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# Variation of CD8+ T-lymphocytes around the bronchial internal perimeter in chronic bronchitis

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*Variation of CD8+ T-lymphocytes around the bronchial internal perimeter in chronic bronchitis. E. Gamble, W. Burns, J. Zhu, T. Ansari, V. De Rose, J. Kips, N.C. Barnes, P.K. Jeffery. ©ERS Journals Ltd 2003.*

**ABSTRACT: The variation of CD8+ cells has been determined around the internal perimeter of intrapulmonary bronchi in smokers with chronic bronchitis (CB), and the amount of tissue required to confidently estimate the true mean has been calculated.**

**Lung specimens were obtained from 10 smokers with CB. Paraffin sections of intrapulmonary bronchi were immunostained and CD8+ cells counted in the epithelium and subepithelium in up to 10 sequential 1-mm segments around the internal perimeter of each airway.**

**The percentage of counts falling between ±20% of the final mean was 43.0% for epithelium and 40.9% for subepithelium. In 90% of subjects, the cumulative mean was stable after examination of subepithelial tissue associated with 5 mm of reticular basement membrane.**

**There is considerable variation in the counts of CD8+ cells between adjacent 1-mm airway mucosal segments in chronic bronchitis. In order to achieve a representative count and to maximise statistical power to detect differences between study populations, subepithelial tissue including a minimum of 5 mm of reticular basement membrane length should be examined.**

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Chronic bronchitis (CB) and chronic obstructive pulmonary disease (COPD) are associated with an inflammatory infiltrate in large and small airways [1–4]. Increased numbers of activated T-lymphocytes and macrophages have been demonstrated by immunohistochemical staining [5]. In the presence of airflow limitation (*i.e.* forced expiratory volume in one second (FEV1) <80% predicted) there is a significant increase in the CD8+ T-lymphocyte subset [6] and FEV1 % pred has been shown to be inversely related to CD8+ cell count [7].

Few studies have examined biopsy variability in smokers with CB. Sampling of airway mucosa sufficient to account for intrasubject variability must be considered when planning any biopsy study. Given the wide variation of biopsy sampling in practice, the authors felt it would be helpful to have further data with which to make informed decisions. Such data have important implications in the planning of studies where bronchial wall tissue parameters are a main end-point. This study examines the variability of CD8+ T-lymphocyte counts in CB around the internal perimeter of intrapulmonary bronchi. The results described herein give an indication of the minimum quantity of tissue that needs to be examined in order to obtain a representative cell count for the entire airway in section.

## Methods

### *Subjects*

Lung resection specimens were obtained from current smokers and one exsmoker with CB undergoing resection for lung tumour. CB was defined as cough productive of sputum occurring on most days of the month for ⩾3 months per year for 2 yrs before the study [8]. Subjects had been free from exacerbations during the month preceding the study; an exacerbation was defined as increased dyspnoea associated with a change in the quality or quantity of sputum leading to the subject seeking additional medical attention. Two patients met the Global Initiative for Obstructive Lung Disease criteria for mild COPD and two for moderate COPD [9]. Mean FEV1 % pred was 89% (range 69–119%). Mean FEV1/forced vital capacity ratio was 75% (range 65–96%). Demographic and lung function data are given in table 1. Appropriate tissue sections were selected from the tissue archive. These had been obtained for and also used in a previously published study [10]. Ten tissue sections from 10 patients including intrapulmonary bronchi cut in transverse section with ⩾7 mm of intact reticular basement membrane (RBM) and associated epithelium and subepithelium were selected. All airways included cartilage and glands. Figure 1 is a photomicrograph of one of the airways used. Paraffin-embedded sections were dewaxed to tap water prior to immunohistological processing. The avidin-biotinylated peroxidase complex technique was used to identify CD8+ cells as described previously [10].

### *Quantification*

An intrapulmonary bronchus cut in transverse section with ⩾7 mm of intact RBM and associated epithelium and subepithelium was selected from each tissue section. CD8+

Table 1. – Subject characteristics

| Subject | Age yrs | Sex | Smoking pack-yrs | FEV1 % pred | FVC % | FEV1/FVC % |
|---|---|---|---|---|---|---|
| A | 58 | M | 49.5 | 77.3 | 100 | 65.2 |
| B | 65 | M | 53 | 94 | 116 | 73 |
| C | 76 | M | 66 | 89 | 98 | 64.6 |
| D | 63 | M | 20 | 72.9 | 94.9 | 77.6 |
| E | 73 | M | 60 | 119 | 78 | 78.6 |
| F | 80 | M | Ex | 98.4 | 77 | 95.5 |
| G | 72 | M | 75 | 83 | 90 | 68 |
| H | 68 | M | 25 | 115 | 107 | 83.7 |
| I | 81 | M | 31 | 70.4 | 91.4 | 68.4 |
| J | 63 | M | 50 | 69 | 71 | 73.2 |
| Mean | 69.9 | | 47.7 | 88.8 | 92.3 | 74.8 |

FEV1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; M: male; Ex: exsmoker.

T-lymphocytes were counted in both the epithelium and subepithelium to a depth of 100 μm in up to 10 sequential segments around the internal perimeter each composed of a 1-mm length of RBM. For one subject, one airway from each of two separate tissue sections was counted to determine variation between airways. Imaging and quantification was carried out with a Zeiss light microscope (Thornwood, NY, USA) and ×40 objective lens with eyepiece graticule by a single observer. The internal airway perimeter was measured using computerised image analysis. The intraobserver coefficient of variation (CV) was 4% for subepithelial and 9% for epithelial CD8+ cells, respectively. The interobserver CV was 5% for subepithelial and 18% for epithelial CD8+ cells, respectively.

### *Statistical analysis*

The CV was calculated as the SD/mean expressed as a percentage. Data were also analysed descriptively. Final mean cell counts were calculated for each subject by dividing the total cell count in all segments by the total RBM length examined. Cumulative means were calculated as the results of counts of each additional segment were added. The proportion of single-segment counts that were within 20% of the final mean for each individual was determined. The number of segments that had been counted before the cumulative mean, remaining within 20% of the final mean value, was also established and at this point the cumulative mean was considered to be stable.

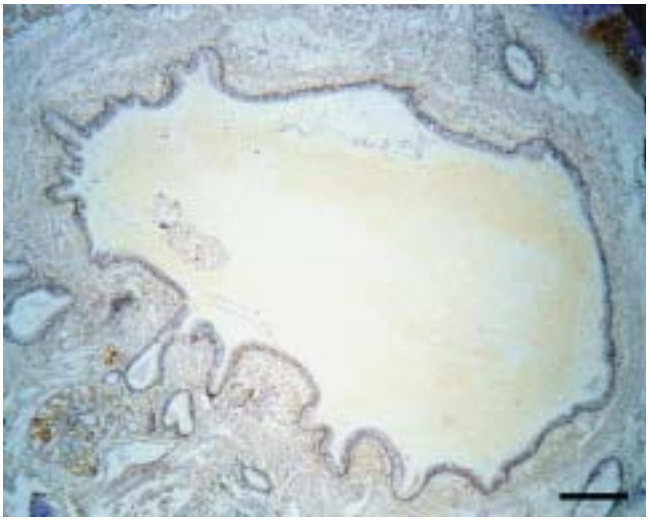

Fig. 1. – Photomicrograph of an airway in cross-section at low power. Scale bar=0.5 mm.

## Results

One bronchus from each of 10 subjects was examined and from these 93×1-mm segments of mucosa were of sufficient quality to be analysed. The mean airway internal perimeter was 12.49 mm (range 7.57–19.55 mm). Figure 2 shows the subepithelial cell count from each segment for each airway for the 10 subjects studied. Of the 93×1-mm segments of mucosa assessed, the CD8+ cell count fell within 20% of the final mean value in 43.0% of segments for epithelium and 40.9% of segments for subepithelium. Counts for the epithelium varied by >50% from the final mean in 24% of 1-mm segments. For the subepithelium, counts varied by 50% from the final mean in 18% of 1-mm segments. Figure 3 shows the single-segment subepithelial cell count/final mean as a percentage for the 10 subjects studied. Although most values cluster around the mean, there are a small number of values that depart markedly from the mean. The mean count was stable for 80% of subjects after examination of 6 mm of epithelial tissue and for 90% of subjects after examination of 5 mm of subepithelial tissue. The mean was stable for all counts after examination of tissue in which a length of 9-mm RBM was included. The CV (using data from 10 segments) for two different airways from the same subject was 4.6% for epithelial and 22.6% for subepithelial counts, respectively. The CV for 10 individual 1-mm segment counts from the

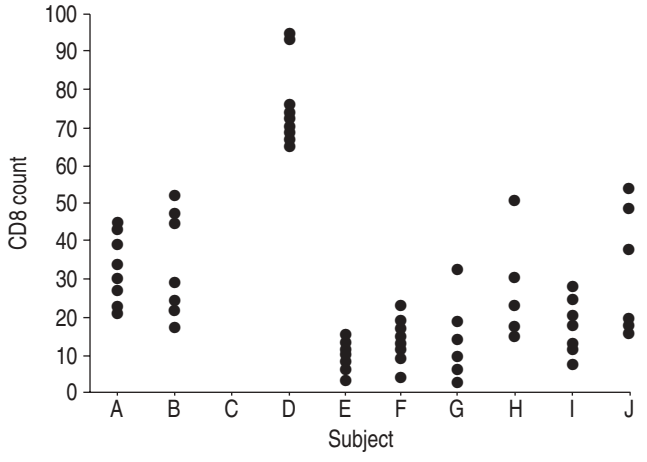


Fig. 2. – Each dot represents the absolute CD8 count for a single 1-mm mucosal segment from subjects A to J.

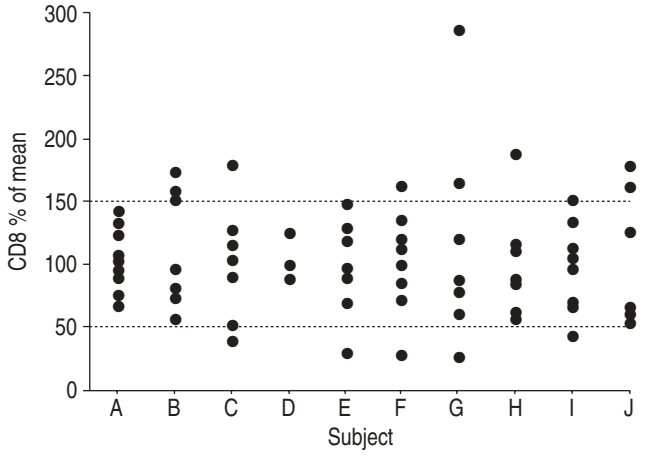


Fig. 3. – Each dot represents the percentage of the final mean CD8 count for that subject for a single 1-mm mucosal segment. Dotted lines indicate the range ±50% of the final mean CD8 count.

same subject was 14.4% for epithelial and 80.8% for subepithelial counts, respectively.

## Discussion

In sampling airway mucosa it is assumed that the biopsy obtained will be representative of the entire airway in cross-section. However, this is not proven, and the intrinsic variability in the distribution of inflammatory cells within airway tissue must be taken into account so that adequate sampling can be performed. Examination of more tissue is likely to provide a more representative cell count for the airway. However, the quantity of tissue that can be sampled and examined is limited by practical considerations.

Few studies have addressed the issue of how much endobronchial biopsy tissue should be examined to obtain a representative cell count and hence to maximise statistical power to detect a difference between study populations. There is a paucity of data and it is unclear whether the results of such studies in asthma can be extrapolated to a group of smokers with CB or COPD. SULLIVAN *et al.* [11] examined the variation in inflammatory cell numbers between step sections of a bronchial biopsy from subjects with asthma, COPD and healthy controls. Inflammatory cells did not follow a normal frequency distribution, so the quantity of tissue required could not be predicted linearly. By descriptive analysis they recommended examination of a zone of RBM of $\geqslant 7$ mm length to a depth of 60 μm (*i.e.* 0.42 $mm^2$). TEN HACKEN *et al.* [12] using bronchial biopsies from healthy controls and subjects with asthma, demonstrated that tissue of 1.0×0.1 mm in size (0.1 $mm^2$), along 1-mm RBM was sufficient to obtain constant cell numbers. SONT *et al.* [13] examined variability between fields within a single section and between different airway sites in patients with asthma. They found satisfactory correlation between two areas of average size 0.035 $mm^2$.

While the variation in cell counts between tissue sections of a single biopsy in COPD has been studied [11], the variation of 1-mm segments (equivalent to a biopsy sample) around the internal perimeter of an airway has not been examined. To the best of the authors′ knowledge, this study is the first to report variation in CD8+ cell count around the internal perimeter of an airway and to use such data to estimate the quantity of tissue that should be examined to provide accurate estimates of inflammatory cell counts. Such knowledge should allow investigators to improve study design and increase the power to detect differences between-study populations.

From the results of the present study, examination of a single 1-mm segment of mucosal tissue gave a subepithelial CD8+ count of acceptable accuracy on only 41% of occasions. A significant minority of 1-mm segments fell wide (>±50%) of the final mean value. These results suggest that subepithelial tissue associated with $\geqslant 5$ mm of RBM should be examined in order to obtain a representative subepithelial CD8+ cell count. The findings of SULLIVAN *et al.* [11] demonstrate that inflammatory cells are not randomly distributed throughout large airway subepithelial tissue but tend to cluster. This could account for a large part of the variation observed in this study between 1-mm tissue samples. Increasing the quantity of tissue assessed will provide a more representative result and the study by SULLIVAN *et al.* [11] indicated that at least three bronchial biopsies, each of 2 mm size should be counted. The present study of intrapulmonary bronchi would support the need to aim for this quantity of tissue. The variability observed herein was greater than that of intraobserver variation. Interestingly, the CV between 1-mm segments from the same airway was substantially higher than the CV between two airways from the same subject indicating the importance of sampling adequately from each airway.

There is considerable variation in counts of CD8+ cells between adjacent 1-mm segments of mucosa in smokers with chronic bronchitis. These results suggest that subepithelial tissue associated with $\geqslant 5$ mm of reticular basement membrane should be examined in order to achieve a representative cell count for the airway and maximise statistical power to detect group differences.

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