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PATHOPHYSIOLOGY

Correlation between Eosinophil Count and Methacholine Challenge Test in Asymptomatic Subjects

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Background. Methacholine challenge test is used to identify airway hyperresponsiveness (AHR). Total eosinophil counts in peripheral blood reflect asthmatic activity and are useful for early detection of exacerbations. Correlation between the two is poorly defined, particularly in asymptomatic subjects. *Objective*. The aim is to define correlation between methacholine challenge test and peripheral blood eosinophilia. *Methods*. All flight academy candidates evaluated in the Israeli Air Force Aero Medical Center between the years 2010 and 2011 were included. Candidates were referred to methacholine challenge test based on a personal history of asthma or wheezing in childhood, a first-degree relative with the diagnosis of asthma, or an abnormal spirometry. All methacholine tests were analyzed retrospectively. *Results*. Asymptomatic subjects with a positive methacholine challenge test demonstrated significantly ($p < .002$) higher levels of absolute eosinophil count compared with the group with a negative methacholine test (0.46 $\pm$ 0.21 vs. 0.21 $\pm$ 0.01 K/μL). Total white blood cell and neutrophil counts were higher in the group with a positive methacholine test compared with the group with a negative methacholine test, but to a lesser extent (7.1 $\pm$ 0.25 vs. 6.6 $\pm$ 0.09 K/μL, $p = .04$ and 3.9 $\pm$ 0.24 vs. 3.5 $\pm$ 0.07 K/μL, $p = .04$, respectively). No association was demonstrated between eosinophil count and severity of AHR expressed by the methacholine concentration evoking a response. *Conclusion*. There is a strong association between eosinophil count and AHR in asymptomatic subjects.

Keywords asthma, diagnosis, eosinophil, methacholine

INTRODUCTION

Eosinophils have been known to be associated with bronchial asthma for over 100 years (1); however, in the past two decades they have received increasing attention. Asthma, particularly of the allergic phenotype, is characterized by marked infiltration of the airway by eosinophils and by airway hyperactivity to various stimuli. Numerous human airway studies have provided consistent evidence for the strong association between the inflammatory nature of asthma and eosinophils (2).

Airway inflammation is present in all patients with asthma, whether the disease is symptomatic or quiescent. This chronic inflammation causes subsequent airway structural changes referred to as remodeling and is in part responsible for hyperactivity of the airways (3). Airway hyperresponsiveness (AHR) is a characteristic feature of asthma but can also be seen in asymptomatic subjects. Methacholine challenge test is one of the most widely used tests to identify this characteristic hyperresponsiveness and thus used for the diagnosis and follow-up of disease severity in symptomatic asthmatic patients. It is a highly sensitive challenge test with a high negative predictive value, though with a low specificity in the range of 8 mg/mL of methacholine or higher (4). The significance of a positive methacholine challenge test in an asymptomatic population is unclear.

Eosinophils play an important role in the pathogenesis of asthma, and airway eosinophilic inflammation is a characteristic feature of asthma, both allergic and nonallergic (5). This can be assessed directly by measurement of eosinophils or related proteins in sputum or indirectly by measurement of the same markers in peripheral blood. Several studies demonstrated the elevation of airway eosinophils in induced sputum or bronchoalveolar lavage in asthmatic patients whether symptomatic or in a preclinical stage (6, 7), in both allergic and nonallergic asthma (8). Furthermore, the airway eosinophil count correlates with the degree of response in challenge tests (6). Similarly, total eosinophil counts in peripheral blood were found to reflect asthmatic activity and are useful for early detection of exacerbations (9). Thus eosinophilia, both in the sputum and in peripheral blood, along with AHR as measured in challenge tests, may serve as markers of airway inflammation, in known asthmatic patients, aiding in treatment regimens. This may also be true in asymptomatic subjects distinguishing a population that might be in risk for respiratory disease. This study evaluated the association between peripheral blood eosinophil counts and the presence of AHR evaluated by the methacholine challenge test.

METHODS

Study Design and Subjects

The study was approved by the ethics committee of the Medical Corps of the Israel Defense Forces. Study

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population included all flight academy candidates evaluated in the Israeli Air Force Aero Medical Center (IAFAC) between the years 2010 and 2011. The candidates were healthy male adolescents, who had previously undergone medical screening to be enlisted in the Israeli Defense Forces. Only those who have no underlying chronic disease are selected for further evaluation for flight training. All candidates undergo further routine medical screening at the IAFAC, including blood tests and spirometry. Detailed personal and family history and a comprehensive physical exam were performed.

Candidates with a history of allergic rhinitis or severe atopic dermatitis are not referred to the IAFAC. All candidates were evaluated by an ear, nose, and throat specialist as part of their routine evaluation. Those with a history raising suspicion for allergic rhinitis or with clinical findings compatible with allergic rhinitis were disqualified and thus excluded from the study. Subjects underwent detailed questioning by an aero medical physician with specific attention to asthma and their primary care physician completed a questionnaire in which he was asked to specifically address any episodes compatible with asthma. All candidates who had a confirmed diagnosis of asthma or any use of an inhaled corticosteroid, inhaled beta-agonist, or inhaled anticholinergics in the 5 years prior to the evaluation were considered to have asthma, were disqualified, and thus were excluded from the study. If more than 5 years elapsed since the use of such medications or since the last episode defined as an asthmatic attack, subjects were referred to a methacholine challenge test. Criteria for referral of candidates to methacholine challenge test were a personal history of asthma or wheezing in childhood (more than 5 years since their evaluation at the IAFAC), a first-degree relative with the diagnosis of asthma, or an abnormal spirometry, as was ascertained in previous studies (10). All candidates who have undergone a methacholine challenge test were retrospectively analyzed for age, gender, smoking status, full blood count including eosinophil count, and spirometry. No additional allergy tests were performed to rule out other allergic conditions and the exclusion of additional allergic conditions was based on the history and physical examination of the candidates.

Measurements and Definitions

Cell Count. White blood cell (WBC) and eosinophil counts were measured by an automated cell counter (automated analyzer—Cell-Dyn1 1600, Abbott Diagnostics, Abbott Park, IL, USA). Absolute eosinophil counts were determined by a hemocytometer.

Spirometry and Methacholine Challenge Protocol. Spirometry was performed using a standardized technique with the best of three attempts recorded. The method of methacholine inhalation challenge by the reservoir bag has been previously described (11). Solutions of methacholine in a citrate buffer were prepared under sterile conditions for each concentration: 0 (diluent), 0.07, 0.2, 0.6, 1.9, 5.67, and 17 mg/mL. All solutions were stored for not more than a week at 4°C. The inhalation challenge was performed using an atomized nebulizer (Pari, Starnberg, Germany) part filled with 6 mL of methacholine solution to produce the aerosol. The aerosolized methacholine was kept in a 10 L reservoir bag and then was inhaled into the respiratory tract of the subject. Spirometric parameters were measured at baseline and repeated 1–5 minutes after each dose of methacholine until at least two technically correct maneuvers were obtained; the better value was recorded. The concentration inducing a 20% drop in forced expiratory volume in 1 second (FEV_1) Peak concentration (PC_{20}) was calculated from the semi-logarithmic dose–response curve. The provocation was continued until the last dose in all subjects.

Statistical Analysis

WBC and eosinophil counts are presented as mean ± SEM. Comparison of the WBC counts between the group of candidates with a positive methacholine test and those with a negative methacholine test was performed by an unpaired Student's *t*-test. Linear regression analysis was performed on the eosinophil cell count in the candidates with a positive methacholine test against PC_{20}. Additionally, the population study was divided according to the indication for referral to the methacholine test and according to the result of the methacholine test. A one-way analysis of variance (ANOVA) test was performed together with the Bonferroni's multiple comparison test in order to analyze the difference between the eosinophil cell counts of the different groups. *P*-values $<.05$ were considered as statistically significant.

RESULTS

A total of 261 flight academy candidates were referred for further testing by means of a methacholine challenge test. Demographic characteristics are presented in Table 1. Most subjects were nonsmoking males. Only 14 were females and only 9 were smokers and all smoked less than 1/2 pack/year. Average age was 17.5 years reflecting the age at which medical screening is performed in army recruits. The primary cause for referral to methacholine test was a family history of asthma (53.5% of the study population) as compared to the criteria of childhood asthma (27%) and/or an abnormal spirometry (19.5%). Subjects with a positive methacholine challenge test demonstrated significantly higher levels of absolute eosinophil counts compared with the group with a negative methacholine test (0.46 ± 0.21 vs. 0.21 ± 0.01 K/μL, $p < .002$). Similarly, the total WBC count and neutrophil count in the group with a positive methacholine test was higher compared to that in the group with a negative methacholine test, but to a lesser extent (7.1 ± 0.25 vs. 6.6 ± 0.09 K/μL, $p = .04$, and 3.9 ± 0.24 vs. 3.5 ± 0.07 K/μL, $p = .04$, respectively). There was no difference between the groups with respect to the other cell types.

Despite the association between the eosinophil count and a positive methacholine test, there was no evidence of a correspondence between the eosinophil count and the

TABLE1.—Demographic details and cell counts of the study population.

	Positive methacholine	Negative methacholine	Statistical significance (p)
Age (years)	17 ± 0.093	18 ± 0.064	.034
Sex (F/M)	1/36	13/211	.22
Smoking (+/–)	3/34	6/218	.047
Total WBC count (K/μL)	7.1 ± 0.25	6.6 ± 0.093	.037
Eosinophil count (K/μL)	0.46 ± 0.21	0.21 ± 0.01	.0021
Neutrophil count (K/μL)	3.9 ± 0.24	3.5 ± 0.08	.04
Childhood asthma eosinophil count (K/μL)	0.21 ± 0.04	0.24 ± 0.02	.28
Abnormal spirometry eosinophil count (K/μL)	0.33 ± 0.09	0.19 ± 0.03	.047
Family history eosinophil count (K/μL)	0.83 ± 0.52	0.20 ± 0.015	.001

Notes: WBC, white blood cell; F, female; M, male. Positive methacholine is defined as PC_{20} lower than 16 μg/mL of methacholine. Negative methacholine is defined as PC_{20} higher than 16 μg/mL of methacholine.

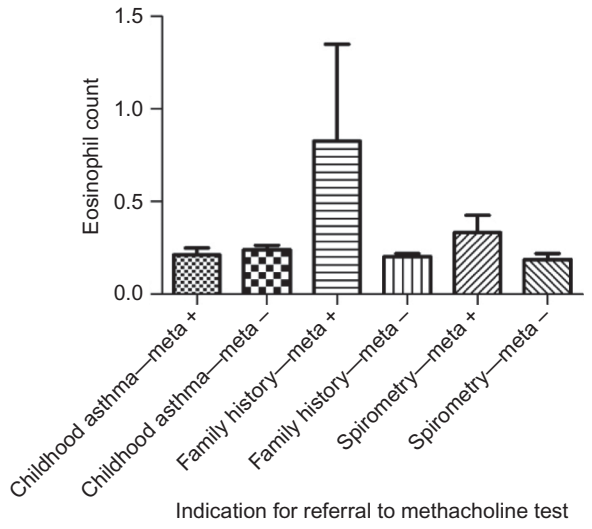


FIGURE1.—Correction between eosinophil count and indication for methacholine test.

severity of the AHR, as is expressed by PC_{20} in the positive methacholine group.

Candidates with a positive methacholine challenge test referred because of family history of asthma had significantly higher eosinophil cell counts compared with those with a negative methacholine test referred for the same indication (0.83 ± 0.52 vs. 0.20 ± 0.015 K/μL, $p = .001$). Similar differences in eosinophil counts were also noted in those referred to methacholine challenge because of an abnormal spirometry, but to a lesser extent (0.33 ± 0.094 vs. 0.19 ± 0.033 K/μL, $p = .047$). These differences in eosinophil counts were not observed in those referred to methacholine challenge test because of childhood asthma or wheezing. Mean eosinophil counts were significantly higher in the group with a positive methacholine challenge test and a family history of asthma compared with all other groups (0.83 ± 0.52 K/μL, $p = .0026$) (Figure 1).

DISCUSSION

The significance of asymptomatic AHR is unclear. The prevalence of asymptomatic AHR in the general population varies in different studies, ranging from 2% to 50%, although most studies report prevalence lower than 15% (12). In our study, 16% of the candidates demonstrated asymptomatic AHR but this population is not similar to that evaluated in other studies, as only subjects with certain indications were referred to a methacholine challenge test, possibly raising the incidence of a positive methacholine challenge test. It is suggested that asymptomatic AHR is associated with airway inflammation and remodeling, and that these are of intermediate level on the spectrum of inflammatory changes that range from minimal to none in subjects with no AHR to the classical changes known to occur in symptomatic asthma. When comparing asthmatic patients and asymptomatic individuals with AHR, bronchial subepithelial fibrosis was found in both cases, but in the AHR individuals it was focal rather than continuous as in the asthmatic patients. After a 2-year follow-up, 4 of the 10 individuals with AHR in a previous study developed asthma symptoms and the appearance of these symptoms was associated with an increase in inflammatory changes in the airways, specifically an increase in the CD4/CD8 ratio and airway fibrosis (13). Another possible explanation for the lack of symptoms in subjects with asymptomatic AHR is that they have a lower level of maximal airway response than mild asthma patients with similar AHR (14).

There are conflicting data regarding whether a positive methacholine test in asymptomatic subjects can predict the future development of asthma. In a study following 199 adolescents for 14 years, asymptomatic AHR was not significantly related to the development of asthma (15), while in a 3-year follow-up study it was demonstrated that subjects with asymptomatic AHR had a greater increase in airway responsiveness over time and a greater frequency of development of asthma symptoms than did subjects who did not demonstrate hyperresponsiveness (16). The risk of developing asthma was found to be proportional to the degree of AHR (17). In the epidemiological study on the genetics and environment of asthma, asthma was diagnosed more frequently in those with asymptomatic AHR (18). These studies and others reporting a high incidence of asthma among individuals with asymptomatic AHR (10) indicate that asymptomatic AHR is a risk factor for asthma. Nonetheless, the likelihood of asthma following a positive methacholine challenge test is dependent largely on the pretest probability, which is derived from age, smoking status, family and personal history, atopic status, physical

exam, and basic tests including spirometry and blood tests (19, 20).

The chronic inflammation of the airway mucosal lining has a central role in the production of the clinical manifestations of asthma, particularly AHR. The eosinophil appears to play a major part in producing this inflammation and mucosal injury. Studies in asthmatics reveal significantly elevated sputum eosinophil counts which were associated with the severity of the AHR, as assessed by PC_{20} in the methacholine challenge test. The sputum eosinophil count was significantly and inversely associated with PC_{20}, accounting for 16% of its total variance (21). Both the sputum eosinophil count and the AHR were associated with uncontrolled asthma, acting as possible markers for the disease activity (22). Thus, the combination of both airway eosinophilia and methacholine challenge test can give a better and more comprehensive tool for the estimation of the severity and extent of airway inflammation and disease activity and in guiding treatment decisions, specifically steroidal treatment and dosage (23, 24). Moreover, eosinophilia may be a diagnostic tool in asthmatic patients during an asymptomatic phase with normal lung functions (25).

Peripheral blood eosinophil count probably is a less accurate marker than sputum eosinophilia for airway inflammation (26); nevertheless in asthmatic patients, peripheral blood eosinophil counts were high and inversely associated with PC_{20}, as seen in the sputum eosinophil count (27). Furthermore, elevated peripheral blood eosinophil counts were associated with AHR, to a lesser extent than in asthma patients, yet significant, in asymptomatic subjects (28). In a 15-year follow-up of children with asymptomatic AHR higher levels of peripheral blood eosinophil and higher rates of developing asthma symptoms were documented (29). These findings are compatible with our finding of significantly higher eosinophil counts in peripheral blood of asymptomatic AHR as compared with asymptomatic subjects without AHR. The eosinophil count was not related to severity of AHR as seen in other studies, mainly sputum eosinophil studies. This might be attributed to the fact that the eosinophil count in peripheral blood is an indirect marker for the inflammatory process in the airway, thus it is not in a linear correlation to the AHR. Despite this, it can be used as a simple, accessible, noninvasive tool in screening asymptomatic subjects for the presence of AHR. This may be particularly useful in cases where the diagnosis of AHR is crucial and methacholine challenge is inconclusive, such as in divers, athletes, or aviation candidates.

Studies of family patterns demonstrated that there is a higher prevalence of AHR among relatives of subjects with asthma. Furthermore, subjects with asymptomatic AHR with a family history of asthma had higher atopic scores than those without a family history of asthma (10). Our results demonstrated higher eosinophil counts in subjects with asymptomatic AHR and a family history of asthma compared with those without AHR. Furthermore, the group with AHR and a family history of asthma had the highest absolute eosinophil count in the study, thus identifying a subpopulation that might be at risk for development of asthma. This is opposed to a personal history of wheezing or asthma in childhood or an abnormal spirometry, which was found to be less specific for the diagnosis of asthma. It is possible that some of the subjects who reported childhood asthma had in fact an intercurrent illness such as bronchitis and thus were not at any elevated risk for future asthma, as opposed to those with a family history of asthma, which is certainly a risk factor for the future development of asthma.

The average eosinophil count in candidates with a positive methacholine challenge test was more than twice the value in those with a negative methacholine challenge test. The clinical utility of this finding is limited, as the eosinophil count cannot replace challenge test as a sole criterion for the diagnosis of asthma. Yet, the higher eosinophil count in this group signifies that these subjects indeed have an atopic tendency and the results of the methacholine challenge probably reflect the presence of silent AHR. The eosinophil count may serve as an aid in those with borderline methacholine challenge test to identify subjects with atopic tendency. This may aid the clinician in the decision whether to initiate treatment in subjects with equivocal symptoms and provocation studies or when making a decision regarding the selection of asymptomatic athletes or military recruits with equivocal provocation studies results.

Both neutrophil count and eosinophil count differed significantly between the two groups, but the difference was significantly greater in the eosinophil count. A recent report by Just et al. (30) identified three asthma phenotypes. Children in cluster 2 were characterized by severe asthma with bronchial obstruction and had more pronounced neutrophilia, whereas those with multiple allergies (cluster 1) had more pronounced eosinophilia. Despite the fact that this study did not include subjects with active asthma, it would seem logical that those with a positive methacholine challenge are closer phenotypically to "cluster 1" because "cluster 2" subjects usually have more significant clinical symptoms. This may the reason for the greater difference in eosinophil counts compared with the difference in neutrophil count in the study population.

This study has several limitations. Because most flight academy candidates are male, the study population included predominantly males and thus the results may not be applicable to females.

This is a retrospective study with no long-term follow-up, thus the incidence of developing asthma amongst the study population is unknown. This requires further follow-up in future studies.

The cutoff for a positive methacholine challenge in the study population was 17 mg/mL. The usual definition of a positive methacholine challenge test is a PC_{20} greater than 8 mg/mL but the standard set in the Israeli Air Force (IAF) is 17 mg/mL in order to further increase the sensitivity of the study, as was previously described (10). The definition of the precise cutoff of PC_{20} for the diagnosis of AHR is debatable and requires further studies.

No correlation was noted between the eosinophil count and the PC_{20} and this may be a limitation regarding the application of the data to the general population. Yet the association between eosinophil count and methacholine challenge results is notable independent of the methacholine concentration used as cutoff.

Despite these limitations, the relatively large cohort of healthy subjects referred to methacholine challenge test in the absence of other potential confounders that may lead to peripheral eosinophilia, such as allergic rhinitis or severe atopic dermatitis, helps draw conclusions relevant to the study question.

Conclusions

By the time symptomatic asthma develops, airway structural and functional changes are largely irreversible. Therefore the best treatment for asthma would be to prevent it entirely, instead of trying to achieve optimal control once it has developed. AHR in asymptomatic population may not by itself be a strong predictor for asthma but when associated with peripheral blood or sputum eosinophilia, atopy, or a strong family history of asthma, it may indicate a population at risk for developing asthma. Eosinophil count may thus be used as an adjunctive diagnostic tool in cases where the diagnosis of asthma is crucial, such as in military candidates and athletes.

Further studies are required in order to evaluate the cost-effectiveness of screening for asthma among asymptomatic individuals and the role of eosinophil count in peripheral blood in this screening process.

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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 this article.

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