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Sputum eosinophilia is more closely associated with airway responsiveness to bradykinin than methacholine in asthma

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Sputum eosinophilia is more closely associated with airway responsiveness to bradykinin than methacholine in asthma. R. Polosa, L. Renaud, R. Cacciola, G. Prosperini, N. Crimi, R. Djukanovic. ©ERS Journals Ltd 1998.

ABSTRACT: Hyperresponsiveness of the airways to various spasmogenic stimuli is a characteristic feature of bronchial asthma. However, the association between the different stimuli to which asthmatic airways are hyperresponsive and airways inflammation is not completely understood.

We have investigated the relationship between airway inflammation and airway hyperresponsiveness in asthma, as assessed by bronchoprovocation tests to methacholine and bradykinin, two well defined bronchoconstrictor agonists. Sputum induction by hypertonic saline and methacholine and bradykinin challenges were performed in 14 nonsmoking subjects with mild-to-moderate asthma.

Airway responsiveness to either agonist did not correlate with sputum neutrophils, lymphocytes, and macrophages. Whilst the absolute number of eosinophilia failed to be significantly related to methacholine responsiveness (r=-0.47; p=0.09), it correlated markedly and significantly with provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second (r=0.72; p<0.01). When expressed as % of total cell counts, sputum eosinophils correlated with both types of responsiveness (r=-0.56; p=0.04 and r=-0.76, p<0.001, respectively). Although the concentration of eosinophil cationic protein (ECP) in the sputum correlated with the absolute numbers of eosinophils (r=0.62; p<0.02), no correlation was found between ECP levels and the airway responsiveness to any of the agonists tested.

In subjects with mild-to-moderate asthma, airway responsiveness to bradykinin is more strongly associated with the magnitude of eosinophilic inflammation in the airways than methacholine. This finding underlines the selectivity of diverse agonists in assessing airway hyperresponsiveness and cellular inflammation in asthma.
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The role of inflammation in asthma is widely appreciated, and airway eosinophilic inflammation is considered to be one of the most important features of the pathology of this disease [1]. The importance of airway eosinophilia as a major marker of disease activity has been documented in a number of studies [2–5]. The eosinophil is believed to be one of the key cells responsible for the development of many of the features of asthma, including damage and shedding of the respiratory epithelium, allergen-induced late asthmatic reactions, and airway hyperresponsiveness [6].

The existence of a possible correlation between the magnitude of airway eosinophilic inflammation and the degree of airway hyperresponsiveness to methacholine or histamine has been controversial, with some studies reporting a correlation between the magnitude of eosinophilic inflammation and airway responsiveness to methacholine or histamine in bronchial biopsies [7] and in bronchoalveolar lavage (BAL) fluid [8, 9] and others failing to establish an association [10–12].

Besides methacholine and histamine, asthmatic subjects have also exaggerated airway responses to bradykinin [13, 14], which is a pro-inflammatory nonapeptide produced *de novo* in body fluids and tissues during inflammatory conditions including bronchial asthma [15]. Methacholine- and histamine-induced bronchoconstriction are likely to be due, primarily, to a "direct" effect of these agonists on specific receptors on the airway smooth muscle. In contrast, the underlying mechanism of bronchoconstriction for bradykinin is mainly "indirect", involving neural reflexes [15].

In order to further improve the understanding of bradykinin effects in asthma we have sought evidence for an association between airway inflammation and responsiveness to bradykinin. We have therefore investigated the relationship between infiltrate of inflammatory cells by means of sputum induction and airway hyperresponsiveness to bradykinin and methacholine in asthmatic subjects.

Methods

Subjects

Fourteen nonsmoking asthmatic subjects (9 female, 5 male) with a mean (±SEM) age of 27.6±2.1 yrs who met the American Thoracic Society's diagnostic criteria of asthma

Table 1. – Demographic details of subjects studied

Subject No.	Sex	Age yrs	Baseline FEV_1 % predicted	PC_{20} bradykinin $mg \cdot mL^{-1}$	PC_{20} methacholine $mg \cdot mL^{-1}$	Regular medication
1	M	31	90	0.05	0.60	S
2	F	23	99	1.69	4.52	
3	F	22	84	0.65	1.09	S, BDP (1,000 μg)
4	F	26	86	1.50	1.97	S, BDP (2,000 μg)
5	M	23	91	0.06	1.09	S
6	F	43	80	3.88	8.11	
7	M	19	99	0.75	10.50	
8	M	38	85	0.12	3.33	S
9	F	25	87	0.08	1.46	S
10	F	36	97	0.20	1.34	S
11	F	37	106	3.08	8.98	
12	M	22	83	0.06	2.72	S
13	F	18	88	4.75	4.25	S, BDP (1,000 μg)
14	F	23	77	0.53	0.25	S
Mean		27.6	89.4	0.45*	2.25*	
SEM		±2.1	±2.2	(0.05–4.75)	(0.25–10.50)	

*: geometric mean (range); FEV_1: forced expiratory volume in one second; PC_{20}: provocation concentration causing a 20% fall in FEV_1; S: salbutamol *p.r.n.*; BDP: beclomethasone dipropionate *b.i.d.*

[16] participated in the study (table 1). All were previously shown to have exaggerated responses to both inhaled methacholine and bradykinin. All the subjects studied were atopic, as defined by positive skin prick tests (>3 mm wheal response) to one or more of seven common airborne allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, mixed grass pollen, mixed tree pollen, mixed weed pollen, cat fur, and dog hair). Their baseline forced expiratory volume in one second (FEV_1) was >75% predicted, and none had ever received oral corticosteroids or theophylline. All were controlled on inhaled β-agonists as required, with the exception of those subjects who also took regular inhaled corticosteroids. Inhaled bronchodilators were withheld for at least 12 h prior to each visit, but subjects were allowed to continue their inhaled corticosteroids as usual. None of the subjects studied had experienced a respiratory tract infection or exacerbation of their asthma for at least 6 weeks before or during the study. The study was approved by the local hospitals ethical committee and written informed consent was given by all the subjects.

Study design

The study consisted of three study days outside the pollen season which were carried out at the same time of the day (09.00 h). On the first day, subjects attended the laboratory to undergo a methacholine challenge to determine the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) from baseline. Two days later sputum induction by hypertonic saline was performed according to a recently validated protocol [17]. The expectorated sputum was immediately collected and processed. On the final visit (5–7 days later), subjects attended the laboratory and had bradykinin challenge, to determine their PC_{20} bradykinin values.

Bronchial provocation

Methacholine chloride and bradykinin acetate salt (Sigma, St Louis, MO, USA) were dissolved in phosphate-buffered saline (PBS) (pH 7.4) to produce increasing doubling concentrations ranging from 0.03–16 $mg \cdot mL^{-1}$ for methacholine and from 0.015–4 $mg \cdot mL^{-1}$ for bradykinin and immediately used for bronchial challenge. The solutions were administered as aerosols generated from a starting volume of 3 mL in a disposable Inspiron Minineb (C.R. Bard International, Sunderland, UK) driven by compressed air at 8 $L \cdot min^{-1}$. Patients inhaled the aerosolized solution in five breaths from functional residual capacity to near total lung capacity *via* a mouthpiece. Patients were trained to reach total lung capacity in 3 s.

FEV_1 was measured using a dry wedge spirometer (Vitalograph, Buckinghamshire, UK). After 15 min rest, three consecutive baseline measurements of FEV_1 were made at intervals of 2 min and the best result recorded. Challenges were preceded by inhalation of PBS and only subjects in whom this caused <10 % decreases from baseline FEV_1 were studied. Immediately after challenge with diluent, increasing doubling concentrations of methacholine or bradykinin were administered. FEV_1 was measured at 1 and 3 min after administration of each concentration of agonist. The challenges were discontinued when FEV_1 had fallen by >20% of the postdiluent value. The bronchial responses to the inhaled agonists were expressed as the PC_{20}, which was derived by linear interpolation from the concentration-response curve constructed by plotting the percentage change in FEV_1 from the post-diluent value against the cumulative concentration of agonist administered on a logarithmic scale.

Sputum induction

Sputum induction was performed according to our published method [17]. Briefly, after stopping β_2-agonists for at least 12 h, subjects inhaled hypertonic saline (4.5%) aerosolized by an ultrasonic nebulizer (UltraNeb 99; DeVilbiss, Feltham, Middlesex, UK) with output set at 3 $mL \cdot min^{-1}$. The subjects wore a nose clip and quietly inhaled aerosol for up to four consecutive 5-min periods. After each inhalation, the subjects rinsed their mouth with water and dried

it with tissue paper to minimize contamination with saliva. They then expectorated the sputum into a Petri dish, which was immediately placed onto ice until processing. FEV_1 was then measured between procedures for safety reasons; if the fall in FEV_1 was >20% of baseline, the challenge was discontinued.

Sputum processing and analysis

Whole sputum was transferred into 50 mL polypropylene tubes (Becton Dickinson, Abingdon, UK), weighed, and an equal weight of 0.01 M dithioerythritol (DTE; Fluka, Gillingham, Dorset, UK) solution added to solubilize the mucus. Specimens were then vortexed for 10 s, rocked for 30 min at room temperature, and vortexed again for 10 s. They were then filtered through a 70 mm strainer (Becton Dickinson) and the collected fluid centrifuged at 400×g for 10 min at 4°C. The supernatants were removed and stored at -20°C. The cell pellets were resuspended in 1 mL PBS without Ca^{2+} and Mg^{2+} and viable cells counted in a haemocytometer. Only samples in which squamous cells comprised <30% of total cells were considered satisfactory for analysis. Differential counting was carried out on cytospins stained with May-Grunwald-Giemsa and 600 cells (excluding squamous cells) counted. Slides were coded and examined by one investigator. Eosinophil counts were expressed as a percentage of the number of total cells and as absolute numbers. Eosinophil cationic protein (ECP) levels were measured in duplicate by a commercially available fluorometric enzyme immunoassay (FEIA; Pharmacia, Uppsala, Sweden) with a sensitivity of 2 $mg \cdot L^{-1}$.

Data analyses

Baseline values of FEV_1 were compared between and within study days by two-factor analysis of variance (ANOVA) followed by Neuman-Keuls test where appropriate.

PC_{20} methacholine and bradykinin values were logarithmically transformed to normalize their distribution and expressed as geometric mean (range). All other variables which were not normally distributed were expressed as median (range). The relationship between methacholine PC_{20} and bradykinin PC_{20} was studied by least squares linear regression after logarithmically transforming the PC_{20} values. All the data for cells and ECP in the sputum samples had a non-normal distribution. Thus, for correlation analyses of the data that included sputum variables and airway responses to bradykinin and methacholine, the Spearman's rank-order test was used. Values of $p<0.05$ were considered to indicate statistical significance.

Results

There was no significant difference in baseline values of FEV_1 between any of the three study days, with mean (±SEM) values ranging from 2.95± 0.20 to 3.10±0.23 L. The geometric mean (range) PC_{20} values for methacho-line and for bradykinin were 2.25 $mg \cdot mL^{-1}$ (0.25–10.50 $mg \cdot mL^{-1}$) and 0.45 $mg \cdot mL^{-1}$ (0.05–4.75 $mg \cdot mL^{-1}$), respectively (table 1). A weak but significant correlation was observed between methacholine PC_{20} and bradykinin PC_{20} ($r=0.54$, $p=0.046$).

Sputum induction was well tolerated by all subjects studied. Inhalation of hypertonic saline caused a mean fall in FEV_1 of 0.24±0.08 L. None of the subjects studied experienced a fall in FEV_1 >20% after sputum induction.

The median (range) inflammatory cell count in sputum was 2.3% (0.6–22.7%) for eosinophils, 21.3% (7.0–35.8%) for neutrophils, 4.0% (0.9–11.3%) for lymphocytes and 69.0% (51.0–91.0%) for macrophages (table 2).

The median (range) ECP concentration measured in the sputum supernatant was 29.1 (5.4–563.7) $ng \cdot mL^{-1}$. The concentration of ECP measured in the fluid phase correlated strongly and significantly with the absolute numbers of sputum eosinophils ($r=0.62$; $p<0.02$).

There was a tendency towards a correlation between methacholine PC_{20} values and the absolute count of eosinophils in sputum, which failed to reach statistical significance

Table 2. – Total and percentage cell counts in the sputum of the subjects studied

Subject No.	Total cell counts* $\times 10^3 \cdot g$ sputum^{-1}	Squamous cells %	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %	ECP $ng \cdot mL^{-1}$
1	1,800	28	68.5	4.0	21.5	6.0	40.8
2	2,300	16	69.0	3.2	26.0	1.8	134.6
3	320	12	75.1	4.3	19.1	1.5	10.0
4	350	22	77.0	1.0	21.0	1.0	5.4
5	547	30	51.0	11.3	15.0	22.7	445.5
6	375	8	70.2	5.1	24.1	0.6	52.8
7	537	25	64.0	8.0	25.3	2.7	5.5
8	1,005	24	52.0	6.0	24.0	18.0	563.7
9	517	10	68.9	3.9	8.0	19.2	71.1
10	427	27	79.0	2.5	12.5	6.0	21.7
11	397	25	67.0	9.1	23.0	0.9	36.0
12	637	11	85.3	3.1	10.1	1.5	8.2
13	1,155	10	91.0	1.3	7.0	0.7	13.3
14	772	18	53.7	0.9	35.8	9.6	22.2
Mean	795.6	19.0	69.41	4.55	19.46	6.59	102.2
SEM	158.5	2.1	3.17	0.83	2.16	2.07	46.9
Median	542.0	20	68.95	3.95	21.25	2.25	29.1

*: excluding squamous cells; ECP: eosinophil cationic protein.

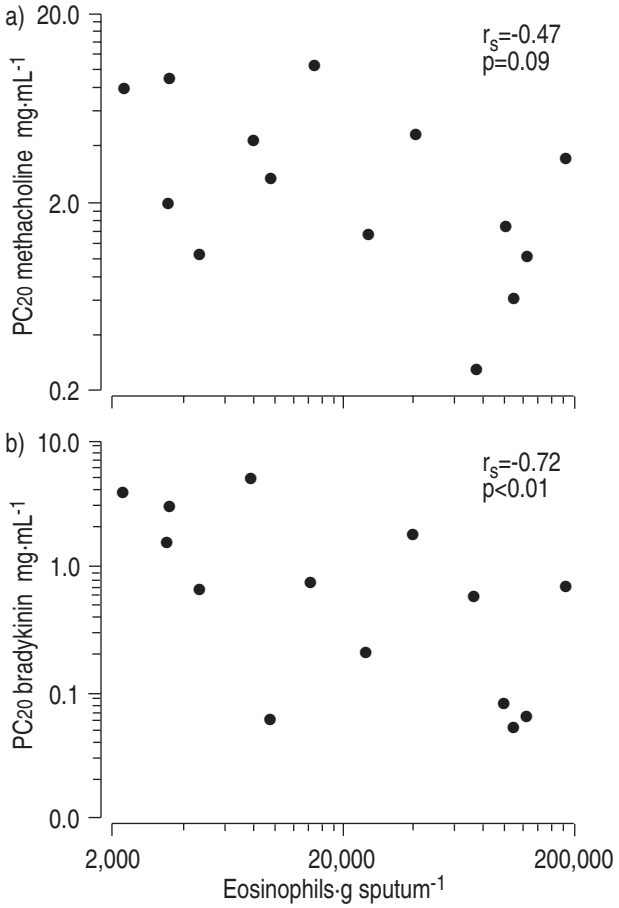

Fig. 1. – Correlation between the number of eosinophils in the sputum and the provocative concentration causing a 20% fall in forced expiratory volume in one second (PC_{20}) of methacholine (a) and bradykinin (b) in asthmatic subjects (n=14). The strength of the association was analysed by Spearman's rank-order test.

(r_s=-0.47, p=0.09, fig. 1a). Indeed, when the sputum eosinophilia was expressed as a percentage of total cell counts, a weak but significant association with the PC_{20} values for methacholine was found (r=-0.56, p=0.04).

However, there was a marked and significant negative correlation between bradykinin PC_{20} values and sputum eosinophils, both in terms of absolute (r=-0.72, p<0.01, fig. 1b) and of percentage counts (r_s=-0.76, p<0.001).

No significant correlation was found between the concentration of ECP in sputum and the airway responsiveness to either of the agonists tested.

Furthermore, we found no significant correlation between the PC_{20} values for methacholine or bradykinin and either the percentage or the absolute count of lymphocytes, neutrophils, and macrophages.

Discussion

It has long been appreciated that airway hyperresponsiveness is not a nonspecific phenomenon and that responses to individual agonists, such as histamine, methacholine, exercise and bradykinin, are not necessarily closely associated [18]. This is likely to reflect different components of asthma pathogenesis which can be identified by increased responses to a given stimulus. Consistent with this notion, in the present study we have shown a difference in the relationship between airways eosinophilia on the one hand and airway hyperresponsiveness to bradykinin and methacholine on the other.

In keeping with previous reports, airway responsiveness to both agonists did not correlate with the number (and percentage) of lymphocytes, neutrophils, and macrophages in asthmatic airways [2, 12, 19]. However, we have shown a strong negative correlation between PC_{20} bradykinin and sputum eosinophils, which was much stronger and significant than the association with PC_{20} methacholine. The presence of ECP in association with an increased number of eosinophils in the sputum reflects that activation and degranulation of eosinophils occurs in the airway mucosa of these subjects. Whilst our findings indicate that airway responsiveness to bradykinin is related to the degree of eosinophilic infiltration, the lack of an association with ECP levels would suggest that the mechanisms involve other eosinophil mediators which, possibly together with their basic protein, contribute to the enhanced airway responses to bradykinin. However, the concentration of ECP in sputum may reflect poorly the eosinophil activation that occurs deep in the airways mucosa, thus explaining why we found no correlation between sputum ECP and airway responsiveness to bradykinin. Although we cannot exclude the possibility that this may be also due to the confounding and variable influence of saliva in induced sputum, two large studies using sputum plugs (known to contain reduced amounts of saliva) have shown a similar lack of correlation between ECP levels and airway hyperresponsiveness [20, 21].

With increasing use of induced sputum as a safe and simple means of assessing airways inflammation, a number of investigators have shown a correlation between sputum eosinophilia and airway hyperresponsiveness [17, 20, 22]. However, other studies have failed to relate eosinophil differential counts in sputum to airway hyperresponsiveness [21, 23]. The reasons for such discrepancies are unclear but may be related to the diversity in clinical and functional characteristics of the subjects studied or to important differences in the study protocol or statistical conduct.

Indeed, some of these problems are well represented in the present paper. For example, we failed to show a statistically significant correlation with PC_{20} methacholine when the absolute count of eosinophils in sputum was used, but this relationship reached statistical significance (although weak) when the sputum eosinophilia was expressed as a percentage of total cell counts. However, our data on bradykinin are far more sound as the marked and significant negative correlation between bradykinin PC_{20} values and sputum eosinophils was maintained both in terms of absolute and percentage counts.

The findings of the present investigation agree with the recent study by Roisman *et al.* [24], in which a significant correlation was found between airway responsiveness to bradykinin (but not methacholine) and eosinophils count in BAL and bronchial biopsies.

The stronger relationship between bradykinin airway responsiveness and airway eosinophilic inflammation cannot be explained by different conditions of airway challenge, as baseline FEV_1, before methacholine and bradykinin challenges were not statistically different. Challenges were

carried out at the same time of the day, thus ruling out a possible influence of circadian variations on airway responsiveness. Bradykinin challenge was carried out after sputum induction to avoid any inflammatory change induced by the pro-inflammatory peptide in airway mucosa. Additionally, the relatively short time interval between methacholine and bradykinin challenges allowed us to reduce the probability that the relationship between the responses to the two agonists was affected by spontaneous intraindividual variations in airway responsiveness.

The discrepancy between the two agonists for the relationship between airway responsiveness and sputum eosinophilia may reside in the difference in their mechanisms of bronchoconstrictor effect. Methacholine elicits bronchoconstriction through a direct action on the airway smooth muscle, whereas inhaled bradykinin causes bronchoconstriction mainly *via* "indirect" mechanisms, involving neural reflexes [15]. Further support for the differential mechanisms of action of bradykinin and methacholine is suggested by the recent demonstration that whole lung antigen challenge elicited a more marked increase in bradykinin airways responsiveness than in methacholine responsiveness and that the shifts in antigen-induced bronchial hyperresponsiveness to the two agonists followed considerably different time courses [25, 26]. These observations of the disparate responses of bradykinin and methacholine in the setting of allergic inflammation suggest that the mechanisms by which bradykinin exerts its effects are particularly sensitive to acute inflammation of the bronchial mucosa, whereas methacholine responsiveness might predominantly be dependent on structural changes of the airways.

Several possibilities exist to explain how eosinophilic inflammation may selectively influence the response of asthmatic airways to bradykinin. Recruitment of eosinophils and release of their granular content is associated with the magnitude of airway epithelial damage and shedding in asthma [3, 27]. Eosinophils are capable of damaging the respiratory epithelium through the action of toxic products such as ECP, which are contained in their granules and secreted upon eosinophil activation [27]. *In vitro*, airway smooth muscle contraction to bradykinin is potentiated by loss of or damage to the airway epithelium [15, 28]. The constrictor effect of bradykinin is attenuated by the release of epithelial-derived prostaglandin E2 [29] and by its degradation by two major bronchial peptidases, neutral endopeptidase (NEP) and kininase II [15]. With damage of the epithelium and the resulting loss of these epithelial-bound peptidases, a selective increase in bradykinin airway responsiveness would be expected, because methacholine is not a substrate for these enzymes [30, 31]. Moreover, damage to the epithelium might also enhance sensory nerve stimulation to the action of bradykinin, resulting in the release of spasmogenic neuropeptides by way of an axon reflex [15].

Another potential explanation for the association between airway hyperresponsiveness to bradykinin and sputum eosinophilia is that eosinophilic inflammation may alter responsiveness to bradykinin by increasing kinin receptor expression. It has been demonstrated that interleukin (IL)-1, which is generated at sites of allergic inflammation [32], can increase the number of bradykinin receptors on human synovial cells [33]. If the expression of bradykinin receptors on target tissues is induced during allergic inflammation, this could help to explain the hyperresponsiveness to bradykinin seen in patients with high eosinophil counts in their sputum.

In asthma, the responsiveness to exogenous bradykinin is more strongly related to sputum eosinophilia than methacholine. Because the present finding underlines the selectivity of bradykinin in assessing airway hyperresponsiveness in relation to the degree of eosinophilic inflammation, in the future it would be of interest to examine the clinical usefulness of bradykinin bronchoprovocation as a marker of the severity of the inflammatory processes in asthma.

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