

TECHNICAL NOTE

Methacholine challenge does not affect bronchoalveolar fluid cell number and many indices of cell function in asthma

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ABSTRACT: Methacholine (MCh) challenge testing is often incorporated into clinical studies prior to performing bronchoscopy as a measure of bronchial hyperresponsiveness (BHR). However, the effect of methacholine on many aspects of bronchoalveolar lavage (BAL) fluid cell count and function have not been fully evaluated.

Ten patients with asthma, maintained on inhaled β_2 -agonists, were studied. Each subject underwent two bronchoscopies in a random order, one preceded by methacholine challenge within 30 min of the BAL. The investigators were blinded to the regimen. Several markers of BAL fluid cell number and function were studied: cell count and differential, histamine, eosinophil products, including eosinophil cationic protein and Charcot-Leyden crystal protein, macrophage production of thromboxane B_2 and leukotriene B_4 , neutrophil lysozyme and lactoferrin, lymphocyte typing and activation markers measured *via* flow cytometry.

No significant differences were noted in any of these markers of cell number or function which could be ascribed to methacholine challenge.

Thus, methacholine challenge does not appear to affect these markers of cell number and function. These findings indicate that a methacholine challenge can be used as a measure of bronchial hyperresponsiveness within 30 min prior to bronchoscopy without altering bronchoalveolar lavage fluid characteristics.

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Bronchoscopy with bronchoalveolar lavage (BAL) is frequently employed in studies assessing the pathogenesis of asthma. The technique is a safe method of quantitating airway inflammation under a variety of conditions in asthma, such as after allergen challenge or at night as a measure of circadian variation of airway inflammation. Methacholine provocation testing is also used extensively to measure bronchial hyperresponsiveness (BHR) in asthma [1], and carried out prior to bronchoscopy in clinical protocols [2].

The possible affect of methacholine challenge on BAL fluid cell count and function has received limited attention. When performed 24 h prior to BAL, SODERBERG *et al.* [3] have shown no change in BAL fluid cell count and differential. However, when methacholine challenge is performed 5 h prior to BAL, increased levels of leukotriene B_4 (LTB $_4$) and prostaglandin D_2 (PGD $_2$) are seen in the BAL fluid of asthmatics as compared to controls [4]. To further clarify the effect of methacholine on BAL fluid characteristics, we investigated the effect of methacholine challenge on cellular number and function when performed within 30 min prior to BAL in subjects with asthma.

Methods

Subjects

Ten asthmatics were recruited from the general Denver, Colorado community. The 10 subjects met diagnostic criteria for asthma [5] for at least 7 yrs prior to the study. The patients asthma state was stable and they had no history of smoking over the past 2 yrs. Exclusion criteria included: use of cromolyn, inhaled or oral corticosteroids within the previous 6 weeks; use of astemizole within the previous 2 months; immunotherapy within the previous 3 months; an upper respiratory infection within the previous month; and any other history of significant nonasthmatic pulmonary disease, other medical illness or noncompliance. Informed consent was obtained for this Institutional Review Board approved protocol.

Protocol

The study involved two visits to the research laboratory. In a random sequence, one visit required a bronchoscopy with BAL alone and one visit required

a methacholine challenge prior to BAL. The patients arrived at the laboratory at approximately 8 a.m. and left the laboratory by 12 noon.

Methacholine challenge

Methacholine challenge was performed as described previously [6]. Methacholine was administered as an aerosol in increasing concentrations (0.0175 to 25.0 mg·dL⁻¹) at 5 min intervals *via* a DeVilbiss 646 nebulizer (Somerset, PA, USA) powered by pressurized air (20 psi) delivered through a Rosenthal-French dosimeter (Baltimore, MD, USA) that was triggered by a solenoid valve set to remain open for 0.6 s. Subjects performed five inspiratory capacity inhalations at each concentration of methacholine, followed by spirometry (Moose, Cybermedic, Louisville, CO, USA) 3 min later. The challenge was stopped after reaching the concentration of methacholine that provoked a 20% reduction in forced expiratory volume in one second (FEV₁) from prechallenge baseline (PC20).

Bronchoscopy with bronchoalveolar lavage

Bronchoscopy with BAL was performed alone or within 30 min of methacholine challenge using procedures identical to those described previously [6]. Prior to the procedure and after methacholine challenge, subjects received 0.4 mg of terbutaline from a metered-dose inhaler (MDI), 60 mg codeine and 0.6 mg atropine intramuscularly. In addition, 4% xylocaine was used to anaesthetize the upper airway and 1% xylocaine was applied to the laryngeal area, trachea and orifice of the right middle lobe or lingula *via* the bronchoscope. Subjects were randomized to undergo bronchoscopy with BAL of the right middle lobe or lingula in the first week with the alternate lobe lavaged in the second week. Bronchoalveolar lavage was performed using five 60 mL aliquots of sterile normal saline at 37°C. Lavage fluid was obtained by immediate gentle hand suction applied to each instilling syringe. Nasal oxygen at 3–4 L·min⁻¹ and pulse oximetry were used to monitor oxygen saturation throughout the procedure. Subjects were monitored post-bronchoscopy with pulse oximetry and periodic physician examination until discharge 4–6 h later.

Bronchoalveolar lavage fluid analysis

Cell count and differential. The lavage fluid was immediately placed on ice, and the aliquots were combined and centrifuged for 10 min at 1,200 rpm and 4°C to separate cells from fluid. Differential cell counts were performed from a known volume of lavage with a Diff-Quick stain (Dade Diagnostics Inc., Aguada, Puerto Rico). Cell counts were carried out with fresh lavage fluid and at least 500 cells were counted to obtain the differential cell count. Results are expressed as cells·mL⁻¹ BAL fluid.

Mast cell studies - histamine. BAL fluid histamine levels

were measured from BAL supernatant using an enzyme-linked immunosorbent assay (ELISA) kit from Biometrica (Newport Beach, CA, USA). The BAL fluid, stored at -70°C was thawed and not concentrated prior to measurement.

Eosinophil studies - eosinophil cationic protein and Charcot-Leyden crystal protein. BAL fluid was placed in separator tubes and incubated at room temperature for 60–120 min prior to centrifugation at 2,000 rpm for 10 min. The fluid was separated, and aliquots of 0.5 mL were frozen at -20°C until used. Eosinophilic cationic protein (ECP) levels were determined using the Pharmacia CAP System ECP fluorescence enzyme immunoassay (FEIA) kit (Pharmacia Diagnostics AB, Uppsala, Sweden). The detection limit of the assay is 0.5 µg·L⁻¹, and the intra- and interassay coefficients of variation were ≤7% and 8%, respectively. Results are expressed in µg·mL⁻¹.

Charcot-Leyden crystal (CLC) protein was measured as described previously [7, 8]. One hundred microlitre aliquots of BAL fluid were placed in a "double-sandwich" radioimmunoassay. Results are expressed in ng·mL⁻¹.

Macrophage studies - leukotriene B₄ and thromboxane B₂ production. The BAL fluid macrophages were resuspended at a concentration of 1×10⁶ macrophages·mL⁻¹ in Dulbecco's modified eagle medium (DMEM) with 10% foetal bovine serum and 100 µg·mL⁻¹ penicillin/100 µg·mL⁻¹ streptomycin. These cells were then plated in a 24 or 48 well plastic culture plate (depending on yield) and allowed to adhere for 2 h at 37°C/10% CO₂. The cells were then washed three times with cold phosphate-buffered saline (PBS) and new media (DMEM/0.1% bovine serum albumin) was then added. The supernatants were then harvested and analysed for the eicosanoids LTB₄ and thromboxane B₂ (TxB₂). The plated cells were again washed three times (PBS Ca⁺⁺ and Mg⁺⁺), lysed (0.2 N NaOH), and protein content per well was determined. All eicosanoids were reported as pg·µg⁻¹ protein.

Eicosanoid production was determined by using enzyme immunoassays [9], and cellular lysate protein was determined by using a modified Lowry assay [10]. LTB₄ was measured as a representative of the 5-lipoxygenase pathway and TxB₂ was measured as a representative of the cyclooxygenase pathway. The LTB₄ antibody was purchased from Advanced Magnetics Inc. (Cambridge, MA, USA), whilst the TxB₂ antibody was a generous gift from F. Fitzpatrick (UCHSC). The sensitivity of the LTB₄ assay was routinely 30 pg·mL⁻¹ and that of TxB₂ was 15 pg·mL⁻¹. Protein values averaged 89.9 µg/1×10⁶ cells.

Neutrophil studies. Neutrophil degranulation was assayed using lactoferrin as a secretory granule marker and lysozyme as a marker for primary and secretory granules. BAL fluid supernatants were collected for enzyme determination. Total enzyme content was determined by freeze-thawed lysates. Lysozyme activity was measured using a turbidometric index as the rate of lysis of *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA)

[11].

Lactoferrin determinations were performed *via* an ELISA technique [12]. Plates were read on Dynatech ELISA reader (Chantilly, VA, USA) and data are reported as the percentage of activity in each unknown as compared to the lysed polymorphonuclear leucocyte (PMN) samples.

Lymphocyte immunophenotyping. To examine BAL fluid for lymphocyte phenotypes, the lavage fluid was first centrifuged at 1,000 rpm to pellet the cells. At this point, the cells were made up to a concentration of 1×10^7 lymphocytes·mL⁻¹ in autologous serum or normal pooled human serum. The procedure for staining the BAL fluid is the same as is used for blood lymphocyte immunophenotyping, including lysing any residual red blood cells (RBCs) and setting the appropriate gates by light scatter and CD45+/CD14- cells to analyse the lymphocyte populations [13]. This method for enumeration of lymphocyte populations was followed, as published by the National Committee for Clinical Laboratory Standards [14]. In brief, the direct two colour immunofluorescent procedure was used, where monoclonal antibodies were mixed with heparinized blood and, after a period of incubation, the RBCs were lysed. CD3 (T3-RD1, Cyto-Stat/Coulter Clone, Coulter Immunology) were used to determine the number of T-cells, CD4 (B4-FITC, Cyto-Stat/Coulter Clone, Coulter Immunology, Hialeah, FL, USA) and CD8 (T8-FITC Cyto-Stat/Coulter Clone, Coulter Immunology) for enumerating T-helper and T-cytotoxic/suppressor cells, respectively. The antibodies human leucocyte antigen-DR (HLA-DR) and CD25, activation markers, were purchased from Olympus, Lake Success, NY.

The percentage of lymphocytes expressing these markers was determined by flow cytometry. A two colour CD45/CD14 reagent (Mo2-RD1/KC56-FITC Cyto-Stat/Coulter Clone, Coulter Immunology) was used to verify the lymphocyte gates, while isotypic controls (Cyto-Stat/Coulter Clone, Coulter Immunology) were used to set the background staining. The specific procedure used was as follows: 10 μ L, or the manufacturer's recommended volume, of single or combined two colour monoclonal antibodies were added to BAL fluid in a 12 \times 75 mm tube. Samples were read on a Coulter Epics Profile 1 flow cytometer. Lymphocytes were gated by their light scatter characteristics and these gates were verified with the CD45/CD14 reagent, such that greater than 99% of the gated cells were CD45+/CD14- cells. The percentage of lymphocytes expressing each of the specific cell surface markers was determined by counting 10,000 cells.

Statistical analysis

The paired t-test was used to compare parameters before and after methacholine challenge which were normally distributed. These data are expressed as mean \pm SEM. However, some parameters were not normally distributed, thus these were compared before and after methacholine

challenge using the Wilcoxon signed rank test. These data are expressed as median with the 25–75 interquartile range (IQ). The normality of the data was tested using the SHAPIRO and WILK [15] test. Assuming a single pairwise comparison and a type one error rate of 5%, the sample size reflects an 80% power for detecting a pairwise difference of one standard deviation [16]. All tests are two-sided with p-values equal to or less than 0.05 considered to be significant.

Results

Subjects

The subject characteristics are shown in table 1. The 10 subjects consisted of five males and five females with an average age of 34 \pm 3 yrs. All patients were maintained on inhaled beta₂-agonists only. The mean duration of asthma was 22 \pm 3 yrs, and three of the 10 subjects experienced nocturnal symptoms resulting in awakening from sleep to use an inhaled beta₂-agonist at least three nights per week.

Spirometry and methacholine challenge testing

The spirometry for each subject before methacholine challenge is listed in table 1. The mean (\pm SEM) FEV₁ for all 10 subjects was 3.1 \pm 0.3 L, 81 \pm 6% predicted. The mean forced vital capacity (FVC) was 4.5 \pm 0.4 L, 91 \pm 5.3%, pred and the mean FEV₁/FVC ratio was 69 \pm 4%. There were no significant differences in spirometry prior to each bronchoscopy. The median PC₂₀ was 0.72 (IQ 0.14–1.6) (table 1).

BAL fluid analysis

Table 1. – Subject characteristics

Sub. No.	Age yrs	Sex	Asthma* yrs	FEV ₁ L	FEV ₁ % pred	PC ₂₀ mg·mL ⁻¹
1	24	M	20	1.7	37	0.14
2	25	M	22	3.0	74	2.8
3	47	F	21	2.4	75	0.45
4	29	F	7	2.0	78	0.98
5	42	M	30	3.4	74	1.2
6	39	M	34	3.3	85	0.13
7	42	M	39	4.5	113	1.2
8	25	M	20	3.0	58	0.18
9	21	F	17	4.5	107	0.01
10	44	F	14	2.5	90	10.0
Mean	34	6/4	22	3.1	81	0.72
\pm SEM	\pm 3		\pm 3	\pm 0.26**	\pm 6	0.14–1.6 [#]

*: history of asthma; **: spirometry listed was performed prior to methacholine challenge. Spirometry performed on alternate day (without methacholine challenge) was within 10% of values listed. #: interquartile range. Sub.: subject; M: male; F: female; FEV₁: forced expiratory volume in one second; % pred: percentage of predicted value; PC₂₀: concentration of methacholine provoking a 20% reduction in FEV₁.

Table 2. — Bronchoalveolar lavage total and differential cell count with and without methacholine challenge

	Without MCh	With MCh	Paired difference	p-value
Total WBC $\times 10^4$	12.2 \pm 2.2	11.7 \pm 1.5	0.4 \pm 1.7 (5.3)	0.8
Eos %	2.2 \pm 0.5	2.3 \pm 0.6	-0.1 \pm 0.9 (2.8)	0.9
Macs %	87.0 \pm 2.1	86.6 \pm 7.2	0.4 \pm 2.4 (7.7)	0.9
PMNs %	2.3 \pm 1.2	1.2 \pm 0.4	0.2* -0.4–2.1	0.3
Lymphs %	9.6 \pm 6.6	10.1 \pm 1.9	-0.5 \pm 1.8 (5.8)	0.8

Values are presented as mean \pm SEM, and SD in parenthesis. MCh: methacholine; WBC: white blood cells; Eos: eosinophils; Macs: macrophages; PMNs: polymorphonuclear leucocytes; Lymphs: lymphocytes. *: median with interquartile range is shown as data are not normally distributed.

Cell count and differential. The mean BAL fluid cell count and differential with and without methacholine challenge is shown in table 2. The mean paired differences and standard deviations are also shown. There was no significant difference between the mean percentage return of BAL fluid with and without methacholine challenge (40.7 \pm 6.7 and 41.7 \pm 6.9 mL, respectively; p=0.88). There were also no significant differences in total cell count, percentages or absolute numbers of BAL fluid eosinophils, macrophages, neutrophils and lymphocytes with and without methacholine challenge.

Mast cell, eosinophil, macrophage and neutrophil prod-

Table 3. — Bronchoalveolar lavage levels of macrophage eosinophil and neutrophil products

Parameter	Without MCh	With MCh	Paired difference	p-value
Mast cells				
Histamine ng·mL ⁻¹	4.8 \pm 0.29	4.8 \pm 0.29	0.13 \pm 0.1	0.95
Eosinophils				
ECP μ g·mL ⁻¹	2.3 \pm 0.3	2.9 \pm 0.8	0.0* 0.0–0.0	1.00
CLC ng·mL ⁻¹	13.5 \pm 6.5	10.5 \pm 5.3	0.5* -2.5–6.4	0.50
Macrophages				
LTB ₄ pg· μ g ⁻¹ protein	4.0 \pm 1.6	8.5 \pm 5.7	2.8 \pm 2.1 (4.3)	0.30
TxB ₂ pg· μ g ⁻¹ protein	10.8 \pm 2.6	10.0 \pm 3.1	-0.6 \pm 3.4 (9.0)	0.90
Neutrophils				
Lactoferrin ng·mL ⁻¹	12.4 \pm 1.3	9.7 \pm 1.3	2.7 \pm 1.6 (5.2)	0.13
Lysozyme μ g·mL ⁻¹	0.05 \pm 0.04	0.03 \pm 0.02	0.00* 0.00–0.00	1.00

Values are presented as mean \pm SEM, and SD in parentheses. MCh: methacholine; ECP: eosinophil cationic protein; CLC: Charcot-Leyden crystal protein; LTB₄: leukotriene B₄; TxB₂: thromboxane B₂; *: median with interquartile range is shown where data are not normally distributed.

Table 4. — Bronchoalveolar lavage fluid lymphocyte analysis. % lymphocytes expressing the marker

Marker	Without MCh	With MCh	Paired difference	p-value
CD3	70.1 \pm 10.5	57.9 \pm 14.5	0.2* -21.8–59.4	0.7
CD4	48.8 \pm 5.1	50.2 \pm 4.9	18.6 \pm 14.2 (40.2)	0.2
CD8	37.9 \pm 4.9	35.4 \pm 4.4	2.5 \pm 2.4 (7.2)	0.3
CD3+/HLA-DR	10.8 \pm 2.4	13.3 \pm 3.9	-1.1 \pm 2.6 (7.3)	0.7
CD4+/HLA-DR	2.4 \pm 0.5	4.9 \pm 2.3	-0.4* -3.7–1.2	0.7
CD8+/HLA-DR	1.8 \pm 0.4	1.5 \pm 0.6	0.3 \pm 0.5 (1.6)	0.6
CD4/CD25	2.4 \pm 0.5	1.8 \pm 0.5	0.06 \pm 0.7 (2.2)	0.4
CD8/CD25	0.3 \pm 0.1	0.3 \pm 0.1	0.1* -0.1–0.2	0.4

Values are expressed as mean \pm SEM, and SD in parentheses. *: median and interquartile range is shown where the data are not normally distributed. MCh: methacholine challenge; HLA-DR: human leucocyte antigen-DR.

ucts. The BAL histamine, ECP, CLC, LTB₄ and TxB₂ levels were not significantly affected by methacholine challenge (table 3), the neutrophil degranulation products lactoferrin and lysozyme were not significantly affected by methacholine challenge (table 3).

Lymphocyte immunophenotypes. The percentage of lymphocytes expressing specific markers and mean paired differences are shown in table 4. Again, no significant differences were noted in BAL fluid CD3, CD4 and CD8. In addition, there were no significant differences in BAL fluid levels of activated T-cells (CD3/HLA-DR, CD4/HLA-DR, CD8/HLA-DR, CD3/CD25, CD4/CD25, CD8/CD25).

Discussion

This study demonstrated that in patients with asthma, no significant change in BAL fluid cell count and function were seen when bronchoscopy was performed 30 min after methacholine challenge testing. Thirty minutes was chosen as the time interval as this order of events is often incorporated into clinical protocols.

Our results are similar to those in the investigation by LAM *et al.* [17], in which methacholine challenge was performed immediately prior to bronchoscopy. They found no change in BAL fluid cell count, differential, histamine, leukotrienes (LTB₄, LTC₄, LTD₄, LTE₄), and prostaglandins (PGD₂, PGE₂ and TxB₂) when bronchoscopy with BAL was performed 10 min after methacholine challenge [17]. In contrast, they found increased BAL fluid PGE₂ and TxB₂ in asthmatic patients immediately after challenge with allergen, suggesting that a significant inflammatory response occurs with allergen but not methacholine.

When bronchoscopy is performed hours after metha-

choline challenge, changes in BAL fluid characteristics are seen. NOWAK *et al.* [4] performed bronchoscopy 5 h after methacholine challenge testing in subjects with and without asthma and found increased BAL fluid LTB₄ and PGD₂ only in those subjects with asthma. LTB₄ and its metabolites exhibited the largest increase (three fold). BEASLEY *et al.* [18] found increased numbers of nucleated cells in the BAL fluid of asthmatics as compared to controls 18 h after methacholine challenge but the percentages of individual cell types, such as lymphocytes, eosinophils, neutrophils and macrophages, were unchanged. In addition, there was an increase in tissue neutrophils from 0 to 2% by electron microscopy in endo-bronchial biopsies of asthmatics 18 h after methacholine challenge as compared to baseline. In contrast, SODERBERG *et al.* [3] did not observe any change in BAL fluid cell count and differential when performed 24 h after methacholine challenge. Thus, it appears that methacholine may cause mild inflammatory changes when performed several hours before bronchoscopy.

The mechanism by which methacholine can induce inflammatory changes over time in the airways is unclear. In addition to bronchial smooth muscle cells, endothelial [19], epithelial [20], neutrophils [21], lymphocytes [22], mast cells [23] monocytes and macrophages [24] have been reported to possess muscarinic acetylcholine receptors. Furthermore, the data available do not distinguish whether the mediator response in BAL fluid is a direct effect of methacholine or of bronchoconstriction itself. The study discussed above by NOWAK *et al.* [4], in which bronchoscopy was performed 5 h after methacholine challenge, did incorporate normal controls, but the maximum dose of methacholine these subjects received was 16 mg·mL⁻¹, which did not induce bronchoconstriction. The lack of mediator response in the normal group suggests that methacholine or bronchoconstriction *per se* does not induce inflammation. These results cannot be confirmed unless bronchoconstriction is induced to a similar level in normal subjects. As our study did not reveal any significant changes in BAL fluid mediators in our asthmatic group at doses clearly sufficient to cause bronchoconstriction, we did not feel it was necessary to study nonasthmatic subjects.

Although methacholine challenge does not cause significant changes in BAL fluid characteristics, allergen challenge results in dramatic changes in lavage fluid. SMITH *et al.* [25] noted increased lavage eosinophils and neutrophils 12 h after allergen challenge, with a significant correlation between the combined cell percentages and the severity of the late asthmatic response. DIAZ *et al.* [26] found that the increase in BAL fluid lymphocytes, neutrophils, eosinophils, eosinophil major basic protein and eosinophil derived neurotoxin was significantly greater 6 h after allergen challenge. BEASLEY *et al.* [18] found increased numbers of CD14 cells in the lavage fluid of asthmatics 18 h after allergen challenge, and this increase was not seen after methacholine challenge. Clearly, allergen challenge, with its associated immediate and late asthmatic responses, results in significant airway inflammation, in contrast to methacholine challenge.

Although methacholine may cause a mild inflamma-

tory response as measured in BAL fluid when performed several hours before bronchoscopy, it does not appear to cause any significant changes in cell count, differential and multiple indices of cell function when performed immediately before BAL. It must be emphasized that these results can be applied only to the particular cell products measured, which were chosen as they are often measured in BAL fluid. Thus, these results should be helpful when designing clinical research studies where bronchial hyperresponsiveness and airway inflammation are measured.

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