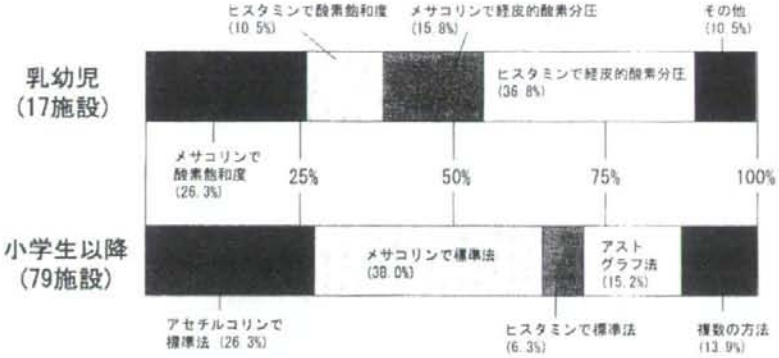

図1　気道過敏性試験を行っている施設での吸入試験法の別

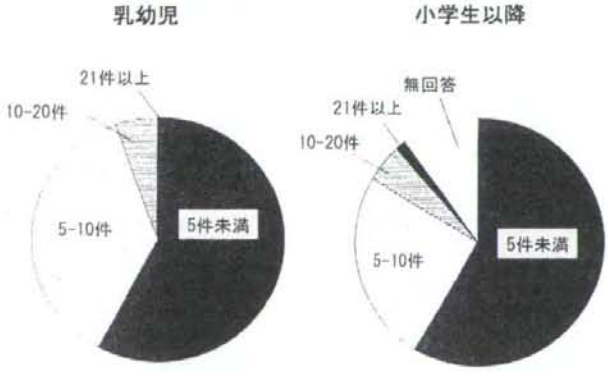

図2　気道過敏性試験の月平均回数

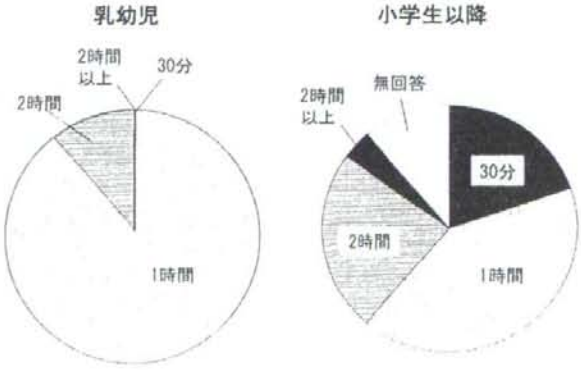

図3　気道過敏性試験にかかる時間

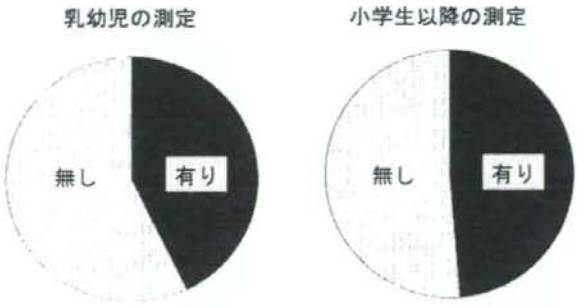

図4　気道過敏性試験時の救急対応の経験

4．考　察

気道過敏性は喘息の病態の基本と考えられ，これまで診断や治療の評価に活用されてきた．しかしながら，小児の領域では，患者の協力が得られないことなどを含む技術的な問題から，敬遠される傾向にあったことは否めない．これを反映してか，これまでに，本邦の小児アレルギーの診療において，気道過敏性の検討がどの程度に用いられているかについての調査も行われなかった．

今回のアンケートの対象は，専門施設以外の病院，医院の医師が大半と考えられるが，気道過敏性試験を行っている施設は9.7%という高率であった．このことは，気管支の慢性の炎症の存在について客観的な評価を行い，その時点での最良の治療を選択するという患者利益を鑑みての結果と思われる．

気道過敏性の検査の開始年齢も，標準法を用いることが困難である乳幼児で，すでに16.4%（回答された病院全体の1.6%）と高率であることがわかった．これは，乳幼児期の喘息の診断，予後評価において，気道過敏性の重要性が認識されている結果と思われた．乳幼児に気道過敏性試験を行っている19施設での負荷薬剤や測定機器の選択には偏りがみられなかったが，ヒスタミンで経皮的酸素分圧測定が36.8%と最も高かった．月平均回数は半数以上が5件未満で，検査にかかる時間は全施設が1時間から2時間であった．これは，患者の安全性や吸入試験に関わる労力を考えれば，納得のいくものであるが，いずれにしても並々ならぬ努力が必要であると思われた．一方，今までに乳幼児の検査中に緊急対応を必要とした症例の経験の有無について，有りが42.9%であったことには留意しなくてはならないが，乳幼児の気道過敏性検査を行っている施設は19施設であるのに対し42施設が回答していることと，以下の小学生以上の結果とほぼ同数であるため，小学生以上での結果が混入している可能性が考えられる．いずれ，この分野だけの検討，調査を行うべきかと思われた．

小学生以降での気道過敏性検査においても，負荷薬剤や測定機器の選択には，比較的，偏りがみられなかった．複数の方法を行って検討している施設が13.9%あり，多角的に気道の評価を行おうとする姿勢であると考えられた．月平均回数は乳幼児と比較して多く，検査にかかる時間は短くなっている．今までに乳幼児の検査中に呼吸困難などの緊急対応を必要とした症例の経験の有無については，有りが48.6%と乳幼児とほぼ同数の結果であったが，前述のごとく，この分野だけの独立した検討，調査を行うべきと思われる．

5．まとめ

回答のあった施設の9.7%にて，小児アレルギーの診療として気道過敏性の検討が行われていることからも，我が国では，乳幼児を含めた小児の喘息の診断，重症度や治療の評価に気道過敏性の測定は十分活用されていると考えられた．

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シンポジウム●学校におけるアレルギー疾患の管理と支援

今後の具体的取り組みの方向を探る
—小児アレルギー科医の立場から

海老澤元宏*

はじめに

文部科学省アレルギー疾患に関する調査研究委員会により，平成16年度の全国の小・中・高校生約1,200万人の各種アレルギー疾患の有病率が報告された．それによると，気管支喘息5.7%，アトピー性皮膚炎5.5%，食物アレルギー2.6%，アナフィラキシー0.14%，アレルギー性鼻炎9.2%，アレルギー性結膜炎3.5%という結果であった．この有病率の解釈において，アトピー性皮膚炎やアレルギー性鼻炎・結膜炎では軽症例が把握できていないと推定されるが，何らかのアレルギー疾患を有する生徒は各疾患のオーバーラップを考慮しても20%以上になると思われる．アレルギー疾患の児童は各クラスに必ず存在すると思われるが，アレルギー疾患に対する学校での正しい理解や適切な対応は不十分である．

本稿では，学校でのアレルギー疾患をもつ生徒への対応の現状と今後の方向性に関して述べてみたい．

I. 気管支喘息に関して

気管支喘息児にとって学校生活において問題となる場面は，①体育や部活動での運動，②動物との接触やホコリの舞う環境での活動，そして③宿泊を伴う校外活動である．①に関しては，運動誘発性喘息という現象が喘息の重症度が上がるにつれて顕在化するからである．特に持久走，サッカーなど，運動強度の強い運動が一定時間続くような場合には喘息が出やすくなる．②に関しては，喘息児のなかには動物アレルギーを合併する者もおり，また掃除などのホコリが舞う環境において喘息発作が誘発されることがあるからである．③の宿泊を伴う校外活動においては，寝具などからのダニの影響やホコリの舞う環境で喘息発作が誘発されることがしばしば認められる．

喘息児は，何もないときには健常児と何ら変わらずに活動できることが多い．しかし，ひとたび発作を起こすと緊急事態である．喘息のコントロールは，以前に比べ吸入ステロイドに代表される薬物療法の進歩により非常に良くなったが，濃厚な治療を受けてかろうじて落ち着いているケースも存在する．学校関係者が喘息児の重症度や治療内容を把握したうえで，症状がコントロールされ学校生活に支障を来していないかなどの評価や，発作時の対応など，親と共通認識をもつことが重要である．気管支拡張薬（β_2刺激薬）のインヘラーの吸入をスペーサーを用いて行うことも緊急時には必要であるが，基本的には本人の判断で行うことになる．学校側として発作時に薬物を用いているかどうかなどの把握も必要である．

II. アトピー性皮膚炎に関して

アトピー性皮膚炎児にとって問題となる学校生活の場面は，①プールでの授業および長時間の紫外線下の活動，②動物との接触，③発汗後である．①に関しては，プールの塩素消毒や強

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い紫外線がアトピー性皮膚炎を悪化させる要因となるからである．②はアトピー性皮膚炎をもつ生徒は動物アレルギーを合併していることもあるからである．③は汗がアトピー性皮膚炎児の症状の悪化要因として，夏を中心にして大きな問題となっているからである．運動などによる発汗後にシャワーができる環境があると，汗による悪化をかなり防ぐことが可能である．

アトピー性皮膚炎の管理も，スキンケアをベースとして，ステロイド外用療法やタクロリムス軟膏により以前よりも格段に改善している．学校生活に支障を来すような症状がある場合には，親とも相談し，適切な治療を促すことも重要である．いじめや不登校の原因になることもあるので注意が必要である．

III. 食物アレルギーに関して

学童期の食物アレルギーは，基本的には即時型の食物アレルギーが中心となる．病型としては，即時型，口腔アレルギー症候群，食物依存性運動誘発アナフィラキシーの3つに分類される．学童期になると，食物アレルギーがアトピー性皮膚炎の悪化につながることはほとんどなくなる．原因としては牛乳，鶏卵，小麦などが多いが，ソバ，ピーナッツ，甲殻類，果物など多岐にわたる．学童期の食物アレルギーは客観的な症状や食物負荷試験によって診断されるべきであり，IgE抗体陽性というだけで除去の指導が行われるべきではない．このことは医療従事者の食物アレルギーへの適切な対応や保護者の正しい理解が必須であることを示している．

学校生活上，最も問題になるのは学校給食である．食物アレルギーへの対応が行われているかどうかは，都道府県や市町村ごとに異なっている．本来であれば，すべての生徒に対して給食が提供されるべきであるが，クラスで1人だけ弁当を食べているというような事態も全国で日常的な状態である．食物アレルギーへの対応の充実の基本は，まず学校関係者に正しい知識をもってもらうことから始めるべきで，そのうえで各給食センターや調理場の実態に合わせて対応できる範囲を定めていく．学校給食においても，全国で毎日のように健康被害が起きていることも事実である．家庭科で食物を取り扱う実習や牛乳パックの回収など，食物アレルギー児にとって健康被害が引き起こされるような場面も学校生活では認められる．さらに一生に一度の修学旅行なども，食物アレルギーが原因で参加できないようなケースも存在する．そのような事例が実際にあることは，われわれが患者側から調査しても，学校側から調査しても，紛れもない事実である．

このように多くの課題を抱えているが，食物アレルギーの診療レベルの改善，保護者と学校関係者への正しい知識の普及により，事態の改善が図られることを期待している．

IV. アナフィラキシーに関して

アナフィラキシーは，アレルギー反応の最重症な症状として，時には命にかかわることもあり，緊急の対応を必要とする．アナフィラキシーの原因として最も多いのは食物アレルギーであるが，そのほかにもハチ刺傷，運動，食物＋運動（食物依存性運動誘発アナフィラキシー）などが原因として挙げられる．有病率は0.14％であるので，各学校に必ずそのような生徒が存在しうると考えられる．アナフィラキシーの症状として最も危険なのは，呼吸器系の症状として呼吸困難（喉頭浮腫，喘鳴など）を呈する場合である．学校関係者がまず行うべきは症状の把握であり，重症度の適切な評価である．緊急時の対応（搬送先の確保，保護者への連絡など）は保護者との間で取り決めておくべきであるが，すべてのケースでそのようなことが行われていないのが現状である．

アナフィラキシーの際に最も有効な薬剤は，アドレナリンの自己注射（エピペン®）である．

表1　学校におけるアレルギー対策の現状

1) アレルギー疾患を有する生徒の急増
　→学校における緊急時の対策の整備
　→アレルギー疾患を有する生徒の基本的人権の保障
2) さまざまなアレルギー疾患の急増
　→学校側の認識，教師の知識が不十分
3) 保護者からの申し出に基づいて
　→保護者による主観が入ったり過剰要求が生じる可能性
4) 学校の方針，施設，教師の知識・熱意などによる対応状況の違い
　→地域・学校・クラスによる格差，混乱

しかし，医療関係者にもその効果が正しく認識されているとはいえず，今後の啓発が必須である．呼吸器症状が出現した際などのアナフィラキシー症状に対しては，患者自身と保護者がエピペン®を使用することは認められているが，学校生活においては薬剤の保管や緊急避難として第三者が使用することも推奨されている．

V. アレルギー性鼻炎・結膜炎に関して

アレルギー性鼻炎・結膜炎をもつ生徒の場合には，スギ花粉の飛散時期の屋外での活動などに対して配慮が必要となることもある．またアレルギー性結膜炎の生徒では，プールの塩素消毒が結膜に対して悪影響を与えることも多く，ゴーグル着用などにより予防することが重要である．治療として両者とも抗ヒスタミン薬が用いられることが多いが，眠気などが出にくい薬剤も処方できるようになってきているので，処方する際に考慮すべきである．

VI. 今後の方向性

以上述べてきたことは，平成20年5月に日本学校保健会から全国の教育委員会に向けて送付する予定の「学校のアレルギー疾患に対する取り組みガイドライン」のなかで触れられており，対応策の中心として示された「学校生活管理指導表（アレルギー疾患用）」が今後主治医と学校側の連絡のツールとして活用されていくものと期待している．学校でのアレルギー対策の最も重要なパートは，医師による正しい診断と患者および保護者への適切な指導である．「学校生活管理指導表（アレルギー疾患用）」の考えの基本にある問題点と解決策を表1と表2に示して，今後の学校でのアレルギー対策の充実を祈念したいと思う．

表2　問題の解決策

1) 医療機関から学校側への正確な情報提供
　→学校生活管理指導表（アレルギー疾患用）
2) 各アレルギー疾患ごとに学校生活における問題点を挙げて配慮の必要性を医師から指示
3) 学校側のアレルギー疾患に関する知識のレベルアップ
　→学校への対応マニュアルの作成
　　• アレルギー疾患の知識の普及
　　• 学校生活管理指導表（アレルギー疾患用）の解説など
4) 緊急時の対応について
　→喘息発作治療薬やエピペン®の管理・使用について
5) アレルギー疾患を有する児の権利を尊重
　→すべての生徒に給食を提供，学校行事への全員参加

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PEDIATRIC ALLERGY AND IMMUNOLOGY

High prevalence and young onset of allergic rhinitis in children with bronchial asthma

Masuda S, Fujisawa T, Katsumata H, Atsuta J, Iguchi K. High prevalence and young onset of allergic rhinitis in children with bronchial asthma.
Pediatr Allergy Immunol 2008: 19: 517–522.

Bronchial asthma and allergic rhinitis often co-exist, and rhinitis is a major risk factor for the development of asthma. However, the reported incidence of allergic rhinitis in asthmatic children varies widely. The aim of this study was to elucidate the incidence of allergic rhinitis, the onset age of chronic upper and lower airway symptoms, and the correlation of these two symptoms in asthmatic children. A cohort of 130 consecutive children (ages 2–10) with asthma was evaluated. A questionnaire regarding upper and lower airway symptoms was filled out by the parents. Objective diagnosis of allergic rhinitis was also made on the basis of rhinoscopy, nasal cytology, nasal challenge, and specific serum IgE (CAP-RAST). Persistent nasal symptoms were present in 83.8% of the asthmatic children. The incidence of allergic rhinitis was 77.7% based on the objective findings. The mean onset age of asthma was 2.8 yr, and that of rhinitis was 2.9 yr. Nasal symptoms started as early as the first year of life in 8.9% of the children. In children with comorbid asthma and allergic rhinitis, rhinitis preceded in 33.7%, asthma preceded in 31.7%, and both started in the same year in 26.7%. In 7.9%, rhinitis was asymptomatic. Concomitant exacerbation of the upper and lower airways occurred in 34.6% of the total 130 children. These results demonstrate that allergic rhinitis manifested early in life in the majority of the asthmatic children. Persistent nasal symptoms in infancy may point to subsequent development of asthma and possible early intervention.

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Key words: bronchial asthma; allergic rhinitis; comorbidity; age of onset; child

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Prevalence of allergic diseases in children is increasing (1, 2) and it is widely accepted that allergic rhinitis and bronchial asthma commonly occur together. However, the reported prevalence of allergic rhinitis in patients with asthma, which is mostly based on a questionnaire to the patients, ranges from 6.2% to 95% (3). This wide variability may be attributed to the fact that there is no standardized questionnaire for the diagnosis of rhinitis and that patients with rhinitis and asthma often 'ignore' their upper respiratory symptoms because of their more prominent lower airway symptoms (3). Defining 'allergic' nasal symptoms in children is even more difficult because of the high frequency of upper respiratory infections in early childhood. In addition, young children rarely complain of nasal symptoms. To overcome the limitation of the questionnaire method, we performed objective diagnosis of allergic rhinitis based on the nasal endoscopy, nasal cytology, nasal provocation test, and serum specific IgE in a cohort of children with asthma. This approach enabled us to effectively utilize a questionnaire and to identify true prevalence of allergic rhinitis in a defined population of childhood asthma.

Here, we found that the majority of children with asthma at a mean age of 5 yr also had

allergic rhinitis and that the onset of allergic rhinitis occurred relatively early in life, either concurrently or prior to asthma, in 60% of the patients.

Subjects and methods

We evaluated a cohort of children < 10 yr of age with atopic bronchial asthma who visited Mie National Hospital's pediatric allergy clinic during consecutive 12 months. We included only the pre-pubertal children because natural outgrow of asthma often seen in adolescent age may confound the analysis of the two comorbid disorders. The diagnosis of atopic asthma was confirmed on the basis of a history of recurrent wheeze and dyspnea, reversible bronchoconstriction, and sensitivity to at least one inhalant allergen as evidenced by a positive CAP-RAST. One-hundred and thirty children (86 boys, 44 girls, ages 2–10; mean: 5.3 yr) who consecutively visited our clinic were enrolled in the study. Classification of the cases according to the international guideline (4) showed 77.9% as mild persistent, 18.6% as moderate persistent, and 3.5% as severe persistent asthma. Informed consent was obtained from the parents of the patients. All the children were found to be sensitized at least to house dust mite (HDM).

A questionnaire was filled out by the parents. It included questions concerning nasal symptoms, onset ages of rhinitis and bronchial asthma, correlation between nasal symptoms and asthma symptoms, and family history of allergic diseases. The parents were also asked whether a physician had ever diagnosed the nasal symptoms as being due to allergies. 'Persistent nasal symptoms' was defined on the basis of a positive answer to the following question: 'Has your child had a long-lasting runny or stuffy nose or episodes of sneezing, apart from colds?' Those who answered 'yes' were asked about the age when the problem started.

After the questionnaire, rhinoscopy, nasal cytology, and allergen provocation tests were performed. The diagnosis of HDM-sensitized allergic rhinitis was based on (i) presence of eosinophils in nasal smears; (ii) positive nasal provocation test with a HDM allergen disc; and (iii) positive specific serum IgE to HDM by CAP-RAST. The children who had at least two positive factors of the criteria were confirmed as allergic rhinitis. Because HDM is the most prevalent inhalant allergen and the majority of patients with perennial allergic rhinitis are sensitized to HDM in Japan (5, 6), the criteria covers most of perennial allergic rhinitis in the country. Although Japanese cedar pollen (JCP) is the major allergen to cause 'seasonal' allergic rhinitis in Japan (7, 8), it does not relate to asthma prevalence (9) and we focused on HDM, not JCP, in this study.

The severity of rhinitis symptoms was determined according to the Japanese practice guideline for nasal allergy (10). In brief, the severity of nasal congestion was classified into four grades, i.e., no congestion was 'none,' congestion of one side was 'mild,' occasional congestion of both sides was 'moderate,' and complete congestion was 'severe.' The severity of rhinorrhea and sneezing was also classified into four grades on the basis of the number of nose blows and sneezes per day, i.e., < 1 was 'none,' 1–5 was 'mild,' 6–10 was 'moderate' and > 10 was 'severe.' The severity of rhinitis was assigned to that of the highest grade in any of the symptoms.

The chi-squared test was used to compare proportions of the data in different groups. A difference in percentages was considered significant when the p-value was < 0.05.

Results

Previous diagnosis of allergic rhinitis

The parents of 75 children (57.7%) answered that their children had been diagnosed as having allergic rhinitis by another physician. In addition, the parents of 11 children thought that their children had had allergic rhinitis although a definitive diagnosis had never been made.

Prevalence and severity of persistent nasal symptoms based on the questionnaire

The replies to the questionnaire revealed that 109 children (83.8%) had had persistent nasal symptoms. The symptoms were mild in 26.9%, moderate in 40.0% and severe in 16.9% of the children. The combined ratio of 'severe' and 'moderate' symptoms was 50.4% for nasal congestion, 23.1% for rhinorrhea and 10.7% for sneezing. Concerning other symptoms, 22.3% of the 130 children had recurrent epistaxis, 46.2% had frequent sniffing, 30.0% had snoring, 40.8% had a cough, and 32.3% had eye symptoms.

Prevalence of allergic rhinitis based on objective findings

Rhinoscopy and nasal cytology were performed in all the children. Eosinophilia in nasal smears was positive in 97 (74.6%) of them. Nasal provocation tests were positive in 45 (84.9%) of 53 children who could be carried out the test.

Seventy-seven children were unable to be performed the test because they were too young. On the basis of the diagnostic criteria, 101 children (77.7%) were confirmed as allergic rhinitis. All the children diagnosed as allergic rhinitis had pale or red swollen mucosa and/or watery discharge with rhinoscopy. Twenty-seven (26.7% of 101 children) had mild, 42 (41.6%) had moderate, and 20 (19.8%) had severe nasal symptoms.

Table 1 summarizes the relationship between prevalence of persistent nasal symptoms found by the questionnaire and objective diagnosis of allergic rhinitis. Positive predictive value for the questionnaire was 81.7% and negative predictive value was 42.9%. Actually, the parents of 12 (11.9% of total subjects) children had not noticed any nasal symptoms in their children despite the presence of typical mucosal findings of allergic rhinitis. Twenty-nine (22.3% of total subjects) children did not have objective findings of allergic rhinitis, although the parents of 20 children complained of persistent nasal symptoms in their children. Six of those 20 children were diagnosed as having sinusitis.

Age at onset of asthma and rhinitis symptoms

As presented in Fig. 1, the distributions of the onset ages of asthma and rhinitis were similar. The mean onset age of asthma in all the patients was 2.8 ± 1.9 yr. In 89 children with both asthma and symptomatic allergic rhinitis, nasal symptoms started at 2.9 ± 1.7 yr of age. In children with allergic rhinitis, nasal symptoms started as early as the first year of life in 9.0% of them and at 2 yr of age in 22.5%. In the patients who were previously diagnosed as having allergic rhinitis by other physicians, the mean age at the diagnosis was 3.5 ± 1.7 yr.

Table 1. Prevalence of allergic rhinitis based on objective findings and persistent nasal symptoms found by the questionnaire

	Persistent nasal symptoms found by the questionnaire		
	+	−	
Allergic rhinitis			
+	89*	12	101 (77.7%)
−	20†	9	29 (22.3%)
Total	109 (83.8%)	21 (16.2%)	130

Positive predictive value and negative predictive value for the questionnaire were 81.7% and 42.9%, respectively.

*Twenty-seven had mild, 42 had moderate, and 20 had severe nasal symptoms.

†Six of them were diagnosed as having sinusitis.

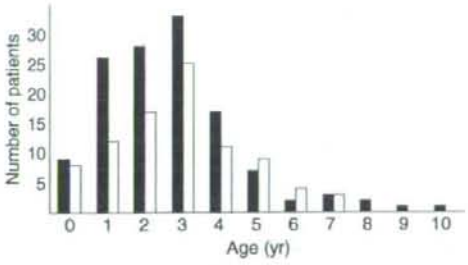

Fig. 1. Distribution of the age at onset of asthma and rhinitis symptoms. Closed bar: onset of bronchial asthma in total children (n = 130, 2.8 ± 1.9 yr). Open bar: onset of nasal symptoms in children with allergic rhinitis (n = 89, 2.9 ± 1.7 yr).

The children with asthma who also had allergic rhinitis were further subdivided into four groups according to which of the two illnesses started first. Of the 101 children who were diagnosed as having allergic rhinitis, the onset of asthma preceded in 32 (31.7%), the onset of rhinitis preceded in 34 (33.7%), and both diseases manifested at the same time in 27 (26.7%) patients. Eight (7.9%) patients had asymptomatic rhinitis. Table 2 summarizes the patients' current age and the age of onset of the illnesses. It is of note that the initial symptoms occurred at an age of 2 yr regardless of the airway site, and that allergic rhinitis started at a young age, either concurrently or prior to asthma, in 61 children (60.4%).

The correlation between upper respiratory symptoms and bronchial asthma

Concomitant exacerbation of the upper airways with the bronchial asthma occurred in 34.6% of the total 130 children. Concomitant exacerbation was experienced in 33.7% of the children with rhinitis. Interestingly, even in the case of children without evidence of rhinitis, 37.9% experienced nasal symptoms when their asthma exacerbated, which may indicate that viral upper respiratory infections had caused asthma exacerbation. Alternate exacerbations of the upper and lower airway symptoms were found only in one case (0.9%).

Family history of allergic diseases

The data on the family history of allergic diseases are shown in Fig. 2. A family history of bronchial asthma was seen significantly more frequently in children whose nasal symptoms started early, before the onset of asthma or at

Table 2. Current age and the onset age of bronchial asthma and nasal symptoms

Group (n)	Current age (mean ± 1 s.d.)	Sex (M/F)	Onset age (mean ± 1 s.d.)	
			Bronchial asthma	Nasal symptoms
Children without allergic rhinitis (asthma alone) (29)	4.2 ± 1.7	19/10	2.3 ± 1.3	–
Children with asthma and allergic rhinitis (101)				
Asthma preceded (32)	6.0 ± 2.1	24/8	2.1 ± 1.5	3.9 ± 1.5
Nasal symptoms preceded (34)	5.6 ± 2.4	21/13	4.2 ± 2.2	2.3 ± 1.6
Both symptoms occur at the same time (27)	4.8 ± 2.3	15/12	2.3 ± 1.6	2.3 ± 1.6
Asymptomatic rhinitis (8)	5.6 ± 2.5	7/1	2.0 ± 1.3	–

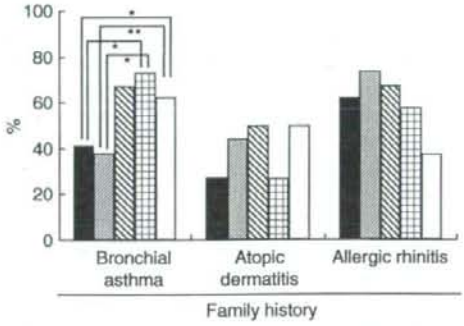

Fig. 2. The prevalence of family history of allergic disease. The patients were divided into the following groups on the basis of comorbidity of rhinitis and relative precedence of the onset: closed bar, asthma alone; Fine hatched bar, asthma preceded; large hatched bar, rhinitis preceded; cross striped bar, both symptoms occurred at the same time; open bar, asthma and asymptomatic rhinitis. $^{*}p < 0.05$, $^{**}p < 0.01$. A family history of asthma was significantly more common in children whose nasal symptoms started early, before the onset of asthma or at the same time with asthma than in those who had asthma alone or rhinitis of later onset.

the same time as the asthma compared with in those who had asthma alone or allergic rhinitis of later onset. There were no statistically significant differences in the prevalence of a family history of atopic dermatitis and allergic rhinitis among the groups.

Discussion

In this study, we found a high incidence of allergic rhinitis, about 80%, in children with asthma at a mean age of 5 yr old. Because the incidence in previous reports varies widely, we employed direct examination to diagnose rhinitis objectively in a cohort of 130 asthma patients < 10 yr of age, all of who visited our pediatric allergy clinic consecutively within a certain period.

It is of note that no nasal symptoms were recognized by the parents of 11.9% of children who had objective findings of rhinitis, which was also evidenced by low negative predictive value, 42.9%, of the questionnaire. These findings suggest that a certain population of asthmatic children without nasal symptoms may have allergic rhinitis. Nasal symptoms of young infants may be unrecognized by the parents, and careful examination is thus important; several reports, although they deal with adults, support this finding. Gaga et al. (11) reported that eosinophil infiltration was present in the nasal mucosa of asthmatic patients even in the absence of rhinitis. Some of the asthmatic patients who considered themselves free of nasal symptoms were demonstrated to have evidence of nasal airway disease (3).

Next, we found that the nasal symptoms in asthmatic children started early in life. Nasal symptoms occurred as early as in the first year of life in 9.0% of asthmatic children with allergic rhinitis, and the mean onset age of rhinitis was 2 yr. In addition, nasal symptoms preceded bronchial asthma in 33.7% of the children, and the two symptoms started at the same time in 26.7%. These results indicate that nasal symptoms start in infancy in a considerable number of asthmatic children. An epidemiological study (12) reported that children whose rhinitis began in the first year of life were more likely to have respiratory symptoms or asthma at age 6 than those without. Leynaert et al. (13) indicated that rhinitis that develops in the first year of life is an early manifestation of an atopic pre-disposition and a risk factor for asthma. A recent large survey for allergic rhinitis in pre-school children using ISAAC [The International Study of Asthma and Allergies in Childhood (14)] written questionnaire also revealed that rhinitis has a strong association with wheezing symptoms, asthma (15).

The present study also showed that one-third of asthmatic children experienced concomitant exacerbation of upper and lower airway symptoms, even those without objective findings of allergic rhinitis. 'Exacerbation of nasal

symptoms' includes not only allergic inflammation but also viral or bacterial infections, such as a common cold or sinusitis. This finding indicates that any nasal symptoms can pre-dispose to exacerbation of asthma.

The mechanisms that connect upper and lower airway allergies are under active investigation. Proposed pathogenetic mechanisms include a nasobronchial reflex, mouth breathing caused by nasal congestion, and pulmonary aspiration of nasal contents (16). In school age children, viruses were detected in 80% of reported episodes of asthma exacerbations (17). Upper airway rhinovirus infection is an important risk factor for asthma exacerbation through various pathophysiologies to cause allergic inflammation in the lower airway, such as ICAM-1 expression (18). It has also been demonstrated that rhinovirus could directly infect lower airways (19). 'Spreading' of allergic inflammation from the upper airway to the lower airway may be induced since local allergic sensitization causes systemic production of allergen-specific IgE and T cells are able to migrate from regional lymph nodes and 'home-in' on other tissues (20).

The 'Allergic Rhinitis and its Impact on Asthma' WHO guideline stresses that asthma and rhinitis should be considered to be 'one disease' to achieve better control of the common 'one airway' diseases (21). Accumulating evidence suggests that treating allergic rhinitis in patients with comorbid asthma reduces the airway hyperresponsiveness and improves lung functions (22–24). Recent studies have demonstrated the efficacy of early intervention for allergic rhinitis in asthmatic patients (25, 26). Corren et al. (27) in a large scale case–control study, showed that treatment of asthmatic patients who had allergic rhinitis with either nasal corticosteroids or second-generation antihistamines was associated with a significantly lower risk of asthma-related emergency room treatment and hospitalization.

In the present study, a family history of asthma was more common in children who had comorbid allergic rhinitis, especially in children who had earlier onset of nasal symptoms. This finding suggests that a genetic pre-disposition may have some impact on the development of allergic rhinitis in early childhood (28). One should also be aware of possible environmental risks for early onset of rhinitis since it has been reported that environmental tobacco smoke was strongly associated with an increased risk of developing allergic rhinitis at age one (29). We could not, however, found a positive correlation between smoking of parents and onset age of persistent nasal symptoms probably because of small number of subjects. Particular attention should be paid to coexistent lower airway symptoms in young children with nasal symptoms. However, the ideal time for early intervention and the most effective treatment strategy remain unknown.

Possible shortcomings exist in this study. First, evaluation of symptoms with questionnaire is prone to recollection bias especially for the onset age of nasal symptoms. This inherent problem in questionnaire, however, may be less problematic in this study because we included young children with mean age of 5 yr and the parents of them would have better memory of toddler ages than those of adolescent or older counterparts would. Second, our diagnostic criteria for 'objective' allergic rhinitis were based on sensitization to a single allergen, HDM, and we may have missed patients with perennial allergic rhinitis who may have been sensitized to other inhalant allergen. However, as described in Subjects and methods, HDM is the most prevalent perennial allergen for both allergic rhinitis and asthma in Japan and sensitization to other allergens without HDM sensitization is very rare except for seasonal allergen, JCP (5, 6, 30).

In conclusion, we found that 77.7% of asthmatic children had allergic rhinitis, and their nasal symptoms started early in life. We reached a diagnosis of allergic rhinitis in young children based on the objective findings. As recognition of rhinitis symptoms in infancy is difficult even for mothers, our findings suggest the importance of careful nasal examination in young children with asthma. A correct diagnosis of allergic rhinitis may be one of the clues for early intervention in respiratory allergic disease in children.

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ORIGINAL ARTICLE

Awarded Article, Annual Meeting of JSA

House Dust Mite Extract Induces Interleukin-9 Expression in Human Eosinophils

Takao Fujisawa[1], Hajime Katsumata[1] and Yoshiko Kato[1]

ABSTRACT

Background: Eosinophils play a pivotal role in allergic inflammation. Recent evidence suggests that they not only function as terminal effector cells but have potential to interact with allergen and initiate immune responses. We investigated cytokine production from eosinophils through direct interaction with a major allergen, house dust mite (HDM) .

Methods: Purified eosinophils from HDM-sensitized or non-sensitized donors were cultured with HDM extract or lipopolysaccharide (LPS) for 18 or 40 h. A panel of cytokine gene expression in eosinophils was examined by means of real-time RT-PCR. Released cytokines in the culture supernatants were assessed with a specific ELISA. In some experiments, HDM was pretreated with protease inhibitors, then added to the culture. Cytokines tested for gene expression were interleukin (IL)-2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 16,17, 18, TGF-β1 and GM-CSF,.

Results: LPS induced small enhancement of GM-CSF gene expression at 18 h. At 40 h, HDM induced about 60-fold enhancement of IL-9 gene expression. IL-9 protein was also detected in the culture supernatants at 60 h. Those reactions were observed regardless of HDM sensitization status of the donors. HDM-induced IL-9 expression was completely inhibited with a serine protease inhibitor, AEBSF, not with a cysteine protease inhibitor, E-64.

Conclusions: Accumulated eosinophils in the airways in asthma may directly react with HDM and produce IL-9 to further promote Th2-type immune responses. Protease-activated receptor 2, a ligand for serine proteases, which contained in HDM, may be involved in the reaction.

KEY WORDS

allergens, eosinophils, house dust mites, interleukin-9, proteinase activated receptor 2

INTRODUCTION

Massive eosinophil infiltration in the airway mucosa is a prominent feature of the pathology of bronchial asthma and multiple evidence suggest that eosinophils are the major effector cells in the pathogenesis of bronchial hyperresponsiveness and airway remodeling in asthma.[1] Among various mediators from eosinophils, major basic protein (MBP) in specific granules has pleiotropic functions to cause bronchial epithelial cell damage,[2] airway hyperresponsivenss,[3] and activation of other inflammatory cells.[4] Cysteinyl leukotrienes cause acute bronchoconstriction, hypersecretion, and promote airway inflammation.[5] Transforming growth factor β (TGF-β) from eosinophils[6] has been shown to be involved in airway remodeling.[7]

Conventional understanding of the role of eosinophils in asthma is the terminal effectors as described above under control of Th2 cells,[8] natural killer T cells,[9] mast cells,[10] and monocytes.[11] Recent evidence, however, suggests that eosinophils not only function as terminal effector cells but as immunomodulatory cells in innate immunity. Eosinophils in the airways have a potential to traffic to regional lymphnodes for antigen presentation to T cells.[12,13]

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Eosinophil-derived neurotoxin (EDN), one of eosinophil granule proteins, induces dendritic cell migration.[14] Eosinophils also directly interacts with allergens; house dust mite (HDM) and birch pollen allergens induce chemotaxis and degranulation,[15] pollen-associated lipid mediators induce chemotaxis and CD11b expression,[16] and an environmental fungus Alternaria protein product induces intracellular calcium mobilization, cell surface expression of CD63 and CD11b, and degranulation.[17] These observations indicate that eosinophils can initiate or modulate allergic immune responses by interacting with exogenous molecules such as allergens.

Thus, to further clarify the potential of eosinophils as initiators of allergic inflammation, we investigated a panel of cytokine production from eosinophils through direct interaction with a major allergen, HDM. Here, we found that eosinophils produce significant amount of IL-9, one of the pivotal Th2-type cytokines in the pathogenesis of asthma, in response to HDM extract.

METHODS

REAGENTS

House dust mite (Dermatophagoides pteronyssinus) extract was purchased from GREER Laboratories (Lenoir, NC, USA). E-coli-derived lipopolysaccharide (LPS) was from Sigma (St. Louis, MO, USA). Protease inhibitors, trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64) and 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) were also purchased from Sigma.

ISOLATION OF EOSINOPHILS

Heparinized peripheral blood was obtained from HDM-sensitized or non-sensitized donors. The former subjects had mild allergic rhinitis and the latter were healthy non-atopic individuals. Sensitization to HDM was defined as CAP-RAST titer to Dermatophagoides pteronysmus >0.7 UA/ml (Phadia, Tokyo, Japan). Eosinophils were isolated by negative selection using anti-CD16 bound micromagnetic beads (MACS™, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as previously described.[18] The purity of eosinophils was more than 99%. This work was approved by the ethical committee of Mie National Hospital and written informed consents were obtained from all subjects.

QUANTITATIVE RT-PCR

Purified eosinophils at 1×10^6/ml in RMPI 1640 medium (Sigma) were cultured with HDM extract at 100 μg/ml or LPS at 1 μg/ml in the presence of 5% heat-inactivated fetal bovine serum (FBS; Sigma) or 5% non-processed FBS for 18 or 40 h. Total RNA was extracted from the cells and reverse transcribed. Real-time PCR was carried out for IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, IL-17, IL-18, and GM-CSF on ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Probe/primer sets for each cytokine were designed using Primer Express software (Applied Biosytems). Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method reported elsewhere.[19] In some experiments, HDM was incubated with a cysteine protease inhibitor, E-64 or a serine protease inhibitor, AEBSF, at 37℃ for 30 min then added to the culture.

ELISA FOR IL-9

Eosinophils at 1×10^6/ml in RMPI 1640 medium with 5% FBS in the presence or absence of HDM were cultured for 60 h and the supernatants were tested for IL-9 protein by a sandwich ELISA. Each well of a 96-well microplate (Immuno Module F8 Maxisorp, Nunc, Roskilde, Denmark) was filled with 50 μl of anti-human IL-9 monoclonal antibody (clone MH9A4, BioLegend, San Diego, CA, USA) at 5 μg/l in carbonate buffer (pH 9.6) and incubated for 18 h at 4℃. After removal of the antibody solution, the wells were washed four times with phosphate-buffered saline containing 0.5 ml/l Tween 20 (PBS-T). PBS containing 0.1% bovine serum albumin (BSA; Sigma) was added to each well, then incubated for 1 h at 25℃. After aspiration, aliquots of human recombinant IL-9 (PeproTech, New Jersey, USA) standards or samples (25 μl each) were added to the wells and incubated for 16 h at 4℃. Each well was washed four times with PBS-T, 50 μl of biotin-conjugated anti-human IL-9 (clone MH9A3, BioLegend) was added and incubated for 1 h at 25℃. After washing with PBS-T, 100 μl of streptoavidin-HRP (GIBCO Industries, Langley, OK, USA) in PBS containing 0.1% BSA was added to each well and incubated for 1 h at 25℃. Following four washing of the assay plates, the immunoreactivity was visualized by addition of 100 μl/well of substrate solution (TMB solution, Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 25℃. The reaction was stopped by the addition of 50 μl of 1 g/l sodium dodecil sulfate to each well, and absorbance was measured at 405 nm. The IL-9 levels were calculated based on the standard curve on each assay plate.

STATISTICAL ANALYSIS

The data were expressed as mean ± SEM of indicated numbers of experiments and p values were determined with ANOVA for multiple groups with Bonferroni post test.

RESULTS

CYTOKINE GENE EXPRESSION OF EOSINOPHILS BY HDM EXTRACT AND LPS

First, we tested whether HDM extract directly induces cytokine gene expression from eosinophils. Because HDM extract may contain LPS and LPS activates eosinophils via TLR-4 and CD14,[20] which is not

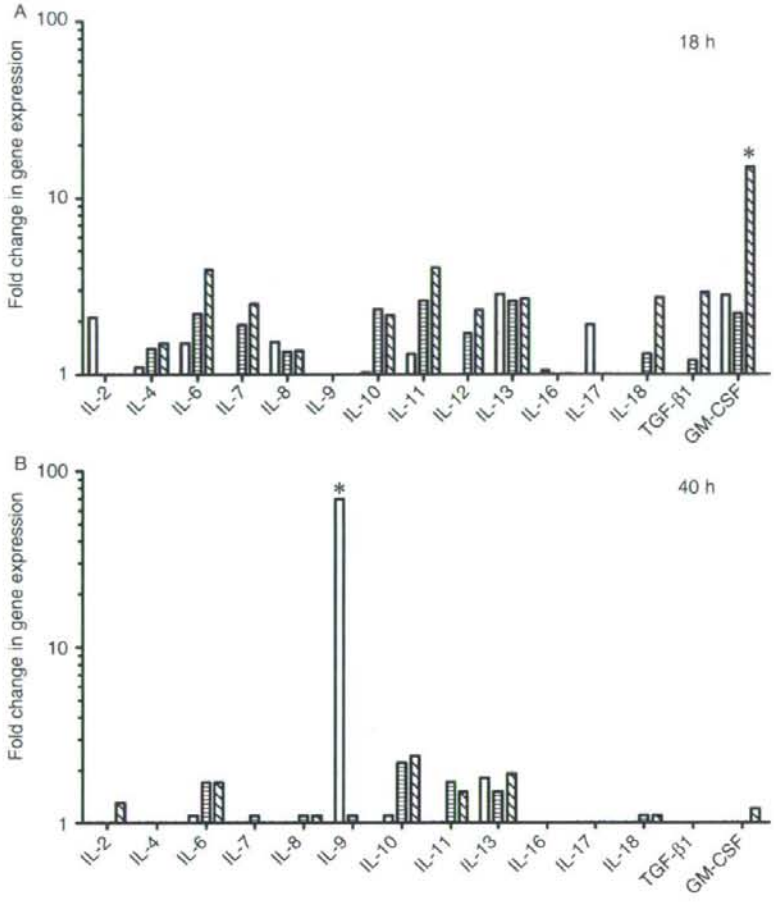

Fig. 1 Cytokine gene expression by HDM extract (open bar), LPS with heat-inactivated FBS (horizontal hatched bar), and LPS with non-processed FBS (diagonal hatched bar). **A**. 19 h culture **B**. 40 h culture. The data represent geometric means of fold changes in gene expression. ($n = 4-5$). * $p < 0.05$

only expressed on the cell surface but present as soluble form in the serum,[21] we also cultured eosinophils with LPS in FBS-containing medium. In addition, to fully induce activation potential of LPS, FBS with no heat inactivation process was also used since complements may be involved in LPS-induced activation.[22,23] At 18 h of incubation, HDM extract did not induce more than 3-fold increase in gene expression of a panel of cytokine tested (Fig. 1A). LPS in combination with non-processed serum induced significant gene expression of GM-CSF (Fig. 1A). At 40 h, surprisingly, HDM induced about 60-fold enhancement of IL-9 gene expression while LPS did not cause significant gene expression of any cytokine (Fig. 1B). We also confirmed with a specific ELISA that HDM extract, not LPS, induced significant IL-9 protein production at 60 h of incubation (Fig. 2).

HDM EXTRACT INDUCED IL-9 PRODUCTION REGARDLESS OF HDM SENSITIZATION STATUS

Since HDM is a major IgE sensitizing allergen, we sought the possible relationship between the presence of HDM-specific IgE antibody and HDM-induced IL-9 gene expression in the subjects. There was, however, no difference in IL-9 gene expression between HDM-sensitized and non-sensitized donors (Fig. 3).

SERINE PROTEASE INHIBITOR ABOLISHED HDM-INDUCED IL-9 GENE EXPRESSION

Many allergens including HDM are found to be proteases. We then examined the effect of protease in-

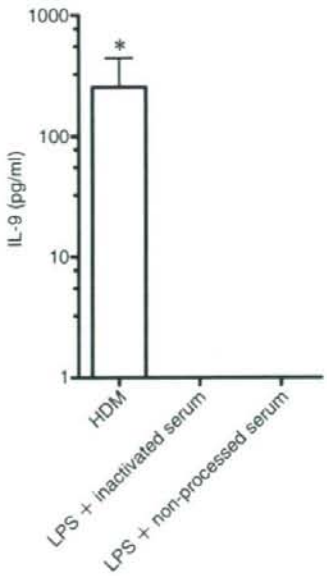

Fig. 2 IL-9 protein concentrations in the supernatants of eosinophils after 60 h of culture with HDM extract (open bar), LPS with heat-inactivated FBS (horizontal hatched bar), and LPS with non-processed FBS (diagonal hatched bar). The data represent geometric means. ($n = 3$). $^*p < 0.05$

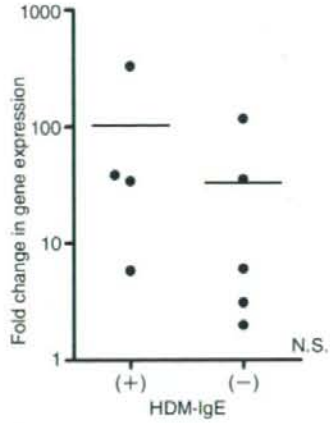

Fig. 3 HDM-induced IL-9 gene expression in HDM-sensitized (HDM-IgE (+)) and non-sensitized (HDM-IgE (−)) individuals. We compared the two groups by Man-Whitney test. N.S.; not significant

hibitors to see whether the cytokine-inducing function of HDM is derived from its proteolytic activity for protease-activated receptors (PARs) expressed on eosinophils.[24] Pretreatment of HDM with a serine protease inhibitor, AEBSF, completely abolished the subsequent IL-9 gene expression with HDM (Fig. 4). On the other hand, a cysteine protease inhibitor, E-64, did not have any effect on the reaction even at the higher concentration. Because of known specificity of proteases to PAR, it is suggested that eosinophils may respond to HDM via PAR-2.

DISCUSSION

In the present study, we showed that eosinophils directly reacted with a major allergen, HDM, and produced significant amount of a pleiotropic Th2-type cytokine, IL-9. Since the stimulatory effect of HDM was completely abolished by pretreatment with a serine protease inhibitor, not with a cysteine protease inhibitor, it is suggested that eosinophils responded to HDM through PAR-2, at least in part.

IL-9 is classified as a Th2-type cytokine originally identified in activated CD4+ T cells.[25] It promotes the proliferation and differentiation of mast cells and hematopoietic progenitors, stimulates the proliferation of activated T cells, and enhances the production of immunoglobulins by B cells.[26] In the context of Th2-type inflammation, IL-9 was reported to induce eotaxin production from airway smooth muscle cells, IL-5 receptor expression from eosinophils,[27,28] and goblet cell hyperplasia.[29] Selective overexpression of the *IL9* gene within the lungs of transgenic mice resulted in massive airways inflammation with eosinophils and lymphocytes as predominant infiltrating cells.[30] Blockade of IL-9 inhibited the development of airway inflammation in a mouse model of asthma.[31] In humans, a close association between the *IL9* gene and bronchial hyperresponsiveness has been suggested.[32] Collectively, IL-9 may play an important role in the pathogenesis of asthma and the potential of eosinophils to produce IL-9 adds another mechanism that underlie exacerbating nature of Th2-type inflammation.

Environmental allergens including house dust mites, fungi, and pollens contain various kinds of proteases and it has been demonstrated that these proteases directly cause activation of epithelial cells and inflammatory cells leading to inflammatory cytokine production[24] and degradation of epithelial tight junctions facilitating further allergen entry[33]. Thus, the proteolytic activities characterize the pathogenic nature of the allergen molecules independent of their IgE-binding epitopes. HDM, the most common allergen in Japan and other area of the world with warm and humid climate, contains cysteine proteases and serine proteases as well as a number of other uncharacterized proteases. Der p 1 and Der f 1, the major allergens in HDM, are cysteine proteases and Der p 3, Der f 3, Der p 9, and Der f 9 are serine proteases.[33-36] Proteases stimulate cells via PARs, 7-transmembrane G protein-coupled receptors. They cleave the amino acids at a specific site of the extracellular N-terminus of the PARs to expose a new N-terminal ligand do-

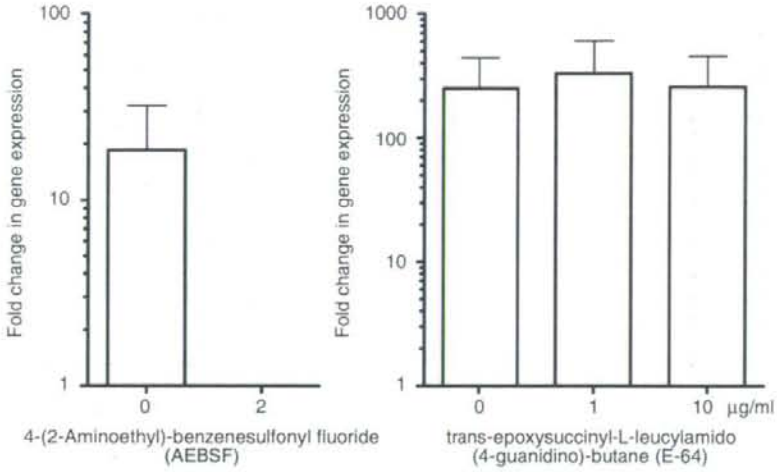

Fig. 4 IL-9 gene expression from eosinophils cultured with HDM extract pretreated at 37°C for 30 min with AEBSF, a serine protease inhibitor, or E-64, a cysteine protease inhibitor. The data represent geometric means of fold changes in gene expression. ($n = 3$).

main that binds to another site on the same molecule, thereby activating the receptor. Four PARs have been identified and amino acid sequences of each cleavage site is specific for the particular PAR. Serine proteases are PAR-2 agonists and we speculate that HDM extract stimulated eosinophils to produce IL-9 via PAR-2 because a serine protease inhibitor, not a cysteine protease inhibitor, blocked the reaction although the results presented here were still insufficient to prove PAR-2-mediated IL-9 production.

We also examined the possibility that HDM-induced IL-9 production from eosinophils was IgE-mediated. Eosinophils express low affinity IgE receptors, CD23, and may express high affinity Fc epsilon receptors[37,38] as well. If the latter is the case, isolated eosinophils from peripheral blood of HDM-sensitized individuals have membrane-bound HDM-specific IgE antibody and have potentials to be activated upon binding of IgE to the allergen. We found, however, that HDM induced the activation was independent of specific IgE to HDM. We also confirmed that that effect was not from contaminated LPS in HDM extract because LPS did not induce IL-9 production even in the presence of the serum.

In the present experiments, we employed the concentrations of HDM and LPS based on a previous study in which the ability of various allergens to cause eosinophil activation and chemotaxis *in vitro* was investigated.[15] One drawback of our study is that the concentration of HDM used in the experiments was rather high and we did not find significant production of IL-9 on stimulation with lower concentrations of HDM (data not shown) and the present findings may not be relevant to clinical situations. However, exposure levels of HDM vary widely[39] and actual concentrations found in the airways are not known. Alternatively, our experimental model may represent other PAR-2 and PAR-2 agonist interactions such as tryptase from mast cells.[24]

In summary, we have shown that eosinophils are capable of interacting with HDM and producing IL-9. These findings suggest that eosinophils may play a role in innate immunity. When eosinophils encounter with an allergen that has serine-specific enzymaic activity, they may promote Th2-type immune response by producing IL-9.

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Neutrophil Proteases Activate Eosinophil Function in vitro

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Key Words
Eosinophils · Superoxide · Elastase · Serine protease · Neutrophils · Cytokines · Chemokines

Abstract
Background: Recent evidence suggests that both neutrophilic and eosinophilic inflammation persist in the airways of patients with severe asthma. Mechanisms for interaction between neutrophils and eosinophils are still to be understood. Since eosinophils express protease-activated receptor 2, neutrophil-derived serine proteases may activate eosinophils. ***Objective:*** We investigated the effect of neutrophil serine proteases on eosinophil effector functions. ***Methods:*** Peripheral blood eosinophils were stimulated with elastase, cathepsin G and proteinase 3. Superoxide generation was quantitated with the cytochrome C reduction method. A panel of cytokines and chemokines in the culture supernatants were measured with a multiplex beads array system. Effects of an elastase inhibitor, sivelestat, and a serine protease inhibitor, PMSF, on the protease-induced reactions were also tested. ***Results:*** Neutrophil proteases significantly induced superoxide production from eosinophils. Elastase was the most potent among them. Sivelestat and PMSF inhibited the reaction. The proteases induced production of IL-6, IL-8, TNF-α and GRO-α, that have a possible connection with neutrophilic inflammation. ***Conclusion:*** Neutrophil proteases activate eosinophils to produce superoxide, proinflammatory cytokines and neutrophilotactic chemokines and may further aggravate airway inflammation in patients with severe asthma.

Introduction

Eosinophilic airway inflammation is a central feature of asthma. A predominant role of neutrophils, however, has been implicated in severe/fatal asthma [1–3]. A recent report from the European Network Study for Understanding Mechanisms of Severe Asthma (ENFUMOSA) states that patients with severe asthma have greater sputum neutrophilia and higher eosinophil-derived mediator concentrations, compared with patients with mild to moderate asthma, suggesting that both neutrophilic and eosinophilic inflammation persist in the airways of severe asthma [4].

Recently, the mechanism for the colocalization of neutrophils and eosinophils in the airways has been studied. Nagata et al. [5] reported that activated neutrophils promote eosinophil transbasement membrane migration (TBM) through a combination of mediators secreted from neutrophils such as leukotriene B4, matrix metalloproteinase 9 and tumor necrosis factor-α (TNF-α).

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In addition to the mentioned mediators, neutrophils contain potent serine proteases such as neutrophil elastase, cathepsin G and myeloblastin (proteinase 3, PR3), which not only cleave exogenous proteins such as bacteria for host defense and endogenous structural proteins for tissue damage and remodeling [6], but activate a variety of cells through protease-activated receptors (PARs), which are 7-transmembrane G protein-coupled receptors. A specific protease cleaves the amino acids at a specific site of the extracellular N-terminus of the molecule to expose a new N-terminal ligand domain that binds to another site on the same molecule, thereby activating the receptor. Eosinophils express PAR-2 [7] and have been reported to be activated with serine proteases such as trypsin [8]. We have also observed that house dust mite extract induced IL-9 expression from eosinophils, possibly through PAR-2 [9]. Therefore, we hypothesized that the neutrophil serine proteases may activate eosinophils through PAR-2 in the context of possible neutrophil-eosinophil interactions in severe asthma. Here, we demonstrate that neutrophil serine proteases induce superoxide generation and proinflammatory cytokine production from eosinophils.

Materials and Methods

Reagents

Human neutrophil elastase was purchased from Athens Research and Technology (Athens, Ga., USA), human neutrophil cathepsin G from Calbiochem (San Diego, Calif., USA) and PR3 from Elstin Products Co. Inc. (Owensville, Mo., USA). Hanks' balanced salt solution (HBSS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer solution and fetal bovine serum (FBS) were obtained from Life Technologies BRL (Grand Island, N.Y., USA). Superoxide dismutase (SOD), gelatin, horseheart ferricytochrome C (type VI) and RPMI 1640 were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Fluo 3-AM was purchased from Dojin Chemical (Kumamoto, Japan). A serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF), was obtained from Sigma. Sivelestat sodium hydrate was a kind gift from Ono Pharmaceutical Co. (Tokyo, Japan).

Eosinophil Isolation

Eosinophils were purified by negative selection using anti-CD16 bound micromagnetic beads (MACS™; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as previously described [10]. The purity of eosinophils was more than 97%. The contaminating cells were neutrophils, and no mononuclear cells or basophils were present.

Superoxide Anion (O_2^-) Generation

Superoxide generation was measured with a cytochrome C reduction method [11]. In brief, freshly isolated eosinophils at 1.25 $\times$ 10^6 cells/ml were resuspended in HBSS with 25 mM HEPES, 0.03% gelatin and 100 μM cytochrome C. The cell suspension (100 μl) was dispensed onto the wells of 96-well tissue culture plates, followed by the addition of various concentrations (0.1 and 1 μg/ml) of elastase, cathepsin G, PR3 or medium alone. The reaction mixture was incubated at 37°C and absorbance was measured at 550 nm over 3 h with a microplate autoreader (Wallac 1420 ARVO MX; PerkinElmer, Waltham, Mass., USA). Each reaction condition was performed in duplicate and against an identical control reaction containing 20 mg/ml of SOD. Due to the 1:1 stoichiometry of cytochrome C reduction and O_2^- generation, the data were calculated using an extinction coefficient of 21.1 M^{-3} cm^{-1} for cytochrome C reduction and expressed as nanomoles of $O_2^-/10^5$ cells minus SOD control and spontaneous O_2^- generation. For inhibition experiments, elastase was premixed with PMSF at 0.1 mM or sivelestat sodium hydrate at 10 μg/ml 30 min prior to incubation with eosinophils and superoxide generation was measured as described above.

Measurement of Intracellular Calcium

Cells were suspended in HBSS with Ca^{2+}, Mg^{2+} and 2% bovine serum albumin (Sigma) at a cell density of 2 $\times$ 10^6/ml. Fluo 3-AM (Dojindo, Tokyo, Japan) was added at a final concentration of 2 μM. After incubation for 20 min, cells were washed and suspended in a buffer containing 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.03% human serum albumin and 25 mM HEPES, pH 7.4, at a concentration of 1.6 $\times$ 10^6 cells/ml. Calcium influx was measured using excitation at 340 and 380 nm with a fluorescence spectrometer (Wallac 1420 ARVO MX; PerkinElmer).

Production of Cytokines and Chemokines from Eosinophils

Eosinophils at 1 $\times$ 10^6/ml in RMPI 1640 with 5% FBS were cultured with elastase, cathepsin G or PR3 at 0.1, 1 and 10 μM at 37°C for 48 h. A panel of cytokines and chemokines in the supernatants were measured by using Luminex multiplex kits (human cytokine 10-plex for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α and GM-CSF; human chemokine 10-plex for eotaxin, GRO-α, IP-10, MCP-1, RANTES, MCP-2, MCP-3, MIP-1α, MIP-1β and MIG; Invitrogen, Carlsbad, Calif., USA) on a Luminex 100 multiplex beads array system (Luminex Corp., Austin, Tex., USA) [12].

Statistical Analysis

Data are expressed as means ± SEM of indicated numbers of experiments and p values were determined with two-way ANOVA for multiple groups with the Bonferroni post test.

Results

Superoxide Generation by Neutrophil Proteases

Elastase and cathepsin G significantly induced O_2^- generation from eosinophils, whereas PR3 had a minimal effect (fig. 1). Elastase appeared to be most potent in the reaction, since the O_2^- reaction kinetics showed that low levels of activation started at 60 min followed by significant elevation at 90 min with elastase and 150 min with