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ASTHMA PREVALENCE AND RISK FACTORS

Morphological and Molecular Characteristics of "Difficult" Asthma

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Background. There are several clinical variants of severe difficult-to-treat asthma: asthma with persistent airflow limitation, brittle asthma, and fatal asthma; but the differences between the pathogenetic mechanisms underlying the disease heterogeneity are unknown. *Objectives.* The aim was to evaluate the morphological and molecular characteristics of brittle asthma type I and asthma with persistent airflow limitation compared to mild-to-moderate asthma, by the analysis of the cellular structure and gene expression in the bronchial mucosa. *Methods.* Bronchoscopic evaluation was performed in 42 asthmatic patients: 10 with brittle asthma, 10 with severe asthma with persistent airflow limitation, and 22 with mild-to-moderate asthma. Morphometric and cytological analyses of the bronchial mucosa were performed. The mRNA levels for the *ADRB2*, *HRH1*, and *CHRM3* genes in the bronchial mucosa were measured by quantitative real-time polymerase chain reaction (PCR). *Results.* A predominance of eosinophils (29.48/mm^2, 95% confidence interval [CI] 25.24–33.72) and neutrophils (40.13/mm^2, 95% CI 32.77–47.49) was observed in patients with mild-to-moderate asthma; however, histiocytes-macrophages (65.80/mm^2, 95% CI 56.95–74.65) and lymphocytes (52.94/mm^2, 95% CI 42.83–63.06) were more common in patients with brittle asthma, and neutrophil counts (81.11/mm^2, 95% CI 58.33–103.89) were significantly increased in subjects with persistent airflow limitation. An increase in the expression of the M_3-cholinoreceptor and the β_2-adrenoreceptor genes was demonstrated in severe asthmatics compared to mild-to-moderate asthma patients. Significantly higher levels of *CHRM3* (57.17%, 95% CI 55.04–59.29) and *HRH1* (38.82%, 95% CI 35.84–41.81) mRNAs were observed in patients with brittle asthma. The level of *ADRB2* gene expression (71.41%, 95% CI 63.54–85.09) was maximal in patients with asthma with persistent airflow limitation. *Conclusions.* There is evidence of significantly different morphological characteristics and molecular mechanisms of inflammation and bronchoconstriction underlying the clinical heterogeneity of severe asthma.

Keywords asthma phenotypes; difficult-to-treat asthma; gene expression; morphology

Introduction

In spite of the marked insights of modern medicine into the pathogenesis of bronchial asthma, there remain unresolved issues regarding therapeutic control and in particular the choice of optimal pharmacotherapy regimes. The diagnosis of "bronchial asthma" encompasses several asthma "phenotypes," which can be defined by the age of onset (early-onset—before 12 years of age, and late-onset), the inflammatory component (eosinophilic or neutrophilic asthma), trigger-related factors (allergic, intrinsic, aspirin-induced, menses-related, nocturnal asthma), and response to treatment (treatment-sensitive, treatment-resistant, corticosteroid-resistant asthma, etc.) (1).

There is a separate group of patients with severe difficult-to-treat asthma in whom optimal therapeutic control cannot be achieved using the maximal recommended doses of inhaled therapy. Difficult asthma is a serious medicosocial problem associated with a high morbidity and mortality and accounts for up to 80% of all the costs for asthma management. Among this cohort of patients, there are also several clinical variants: those with a persistent pattern of airway obstruction (or asthma with persistent airflow limitation), patients with unstable or brittle asthma, and those patients with fatal or near-fatal asthma (2). Clinical and functional characteristics of these phenotypes are very distinguishable, suggesting a difference between the pathogenetic mechanisms underlying difficult asthma heterogeneity. Bronchial hyperresponsivness (BHR) is one of the basic factors responsible for the functional differences of the difficult asthma phenotypes. The M_3-cholinoreceptor and the H_1-histamine receptor are the basic receptors of smooth muscle contraction, whereas β_2-adrenoreceptor stimulation leads to bronchodilatation. Dysfunction of these bronchoconstrictor receptors, which are widely presented on regulatory inflammatory cells, causes modification of bronchial smooth muscle tone and is one of the causes of the BHR phenomenon.

Bronchoscopic evaluation of asthmatic patients has revealed some differences in bronchial inflammation in patients with different clinical phenotypes of the disease. Wenzel et al. revealed neutrophilic infiltration of the bronchi in severe asthma patients (3). An increase of eosinophils in transbronchial biopsy specimens obtained at 4 AM in combination with decreased lung function traits was demonstrated in patients with nocturnal asthma (4). Carroll et al. found high levels of neutrophils in the bronchial tissue of patients with fatal asthma, if death occurred within 2 h following a sudden asthma attack (5).

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It is likely that there are various types of inflammatory processes (with prevalence of different effector cells) or differences in bronchoconstriction gene expression underlying the varying asthma clinical phenotypes. This could explain the absence of therapeutic control in some patients in spite of therapy. Therefore detection of the mechanisms determining the clinical variant of asthma may enable more effective and individualized treatment regimes.

The aim of this study was to evaluate the morphological and molecular characteristics of brittle asthma type I and asthma with persistent airflow limitation, by analyzing the cellular structure and gene expression in the bronchial mucosa.

Methods

Study Group

A cross-sectional open-label study was conducted. It was approved by a local ethical committee and informed consent was obtained from all patients included in the study. All subjects met the following inclusion criteria: (1) males and females aged $\geq$18 and $\leq$65; (2) high compliance with treatment (according to the investigator's opinion); (3) for brittle asthma type I: $\geq$40% diurnal variation of peak expiratory flow (PEF) for $\geq$50% of the time for a period of at least 150 days despite therapy with at least 1500 μg of beclomethasone daily (or equivalent); (4) for asthma with persistent airflow limitation: persistent airway obstruction (forced expiratory volume in one second [FEV1] $\leq$60% predicted), with or without episodes of sudden deterioration; regular (at least 1 year) oral corticosteroid treatment at doses of 5 to 20 mg of prednisolone (corticosteroid-dependence); (5) for mild-to-moderate asthma: diurnal PEF variability $\leq$30% all the time during the preceding month; regular therapy of inhaled corticosteroid in a dose $\geq$500 μg of beclomethasone daily (or equivalent) during the preceding month; and positive skin prick test for at least one indoor aeroallergen.

The three patient groups that were included in this study are (1) brittle asthma type I (total 10; 3 males, 7 females); (2) asthma with persistent airflow limitation (total 10; 3 male, 7 females); and (3) mild-to-moderate asthma (total 22; 2 males, 20 females).

The exclusion criteria were (1) current or recent exacerbation of disease; (2) past or present diseases that, as judged by the investigator, may affect the outcome of this study (these diseases include, but are not limited to, cardiovascular disease, malignancy, hepatic disease, renal disease, hematologic disease, neurological disease, endocrine disease, and pulmonary disease); (3) potential hazard of instrumental investigation for the patients (in the investigator's opinion); and (4) history of tobacco use of $>$10 pack-years.

Procedures

All subjects underwent a routine medical history and physical examination including a PEF measurement. Spirometry (MasterScope, Jaeger), was performed on all subjects according to the American Thoracic Society (ATS) guidelines (6), using predicted values from Quanjer et al. (7). BHR measurement was performed in patients with a prebronchodilator FEV_1 $\geq$70% predicted (MasterScope, Jaeger); test conditions met criteria stated in ATS guidelines (8). Functional tests were performed in the morning, at least 8 h after any short-acting β_2-agonist and 48 h after long-acting β_2-agonist inhalation. Every patient did a minimum of three acceptable maneuvers (the maximum number of maneuvers was eight). Acceptable repeatability is achieved when the difference between the largest and the next largest forced vital capacity (FVC) is 0.150 L and the difference between the largest and next largest FEV_1 values is 0.150 L. BHR measurement was performed using methacholine in doubling concentrations (0.0625 to 16.0 mg/ml); the test is considered positive when the FEV_1 falls more than 20% from the baseline (the value of PC_{20} is the methacholine concentration that resulted in a 20% fall in FEV_1) and PC_{20} $<$16.0 mg/ml.

All patients underwent a bronchoscopic evaluation with an endobronchial biopsy to measure the degree and type of inflammation in their airways. Patients were hospitalized for 3e days to reduce a risk of procedure-related complications. Clinical examination and spirometry were performed prior to the procedure. Premedication consisted of atropine (0.1%; 1 ml) subcutaneous and nebulized short-term β_2-agonist (Berodual; 1 ml). Lidocaine was used for local anesthesia in the upper and lower airways. Vital signs were monitored throughout the procedure.

The bronchoscope (BF1T20; Olympus, Japan) was passed through the oropharynx; two endobronchial biopsies were obtained from the proximal bronchus of the upper lobe of the right lung. Biopsy specimens (first tissue sample) were collected in formalin (10%), than passed over methyl alcohol with increasing concentrations and filled up in paraffin. Tissue sections (5 to 7 μm thick) were stained with hematoxilin-eosin. The preparation of the tissue samples was done by one specialist to reduce any possible effects on the results. Plain microscopy of the bronchial mucosa was done under low magnification ($\times$200); the density of the cover slide epithelium and its different cells (ciliated, goblet, basal epithelium cells) and relative volume of glands and connective tissue were estimated by point-by-point measurements using Avtandilov's grid. The height of the epithelium stratum and the thickness of the basal membrane were measured by ocular micrometer. Inflammatory cells were counted in a 1-mm^2 area of the lamina propria of the bronchial mucosa using Adobe Photoshop 7.0 (Adobe Systems, USA). To advance the reproducibility of the morphological analysis, at least three trained morphologists read the slides.

We obtained poor-quality biopsy samples for one patient with brittle asthma, four patients with asthma with persistent airflow limitation, and five patients with mild-to-moderate asthma; therefore these patients were excluded from subsequent analysis.

Biopsy specimens for molecular analysis (second tissue sample) were collected in test tubes and placed in liquid nitrogen immediately. Total RNA was extracted using TRI Reagent kit (Molecular Research Center, USA) and dissolved in deionized RNase-free water. To synthesize cDNA, reverse transcription was performed with the use of the Reverta kit (Amplisense, Russia). Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was used to measure mRNA levels for the β_2-adrenoreceptor (*ADRB2*), the M_3-cholinoreceptor (*CHRM3*), the

H_1-histamine receptor (*HRH1*), and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes. iQ SYBR Green Supermix (Bio-Rad, USA) was used to perform RT-PCR, which was run on "Bioms-1" PCR-machine (Biomedsib, Russia). The Bioms-1 software was used to determine the threshold cycle (C_t) for each individual reaction and the average between three technical replicates was taken for subsequent estimates. The *ADRB2*, *HRH1*, and *CHRM3* mRNA levels were then recalculated as relative to the *GAPDH* mRNA level.

Statistical Analysis

Data were analyzed using "Statistica for Windows 6.0" software (Statsoft, USA). Because the quantitative data were not normally distributed, nonparametric Kruskal-Wallis test was used to compare the mean values between the groups. Discriminant analysis was performed to estimate the contribution of the studied parameters to the asthma phenotypes appearance. Spearman rank correlation was used to identify relationships between the asthma signs. The data are presented as mean and 95% confidence intervals. Significance of differences was accepted at $p < .05$.

Results

Clinical and functional characteristics of the patients are shown in Table 1. All subjects were matched in age and asthma duration. Patients with severe asthma had significantly more frequent occurrence of day- and night-time symptoms, as well as more frequent emergent medication use. Severe asthmatics also had significantly lower lung function parameters FEV_1 and PEF as compared to the mild-to-moderate patients. Bronchial reactivity in methacholine challenge test was positive in all patients (with prebronchodilator $FEV_1 \geq 70\%$ predicted) and was significantly higher in severe patients. Daily variability of PEF in patients with brittle asthma type I was 44.44% (42.14–46.74%), reflecting disease lability; these patients called an ambulance 4.2 (3.5–4.6) times a year. Exacerbation was more frequent in severe asthmatics, resulting in hospitalization in 50% of the cases. All patients with mild-to-moderate and brittle asthma had atopic disease, as confirmed by skin-prick tests to common allergens and serum immunoglobulin E (IgE) level, whereas among patients with severe asthma with persistent airflow limitation, four asthmatics had a low level of serum IgE and a negative skin-prick tests to allergens. The most common triggers in all the groups were physical exercise, infection of respiratory tract, and allergens (50% of patients with brittle asthma type I had polyvalent sensitization). Fifty percent of the patients with brittle asthma type I (five patients) and 50% of the patients with severe asthma with persistent airflow limitation (five patients) had relatives with asthma. Two patients with mild-to-moderate, three patients with persistent airflow limitation, and four patients with brittle asthma had aspirin intolerance; among them, four individuals (two with brittle asthma and two with asthma with persistent airflow limitation) had aspirin triad.

Morphological evaluation of the bronchial tissue revealed typical changes for asthma: edema and garneting of the collagen fibers of the lamina propria of the bronchial mucosa, subbasement membrane thickening, polymorphic cell infiltrate under the basement membrane, epithelial damage, and exfoliation. Marked smooth muscle hyperplasia in the lamina propria of the bronchial mucosa was specific for brittle asthma type I. Comparative morphometry is presented in Table 2.

Comparison of cell infiltrate revealed a predominance of eosinophils and neutrophils in patients with mild-to-moderate asthma; however, histiocytes-macrophages and lymphocytes were more common in patients with brittle asthma, and neutrophil counts were significantly increased in subjects with persistent airflow limitation (Figure 1).

Linear discriminant analysis was applied to find out which classifiers can separate the three groups of patients with severe asthma. Discriminant functions were calculated with the use of the following signs and traits as the classifiers: the density of the cover slide epithelium, the ciliated epithelium, the goblet cells, and the basal epithelium; the relative volume of the glands and the connective tissue; the height of the epithelium stratum, the thickness of the basal membrane; and the counts of inflammatory cells in the bronchial mucosa. Backward-stepwise analysis was applied, which sequentially removes classifiers with the least impact on the model as

Table 1.—Clinical and functional characteristics of studied subjects.

Parameters	Groups of patients with asthma		
	Mild-to-moderate asthma ($n = 22$)	Severe asthma brittle I type ($n = 10$)	Severe asthma with persistent airflow limitation ($n = 10$)
Age (years)	43.68 (38.09–49.27)	43.40 (31.59–55.21)	54.10 (47.55–60.65)
Duration of disease (years)	8.27 (5.02–11.53)	15.50 (7.41–23.58)	11.30 (6.63–15.97)
Mean daytime symptoms (per day) during last week	0.86 (0.54–0.96)	3.72 (2.80–4.64)*	3.66 (3.28–4.03)*
Mean nighttime symptoms (per day) during last week	0.09 (0.07–0.12)	2.06 (1.49–2.62)*	2.15 (1.71–2.58)*
Mean rescue medication use (per day) during last week	0.99 (0.69–1.23)	5.88 (3.34–8.42)*	8.28 (5.99–10.57)*
Exacerbations during last 12 months	2.28 (1.46–3.11)	3.60 (2.55–4.65)*	4.00 (3.00–4.99)*
IgE, IU/ml	246.97 (127.45–366.50)	309.60 (230.65–388.55)	108.45 (34.64–182.25)#
FEV_1,% of predicted	74.80 (70.24–79.35)	55.06 (45.49–64.63)*	56.04 (46.87–65.20)*
PEF,% of predicted	78.66 (74.46–82.87)	64.83 (50.32–79.33)	51.27 (42.99–59.54)*#
PC_{20}, mg/ml✶	3.34 (1.64–5.04)	0.24 (− 0.19–0.68)*	—✶

Note. Data are mean with 95% confidence intervals.
*p <.05 vs. mild-to-moderate asthma.
#p <.05 vs. asthma brittle I phenotype.
✶—, test was performed only in patients with prebronchodilator $FEV_1 \geq 70\%$ predicted.

TABLE 2.—Comparative morphometry of bronchial tissue samples.

Parameters	Groups of patients with asthma		
	Mild-to-moderate asthma ($n = 22$)	Severe asthma brittle I type ($n = 10$)	Severe asthma with persistent airflow limitation ($n = 10$)
Density of cover slide epithelium, mm^3/mm^3	0.26 (0.23–0.29)	0.12 (0.11–0.14)*	0.14 (0.08–0.21)*
Density of ciliated epithelium, mm^3/mm^3	0.12 (0.10–0.15)	0.05 (0.05–0.06)*	0.06 (0.02–0.10)*
Density of goblet cells, mm^3/mm^3	0.08 (0.07–0.09)	0.02 (0.01–0.03)*	0.04 (0.02–0.06)*
Density of basal epithelium, mm^3/mm^3	0.06 (0.05–0.07)	0.05 (0.03–0.06)	0.04 (0.02–0.06)
Relative volume of glands,%	66.30 (61.15–71.46)	32.16 (26.09–38.24)	24.59 (10.44–38.73)*
Relative volume of connective tissue,%	34.58 (29.90–39.25)	68.22 (62.06–74.38)*	57.13 (45.60–68.66)*
Height of epithelium stratum, micrometers	63.30 (55.39–71.21)	56.31 (46.56–66.06)*	23.17 (14.01–32.33)*#
Thickness of basal membrane, micrometers	8.47 (7.15–9.79)	18.77 (12.44–25.10)*	26.42 (22.56–30.28)*#

Note. Data are mean with 95% confidence intervals.
*$p < .05$ versus mild-to-moderate asthma.
#$p < .05$ versus asthma brittle I phenotype.

determined by individual *F*-remove statistics and corresponding *p* values. Overall and individual Wilks' λ statistics reflecting the discriminatory power of a set of classifiers and separate variables, respectively, were calculated. Three pairwise discriminant functions were calculated for possible pairs of stratification between the groups of patients, and two of them were significant overall. Patient stratification in two roots of significant discriminant functions is presented in Figure 2. Three morphological traits had statistically significant impact on the patient stratification (overall Wilks' $\lambda = 0.02429$; $F(24, 54) = 12.187$; $p < .00001$): height of the epithelium stratum ($\lambda = 0.043$; $p = .0004$), thickness of the basal membrane ($\lambda = 0.77$; $p = .03$), and density of neutrophils ($\lambda = 0.03$; $p = .05$).

Analysis of expression of inflammatory and bronchoconstriction genes (*CHRM3*, *HRH1*, *ADRB2*) was performed in bronchial tissue samples obtained by biopsy. An increase in expression of the *CHRM3* and *ADRB2* genes was demonstrated in severe asthmatics when compared to mild-to-moderate patients (Figure 3). Significantly higher mRNA levels of *CHRM3* and *HRH1* was observed in patients with brittle asthma. The level of *ADRB2* expression was maximal in patients with asthma with persistent airflow limitation.

Linear discriminant analysis was applied in the same way as above, with use of the mRNA levels of the studied genes as the classifiers. Two genes, *ADRB2* and *CHRM3*, were found to be statistically significant stratifies of the severe asthma patients (Figure 4).

DISCUSSION

To the best of our knowledge, this is the first study of morphological and molecular characteristics of bronchial tissue in patients with different clinical phenotypes (brittle type I and asthma with persistent airflow limitation) of severe difficult-to-treat asthma. Our study has demonstrated that the functional differences of these phenotypes may be based in the underlying inflammatory process and differences in gene expression.

The data presented have revealed signs of structural changes in severe asthma (high relative volume of connective tissue, the thickening of the basal membrane, the decrease of cover slide epithelium thickness); the effector cell (neutrophils and eosinophils) infiltration was lower in patients with brittle type I asthma when compared to the mild-to-moderate asthmatics; and neutrophil counts were significantly increased in subjects with persistent airflow limitation.

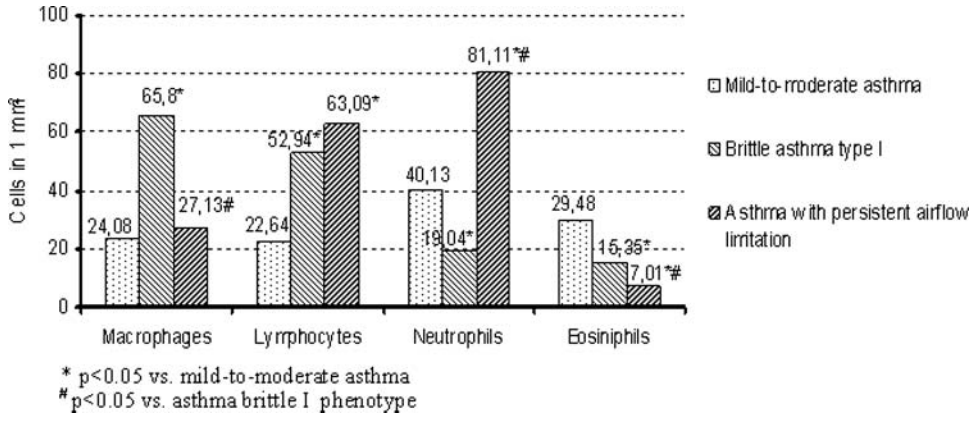

FIGURE 1.—Characteristics of polymorph cell infiltrate of bronchial mucosa.

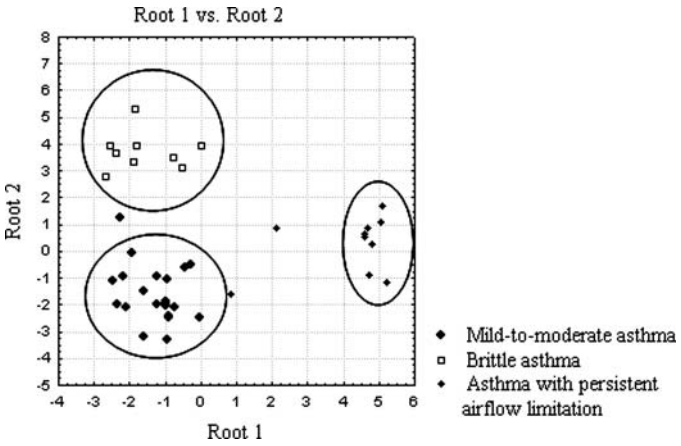

FIGURE 2.—Localization of the studied patients in the dimension of roots based on discriminant function built on morphological characteristics. Overall Wilks' $\lambda = 0.02429$, $F(24, 54) = 12.187$, $p < .00001$. For variables in the model, height of epithelium stratum: Wilks' $\lambda = 0.043$, $p = .0004$; thickness of basal membrane: Wilks' $\lambda = 0.77$, $p < .03$; density of neutrophils: Wilks' $\lambda = 0.03$, $p = .05$.

Undoubtedly, long-term use of higher doses of inhaled corticosteroids and frequent courses of systemic corticosteroids in severe asthma influence the course of the persistent inflammation and the effector cell counts as compared to the mild-to-moderate patients. At the same time, eosinophil counts in brittle asthma patients (15.35/mm^2, 95% confidence interval [CI] 10.45–20.26) are higher than those in patients with corticosteroid-dependent asthma. A comparison with data published elsewhere (9) allows us to suggest the appearance of eosinophilic inflammation in brittle type I asthma. In contrast, in subjects with severe asthma with persistent airflow limitation regularly taking oral corticosteroids, we found high levels of neutrophils. This finding is in accordance with a previous study (10) that demonstrated that the treatment of severe asthmatics with oral corticosteroids for 1 year increases neurtophil counts in biopsy and lavage specimens. Persistent eosinophilic infiltration in patients with brittle type I asthma can result from a defect of apoptosis of these cells (11) and lead to the symptomatic course of asthma and therapy-resistance of the disease. Active remodeling processes in patients with severe asthma can result from persistent inflammation in spite of therapy with high levels of inhaled steroids (12, 13)

Marked smooth muscle hyperplasia in patients with brittle asthma in our study can be another aspect of this clinical phenotype, taking into account the increased bronchial hyperresponsiveness of these patients. Undeniably, these results need to be validated by other techniques (for example immunohistochemistry analysis).

Thus atrophic changes, persisting inflammation, and marked structural changes were demonstrated in patients with severe asthma in spite of intensive therapy. Also, marked morphological differences were observed in patients with brittle asthma type I and those with severe asthma with persistent airflow limitation.

Another aim of this study was to estimate the activity of the genes involved in the development of inflammation and bronchoconstriction in patients with different phenotypes of severe asthma.

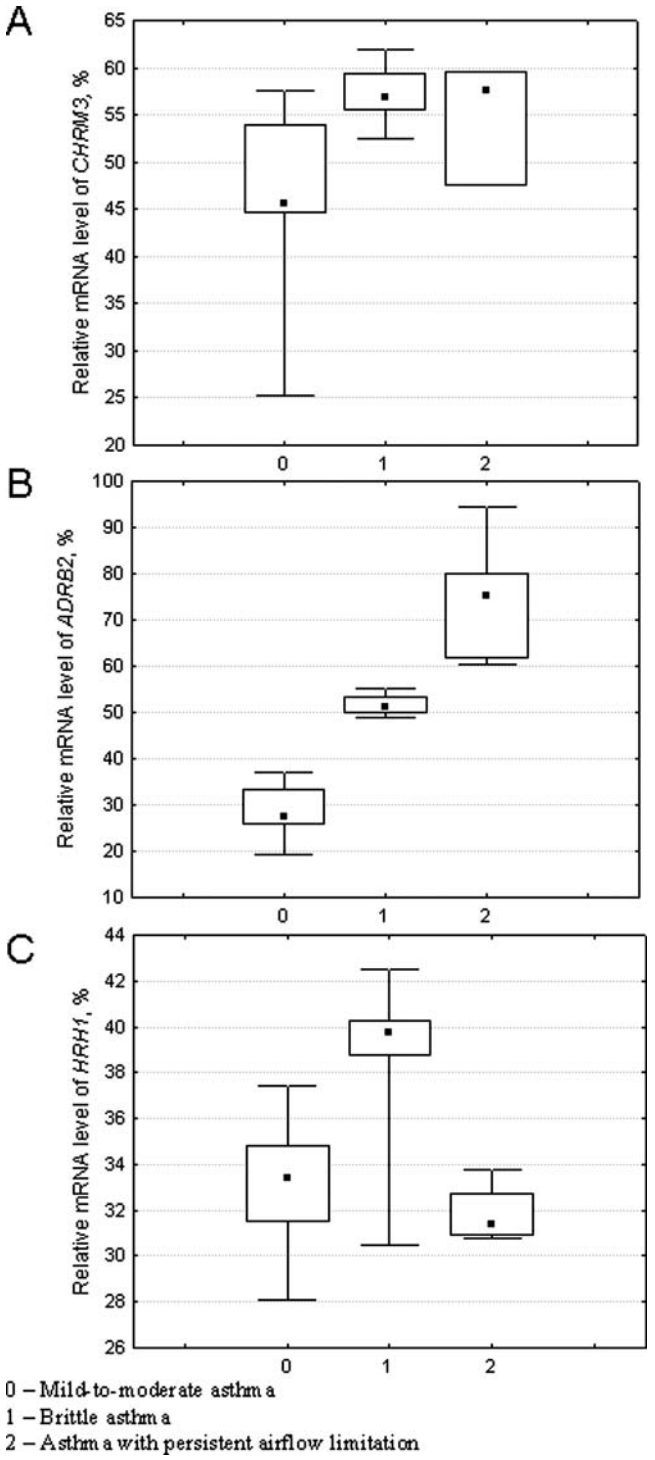

FIGURE 3.—Relative mRNA levels of M_3-cholinoreceptor (A; $p = .0002$), β_2-adrenoreceptor (B; $p < .0001$), H_1-histamine receptor (A; $p = .02$) genes in bronchial mucosa (Kruskal-Wallis ANOVA). Data are presented as median/quart/range.

The increased expression of the M_3-cholinoreceptor and the H_1-histamine receptor genes was observed in patients

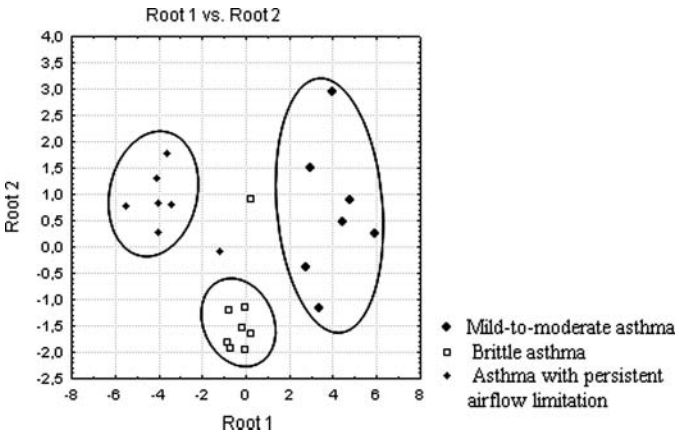

Figure 4.—Localization of the studied patients in the dimension of roots based on discriminant function built on *ADRB2* and *CHRM3* expression levels. Overall Wilks' $\lambda = 0.039$, $F = 22.82$, $p < .0001$. For variables in the model, *ADRB2* mRNA: Wilks' $\lambda = 0.43$, $p < .0001$; *CHRM3* mRNA: Wilks' $\lambda = 0.06$, $p = .03$.

with brittle asthma when compared to the other two groups. Stimulation of the M_3-cholinoreceptor and the H_1-histamine receptor results in bronchial smooth muscle contraction and this could be one of the factors underlying increased bronchial hyperreactivity in brittle asthmatic patients. This is also confirmed by the correlation observed between the *CHRM3* expression and daily variability of PEF (Spearman rank test $r = .86$; $p = .0006$).

It is well known that macrophages express the M3-cholinoreceptor, which takes part in their chemotaxis (14, 15). In our study, we have revealed the maximal level of histiocytes-macrophages in the bronchial mucosa in patients with brittle asthma, and its correlation with M_3-cholinoreceptor gene expression ($r = .41$; $p = .043$). Another possible explanation of the *HRH1* overexpression is the effect of active allergic inflammation, because lymphocytes, eosinophils, and dendritic cells also express this histamine receptor.

We found that the expression of the *ADRB2* gene was increased in patients with severe asthma when compared to mild-to-moderate asthmatics; the maximal level of β_2-adrenoreceptor mRNA was observed in patients with severe asthma with persistent airflow limitation. This can reflect the response to the medication regime: corticosteroids are able to increase *ADRB2* expression through nuclear factor (NF)-κB inactivation (16–18). In addition, the β_2-adrenoceptor is also expressed in many proinflammatory and immune cells, including mast cells, macrophages, neutrophils, lymphocytes, and eosinophils, and provides the stimulation of the anti-inflammatory effect by blocking the release of mediators and proinflammatory cytokine synthesis. However, the overexpression of the β_2-adrenoreceptor revealed in severe patients does not lead to the anti-inflammatory and the bronchodilatory effects expected.

Thus, we have shown that bronchial biopsy specimens obtained from patients with several different clinical phenotypes of difficult-to-treat asthma (brittle asthma type I and severe asthma with persistent airflow limitation) display distinct inflammatory and gene expression profiles. We propose that these differences may underline the clinical heterogeneity of severe asthma and may provide novel targets in the search for new therapeutic strategies.

Because the results were obtained on a limited set of patients, it is essential to obtain independent confirmations in larger sample groups. We expect that the results may, in part, be a result of the effects of steroids taken in various doses by the severe asthma patients. Nevertheless, we believe that our data provide a new insight into the pathophysiological basis of "difficult" asthma.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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