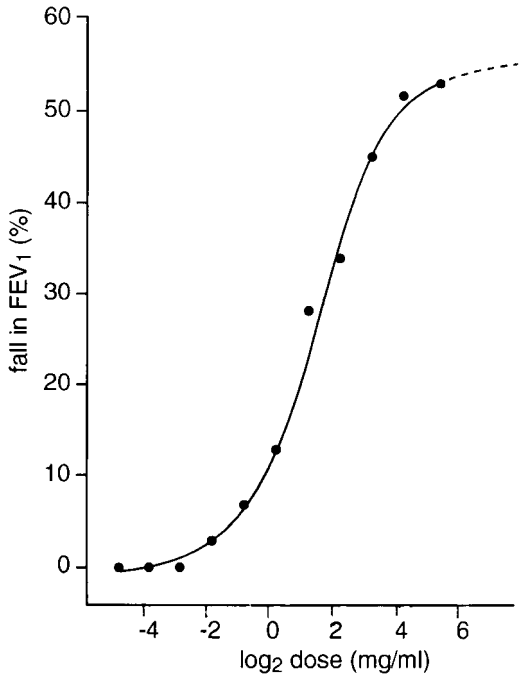

Fig. 1. Example of curve fit with cumulative Gaussian distribution function to a methacholine bromide dose-response curve. Plateau was obtained by extrapolation. Dose values are presented as methacholine chloride equivalents. FEV_1, forced expiratory volume in 1 s.

dose) and 52.95% FEV_1 (range 23.8–80.5% FEV_1), respectively. Median and range of $\log_2 PC_{20}$ and sGaw were 0.08 mg/ml (range −5.18–7.98 mg/ml) and 0.63 s/kPa (range 0.25–2.28 s/kPa), respectively.

Correlation among cellular indexes, FEV_1, hyperresponsiveness, and sGaw. The median values and ranges for $EG1^+$ (total) eosinophils and $EG2^+$ (activated) eosinophils were 11.6 cells/mm BM (range 1.6–68 cells/mm BM) and 3.5 cells/mm BM (range 0–29.3 cells/mm BM), respectively. The correlation among the log dose-response indexes, sGaw, and (activated) eosinophils is indicated in Table 1.

The median thickness of the BM was 10.6 μm (range 6.1–14.5 μm). BM thickness was not significantly correlated with lung function or dose-response indexes.

The correlation between baseline FEV_1 (%predicted) and cellular or dose-response indexes was also not significant.

In addition, we found no significant correlation between the total number of eosinophils ($EG1^+$) and $\log_2 PC_{20}$ or R. However, the number of activated eosinophils ($EG2^+$) was, although weakly, significantly correlated to the P value (Table 1: $r = 0.62$, $P < 0.05$; Fig. 2). No correlation was found between the total number of eosinophils and the P value. The sGaw was negatively correlated with the number of activated eosinophils (Table 1; $r = -0.52$, $P < 0.05$) but not with the total eosinophil number.

Table 1. *Spearman rank correlation coefficients between number of total and activated eosinophils and sensitivity ($\log_2 PC_{20}$), reactivity (%FEV_1/doubling dose), plateau value, and specific airway conductance*

	$\log_2 PC_{20}$	Reactivity	Plateau	sGaw
$EG1^+$	−0.10	0.28	0.37	−0.40
$EG2^+$	−0.32	0.12	0.62*	−0.52*

PC_{20}, provocative concentration that causes 20% fall of baseline forced expiratory volume in 1 s (FEV_1); sGaw, specific airway conductance; $EG1^+$, total eosinophils ($n = 20$); $EG2^+$, activated eosinophils ($n = 20$). *$P < 0.05$.

DISCUSSION

In the present study, we have demonstrated a significantly positive correlation between the number of activated eosinophils in the lamina propria, an important cellular marker in bronchial mucosal inflammation, and the P value. Furthermore, the sGaw was significantly negatively correlated with the number of activated eosinophils. No significant correlations could be demonstrated between basal membrane thickness and either lung function or dose-response indexes.

In a previous study (1), our laboratory has shown that our model was preferable to other methods, which mostly used only part of the data points. The CGD model both enabled smoothing out of random fluctuations in the data points and yielded reliable curve indexes (R and P value) where no experimental P values could otherwise be reached. In eight of the MDR curves in this study, a P could be accurately estimated by averaging the last three PC values. In the other cases, the value had to be extrapolated with the CGD model fit.

Interestingly, only the number of activated eosinophils, not the total number, was significantly correlated with the P value. In previous studies with bronchial biopsies from asthmatic patients, the relationship between eosinophils and the degree of airway hyperresponsiveness (PC_{20} methacholine) has been investigated. Bradley et al. (6) have found a negative correlation

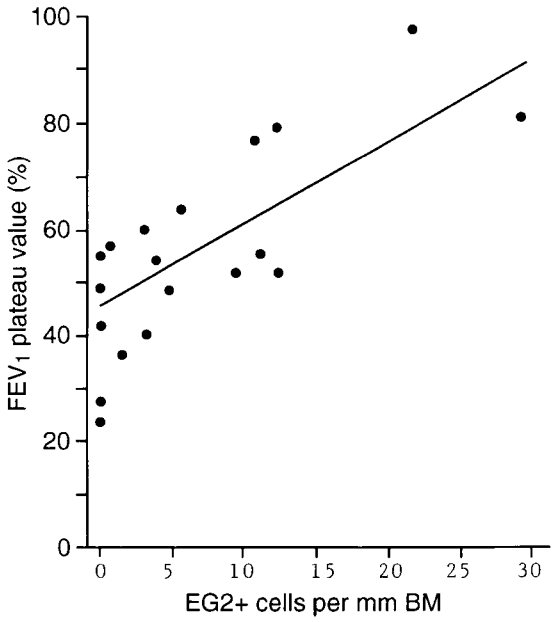

Fig. 2. Plateau values in relation to number of activated ($EG2^+$) eosinophils per millimeter of basement membrane (BM) ($r = 0.62$, $P < 0.05$).

between the number of $EG2^+$ eosinophils and $\log_2 PC_{20}$ when comparing subjects with asthma with control subjects. Djukanovic et al. (11), however, were unable to demonstrate a correlation between the number of activated eosinophils and PC_{20} methacholine, and neither were we.

A recent study (7) showed a significant negative correlation between the total number of intraepithelial eosinophils and PC_{20} methacholine values. These findings were in line with another study (13) that showed that the total number of inflammatory cells and mast cells was significantly negatively correlated with PC_{20} methacholine. Although we also found negative correlation coefficients for the relationship between $\log_2 PC_{20}$ and both the total number of eosinophils and the number of activated eosinophils, these coefficients did not reach significance. Although a reasonable number of sections per patient were analyzed, the relatively small sample size and random measuring errors may have influenced the correlations. Jeffery et al. (17) have found that counting all the inflammatory cells, not only in the lamina propria but also in the submucosa, did not improve correlations. It is, therefore, unlikely that the fact that we restricted our findings to the lamina propria may have influenced the correlations.

The maximal response P has been considered as determined by "postjunctional" mechanisms, such as smooth muscle contractility, viscous and elastic loads on airway smooth muscle shortening, swelling of the airway wall, and intraluminal exudate (22). The significant relationship of the number of activated eosinophils with the P value may reflect the influence of inflammation on airway lumen, in this case being the residual lumen after maximal bronchoconstriction. S changes are associated with a horizontal shift of MDR curves, as shown in Fig. 1. The position with respect to the log dose axis is then most accurately defined as the PC at 50% of the P value. This means that $\log_2 PC_{20}$ is dependent not only on the position of the curve along the horizontal axis but also on the P value. We found, on one hand, a nonsignificant relationship between $EG2^+$ and $\log_2 PC_{20}$ but, on the other hand, a significant correlation between $EG2^+$ and P. Thus most probably the primary $EG2^+$-related effect was on airway lumen diameter and not on S.

We used sGaw, derived from Reff and intrathoracic gas volume, as another index, being representative of the size of the airway lumen. No unique index for resistance exists in cases of unequal ventilation, which may be present in the patient category we investigated. Mean resistance within the breathing cycle is then best represented by Reff (16). Moreover, sGaw is less dependent on lung volume than are other resistance indexes. The significant relationship that we found between sGaw and the number of activated eosinophils, indicating that a decrease in specific conductance coincides with an increase in the number of activated eosinophils, can also possibly be explained by the swelling of the airway wall and the presence of intraluminal exudate. The weaker correlation of $EG2^+$ with FEV_1 (%predicted) may probably be explained by the fact that this variable is less directly related to airway lumen or various aspects of lung mechanics, e.g., elastic properties of lung parenchyma and central airways.

Our findings support suggestions put forward in a recent study by Crimi et al. (10). Similar to our findings, they did not find a correlation between ECP contents and PC_{20}. They suggested that airway remodeling may play a role. The correlations we found between activated eosinophils and both P and sGaw do support their suggestions.

Although Jeffery et al. (17) state that it is more appropriate to look at relative proportions of various inflammatory cells present rather than at the total number of activated eosinophils, we found no significant correlations between cells as antigen-presenting cells ($CD1a^+$ cells), T lymphocytes ($CD4^+$ cells), and mast cells ($tryptase^+$ cells) on bronchial hyperreactivity or lung function indexes (data not shown).

We only investigated eosinophils in bronchial biopsies and not in bronchoalveolar lavage fluid or sputum. Earlier investigations in bronchial lavage fluid also found the degree of eosinophil activation, rather then the total number of eosinophils, to reflect inflammation in asthma (2). The same conclusion was drawn from studies in sputum (20, 23). Our findings, therefore, support the hypothesis that activated eosinophils and/or their mediators cause bronchoconstriction, enhance airway inflammation and epithelial damage, and possibly cause an increase in airway hyperresponsiveness.

In conclusion, the P value and also baseline sGaw are weakly, although significantly, related to the number of activated eosinophils in the bronchial mucosa. These results suggest a direct relationship between bronchial mucosal inflammation characterized by eosinophil activation and the decrease in airway lumen. Our results stress the importance of modeling the entire MDR curve when characterizing airway hyperresponsiveness in patient follow-up.

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