

## Intrasubject variability in airway inflammation sampled by bronchoalveolar lavage in stable asthmatics

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*Intrasubject variability in airway inflammation sampled by bronchoalveolar lavage in stable asthmatics. C. Ward, P.V. Gardiner, H. Booth, E.H. Walters. ©ERS Journals Ltd 1995.*

**ABSTRACT:** Despite the increasing complexity of bronchoalveolar lavage (BAL) studies in asthmatics there are few published data concerning the variability of inflammatory parameters measured using this technique.

We studied the intrasubject variability of cellular and solute parameters in 20 clinically stable, symptomatic, mild-to-moderate asthmatics, in repeat 180 mL BAL procedures performed 1 month apart. During the study, there was no change in disease activity or medication.

Mean (sd) forced expiratory volume in one second (FEV<sub>1</sub>) was 3.2 (1.09) L at the first BAL and 3.05 (0.98) L at the second. The geometric mean dose of methacholine provoking a 20% decrease in FEV<sub>1</sub> (PD<sub>20</sub>) was 23 µg (range 2–1,170 µg) at the first BAL and 28 µg (range 2–440 µg) at the second. There was considerable variability in the BAL cellular and solute parameters measured over the two procedures. Estimates of power calculated for subsequent studies involving this type of subject group were made from the observed variability.

Sample sizes of less than 15 mean that differences have to be large in order to be detected in repeat BAL samples. However, there is little improvement in the power of BAL studies for sample sizes greater than 20, indicating that there is little gain in recruiting more than this number of subjects.

Thus, our study indicates that although studies of the pathophysiology underlying asthma using BAL require considerable commitment, they are practicable.

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The technique of bronchoalveolar lavage (BAL) is widely used as a research tool and has proved useful in studying the airway inflammation widely thought to underlie the pathophysiology of asthma [1]. Increased numbers of inflammatory cells in asthmatics as compared to normals, have been consistently documented [2] and, more recently, studies involving multiple BAL procedures have aimed to assess the effects of pharmacological intervention on inflammation [3–6].

However, despite the increasing complexity of such BAL studies, there are very few published data relating to the intrasubject variability of even the most basic BAL inflammatory parameters. Without these, there are difficulties in interpreting the results obtained with repeat BAL studies of therapeutic intervention or even in planning such studies. Without knowing the scale of such "background" variability, the numbers of suitable subjects needed to detect a significant change cannot be calculated.

We have examined the intrasubject variability of cellular and solute parameters in repeated 180 mL BAL procedures performed 1 month apart in 20 clinically stable, although symptomatic, asthmatics. During the study there was no change in disease activity or medication.

These are the sort of patients most likely to be involved in prospective studies of the effects of asthma therapy on airway pathology.

### Materials and methods

#### Subjects

A total of 20 asthmatic subjects were recruited (table 1). Nine were atopic, and the median age was 34 yrs (range 25–51 yrs). To be included, individuals had to give a history of current asthma symptoms but with no exacerbation in the previous 3 months, no chest infection in the previous 6 weeks, and no change in type or dose of medication in the previous 6 months.

#### Physiology and methacholine challenge

All lung function measurements were made at the same time of day and at least 8 h after any inhaled  $\beta_2$ -agonists had been used. Inhaled corticosteroids were taken at the usual time.

Table 1. – Demographic details of patients who were recruited for the study

Pt No.	Atopy yes/no	Age yrs	BDP* $\mu\text{g}\cdot\text{day}^{-1}$	BAL <sub>1</sub> FEV <sub>1</sub> L	BAL <sub>2</sub> FEV <sub>1</sub> L	BAL <sub>1</sub> PD <sub>20</sub> $\mu\text{g}$	BAL <sub>2</sub> PD <sub>20</sub> $\mu\text{g}$
1	No	53	400	4.03	3.70	110	440
2	Yes	29	0	3.08	3.14	2	3
3	No	18	0	3.32	3.19	22	19
4	Yes	36	0	3.36	3.35	5	12
5	Yes	30	400	4.48	3.54	30	107
6	No	33	0	3.68	3.94	85	9
7	Yes	21	400	3.41	2.67	2	7
8	No	20	0	5.62	5.65	163	200
9	Yes	22	0	4.72	3.98	17	6
10	Yes	44	400	2.04	2.09	10	25
11	No	34	200	2.99	3.08	70	36
12	No	55	200	1.22	1.40	50	265
13	No	60	0	1.60	1.27	1170	91
14	No	41	200	2.44	2.49	10	11
15	Yes	59	0	2.50	2.56	2	250
16	No	25	400	3.37	3.36	3	2
17	Yes	25	400	2.35	2.11	2	6
18	No	59	0	2.08	2.70	18	24
19	Yes	34	0	3.87	3.60	75	46
20	No	42	400	3.83	3.14	500	56
		37# ( $\pm 14$ )		3.20# ( $\pm 1.09$ )	3.05# ( $\pm 0.98$ )	23## (2–1170)	28## (2–440)

Pt: patient; BDP: beclomethasone dipropionate; FEV<sub>1</sub>: forced expiratory volume in one second; BAL<sub>1</sub> and BAL<sub>2</sub>: at first and second bronchoalveolar lavage, respectively; PD<sub>20</sub>: dose of methacholine provoking a 20% decrease in FEV<sub>1</sub>. \*: inhaled steroid usage; #: mean ( $\pm$ SD); ##: geometric mean (and range).

Spirometry was performed using a heated Jaeger "screen-mate" pneumotachometer (Eric Jaeger UK Ltd, Market Harborough, UK). Each measurement was performed in triplicate and the highest value recorded.

Airway responsiveness to inhaled methacholine was measured using a previously described dosimeter technique [7]. Briefly, cumulative doubling doses of methacholine from 3 to 6,400  $\mu\text{g}$  were administered at 5 min intervals until there was a decrease in forced expiratory volume in one second (FEV<sub>1</sub>) exceeding 20%. Results were expressed as the dose of inhaled methacholine estimated to provoke a 20% decrease in FEV<sub>1</sub> (PD<sub>20</sub>) calculated by linear interpolation between the last two points on the dose-response curve.

#### Study design

Two bronchoscopies were performed 1 month apart, during which time the patients remained clinically stable, with no change in active medication. Airway responsiveness was measured 5 days before each bronchoscopy.

#### Bronchoscopy and bronchoalveolar lavage

Fibreoptic bronchoscopy was performed after pre-medication with inhaled salbutamol (200  $\mu\text{g}$ ) from a metered-dose inhaler, intravenous atropine (0.6 mg), and midazolam (2–10 mg). Topical 1.5% lignocaine was

used to anaesthetize the bronchial tree as required. After wedging the bronchoscope in a subsegment of the middle lobe, three 60 mL aliquots of phosphate buffered saline, warmed to 37°C, were instilled *via* a hand operated syringe. The fluid was then immediately aspirated into a siliconized container at a negative pressure of approximately -80 mmHg. The BAL fluid was immediately transported to the laboratory at 4°C for processing and analysis.

#### Cell processing

Total cell counts were performed on the unfiltered BAL fluid using a modified Neubauer haemocytometer. Differential cell counts were made by processing duplicate 1 mL aliquots of unfiltered BAL aspirate using the glass cover method [8]. Differential cell counts were performed in duplicate by two experienced observers counting 1,000 cells each and the results were averaged. In addition, duplicate cytocentrifuge preparations were made using 200  $\mu\text{L}$  of unfiltered BAL aspirate (Shandon Cytospin II, 500 rpm, 2 min). These were fixed in Carnoy's fluid for 45 min at ambient temperature and reserved for subsequent mast cell staining.

BAL fluid was then filtered through a 200  $\mu\text{m}$  stainless steel mesh and cells pelleted at 100 $\times$ g max for 15 min. Aliquots of the BAL supernatant were stored under liquid nitrogen for subsequent assay of tryptase and albumin.

### Mast cell staining

Following fixation, duplicate cytopspins were incubated in 1% Alcian blue in 3% acetic acid for 16 h. Slides were washed in 3% acetic acid and counterstained with 0.5% neutral red. An estimated 5,000 cells were examined using a field scanning technique using light microscopy. "Mast cells" were identified by their content of granules, which characteristically stain metachromatically with Alcian blue.

### Tryptase levels

Levels of mast cell tryptase in BAL fluid were determined by radioimmunoassay using a monoclonal antibody specific for tryptase (AA5) [9] coupled to cyanogen bromide activated agarose (Pharmacia, Milton Keynes, UK). Duplicate 50  $\mu\text{L}$  samples of BAL fluid concentrated 10 times by freeze drying or tryptase standards (2–50  $\mu\text{g}\cdot\text{mL}^{-1}$  in 50  $\mu\text{L}$ ; Pharmacia) were incubated with AA5-agarose (2.5 mg in 100  $\mu\text{L}$ ) and shaken continuously for 16 h at room temperature. Tubes were washed with 0.9% saline and 25  $\mu\text{L}$  of  $^{125}\text{I}$ -antitryptase monoclonal antibody (Pharmacia) was added for 16 h. Following further washing, the radioactivity in each tube was counted in a gamma-counter. The assay was sensitive to a limit of 0.09  $\text{ng}\cdot\text{mL}^{-1}$  with 95% limits of agreement of  $\pm 0.096$   $\text{ng}\cdot\text{mL}^{-1}$ . The concentration of the BAL fluid was taken into account in the subsequent expression of the results.

### Albumin measurements

Levels of albumin in BAL fluid were measured directly using a COBAS autoanalyser. The limit of detection for the assay was 1  $\mu\text{g}\cdot\text{mL}^{-1}$ .

### Statistical analysis

Minitab software (CLE. COM Ltd, Birmingham UK, Lawes agricultural trust UK), was used for statistical analyses. The standard deviations of the differences between the repeated measurements 1 month apart were used to calculate the sample sizes necessary for a subsequent study to detect a significant change in inflammatory indices for paired data. For the calculations, a power of 80% and a significance level of  $p$  equal to 0.05 were specified. These were used because they are the values conventionally designated in the majority of sample size calculations made for clinical pharmacological research.

The following equation was used:

$$n = \left[ \frac{\text{SD} (Z_{1-\beta} + Z_{1-\alpha/2})}{\text{detectable difference}} \right]^2$$

where:  $n$ =number of subjects required;  $\text{SD}$ =standard deviation of the differences in the paired data between the second and first study;  $Z_{1-\beta}$ =0.8416 (a constant term to specify the power; here 80%);  $Z_{1-\alpha/2}$ =1.96 (a constant term to specify the significance level; here 0.05); detectable difference=the difference in an inflammatory parameter that can be detected with statistical significance using repeated BAL.

The equation can, therefore, be simplified and rearranged to relate the background variability in inflammatory indices ( $\text{SD}$ ), the sample size required and detectable differences:

$$\begin{aligned} \text{detectable difference} &= (1.96+0.8416) \times \text{SD} / \sqrt{n} \\ &= 2.80 \times \text{SD} / \sqrt{n} \end{aligned}$$

Hence, for example, if preliminary studies indicated that the standard deviation of the differences differential count (as a percentage) was 16, then with a sample size of 15 subjects in an intervention study, the minimal detectable difference would be  $2.8 \times 16 / \sqrt{15}$ , or approximately 12%. Any change less than that would be less likely to achieve statistical significance.

In determining the sample size calculations, alternative criteria could be adopted. If these were more rigorous, *e.g.* 90% power and significance level of  $p$  equal to 0.01, the relationship between sample size required and detectable difference would be shifted to the right, such that for a given detectable difference more subjects would be required.

## Results

### Physiology and methacholine responsiveness

There was no significant change in baseline spirometry between the two bronchoscopic procedures (table 1). Mean (SD) FEV<sub>1</sub> was 3.2 (1.09) L at the time of the first BAL and 3.05 (0.98) L at the second. There was, however, variability in the spirometry, consistent with symptomatic asthma. Ninety five percent limits of agreement, calculated as suggested by BLAND and ALTMAN [10], were -0.624–0.928 L, *i.e.* in 95% of subjects the second of a further pair of FEV<sub>1</sub> measurements would be expected to be between 624 mL lower and 928 mL higher than the first.

Similarly, on average, there was very little change in PD<sub>20</sub>FEV<sub>1</sub> to methacholine between the procedures. Geometric mean PD<sub>20</sub> was 23  $\mu\text{g}$  (range 2–1,170  $\mu\text{g}$ ) at the time of the first BAL and 28  $\mu\text{g}$  (range 2–440  $\mu\text{g}$ ) at the second. There was, however, variability in the individual PD<sub>20</sub> measurements, again consistent with symptomatic asthma. Ninety five percent limits of agreement (expressed as a ratio) were 0.08–13, *i.e.* the variability of the data would lead us to expect that in 95% of subjects a second PD<sub>20</sub> measurement would be 0.08–13 times the first.

### Total and differential BAL cell count

Paired data were available for analysis in 18 of the 20 subjects studied. There were no significant changes in any of the cell parameters measured and mean values were similar for the two BAL procedures (table 2). However, there was considerable variation, reflected in the large standard deviations. Figure 1 illustrates this variability by means of a plot of the differences in values between procedures against the mean counts, using the method suggested by BLAND and ALTMAN [10].

Table 2. – Descriptive statistics for results of cell counts and solute assays from BAL procedures performed 1 month apart with no change in active treatment

	BAL1	BAL2
Return volume mL	79 (31)	96 (24)
Total cell count $\times 10^3 \cdot \text{mL}^{-1}$	136 (70)	182 (101)
Macrophages %	65 (14)	70 (14)
Lymphocytes %	29 (15)	26 (14)
Eosinophils %	1.9 (2.1)	2 (2.5)
Neutrophils %	2 (2)	1.4 (0.9)
Epithelial cells %	2 (2.7)	1.3 (3.0)
Mast cells %	0.22 (0.27)	0.37 (0.50)
Albumin $\mu\text{g} \cdot \text{mL}^{-1}$	74 (42)	76 (40)
Tryptase $\text{ng} \cdot \text{mL}^{-1}$	2.7 (7.5)	1.3 (2.6)

Values are presented as mean, with SD in parenthesis. BAL1 and BAL2: first and second bronchoalveolar lavage, respectively.

### Tryptase and albumin concentrations

There were no significant changes in either of the BAL solutes measured and mean values were similar for the two BAL procedures (table 2). However, there was considerable variation between pairs of measurements as illustrated graphically in figure 2.

### Sample size calculations

Figure 3 is a plot of an estimate of the sample sizes required for a range of specified "detectable differences" in BAL total cell counts and respective percentage cell counts for paired data. The figure was generated using the observed standard deviation of the differences between the two BAL procedures, *i.e.*  $77.6 (\times 10^3 \cdot \text{mL}^{-1})$  for total cell count, 15.9% for macrophage differential count, 12.7% for lymphocyte count, 1.0% for eosinophil count and 0.45% for mast cell count, and the equation quoted in the methods for detectable difference. Thus, for example, for the percentage alveolar macrophage counts with a SD of 15.9 and a specified sample size of  $n=15$ , we would expect to be able to detect a statistically significant difference in paired samples of at least 11.5% at  $p=0.05$ , with 80% power.

Figure 4 is a plot of an estimate of the sample sizes needed for a range of required detectable differences in BAL tryptase and albumin concentrations. Again, the observed standard deviations of the differences in BAL

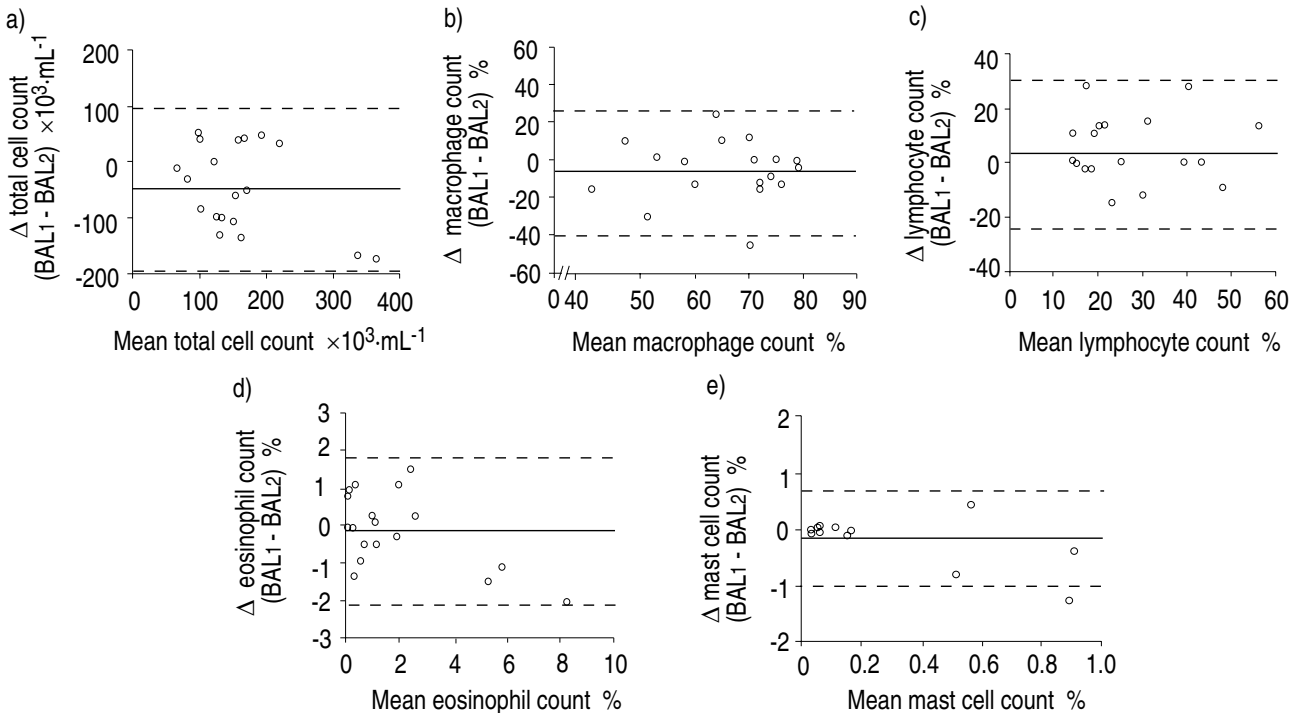


Fig. 1. – Plots of difference between BAL procedures (BAL1-BAL2) against mean values for: a) total cell count; b) % alveolar macrophages; c) % lymphocytes; d) % eosinophils; and e) % mast cells. The solid line represents the mean of the differences between procedures. The dashed line represents 2 SD of the differences between procedures (95% limits of agreement). BAL1 and BAL2: first and second bronchoalveolar lavage, respectively.

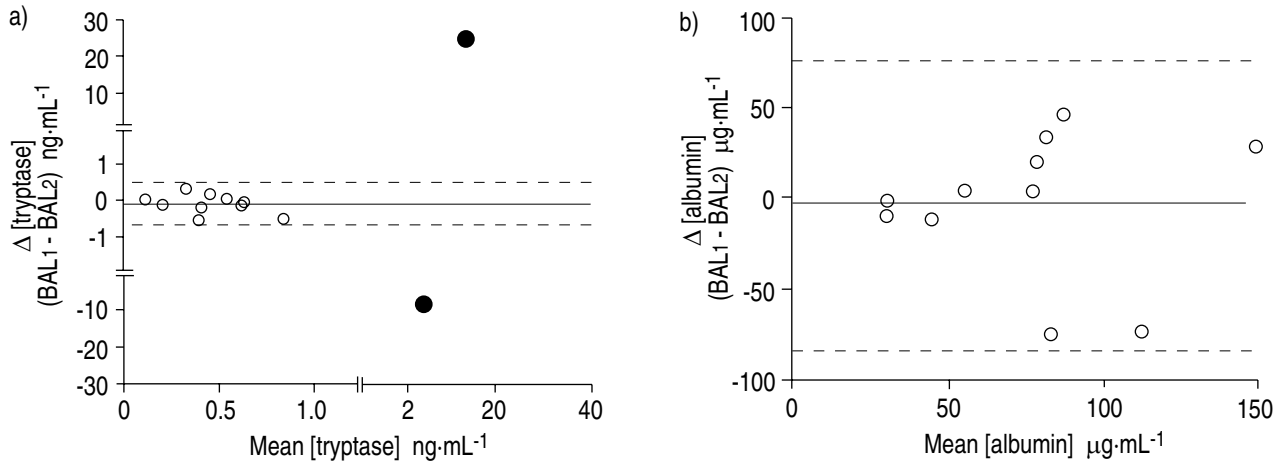


Fig. 2. – Plots of differences between BAL procedures (BAL1-BAL2) against mean values for solute assays: a) tryptase concentrations; and b) albumin concentration. The solid line represents the mean of the differences between procedures. The dashed line represents 2 SD of the differences between procedures (95% limits of agreement). Solid circles represent outliers that were not included in the calculation of mean and SD. BAL1 and BAL2: first and second bronchoalveolar lavage respectively.

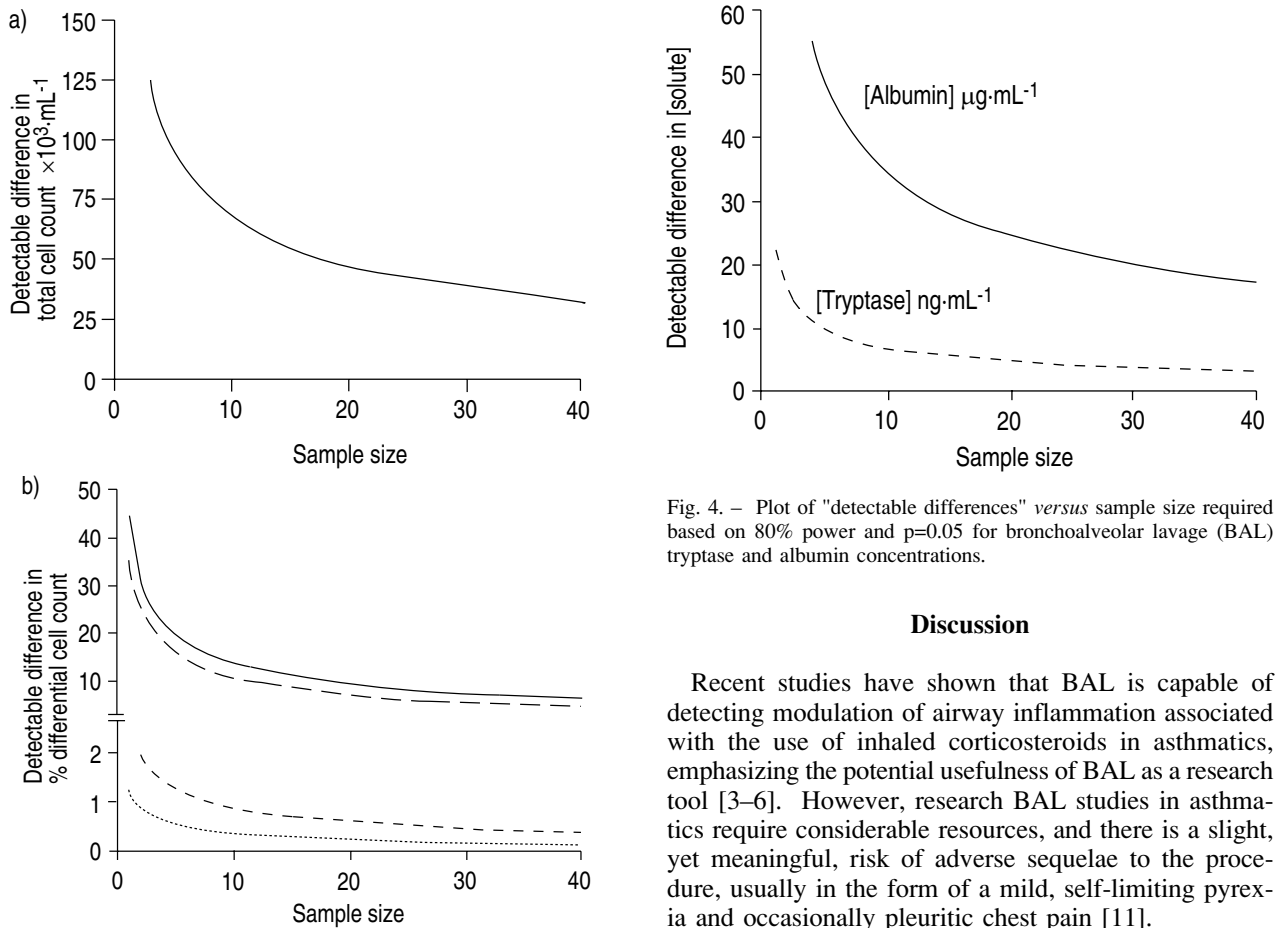


Fig. 3. – Plot of "detectable differences" versus sample size required based on 80% power and  $p=0.05$  for: a) bronchoalveolar lavage (BAL) total cell count; and b) percentage differential cell count for alveolar macrophages, lymphocytes, eosinophils and mast cells. — : macrophages; — — : lymphocytes; - - : eosinophils; ..... : mast cells.

Fig. 4. – Plot of "detectable differences" versus sample size required based on 80% power and  $p=0.05$  for bronchoalveolar lavage (BAL) tryptase and albumin concentrations.

**Discussion**

Recent studies have shown that BAL is capable of detecting modulation of airway inflammation associated with the use of inhaled corticosteroids in asthmatics, emphasizing the potential usefulness of BAL as a research tool [3–6]. However, research BAL studies in asthmatics require considerable resources, and there is a slight, yet meaningful, risk of adverse sequelae to the procedure, usually in the form of a mild, self-limiting pyrexia and occasionally pleuritic chest pain [11].

It is, therefore, necessary that if BAL studies are undertaken, they should have the necessary power to adequately address the hypotheses under investigation. This is consistent with the growing awareness that the use of adequate sample sizes in medical research is an ethical issue, as well as being fundamental to sound experimental design [12]. Indeed, many institutional Ethics Committees, including our own, insist on an adequate power calculation for the expected changes being sought

solute concentrations between the two procedures were used *i.e.* 7.95 ng·mL<sup>-1</sup> for tryptase and 39.5 µg·mL<sup>-1</sup> for albumin.

before granting permission for studies. In this context, the lack of previous data documenting background within-group variability of BAL data in asthmatics could prove a barrier to an adequate and ethical protocol being submitted. At the same time, it is unreasonable to recruit more patients than are needed. For these reasons, we felt that it was important to prospectively identify the power of BAL studies in asthmatics, given our interest and activity in this field of research. We are unaware of any other similar studies so far.

Our data would suggest that in the type of mild-to-moderate but symptomatic asthmatic population that we studied, use of sample sizes much less than 15 mean that differences have to be inappropriately large to be easily detected in repeat BAL samples, even after a potent therapeutic intervention. Recent studies of inflammation in asthma using BAL have used widely varying subject numbers, with some as low as five [13]. Our results indicate that the sample sizes of previous studies should be borne in mind when their findings are interpreted.

Our results also suggest, however, that there is little improvement to be gained in the power of BAL studies by increasing subject numbers above 20. Reassuringly, this indicates that though BAL studies require considerable commitment, they are certainly practicable.

Our patients were all clinically stable at the time of recruitment and active therapy was not changed during the study. More severe asthmatics or clinically unstable subjects may demonstrate greater variability for biological and technical reasons. This should be considered in the study designs involving such subjects, as large sample size numbers may be required for the detection of changes in inflammatory parameters. However, the types of patients with asthma included in this intrasubject variability study are likely to be typical of those involved in future drug studies. Thus, they were relatively young, with reasonably well-preserved lung function, receiving little or no inhaled corticosteroid, and with quite marked bronchial hyperresponsiveness and variability in lung function. They would be chosen for a therapeutic intervention study largely on these criteria, so that it would be ethical to increase or change their medication, and it would be reasonable to expect that a significant improvement could be obtained both in clinical and bronchoscopic end-points. This study gives the limits of what can be reasonably expected from a study using BAL to determine improvement in asthmatic airway inflammation.

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