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Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate

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Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate. M. Duddridge, C. Ward, D.J. Hendrick, E.H. Walters. ©ERS Journals Ltd 1993.

ABSTRACT: Using serial bronchoalveolar lavage (BAL), we have studied changes in the airway inflammatory cell populations in 20 asthmatic patients, before and after treatment with inhaled beclomethasone dipropionate (BDP), 2,000 μg daily in an uncontrolled study.

There was a significant improvement in asthma severity, as measured by symptom score and airways responsiveness, and there were significant reductions in the total BAL eosinophil, epithelial cell and mast cell counts, with a significant increase in the percentage BAL lymphocyte count. No significant correlations were found between the changes in airway inflammatory cell numbers and the reduction in asthma severity. In contrast, the fall in ROS generation by the pulmonary macrophage and granulocyte populations was nonsignificant, but the improvement in airways responsiveness was positively correlated to the reduction in the unstimulated pulmonary macrophage activity.

Although these data are uncontrolled, the results are compatible with previous studies in suggesting an effect of steroids on the eosinophil, mast cell and epithelial cell in asthmatic airways. They also highlight the probable importance of the luminal lymphocyte population and pulmonary macrophage activation within the asthmatic airway, the beneficial modulatory effect of inhaled BDP treatment upon them, and the relative steroid-resistance of pulmonary inflammatory cell activity.
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Asthma, until recently, has principally been considered as an intermittent, paroxysmal bronchospastic disorder, which could be relieved by sympathomimetic agents. Over the last decade, clinical asthma has become increasingly accepted as a manifestation of an inflammatory disease of the airways [1–5], with a change in the emphasis on its treatment towards anti-inflammatory agents [6, 7].

An inflammatory infiltrate of eosinophils, mast cells and mononuclear cells has been reported in bronchial biopsies from patients both with mild and with asymptomatic asthma [8–12]. Increased numbers of inflammatory cells have also been found in bronchoalveolar lavage (BAL) fluid from mild stable asthmatics. Although the total cell count may be in the normal range [13–15], or elevated [16], an eosinophilia has been consistently reported [13–16] and related to the degree of nonspecific airways responsiveness [16, 17]. Neutrophilia [13] is not a common feature, although the percentage neutrophil count has been positively correlated with the degree of airways responsiveness [14], as has the percentage of mast cells [16–18], and epithelial cells [16]. A lymphocytosis has also been recognized [13, 14], but negatively correlated to airways responsiveness [14], with the CD8 ("suppressor") lymphocyte phenotype principally responsible for this relationship [19]. We have also previously reported both BAL neutrophil and pulmonary macrophage reactive oxygen species (ROS) generation to be increased in asthmatics, with the latter positively correlated to airways responsiveness [14].

Little is known of the effect of anti-inflammatory agents, in particular inhaled steroids, on the inflammation present in asthmatic airways. Ciliogenesis and the repair of epithelial damage have been demonstrated in the bronchial mucosa during inhaled steroid treatment [20, 21], and a decreased mucosal lymphocyte count has been noted [22]. More recently, oral prednisolone was shown to reduce the percentage of BAL mast cells and their release of histamine [23]. In the only previous study to have shown an effect of a putative inhaled anti-inflammatory agent on the BAL inflammatory cell population of asthmatic airways, cromolyn sodium was shown to reduce the eosinophil count over a four week period, as well as the immunoglobulin A (IgA)/albumin ratio [24].

We have investigated, by serial BAL, the inflammatory cell population of the airways in 20 volunteer asthmatic patients, clinically requiring inhaled steroid therapy as prophylactic treatment, in order to determine whether changes in the inflammatory cell population would accompany the improvement in nonspecific airways responsiveness that has been reported [25]. This was an uncontrolled study, at the constraint of our Ethics Committee, since it was considered unethical to have a placebo limb where the indication for entry to the study was the need for an increase in active treatment. Total and differential cell counts were performed, and ROS release from unstimulated and latex-stimulated aliquots of mixed BAL cells was measured. Lucigenin-amplified chemiluminescence (CL) was used as a marker for pulmonary macrophage activity, and luminol-amplified CL as a marker for granulocyte activity [26]. The changes both in cell numbers and in activity accompanying treatment were noted.

Methods

Twenty volunteer asthmatic patients were recruited to undergo serial BAL studies prior to and following 2–3 months of treatment with inhaled beclomethasone dipropionate (BDP) 1,000 μg twice daily. All subjects were assessed as requiring either the introduction of prophylactic inhaled steroid therapy or an increase in their inhaled steroid treatment at recruitment. Full informed written consent was obtained prior to each investigation, and the study was performed with the permission of the Newcastle Ethics Committee. Permission was obtained to perform two serial studies only, and the need for increased treatment was stipulated. The guidelines of the American Thoracic Society for BAL in asthmatic subjects were followed [27], such that forced expiratory volume in one second (FEV_1) was >1.5 *l* and >60% of predicted prior to the BAL procedure.

Patients

Subject details are shown in table 1. All subjects gave a history of variable bronchospasm and either a >15% diurnal variation in peak expiratory flow (PEF) or FEV_1 and/or a >15% increment in FEV_1 in response to inhaled salbutamol, 200 μg. None had a history of respiratory tract infection, nor acute exacerbation, nor had required oral steroid treatment, within the preceding 8 weeks. In five subjects, the second BAL study was delayed until at least 6 weeks after resolution of an acute upper respiratory tract infection, all being treated with oral antibiotics, and one with a 1 week course of oral prednisolone, 30 mg daily (6 weeks prior to BAL). A second subject was treated with oral prednisolone, 30 mg daily for 1 week (6 weeks prior to BAL), following an exacerbation of asthma in the workplace. All subjects were treated with inhaled salbutamol, 200 μg, as required for relief of bronchospasm, and no other treatment for asthma was being taken concurrently.

Protocol

Serial investigations were performed a median of 11 weeks (range 6–47 weeks) apart at the same time of day. All patients were reinstructed in inhaler technique, a spacer device used if necessary, and requested to rinse their mouth after BDP inhalation. During therapy, one patient reported weight gain of 1 kg, another a transient hoarse voice, and a third a sore throat without clinical evidence of oral candidiasis. Following BAL, subjects were observed overnight. Five episodes of aseptic febrile reactions with or without pleuritic pain were noted in three subjects, and were routinely treated with antipyretics, analgesics and oral antibiotics, and in two with a short course of oral prednisolone (in one subject after the second BAL study). Isolated pleuritic chest pain occurred in a further two subjects, and was treated with analgesics alone. All episodes resolved within 24 h.

Symptom score

We used the scoring system documented in table 2. Patient scores were assessed over the week prior to BAL by a physician without knowledge of previous findings.

Airways responsiveness

One to five days prior to BAL, baseline FEV_1 was measured (expressed as a percentage of that predicted for sex, age, and height) and an inhalation methacholine challenge test performed with the administration of doubling incremental doses of methacholine (from 3.125 μg to a maximum of 6.4 mg) every 5 min, using a locally designed microprocessor controlled dosimeter [28]. The mean of the best three of six satisfactory FEV_1 manoeuvres was recorded after each dose, and the provocative dose of methacholine producing a 20% fall in FEV_1 ($PD_{20}FEV_1$) was obtained by interpolation. All tests were performed between 11.00 and 15.00 h, and inhaled salbutamol was withheld for a minimum of 6 h before each challenge test.

Bronchoalveolar lavage

Fibreoptic bronchoscopy (Olympus BF P10, Keymed, UK) was undertaken with subjects receiving supplemental oxygen at 4 $l \cdot min^{-1}$ and being cardiographically monitored, following premedication with inhaled salbutamol 200 μg, and atropine 0.3–0.6 mg and Diazemuls (Kalibrium, UK) 12.5–30 mg given intravenously. Local anaesthesia with lignocaine was achieved as described previously [29]. BAL was performed with the bronchoscope wedged in a segmental or subsegmental bronchus of the middle lobe (36 procedures in the medial segment), using three 60 ml aliquots of isotonic phosphate-buffered saline (pH 7.4) prewarmed to 37°C. Each aliquot was immediately aspirated, under 60–80 mmHg negative pressure, into siliconized glassware on ice. Following collection, the BAL aspirate was transported to the laboratory within 30 min for analysis.

Table 1. – Patient details

Pt no.	Age yrs	Sex	Smoking status	Atopic status	FVC % pred	TLCO % pred	Study period wks
1	21	F	NS	SPT, IgE, Hx	-	-	10
2	20	M	NS	Non-atopic	121	116	16
3	52	F	ES 6 months 10·day^{-1}, 4 yrs	Non-atopic	76	97	11
4	29	M	ES 6 weeks 20·day^{-1}, 19 yrs	SPT, IgE, Hx	107	106	11
5	28	F	ES 24 months 10·day^{-1}, 11 yrs	SPT, IgE, Hx	103	103	9
6	36	F	ES 10 yrs 20·day^{-1}, 10 yrs	SPT, IgE, Hx	106	102	9
7	26	M	NS	SPT, IgE, Hx	101	127	23
8*	37	F	NS	Non-atopic	131	119	12
9*	45	F	S 10+·day^{-1} 30 yrs	SPT, IgE, Hx	104	90	14
10	29	M	NS	SPT, IgE, Hx	72	105	11
11*	36	M	S 10·day^{-1} 20 yrs	SPT, Hx	104	122	9
12	27	M	ES 2 months, 2·day^{-1}	SPT, IgE, Hx	102	90	9
13	52	F	S 5·day^{-1} 30 yrs	SPT	106	94	8
14	48	F	NS	SPT	107	135	9
15*	48	M	ES 2 months, 2·day^{-1}	SPT, IgE, Hx	61	101	14
16	34	M	ES 2 months 20·day^{-1}, 18 yrs	Non-atopic	107	99	28
17	40	M	ES 5 yrs 20·day^{-1}, 20 yrs	SPT	105	111	10
18	30	F	NS	SPT, IgE, Hx	104	122	7
19	37	F	ES 2 months, 5·day^{-1}	SPT, IgE, Hx	120	102	6
20	27	F	NS	SPT, Hx	118	88	47

NS: nonsmoker; ES: ex-smoker period of cessation, smoking history; S: current smoker, smoking history; SPT: +ve skin prick tests to a variety of common aeroallergens; IgE: elevated serum total immunoglobulin E or radioallergosorbent test (RAST) to house dust mite; Hx: clinical history of atopic eczema, rhinitis or asthma; % pred: % predicted at recruitment; FVC: forced vital capacity; TLCO: transfer capacity of the lung for carbon monoxide, single breath carbon monoxide method; *: subjects treated with low dose inhaled beclomethasone dipropionate (BDP,) <200 μg daily for >6 months at recruitment.

Table 2. – Symptom score

Asymptomatic	0
Occasional (<daily) mild symptoms with exertion/allergen exposure	1
Daily symptoms	2
Early morning awakening with symptoms (±above)	3
Regular nocturnal symptoms (±above)	4

Cell counts

A total cell count was performed using a haemocytometer and viability assessed by acridine orange/ethidium bromide staining [30]. Differential cell counts (Wright Giemsa) were made on cytospin slides of unfiltered BAL aspirate (Shandon Cytospin II, Shandon Southern Instruments, UK). Nonspecific esterase staining was used to confirm the pulmonary macrophage and lymphocyte percentage counts. Cytospin slides were also stained for mast cells: following a 30 min fixation in Carnoy's fluid, slides were stained in Alcian blue for 16 h and counterstained with 1% neutral red. Five thousand cells were enumerated by each observer, and the number of positive mast cells counted.

In view of our previous finding that small volume BAL aspirates were unrepresentative [31], it was prospectively decided to exclude subjects from analysis of the changes in BAL parameters if the total BAL aspirate volume was lower than 40 ml. This occurred in a single subject.

Cellular reactive oxygen species release

BAL aspirate was filtered [32], and the cells harvested (200 × g, 10 min) and resuspended in cell medium 199, without phenol red or glutamine (Gibco, UK), at 10^6·ml^{-1}. Further cytospin slides of these filtered cell suspensions were prepared and a differential cell count performed for the later expression of the ROS release per 10^3 cells of interest. Aliquots of 3.0×10^5 mixed BAL cells were incubated with either 400 μl 10^{-4} M luminol or lucigenin (Sigma Chemicals, UK) (to assess granulocyte and pulmonary macrophage ROS release, respectively [26]) at 37°C in a final volume of 1 ml, and the peak unstimulated CL (cps) measured in a luminometer (Lumac M2010, Sonco, UK). Maximally stimulated CL was similarly measured for aliquots of 1.5×10^5 mixed BAL cells,

with 100 μl 5% latex particles (1.09 μm diameter, Sigma Chemicals, UK) as the stimulus. The mean of duplicate measurements for both CL amplifiers was expressed as either the total CL per aliquot of mixed BAL cells, the $CL \cdot 10^{-3}$ pulmonary macrophages or granulocytes for lucigenin and luminol respectively, or the pulmonary macrophage or granulocyte CL per ml of BAL aspirate ($CL \cdot 10^{-3}$ cells × number of cells per ml of BAL aspirate $\times 10^{-3}$).

Statistical analysis

The total cell count, pulmonary macrophage and lymphocyte counts (per ml of BAL aspirate) and unstimulated and stimulated CL measurements were $\log_{10}$ normally distributed, and the geometric means and 95% confidence interval (95% CI) are given. Percentage differential cell counts, polymorph neutrophil, eosinophil, epithelial cell and mast cell counts were square-root transformed [33] in order to normalize the data, and the squared means and 95% CI are given. Geometric means and 95% CI are also given for the $PD_{20}FEV_1$ methacholine measurements. Paired t-tests were used to assess the changes in cell numbers and activity. One-tailed tests were used to test the hypotheses that decreases in the number of eosinophils, neutrophils, epithelial cells and mast cells, but an increase in lymphocyte numbers, and a fall in cellular ROS release would be found accompanying inhaled BDP treatment, as discussed in the introduction.

Linear regression was used to determine the relationships between changes in symptom score, baseline FEV_1 and $PD_{20}FEV_1$, and between changes in these parameters and changes in BAL findings. The effect of atopic status, smoking status and duration of treatment was examined using multiple regression [34], as were the relationships between changes in ROS release and changes in cell numbers per ml of BAL aspirate (stepwise procedure). All r^2 (or R^2) values quoted are the adjusted values to take into account chance contributions of each predictor variable included in the regression.

Results

Clinical parameters

Both the symptom score and $PD_{20}FEV_1$ methacholine significantly improved following treatment as shown in table 3 and figure 1. In four subjects an interpolated $PD_{20}FEV_1$ was not attained after treatment with BDP 2,000 μg daily. In these cases, the extrapolated $PD_{20}FEV_1$ was greater than 12.8 mg methacholine, and this figure was used for expression of the results. The mean 6% increase in baseline FEV_1 approached statistical significant (p=0.055).

The mean volume of BAL aspirate recovered at each study was not significantly different, being 90 ml (95% CI 78–102 ml) and 94 ml (82–106 ml) at the first and second BAL respectively. Mean cell viability was 94% (95% CI 93–96%), with no difference between the two studies; the lowest viability was 90%.

Cell percentages

Table 4 shows the changes in the percentage differential cell counts accompanying treatment with high dose inhaled BDP. There were significant falls in the percentage of pulmonary macrophages and eosinophils, with a significant increase in the percentage of lymphocytes, which was higher in the non-current smokers (mean 4.8%, 95% CI 1.6–8.0%).

Cell numbers

The changes in the actual cell numbers per ml of BAL aspirate are shown in table 5. There were significant falls in the number of eosinophils, epithelial cells and mast cells. The increase in lymphocyte numbers did not attain significance in the whole group. Because of the known effect of smoking on BAL cell numbers, the changes in the non-current smokers were also analysed separately.

Table 3. – Clinical parameters before and following treatment with inhaled high dose beclomethasone dipropionate, 2,000 μg daily

		Pre high dose inhaled BDP	Post high dose inhaled BDP	Change with high dose# inhaled BDP
Symptom score	mean	3.1	0.9	2.3**
	(95% CI)	(2.6–3.6)	(0.4–1.3)	(1.8–2.9)
Baseline FEV_1+ % pred	mean	88	94	6%†
	(95% CI)	(77–100)	(84–104)	(-1–13)
$PD_{20}FEV_1$ methacholine	μg			
	geometric mean	132	254	1.92 fold increase*
	(95% CI)	(53–330)	(79–816)	(1.04–3.61 fold increase)

+: no inhaled salbutamol in previous 6 h, % predicted for age, height, sex; #: mean and 95% CI of change for each individual; *: p<0.05; **: p<0.0001; †: p=0.055; BDP: beclomethasone dipropionate; 95% CI: confidence interval; FEV_1: forced expiratory volume in one second; $PD_{20}FEV_1$: provocative dose of methacholine producing a 20% fall in FEV_1.

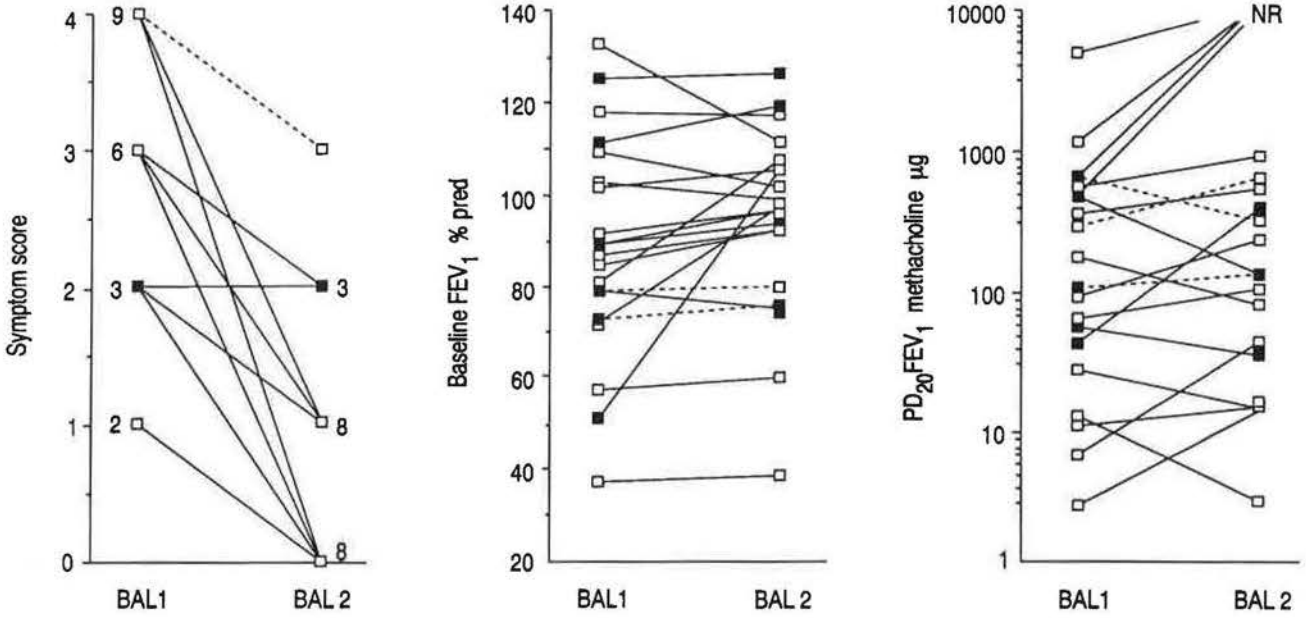

Fig. 1. – Symptom score (see table 2), baseline FEV_1 (% of predicted) and $PD_{20}FEV_1$ methacholine (µg) before (BAL 1) and after (BAL 2) a median 11 weeks (range 7–47 weeks) treatment with inhaled high dose BDP, 2,000 µg daily, in 20 asthmatic patients. Numbers beside symbols for symptom score analysis represent number of subjects. □: atopic patients; ■: non atopic patients; - - - - - -: current smokers; ———: noncurrent smokers. FEV_1: forced expiratory volume in one second; $PD_{20}FEV_1$: provocative dose of methacholine producing a 20% fall in FEV_1; NR: non responsive; BAL: bronchoalveolar lavage; BDP: beclomethasone dipropionate.

Significant falls in the number of eosinophils (mean $0.7\times10^3\cdot ml^{-1}$, 95% CI 0.0–1.3, $p<0.05$) and mast cells (mean $0.14\times10^3\cdot ml^{-1}$, 95% CI 0.01–0.27, $p<0.05$) were still found, although the fall in the number of epithelial cells was no longer significant. However, there was a significant increase in the number of lymphocytes (mean $7.8\times10^3\cdot ml^{-1}$, 95% CI -1.6–17.2, $p<0.05$) in the noncurrent smokers.

Reactive oxygen species generation

Although there was a trend towards a decrease in cell activity, there were no significant falls in unstimulated ROS release as measured by luminol or lucigenin CL, however it was expressed. Table 6 shows the findings for $CL\cdot10^{-3}$ granulocytes (luminol) or pulmonary macrophages (lucigenin). The only significant fall in stimulated ROS release was for granulocyte CL per ml of BAL aspirate, with a mean 1.78 fold decrease (95% CI 0.91–3.51, $p<0.05$).

Relationship between clinical parameters

A weak, but significant, relationship was found between the fall in symptom score and increase in baseline FEV_1 ($r^2=0.219$, $p<0.05$), and between the fall in symptom score and improvement in $PD_{20}FEV_1$ ($r^2=0.274$, $p<0.05$) (data not shown). No relationship was found between the increase in baseline FEV_1 and improvement in $PD_{20}FEV_1$, nor was an effect of atopic or smoking status, or treatment duration, shown on these relationships.

Relationship between clinical parameters and cell populations

No relationship was found between the changes in the cellular profiles and changes in symptom score, baseline FEV_1 or $PD_{20}FEV_1$, nor was there an effect of atopic status, smoking status or treatment duration. However, there was a significant relationship between the improvement in airways responsiveness and the fall in the unstimulated pulmonary macrophage activity per aliquot of mixed BAL cells ($r^2=0.203$, $p<0.05$). There were also significant negative relationships between the fall in symptom score and fall in stimulated granulocyte CL per ml of BAL aspirate ($r^2=-0.250$, $p<0.05$), and between the increase in baseline FEV_1 and fall in stimulated granulocyte CL per ml of BAL aspirate ($r^2=-0.225$, $p<0.05$): *i.e.* the smaller the fall in granulocyte activity the greater the improvement in symptom score or baseline FEV_1.

Relationship between changes in cells and ROS release

The fall in unstimulated granulocyte activity per aliquot of mixed BAL cells was significantly related to the fall in the mast cell count ($r^2=0.248$, $p<0.05$). The fall in stimulated $CL\cdot10^{-3}$ pulmonary macrophages was significantly related to the fall in the eosinophil count ($r^2=0.307$, $p<0.01$). The fall in stimulated granulocyte CL per ml of BAL aspirate was significantly related to the fall in the epithelial cell count ($r^2=0.211$, $p<0.05$).

Table 4. – Differential percentage cell counts before and following treatment with inhaled high dose beclomethasone dipropionate, 2,000 μg daily

	Pulmonary macrophage %	Lymphocyte %	Polymorph neutrophil %	Eosinophil %	Epithelial cell %	Mast cell %
Pre high dose# inhaled BDP	85.5 (82.3–88.7)	9.7 (7.9–11.6)	0.9 (0.7–1.3)	0.6 (0.3–1.1)	1.9 (1.3–2.6)	0.08 (0.02–0.16)
Post high dose# inhaled BDP	81.3 (76.1–86.8)	12.5 (8.7–17.1)	1.5 (0.9–2.3)	0.3 (0.2–0.6)	1.4 (0.8–2.2)	0.05 (0.02–0.11)
Decrease % with high dose## inhaled BDP	4.3* (0.7–7.9)	-4.1** (-7.1 to -1.1)	-0.8 (-1.8–0.1)	0.4* (0.0–0.8)	0.4 (-0.5–1.3)	0.05 (-0.02–0.13)

#: squared mean (95% CI) of square root transformed data; ##: mean (95% CI) of decreased percentage cell count for each individual; *: $p<0.05$ for fall in cell counts; **: $p<0.005$ for increase in percentage lymphocytes. Note: a negative decrease in % cell counts is an increase. BDP: beclomethasone dipropionate.

Table 5. – Cell numbers ($\times 10^{-3}$) per ml of BAL aspirate, before and following treatment with inhaled high dose beclomethasone dipropionate, 2,000 μg daily

	Total[1] cell count	Pulmonary[1] macrophage	Lymphocyte[1]	Polymorph[2] neutrophil	Eosinophil[2]	Epithelial[2] cell	Mast[2] cell
Pre high dose[a] inhaled BDP	187 (137–256)	160 (114–225)	16.7 (13.4–20.8)	1.8 (1.2–2.4)	1.1 (0.6–1.8)	3.5 (2.4–4.8)	0.13 (0.04–0.26)
Post high dose[a] inhaled BDP	163 (117–227)	132 (92–190)	17.8 (11.5–27.4)	2.3 (1.5–3.4)	0.5 (0.2–0.9)	2.1 (1.3–3.2)	0.07 (0.03–0.13)
Decrease in cell numbers with[b] high dose BDP	20 (-33–72)	28 (-24–80)	-8.6 (-20.2–2.9)	-0.9 (-2.0–0.2)	0.6* (0.0–1.2)	1.4* (0.1–2.7)	0.12* (0.01–0.23)

a1: geometric mean (95% CI) cell counts $\times 10^{-3} \cdot ml^{-1}$ BAL aspirate; a2: squared mean (95% CI) square root transformed cell counts $\times 10^{-3} \cdot ml^{-1}$ BAL aspirate; b: mean (95% CI) decreases in cell numbers $\times 10^{-3} \cdot ml^{-1}$ BAL aspirate; *: $p<0.05$ for decreases in cell numbers; BAL: bronchoalveolar lavage; BDP: beclomethasone dipropionate. Note: a negative decrease in cell numbers is an increase.

Table 6. – Cellular reactive oxygen species release ($cpm \cdot 10^{-3}$ cells) before and following treatment with inhaled high dose beclomethasone 2,000 μg daily

	Pre high dose[a] inhaled BDP	Post high dose[a] inhaled BDP	Decrease with high dose[b] inhaled BDP
Unstimulated luminol amplified CL per 10^3 granulocytes	305 (135–689)	231 (102–521)	1.35 fold decrease* (0.76–2.38 fold decrease)
Stimulated luminol-amplified CL per 10^3 granulocytes	3,240 (2060–5100)	2,580 (1530–4330)	1.21 fold decrease** (0.75–1.96 fold decrease)
Unstimulated lucigenin-amplified CL per 10^3 pulmonary macrophages	22.3 (11.0–45.3)	17.2 (10.3–28.8)	1.18 fold decrease† (0.68–2.06 fold decrease)
Stimulated lucigenin-amplified CL per 10^3 pulmonary macrophages	554 (368–834)	495 (318–770)	1.10 fold decrease†† (0.65–1.84 fold decrease)

a: geometric means (95% CI); b: mean (95% CI) of difference in log transformed data; *: $p=0.14$; **: $p=0.21$; †: $p=0.26$; ††: $p=0.36$; CL: chemiluminescence; BDP: beclomethasone dipropionate.

Unstimulated and stimulated pulmonary macrophage CL per ml of BAL aspirate were both significantly associated with changes in cell counts (R^2=0.688, p<0.001 and R^2=0.579, p<0.005, respectively). The fall in unstimulated pulmonary macrophage CL per ml of BAL aspirate was related both to the fall in the epithelial cell count (t-ratio=4.55, p<0.001), and negatively to the increase in lymphocyte cell count (t-ratio =-4.50, p<0.001). Similarly, the fall in stimulated pulmonary macrophage CL per ml of BAL aspirate was related both to the fall in the eosinophil cell count (t-ratio=3.86, p<0.005) and to the fall in the pulmonary macrophage cell count (t-ratio=2.46, p<0.05) and negatively to the increase in the lymphocyte cell count (t-ratio=-2.72, p<0.05): *i.e.* the smaller the increase in the lymphocyte cell count the larger the fall in cell ROS release.

Discussion

In this uncontrolled study, successful treatment of asthmatic patients with high dose inhaled BDP (in terms of improvement in symptom score and airways responsiveness) was accompanied by a significant fall in the number of BAL eosinophils, epithelial cells and mast cells, with a significant increase in the percentage of lymphocytes. The study was uncontrolled due to the constraint of our Ethics Committee, which felt that giving placebo instead of effective inhaled steroid was inappropriate in a symptomatic patient population. We cannot, therefore, say that the effects seen were necessarily caused by high dose inhaled BDP, although this is likely. However, our findings are compatible with earlier cross-sectional studies in asthma [13–19], and suggest that the BAL changes were related, either primarily or secondarily, to the improvement in airways responsiveness. It is to these changes in airways responsiveness that our BAL findings have been related, whether the changes in airways responsiveness were just fortuitous, rather than (probably) being a therapeutic effect of the inhaled BDP.

The improvement in airways responsiveness, an approximate doubling in $PD_{20}FEV_1$, is comparable to that previously described over similar periods using similar doses of inhaled steroid [25, 35, 36]. The improvement was reported to have reached a plateau by 2–3 months [25, 36], suggesting that our duration of treatment was likely to have been optimal for examination of the airway cellular population. Recent biopsy studies [37, 38], have found similar changes in mucosal and submucosal inflammatory cells (eosinophils and mast cells) to those found in this BAL study, with shorter treatment terms.

Previous BAL studies have shown a decrease in the percentage of mast cells in the asthmatic airway after oral corticosteroids [23], whereas a fall in the percentage eosinophil count has been described following oral steroids in the Basenji-greyhound dog model of asthma [39], and inhaled disodium cromoglycate in man [24]. Recently, ADELROTH *et al.* [40] found a fall in BAL levels of eosinophilic cationic protein, without changes in the eosinophil population of the airways, following inhaled steroid therapy. Some eosinophil functions (*i.e.* the release of cationic protein) may, therefore, be affected by inhaled steroids before a reduction in their chemotaxis into the asthmatic airway, although this was not true of granulocyte ROS release in this present study.

The present description of a fall in the number of eosinophils, epithelial cells and mast cells in BAL, with improvement in airways responsiveness, is consistent with previous findings of positive correlations between the severity of airways responsiveness and the percentage or number of eosinophils [16, 17], epithelial cells [11, 16] and mast cells [16–18] in BAL aspirate from asthmatic patients. If these relationships were functionally relevant, then with improvement in airways responsiveness on inhaled BDP treatment, the observed reductions in the airway inflammatory cell population might have been expected.

The novel finding of an increase in the percentage of BAL lymphocytes found to accompany the improvement in airways responsiveness can be explained by the negative correlation previously described between the BAL lymphocyte count, in particular the CD8+ count, and the severity of airways responsiveness [14, 19]. In allergen challenge models, a lymphocytosis has also been described in late asthmatic reactors, principally accounted for by CD4+ cells [41, 42]. In contrast, in subjects with only an early phase reaction, GONZALEZ *et al.* [42] found both an increase in the percentage and number of CD8+ lymphocytes associated with a fall in the percentage of CD4+ lymphocytes. They suggested that the airway luminal CD8+ lymphocytes may be modulating the asthmatic reaction, with which our earlier observation would concur [19]. The decrease in bronchial biopsy submucosal T-lymphocytes described by DJUKANOVIC *et al.* [37] is not necessarily in disagreement with our own findings, as the luminal and mucosal lymphocyte populations may be separately regulated [43]. Our current data would further support the potential functional importance of the lymphocyte, or subset of lymphocytes, in modulating asthma severity. An interaction with macrophages is suggested by the association between the fall in pulmonary macrophage ROS generation and a fall in the lymphocyte count.

The absence of a significant fall in cellular ROS release, apart from the fall in stimulated granulocyte CL per ml of BAL aspirate (which could be accounted for by the fall in eosinophil numbers), was unexpected, in view of the positive correlation previously described between stimulated pulmonary macrophage ROS release and airways responsiveness [14]. Furthermore, CALHOUN and BUSH [44] have recently shown an increase in both spontaneous and stimulated ROS release by BAL cells, following allergen challenge. Neither premedication with salbutamol (unpublished observation) nor the amount of lignocaine used [29] should have influenced cellular ROS release *ex vivo*. Our assay would appear suitable for demonstrating potential therapeutic effects, since we have demonstrated significant falls in cellular ROS release in sarcoidosis following treatment with oral prednisolone [45], and have shown *in vitro* inhibition of ROS release from normal BAL cells by BDP (threshold 10^{-6} M). The

failure to detect an improvement may, therefore, be a consequence of the steroid dose delivered to the airway and/or a difference in steroid sensitivity between the effects on cellular ROS generation and airway cell numbers. The possibility that there may be steroid-modulable and relative steroid-resistant aspects to the pathophysiology of asthma is intriguing and warrants further investigation, as it may have therapeutic implications.

However, the fact that the degree of improvement in airways responsiveness found in this study was positively correlated to the fall in the unstimulated pulmonary macrophage activity (per aliquot of mixed BAL cells) provides further evidence for the potential role of the pulmonary macrophage in regulating airways responsiveness. This suggests a role for inhaled steroids in modulating pulmonary macrophage activity, whether directly or indirectly. That the falls in different facets of pulmonary macrophage activity were related to the reduction in BAL eosinophils, epithelial cells and lymphocytes (and the reduction in granulocyte activity were related to falls in BAL mast cells and epithelial cells) suggests a complex network of cellular interactions in the asthmatic airway.

In conclusion, although these data are uncontrolled for the use of inhaled BDP, the results are compatible with previous cross-sectional BAL studies in asthma, and recent longitudinal biopsy studies, and suggest an effect of steroids on the eosinophil, mast cell and epithelial cell in asthmatic airways. Clearly, BAL offers a complimentary insight into the milieu of the asthmatic airways, as shown by our novel findings highlighting the potential importance of the luminal lymphocyte population and pulmonary macrophage activity, their relationship to airways responsiveness, and the modulatory effect of inhaled steroid treatment upon them, as well as the relative steroid-resistance of pulmonary inflammatory cell activity, compared to changes in cell numbers.

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References

1. Nadel JA. – Inflammation and asthma. *J Allergy Clin Immunol* 1984; 73: 651–653.
2. Chung KF. – Role of inflammation in the hyperreactivity of the airways in asthma. *Thorax* 1986; 41: 657–661.
3. Barnes PJ. – The changing face of asthma. *Q J Med* 1987; 63: 359–365.
4. Holgate ST, Finnerty JP. – Recent advances in understanding the pathogenesis of asthma and its clinical implications. *Q J Med* 1988; 66: 5–19.
5. Reed CE. – Basic mechanisms of asthma. Role of inflammation. *Chest* 1988; 94: 175–177.
6. Sheffer AL (Ed). – International consensus report on diagnosis and management of asthma. *Eur Respir J* 1992; 5: 601–641.
7. Duddridge M, Walters EH. – The prophylactic anti-inflammatory treatment of asthma: its variation throughout the world. *In*: O'Byrne PM, ed. Asthma as an inflammatory disease. New York, Dekker, 1990, pp. 283–312.
8. Dunhill M. – Pulmonary pathology. Edinburgh, Churchill Livingston, 1987, pp. 61–79.
9. Cutz E, Levison H, Cooper DM. – Ultrastructure in airways in children with asthma. *Histopathology* 1978; 2: 407–411.
10. Lozewicz S, Gomez E, Ferguson H, Davies RJ. – Inflammatory cells in the airways in mild asthma. *Br Med J* 1988; 297: 1515–1516.
11. Beasley R, Roche WR, Roberts JA, Holgate ST. – Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989; 139: 806–817.
12. Jeffrey PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. – Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 1989; 140: 1745–1753.
13. Godard P, Aubas P, Calvayrac P, Taib J, Michel FB. – Endoscopie et lavage bronchiolo-alveolaire chez l'asthmatique allergique. *Nouvelle Presse Med* 1981; 10: 3141–3148.
14. Kelly CA, Ward C, Stenton SC, *et al.* – Number and activity of inflammatory cells in bronchoalveolar lavage fluid in asthma and their relation to airway responsiveness. *Thorax* 1988; 43: 684–692.
15. Chan-Yeung M, LeRiche J, Maclean L, Lam S. – Comparison of cellular and protein changes in bronchial lavage fluid of symptomatic and asymptomatic patients with red cedar asthma on follow-up examination. *Clin Allergy* 1988; 18: 359–365.
16. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. – Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137: 62–69.
17. Kirby JG, Hargreave FB, Gleich G, O'Byrne PM. – Bronchoalveolar lavage cell profiles of asthmatic and non-asthmatic subjects. *Am Rev Respir Dis* 1987; 136: 379–383.
18. Flint KC, Leung KBP, Hudspith BN, *et al.* – Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *Br Med J* 1985; 291: 923–926.
19. Kelly CA, Stenton SC, Ward C, *et al.* – Lymphocyte subsets in bronchoalveolar lavage fluid obtained from stable asthmatics, and their correlations with bronchial responsiveness. *Clin Exp Allergy* 1989; 19: 169–175.
20. Heino M, Karjalainen J, Ylikoski J, Laitinen A, Laitinen LA. – Bronchial ciliogenesis and oral steroid treatment in patients with asthma. *Br J Dis Chest* 1988; 82: 175–178.
21. Lundgren R, Soderberg M, Horstedt P, Stenling R. – Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J* 1988; 1: 883–889.
22. Thiringer G, Eriksson N, Malmberg R, Svedmyr N, Zettergren L. – Bronchoscopic biopsies of bronchial mucosa before and after beclomethasone dipropionate therapy. *Scand J Respir Dis* 1977; (Suppl. 101): 173–177.
23. Millar AB, Hudspith BN, Lau A, Pearce F, Johnson NMcI. – A mechanism for the role of steroids in treatment of asthma? *Thorax* 1989; 44: 359P (Abstract).
24. Diaz P, Galleguillos FR, Gonzalez MC, Pantin CFA, Kay AB. – Bronchoalveolar lavage in asthma: the effect of disodium cromoglycate (cromolyn) on leukocyte counts, immunoglobulins, and complement. *J Allergy Clin Immunol* 1984; 74: 41–48.
25. Kerrebijn KF, van Essen-Zandvliet EEM, Neijens HJ. – Effect of long-term treatment with inhaled corticosteroids and beta-agonists on the bronchial responsiveness in children with asthma. *J Allergy Clin Immunol* 1987; 79: 653–659.
26. Ward C, Kelly CA, Stenton SC, *et al.* – The relative contribution of bronchoalveolar macrophages and neutrophils to

lucigenin- and luminol-amplified chemiluminescence. *Eur Respir J* 1990; 3: 1008–1014.
27. NHLBI workshop summary. – Summary and recommendations of a workshop on the investigative use of fibreoptic bronchoscopy and bronchoalveolar lavage in asthmatics. *Am Rev Respir Dis* 1985; 132: 180–182.
28. Connelly MJ, Avery AJ, Walters EH, Hendrick DJ. – The use of sequential doses of inhaled histamine in the measurement of bronchial responsiveness: cumulative effect and distortion produced by shortening the test protocol. *J Allergy Clin Immunol* 1988; 82: 863–868.
29. Duddridge M, Kelly CA, Ward C, Hendrick DJ, Walters EH. – The reversible effect of lignocaine on the stimulated metabolic activity of bronchoalveolar lavage cells. *Eur Respir J* 1990; 3: 1166–1172.
30. Parks DR, Byran VM, Oi VT, Herzenberg LA. – Antigen specific identification and cloning of hybridomas with a fluorescence activated cell sorter. *Proc Natl Acad Sci* 1979; 76: 1962–1966.
31. Duddridge M, Kelly CA, Ward C, Hendrick DJ, Walters EH. – Small volume bronchoalveolar lavage is not optimal for sampling airway inflammatory cells in asthma. *Am Rev Respir Dis* 1989; 139: A459 (Abstract).
32. Kelly CA, Ward C, Bird G, Hendrick D, Walters H. – The effect of filtration on absolute and differential cell counts in fluid obtained at bronchoalveolar lavage. *Respir Med* 1989; 83: 107–110.
33. Armitage P, Berry G. – Statistical methods in medical research. Oxford, Blackwell Scientific Publications, 1987; pp. 358–370.
34. Armitage P, Berry G. – Statistical methods in medical research. Oxford, Blackwell Scientific Publications, 1987; pp. 296–357.
35. Kraan J, Koeter GH, van der Mark ThW, *et al.* – Dosage and time effects of inhaled budesonide on bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137: 44–48.
36. Haahtela T, Jarvinen M, Kava T, *et al.* – Comparison of a β_2-agonist, terbutaline, with an inhaled corticosteroid, budesonide, in newly detected asthma. *N Eng J Med* 1991; 325: 388–392.
37. Djukanovic R, Wilson JW, Britten KM, *et al.* – Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992; 145: 669–674.
38. Jeffery PK, Godfrey RW, Adelroth E, *et al.* – Effects of treatment on airway inflammation and thickening of basement reticular collagen in asthma. A Quantitative light and electron microscopic study. *Am Rev Respir Dis* 1992; 145: 890–899.
39. Darowski MJ, Hannon VM, Hirshman CA. – Corticosteroids decrease airway hyperresponsiveness in the Basenji greyhound dog model of asthma. *J Appl Physiol* 1989; 66: 1120–1126.
40. Adelroth E, Rosenhall L, Johansson SA, Linden M, Venge P. – Inflammatory cells and eosinophil activity in asthmatics investigated by bronchoalveolar lavage. The effects of anti-asthma treatment with budesonide or terbutaline. *Am Rev Respir Dis* 1990; 142: 91–99.
41. Metzger WJ, Zavala D, Richerson HB, *et al.* – Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. Description of the model and local airway inflammation. *Am Rev Respir Dis* 1987; 135: 433–440.
42. Gonzalez MC, Diaz P, Galleguillos FR, *et al.* – Allergen-induced recruitment of bronchoalveolar helper (OKT4) and suppressor (OKT8) T-cells in asthma. Relative increases in OKT8 cells in single early responders compared with those in late-phase responders. *Am Rev Respir Dis* 1987; 136: 660–664.
43. Djukanovic R, Feather I, Gratziou C, *et al.* – Effects of seasonal allergen exposure on airways inflammation and symptoms (Abstract). *Clin Exp Allergy* 1993; 23 (Suppl. 1): 79.
44. Calhoun WJ, Bush RK. – Enhanced reactive oxygen species metabolism of airspace cells and airway inflammation follow antigen challenge in human asthma. *J Allergy Clin Immunol* 1990; 86: 306–313.
45. Duddridge M, Ward C, Hendrick DJ, Walters EH. – Response to oral corticosteroid treatment in sarcoidosis as assessed by serial bronchoalveolar lavage. *Thorax* 1990; 45: 329P (Abstract).