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EDITORIAL

Bronchial biopsies and airway inflammation

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Within the last 10 yrs there has been an explosion of interest in the results of biopsy of the airway lining which, whilst invasive, is safe providing it is performed by an experienced bronchoscopist following the published recommendations [1, 2]. The airway mucosa which is sampled comprises surface epithelium and its supportive subepithelial tissue, often referred to as the lamina propria. The epithelium is continuous from the lining of the nose to the wall of the respiratory (alveolar) portion of the lung. It forms the barrier between external and internal environments, is the site of first interaction between environmental pollutants and/or allergens and host tissue, and the site at which immune responses and reactions are initiated. Data obtained by biopsy of the bronchi have established beyond doubt that asthma is an inflammatory condition and, more recently, that chronic bronchitis is also. There are now sufficient data for biopsies to act as the basis for the validation of less invasive techniques, such as airway lavage, analysis of spontaneous or induced sputum, and the more recent technique of bronchial brush biopsies; this last being the subject of a paper by RIISE *et al*. [3] published in this issue of the Journal.

Whilst bronchial and nasal biopsy have been much used as a technique to research the basic cellular, immunological and molecular abnormalities of airway diseases, major goals for its clinical application remain the more accurate diagnosis of airway disease and monitoring of the efficacy of more specific therapy to treat airway inflammatory conditions, such as asthma, chronic bronchitis (CB), chronic obstructive pulmonary disease (COPD), and cystic fibrosis. The technique of bronchial biopsy has also proved invaluable in the assessment of multiple biopsies to determine the onset of tissue rejection following lung transplantation [4]. Biopsies can also be prepared as explant cultures to enable epithelial outgrowths to be studied for their response to pollutants/allergens and for their capacity to produce cytokines of relevance to allergic inflammation [5]. Bronchial biopsies have provided much novel information concerning: 1) changes which persist in the stable (asymptomatic) phase of airway inflammatory disease; 2) changes associated with exacerbations, *i.e*. the appearance of/or an increase in symptoms which require the individual to seek medical attention or alter his/her treatment; 3) alterations associated with the response to allergen or to occupational exposure; and 4) reversibility of the inflammatory lesion following treatment or removal of the irritant or occupational agent. Following ethics approval and informed written consent, studies in normal healthy volunteers have also been conducted, which provide an invaluable baseline for comparison with disease. The capacity and agreement of the subject to perform repeated biopsy with time allows longitudinal and cross-sectional studies to be performed. Flexible fibreoptic bronchoscopy has provided the opportunity to study early changes in mild disease or following exposure to allergen, and to distinguish these relatively early events from those observed post-mortem.

The studies of SALVATO [6], GLYNN and MICHAELS [7], LUNDGREN [8] and LAITINEN *et al*. [9] were pioneering studies, in which relatively large (3–4 mm) samples of the bronchial mucosa were obtained using the rigid bronchoscope. They demonstrated the mucosal inflammation of asthma, contrasted the appearance of the mucosa in asthma and chronic bronchitis, reported the efficacy of long-term treatment with corticosteroid, and showed the marked damage to surface epithelium. With the advent of the flexible fibreoptic bronchoscope, there has been a keenness to biopsy the airways in asthma and to renew studies which compare clinically distinct airway conditions.

Initial electron microscopic studies of biopsies in mild stable ongoing asthma reported the involvement and degranulation of mast cells and eosinophils [10], highlighted a controlling role for the lymphocyte in the inflammatory response, and demonstrated an association between loss of the surface epithelium and airways hyperresponsiveness (AHR) [11]: most, but not all, of these features were previously described in end-stage (fatal) asthma [12–16]. These features of inflammation were shown to occur even in patients with newly diagnosed asthma [17], and the observations of bronchial biopsies have emphasized the importance of considering early intervention with inhaled anti-inflammatory treatment. Homogenous thickening of the "basement membrane" (a subepithelial reticular layer also referred to as the lamina reticularis), recognized by pathologists as a key characteristic change of asthma, was also shown to occur early on in mild disease [11, 18], and is in contrast to the lack of thickening in COPD [19, 20]. The thickening of the reticular basement membrane in asthma was suggested to represent "subepithelial fibrosis", and was associated with the close presence of myofibroblasts [18, 21]. The activation of T-lymphocytes of the CD4+ subset and also of eosinophils, and the negative association of the last with AHR was demonstrated in studies which examined bronchial biopsies in relatively large numbers (*i.e*. more than 20) of subjects [22, 23]. The upregulation of gene expression for the proinflammatory cytokine, interleukin (IL)-5, in association with the CD4+ T-lymphocyte, particularly in symptomatic asthmatic subjects [24], led to the development of the concept of a prevailing Th_2 allergic inflammatory profile in asthma in which IL-4, IL-5 and IL-10

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predominate [25, 26]. These morphologically based studies have been verified by application of reverse transcriptase-polymerase chain reaction (RT-PCR) applied to bronchial biopsies.

Asthma is unlikely to represent a single entity; biopsy studies have addressed this point by examination and comparison of the airway mucosa in extrinsic (allergic), intrinsic (nonallergic), aspirin sensitive and occupational forms of the condition. The profile of inflammatory cell and cytokine gene expression appears to be similar in these forms of asthma [27–30], although the presence or absence of IL-4 protein has been debated in intrinsic asthma [29, 31].

There has recently been a renewed interest in the power of bronchial biopsies to discriminate between asthma and chronic bronchitis, and to determine whether there is a tissue change particularly associated with reduced airflow and its accelerated decline with age in COPD [19, 32–34]. These studies of biopsies in bronchitis have confirmed the inflammatory cell nature of the disorder, reported earlier in resected lung material by MULLEN and co-workers [35, 36]. Like asthma, analysis of bronchial biopsies has shown that T-lymphocytes appear to be involved in chronic bronchitis also, and there is evidence of their early and persistent activation [32]. Unlike asthma, macrophages are a prominent cell type in COPD [32], and the CD8+ T-lymphocyte subset (rather than the CD4+ subset) predominates [37, 38]. O'SHAUGHNESSY and co-workers [39] have recently demonstrated a statistically significant negative association between the CD8+ population and reduced airflow in smokers, as determined by measurements of forced expiratory volume in one second (FEV_1) in smokers, and there are greater numbers of T-lymphocytes and macrophages in subjects with airflow obstruction [39, 40]. Exacerbations of bronchitis are associated with an increased number of eosinophils [41], although their numbers are small in relation to asthma and it has been suggested that they do not degranulate [42]. The inflammation of chronic bronchitis is also associated with upregulation of cell-surface adhesion molecules (CAMS) [43], and inflammation appears to persist following smoking cessation if the production of sputum continues [44].

Immunohistological and molecular (*in situ* hybridization) studies of bronchial or nasal biopsies taken sequentially following experimental or natural exposure to allergen have shown increased involvement of CD4+ T-cells, mast cells and eosinophils and IL-4, IL-5 and IL-10 [25, 45–48], and there has been study of the expression of CAMS associated with these inflammatory events [49–51]. Recently, GIZYCKI *et al*. [52] have shown by electron microscopy that myofibroblasts increase substantially in number in response to allergen, and that transitional ultrastructural forms between fibroblast and bronchial smooth muscle are found, indicating a possible mechanism for the formation of the increased bronchial smooth muscle mass, a key feature of airway wall remodelling associated with fatal asthma. Occupational exposure to toluene diisocyanate (TDI) resembles allergen exposure in respect of the tissue eosinophilia but, in contrast, there is evidence from bronchoalveolar lavage (BAL) and biopsy of a marked recruitment of neutrophils [30, 53, 54].

Biopsies taken before and following treatment demonstrate the efficacy of inhaled [55–58] or oral corticosteroid [59] in their reduction of allergic inflammation and the associated symptoms of asthma. In a recent study, HOSHINO and NAKAMURA [60] have reported that inhaled corticosteroid was also effective at reducing inflammation, associated symptoms and improving lung function in a group of nonallergic asthmatics. Biopsies also allow investigation of steroid-resistant asthma in order to improve understanding of the reasons for a lack of responsiveness to treatment [61]. Biopsy studies have already demonstrated the anti-inflammatory potential of a number of non-steroid-based agents, such as theophylline [62, 63], in antiasthma treatment, and have the power to identify other more specific treatments aimed at reduction of the activated CD4+ T-lymphocyte and IL-4/IL-5 cytokine involvement. The study by FINNERTY *et al*. [64] published in the present issue of the Journal provides evidence of the efficacy of theophylline in a placebo-controlled parallel group study, in which airway inflammatory cell number and associated IL-4 protein were reduced after 6 weeks of treatment. In this last study, there was also a trend to reduction in IL-5 protein which did not achieve statistical significance, probably due to a combination of large subject variation and the relatively small numbers assessed (see below). In occupational asthma, the potential for reversal of inflammatory and structural changes following cessation of exposure to TDI is debated [30, 65], and the usefulness of anti-asthma treatment in this condition needs to be investigated. Such studies will also be required in chronic bronchitis and will allow, in the short-term, examination of the association of reduced inflammation with reduction of sputum and, in the long-term, with slowing of the rate of decline in lung function.

There are many subtleties in the sampling and interpretation of biopsies. Bronchial biopsies sample mainly the proximal conducting airways, and it has perhaps been surprising to find the extent of inflammation and remodelling which occurs in relatively large airways in asthma. Recent studies indicate that inflammatory changes present in large airways may reflect those present in small airways and, perhaps also, the alveolar walls [66, 67]. Biopsies sample only a fragment of the surface of a very large bronchial tree and it has been reassuring to note that the largest contribution to variation is between subjects rather than between distinct generations of airway [55, 68, 69]. It is estimated that 15 subjects per group should provide sufficient statistical power to detect most of the changes of interest in biopsies of inflamed airways [70]. Most biopsy studies reported in the literature, understandably, have not managed to obtain this number, which means that essential information is being lost to interpretation. For example, the trend to a reduction of IL-5 in response to theophylline, reported by FINNERTY *et al*. [64] in the present issue of the Journal, would probably be statistically significant if the numbers of subjects had been larger.

Surprisingly, variation between biopsies taken from a single airway is no greater than that between step sections of a single biopsy, and it is probably important to sample several random sections of each biopsy to adequately represent the inflammatory cell of interest. It will also be important to quantify, separately, distinct zones of the bronchial mucosa in any one section of a biopsy. The inflammatory cell which predominates within the surface epithelium may be quite different to that in the tissue immediately beneath it or that which surrounds the

bronchial glands. For example, the lavage fluid obtained from subjects with smokers bronchitis is rich in neutrophils [71], and yet it is our experience that the subepithelial tissues of bronchial biopsies obtained from these subjects shows a scarcity of this cell type. However, examination following application of an antibody directed against neutrophil elastase shows an abundance of positivity within the surface epithelium, which is not found beneath it (O'Shaughnessy *et al.* unpublished).

This may, in part, explain the difference between the results of BAL and biopsy and it highlights the value of bronchial brush biopsies if the surface epithelium is to be preferentially sampled. Riise *et al.* [3] demonstrate that the bronchial brush method, whose sample contains more than 90% surface epithelial cells, can detect group differences in respect of percentage eosinophils between asthmatics given β_2-agonists intermittently and those given inhaled corticosteroids regularly, and that the percentage of intraepithelial eosinophils has an association with BAL levels of eosinophil cationic protein (ρ=0.73; $p<0.01$). Interestingly, there is an inverse association of the percentage eosinophils in the brush specimens with the provocative concentration of agonist causing a 20% fall in FEV_1 (PC_{20}) (ρ= -0.67; $p<0.003$).

The correlations between cellular findings of asthmatics at BAL and biopsy are known to be poor, and those between bronchial brush biopsies and counts of surface epithelium obtained by biopsy are not known, and these associations should be examined in the future. There is an association between counts of eosinophils in sputum and biopsy, suggesting that analyses of sputum and bronchial brush biopsies may be less invasive ways to assess eosinophil infiltration of the airway wall but the results of correlation for other cell types are disappointing [72].

It is the author's biased opinion that bronchial biopsy still remains the gold standard for the assessment of ongoing inflammatory events in the airway mucosa. However, to obtain reliable results, the biopsy must be of adequate size and quality, and the experience of the bronchoscopist and his research team is paramount in this regard. There is always a learning curve during which "trial" biopsies must be taken before commencing any study. The choice of fixative, the embedding medium and method of analysis obviously varies depending on the research or diagnostic question being asked. We have found that brief (2 h) fixation in weak (2%) freshly prepared paraformaldehyde followed by snap-freezing provides good tissue morphology and the capacity to conduct immunohistological studies, as well as to carry out molecular studies involving *in situ* hybridization. However, such fixation may also destroy certain epitopes of interest, such as those associated with CAMS. Embedding in glycol methacrylate and thin (1–2 µm thick) section gives the appearance of greater resolution and allows adjacent sections of one and the same cell or tissue structure to be studied to examine for co-localization of proteins [73]. Fixation in glutaraldehyde and embedding in epoxy resin provides the best preservation for electron microscopic studies but water soluble embedding media provide a useful compromise between morphology and immunolabelling at the electron microscopic level. The size and quality of the biopsy is dependent upon the size of biopsy forceps and, particularly, their sharpness. Furthermore, operator skill and experience, and the need for provision of a dedicated research bronchoscope and the frequent replacement of biopsy forceps is often overlooked. For example, loss of surface epithelium is determined by the interaction of inherent epithelial fragility, the bronchoscopists skill, quality and type of biopsy forceps, and speed of fixation and/or freezing technique. In contrast to chronic bronchitis, the fragility of this surface layer in asthma often results in little of it remaining for analysis. Ice crystal damage due either to omission of a cryoprotection step or to relatively slow freezing rates is a common difficulty, not fully appreciated. In this case, whilst immunostaining is possible, quantification may not be, due to large ice crystal-induced gaps in the tissue. Unfortunately, several publications clearly demonstrate this common freezing artefact!

In spite of the difficulties outlined above and the restriction of bronchial biopsy to the investigation of relatively large airways in mild disease, I believe the technique will continue to be applied. This will ultimately benefit the patient, by providing not only information useful to diagnosis and monitoring of inflammatory disease but also novel data concerning the relationship between the inflammatory infiltrate, indices of airways hyperresponsiveness, symptoms and lung function [74], and a greater understanding of basic abnormalities of airway inflammatory disease such that new therapeutic modalities can be designed to treat or prevent its onset.

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