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Do bronchial biopsies represent mast cell density in airways?

A stereological study

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Abstract

Endobronchial biopsy specimens may not adequately represent inflammatory cell counts throughout the airway wall. This study aimed to compare mast cell density in biopsies and airway sections using both stereological and non-stereological methods.

Post-mortem biopsies and adjacent transverse sections were obtained from a mean of 5 proximal airways per case in 10 subjects who had died of non-respiratory causes. Tryptase-positive mast cells were measured stereologically in 30μm sections and non-stereologically in 5μm sections using an optical disector (cells/mm^3) and cell profiles (cells/mm^2), respectively. Reference areas included inner and total airway wall and to 100μm below the basement membrane.

Case means based on 4 or more biopsy sites significantly correlated with those on transverse sections only for counts over the inner airway wall, using both stereological and non-stereological methods. Cells/mm^3 and cells/mm^2 were significantly correlated within all reference areas.

When endobronchial biopsies are obtained from at least 4 proximal airways per case, inter-subject comparisons of mean mast cell density in the inner airway wall are as well represented by counts on biopsies as they are on transverse sections. This is the case using either three-dimensional, stereological or two-dimensional, non-stereological methods.

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Key words: asthma, biopsy, inflammation, stereology

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Introduction

Development of the flexible fibreoptic bronchoscope in 1967 [1] followed by the resolution of concerns about the safety of its use [2] led to an acceleration in the use of endobronchial biopsy to examine inflammation in the airways of patients with asthma. Since the mid 1980s the perceived success of endobronchial biopsies in demonstrating submucosal inflammation and the effects of treatment [3-16] plus the limitations or complications of indirect methods [10, 17, 18] have helped consolidate bronchial biopsy as the "gold standard" for investigating airway inflammation in vivo, in asthma.

Nevertheless, doubt has remained about the representativeness of biopsies, with authors suggesting that "biopsy specimens – being small – may not be 'valid' for the airways as a whole" [6, 10, 19]. Concerns about the anatomical sampling limitations of taking biopsies only from carinal sites [20] have added to concerns about the limitations of size, depth and sampling only from proximal airways [21].

Studies examining the density of inflammatory cells in the airways in asthma using airway transverse sections from post-mortem tissue, sample the bronchial tree more comprehensively than do studies using bronchial biopsies. Since most biopsy studies reporting the density of inflammatory cells have been in mild cases of asthma [3, 4, 6-16, 22], comparisons with post-mortem studies are effectively limited to the small number of such studies which have compared transverse sections from cases of less severe, non-

fatal asthma and controls [23, 24]. Results of biopsy studies in mild asthma have not been entirely consistent with either results of the post-mortem studies using cases of non-fatal asthma, or with each other.

Another methodological consideration is whether the density of inflammatory cells in endobronchial biopsy specimens should be assessed by means of traditional 2 dimensional (2D) "area profile counts" or 3 dimensional (3D) "stereology-based" methods [20]. At the heart of this issue is the question of potential bias. Since the probability of a cell's profile appearing in a 2D section is directly related to the cell's size, shape and orientation relative to the cutting plane, there is an inherent risk that area profile counts are biased in favour of certain cells [25, 26]. 3D approaches render the counts free of such biases as they involve counting cells of interest in 3 dimensional volumes to give cells/mm^3. In practical terms, this relies on application of the disector principle [27]. Very few studies of airway inflammation in asthma have adopted stereological approaches [26, 28-30] and of these only three have used a disector to enumerate cell density. No such studies appear to have used the optical disector [31-33] in thick sections to measure the density of inflammatory cells in actual 3D space.

What is unclear at this stage is the extent and nature of the relationship (if any) between (i) estimates of inflammatory cell density obtained from endobronchial biopsies at the carinae with inflammatory cell density across the whole airway wall in the vicinity of the biopsy site and, (ii) estimates of

inflammatory cell density obtained from samples of these tissues with the use of 3D compared with the more commonly used 2D approaches.

The aims of the present study were to compare the density of mast cells in endobronchial biopsies and transverse sections of large airways from corresponding sites using both stereological 3D and non-stereological 2D techniques.

Methods

Word count : 992

Whole lungs (8 left, 2 right) were obtained at postmortem from ten subjects (6 male / 4 female, ages 14 – 44 years) who had died from non-respiratory causes. Consent for the study of these tissues was obtained from the next-of-kin and with the approval of the ethics committee of Sir Charles Gairdner Hospital. Medical histories were only available from four subjects and in each case excluded the diagnosis of asthma. Lungs were fixed in inflation with 10% neutral buffered formalin at a pressure of 25cm H_2O.

Large airways were biopsied under direct vision at the (sub)carinae using standard cupped biopsy forceps. Following each biopsy, a transverse block 2-3mm long was cut from one of the airway branches immediately adjacent the biopsy site. This procedure was followed progressively from the main lobar bronchi down to 5^{th} or 6^{th} generation airways, taking at least one biopsy and paired transverse block at each level (Figure1).

For each subject, six biopsies and their paired transverse airway blocks were selected at random from those collected. Each biopsy and transverse airway block was embedded separately in paraffin wax and sections were cut at thicknesses of both 5μm and 30μm. Sections were stained with anti-mast cell tryptase monoclonal antibody AA1 (Dako, Australia) using the immunoperoxidase technique as previously described [34]. Sections with preparation artefacts were discarded or replaced.

Cell Counting

All cell counts were conducted using the Computer Assisted Stereology Toolbox (CAST)grid ® system (Olympus, Denmark), using an oil immersion lens at x1000 magnification and numerical aperture setting of 1.35 to minimise the depth of field. Optimal sampling fractions (i.e. the fraction of the area of interest sampled by uniformly spaced, randomly positioned (UR) fields) of the airway wall compartments were based on pilot counts of mast cells on transverse sections. These were conducted on at least 7 trial sampling fractions of 1%, 2%, 5%, 10%, 17% and 25%.

Estimates of 2D mast cell density in 5μm transverse airway sections

Both the inner airway wall (WAi), from the epithelial basement membrane to the outer border of the airway smooth muscle layer, and the total airway wall (WAt), from the epithelial basement membrane to the outer border of the adventitia [35] were delineated on CAST, and, within these reference areas, samples of uniformly spaced, randomly positioned (UR) 3084.3μm^2 fields

comprising 17% of WAi and 5% of WAt were generated. These fields were presented sequentially on the high resolution CAST monitor and positively stained, nucleated cell profiles in focus were counted within a single focal plane with the aid of an unbiased counting frame [36]. The count was repeated on a second, independent sample of UR fields and the mean of the two independent estimates was calculated.

Estimates of 3D mast cell density in 30μm transverse airway sections using the optical disector

The optical disector uses a probe which is a predetermined sampling volume within the tissue of interest. The upper and lower borders are preset to avoid the cut edges of the thick tissue sections and lateral borders are set to allow convenient sampling, depending on the density of objects to be counted. To allow for deformation during tissue processing, the actual thickness of each nominal 30μm thick section was estimated optically as the difference in the height of the microscope stage (using microcator readings) from where the tissue first came into focus to where the tissue went out of focus. The average of ten random points within delineated areas was calculated. Guard areas 4μm and 5μm thick were established at the top and bottom of each section respectively (Figure 2), with the balance of the section's mean thickness being set as the height of the optical disector probe. This resulted in an average disector height of 17μm. Further guard areas 14 - 18μm wide were established around the sides of the disector.

To count positively stained cells appearing in a field, the focal plane with its associated unbiased counting frame was scanned slowly down through the tissue. Cells first coming into focus within the disector height were counted provided that they fell entirely within the counting frame or touched only its acceptance lines and not its forbidden lines (Figure 3).

Within each airway section the number density (N_v) of mast cells (cells/mm^3) was estimated for WAi and WAt by : $N_v = [(10^9 \Sigma Q) / (a.h. \Sigma P)$
where ΣQ = total number of positively stained cells counted in the sample, ΣP = total number of fields in the sample, a = area of the unbiased counting frame (μm^2), and h = disector height (μm)

Estimates of 2D and 3D mast cell density in endobronchial biopsy sections
Independent 17% samples of UR fields were generated over two reference areas; WAi, where definable and an area to a depth of 100μm below the basement membrane, similar to previous studies [3, 6, 7, 16]. 2D counts in 5μm biopsy sections were conducted as for 5μm transverse sections to give cells/mm^2. 3D cell counts in 30μm biopsy sections were conducted using the optical disector as for 30μm transverse sections to give cells/mm^3.

Data analysis
Pearson's correlation coefficient was used to test the relationship between mast cell densities in biopsies and mast cell densities in associated airway transverse sections, and the relationship between 2D and 3D counts. Mean r values, where shown, are Fisher Z weighted means. Otherwise, where

appropriate, data are presented as mean ± standard deviation. Intraclass correlation coefficients (ICC) were also used to assess the relationship between comparable counts on biopsies and transverse sections. Differences between comparable biopsy and transverse section counts were assessed by Bland-Altman plots. The effect of the number of biopsies used was also examined by repeating the analyses using data from 1 – 4 biopsies. Correlations were with the inner airway wall on all transverse sections. Comparisons of results on biopsies and transverse sections were also made between cases grouped by cell counts obtained on transverse sections. Students t-test was used to assess differences between these groups.

Results

Estimates of intraobserver variability for delineation of reference areas were all less than 1%. On transverse sections, the mean count areas assessed per section were 1.33 mm^2 for the inner airway wall and 2.13 mm^2 for the total airway wall. On biopsies, the mean count areas assessed per section were 0.29 mm^2 for the inner airway wall and 0.12 mm^2 to a depth of 100μm below the basement membrane.

Relationships between mast cell density in endobronchial biopsies and adjacent airway transverse sections within cases

Table 1 shows mean intracase correlations between 2D and 3D mast cell densities in individual biopsies and 2D and 3D mast cell densities in corresponding airway transverse sections taken adjacent to the biopsy site. In

general, mean r values were low, with no consistent relationship between measurements regardless of the airway compartment measured or the type of measurement (2D or 3D) employed.

Relationships between case means of mast cell density in biopsies and adjacent airway transverse sections

Mean mast cell densities over the inner airway wall in biopsies showed consistent, significant positive correlation with mean mast cell densities over the inner airway wall on airway transverse sections (Table 2). There was no systematic difference between comparable biopsy counts and transverse section counts for the inner airway wall and differences were within Bland-Altman 95% limits of agreement in more than 90% of comparisons. This was irrespective of whether cell counts were done in 2D or 3D. In general however, the biopsy counts were not significantly correlated with mean mast cell density over the total airway wall on transverse sections.

Mean mast cell densities to a depth of 100μm below the basement membrane in biopsies showed no consistent, significant correlation with mean mast cell densities over the inner airway wall on airway transverse sections, and no significant correlation with mean mast cell densities over the total airway wall on airway transverse sections (Table 2).

The effect of using 1, 2, 3 or 4 endobronchial biopsies per case

For the inner airway wall, the density of mast cells based on a single endobronchial biopsy did not correlate with the case mean on transverse

sections (Table 3). Although mean 2D counts on biopsies showed some significant correlations with case means on airway transverse sections when 2 or 3 biopsies were used, correlation coefficients were not consistently significant for both 2D and 3D counts unless at least 4 biopsies were used to determine the case mean.

Relationship between mean 2D mast cell densities and mean 3D mast cell densities

In biopsies, mean 2D mast cell density was significantly correlated with mean 3D mast cell density for counts over both the inner airway wall (Figure 4a) and to a depth of 100μm below the basement membrane (Figure 4b). In transverse sections, mean 2D mast cell density was significantly correlated with mean 3D mast cell density for counts over both the inner airway wall (Figure 4c) and over the total airway wall (Figure 4d). In general, 2D and 3D counts on airway transverse sections were more strongly correlated than were 2D and 3D counts on biopsies.

Relative variability of 2D and 3D mast cell densities on endobronchial biopsies and airway transverse sections

Mean coefficients of variation (CVs) in mast cell density were similar for 2D counts (32%) and 3D counts (37%), but were significantly smaller for counts on transverse sections (30%) than for counts on biopsies (39%). On transverse sections, mean CVs were smaller for counts over the inner airway wall (23%) than over the total airway wall (39%). On biopsies, mean CVs were

similar for counts over the inner airway wall (39%) and to depth of 100μm below the basement membrane (39%).

Comparisons between case groups

The usefulness of biopsies to detect differences between case groups was examined by comparing cases grouped according to mast cell densities in the inner airway wall on all transverse sections. Both 2D and 3D mast cell counts over the inner airway wall on biopsies showed similar significant differences between the groups as did counts over the inner airway wall on transverse sections (Table 4). However, biopsy counts confined to a depth of 100μm below the basement membrane did not show significant differences between the two groups.

Discussion

Endobronchial biopsy has been widely used as a research tool to assess airway inflammation in asthma [3, 4, 6-16, 22]. Its use as a tool in clinical research has recently become the subject of review [20]. The present study attempts to address the issue of whether endobronchial biopsy specimens provide adequate representation of the distribution of inflammatory cells in the airway wall as a whole. It reports a direct comparison of the density of inflammatory cells in endobronchial biopsies and airway transverse sections from the same proximal airways, compares 2D cell profile counts with 3D stereological counts of airway inflammatory cells, and uses an optical disector to enumerate the density of airway inflammatory cells in actual 3D space. In

addition, it addresses empirically the issue of the minimum number of endobronchial biopsies required to adequately represent the density of inflammatory cells in proximal airways.

The study found that, for a given case, any single biopsy is unlikely to be representative of mast cell density across the airway wall in the vicinity of the biopsy site or in the proximal airways generally. However, four or more biopsies from different sites can be used to generate a case mean which is likely to be representative of mast cell density across the inner airway wall in proximal airways for the purpose of comparing cases. Both 3D stereological and 2D non-stereological methods of assessing mast cell density in proximal airways resulted in similar conclusions. There was little evidence that intercase comparisons of mast cell density in endobronchial biopsies parallel intercase comparisons of mast cell density across the total airway wall in proximal airways, or that measurements of mast cell density confined to a depth of 100μm or less below the basement membrane on endobronchial biopsies can adequately represent mast cell density across the remainder of the airway wall in proximal airways.

The use of a single endobronchial biopsy

A number of biopsy studies of airway inflammation appear to have relied upon the use of a single biopsy specimen [8, 13-15]. In the present study, mean intracase correlations between mast cell density in biopsy sections and adjacent airway transverse sections were very low. This suggests that within a case, any given endobronchial biopsy from a (sub)carina is unlikely to reflect

mast cell density across the airway wall generally in the vicinity of the biopsy site. Such a finding is not unexpected given previously observed intrasubject variations in the density of inflammatory cells within sections [37] and between sections [8, 38] from the same biopsy site, and between biopsy sized segments within an airway section [39].

Furthermore, correlation between mast cell density in one randomly chosen biopsy per case with the corresponding case mean of mast cell density in airway transverse sections did not suggest that a single biopsy is likely to adequately reflect mast cell density in the remainder of the bronchial tree for the purposes of intercase comparison. In this study single biopsies reflected, at best, about 30% of the variance in mean mast cell density in the inner airway wall on transverse sections from proximal airways.

These results are consistent with the finding in a recent study by Gamble *et al*. [40] that sampling more than one airway level is preferable in order to discriminate case groups in COPD on the basis of inflammatory cell density in bronchial biopsies.

Case means from multiple endobronchial biopsies

(i)The inner airway wall

The inherent difficulties and potential risks of taking multiple biopsies in vivo and the problem of occasional artefactual damage tend to restrict the number of biopsies used in research settings. Biopsy studies of airway inflammation have generally used only two or three biopsies per case to enumerate a

particular cell type [3, 4, 6-10, 12-16], and have often relied on 2D cell profile methods, expressing density as cells/area [4, 8-12, 14-16].

Results of the present study indicate a progressive strengthening of the relationship between mast cell counts in the inner airway wall on biopsies and transverse sections as more biopsies are used. Single biopsies reflected less than 30% of the variance in mast cell density in all transverse sections. However, this proportion increased as more biopsies were used to generate a case mean until, using four or more, it exceeded 50%, ranging upwards to about 70%. Single biopsies were not significantly correlated with transverse sections at all, while the only significant correlation between two biopsies and all transverse sections was confined to 2D counts in both, which are potentially biased. In contrast, 2D case means obtained by using three biopsies were significantly correlated not only with 2D counts on transverse sections, but also with 3D counts, which arguably give the best unbiased estimates of cell density for the proximal airways. However, the most consistent results were obtained using four biopsies or more, since mean mast cell densities in the inner airway wall on biopsies and transverse sections were significantly correlated regardless of whether 2D or 3D counts were used. These observations are supported by intraclass correlations, which tend to accentuate the differences between using four or more biopsies and fewer than four biopsies.

In essence, our findings suggest that mast cell density across the inner airway wall in proximal airways is well represented by the mean of four or more

biopsies. The mean of two or three biopsies may be adequate, but there is more uncertainty associated with the use of three biopsies than with four or more, and much more uncertainty with two. A single biopsy seems unsatisfactory.

(ii) The total airway wall

On the other hand, the results did not indicate that intercase comparisons of mean mast cell density over the inner airway wall on biopsies parallel intercase comparisons of mast cell density over the *total* airway wall on transverse sections to a similar degree. Even when using all available biopsies, the resulting case mean generally reflected only 30% to 40% of the variance in mean mast cell density over the total airway wall on transverse sections. In part, this would have been due to greater heterogeneity in the distribution of mast cells over the total airway wall in proximal airways when compared with the inner airway wall [23] as a result of differences between these compartments in the presence or distribution of structural elements such as smooth muscle, cartilage plates, submucosal mucous glands and blood vessels.

(iii) Cell counts to a depth of 100μm below the basement membrane on endobronchial biopsies

A number of biopsy studies reporting the density of airway inflammatory cells, including mast cells, have based their results explicitly on cell counts taken to a depth of approximately 100μm or less below the basement membrane [3, 6, 7, 16]. Establishing such a reference area in biopsies is almost always

possible as there is usually sufficient countable tissue present, and may be largely a matter of convenience since a ‘10 x 10’ eyepiece graticule represents a width of 100μm when using a x100 objective lens in light microscopy.

Results of the present study did not indicate that intercase comparisons of mean mast cell density based on such a reference area in biopsies parallel intercase comparisons of mast cell density over the inner airway wall generally, or the total airway wall generally, in proximal airways. Mean mast cell density to a depth of 100μm below the basement membrane on biopsies reflected, on average, only about 32% of the variance in mean mast cell density in the inner airway wall on transverse sections, and about 11% of the variance in the total airway wall. Comparison of mean mast cell densities based on this reference area in biopsies did not reflect the significant differences between subjects grouped by mean cell density in the inner airway wall.

Mast cells are widely distributed across the inner airway wall, including the smooth muscle layer [23]. Although some authors have implicitly endorsed cell counts in biopsies to a depth of 100μm - 125μm below the basement membrane, mostly for a variety of other inflammatory cells [40-42], a potential problem with using a narrow band of tissue of fixed absolute width when enumerating mast cells is that it will represent a variable fraction of the width of the inner airway wall at the biopsy site; generally a smaller fraction for larger airways. It may include the smooth muscle layer in the smaller proximal

airways, but not in the larger ones. It would therefore seem advisable when counting mast cells within the inner airway wall, to extend the count area to the outer border of the smooth muscle layer. However, while the probability of obtaining 'assessable' smooth muscle in an endobronchial biopsy specimen may be about 70% in segmental airways, it may be much less in the lobar bronchi [43, 44].

2D vs 3D counts

(i) Some theoretical and practical considerations.

Differences of opinion exist among investigators regarding the relative merits of using stereological, 3D methods as against non-stereological, 2D methods to assess the density of inflammatory cells in airway tissue [20, 45]. The two methods produce entirely different quantities. The use of uniform randomised sampling and 3D counting in thick histological sections with an optical disector enables a direct and unbiased estimate of the number density of cells within tissues, expressed as cells per unit volume. On the other hand, 2D counts provide an indirect indication of the number density of cells, based on cell profiles per unit area. Moreover, as demonstrated recently in a study comparing 2D and 3D quantification of inflammatory cells in bronchial biopsies by Fehrenbach *et al.* [26], such counts may be subject to bias since the probability of a cell's profile being included in a thin histological section is proportional to cell height, shape and orientation perpendicular to the plane of the section.

Counting cells in 3D with an optical disector requires the use of relatively sophisticated, specialised and expensive apparatus, such as the CASTgrid ® system (Olympus, Denmark) used in the present study. This consisted of an upright light microscope fitted with a video camera, a microcator and a motorised stage driven by a joystick; an XYZ reader; a computer with a high-resolution monitor, and associated CASTgrid software. The actual disector method of counting cells with this equipment is however, relatively simple (Figure 3). We found that counting cell profiles in 2D was simpler, faster and cheaper than counting in 3D with an optical disector, and by requiring only single thin sections, was more economical in the use of limited biopsy tissue. Thin sections were also less prone to tissue disruption in processing.

Nevertheless, we also found that some features of a stereology workstation could be used to good effect in counting inflammatory cell profiles in 2D, as well as counting in 3D: computerised delineation of the reference area, uniform random sampling of the reference area using the meander sampling facility, counting cell profiles with the aid of an unbiased counting frame, viewing serially presented high power fields (x1000 plus) on a high resolution monitor, use of the navigator facility for easy visualisation of field location within the reference area, marking counted cells with customised icons, and automatic logging of relevant count data.

Use of the above techniques in 2D counts does not rule out the potential bias which is inherent in such counts. However, delineation of reference areas at low magnification, followed by UR sampling and counting with an unbiased

counting frame and associated inclusion rules at high magnification, may rule out other subtle biases pertaining to identification and inclusion of cells when relying on more traditional manual 'randomisation' of fields and the use of an eyepiece graticule.

(ii) The findings of this study

As would be expected, 2D methods and 3D methods produced very different sets of numbers in the present study; the order of magnitude of mean mast cell densities being mostly 10^1 cells mm^{-2} for 2D counts, and 10^3 cells mm^{-3} for 3D counts. Nevertheless, corresponding 2D and 3D case means displayed very strong positive correlations. This relationship was generally tighter in airway transverse sections where mean 2D counts reflected, on average, about 86% of the variance in mean 3D counts in both the inner and total airway walls. In biopsies, mean 2D counts across the inner airway wall reflected about 74% of the variance in comparative 3D counts. In counts to a depth of 100μm below the basement membrane, this proportion was approximately 45%.

When all available biopsies were used, both 2D and 3D counts resulted in similar comparisons between case means on biopsies and case means on transverse sections. In these comparisons, the strongest correlations occurred between counts across the inner airway wall, where 2D and 3D counts on biopsies, were each closely related to both 2D and 3D counts on transverse sections. However, when only 3 biopsies were used to produce a

case mean, the relationship persisted for 2D counts on biopsies, but not for 3D counts.

It is also notable that when cases were grouped according to means of mast cell density across the inner airway wall on transverse sections, 2D and 3D counts resulted in the same grouping, and that comparisons between these groups were preserved in both 2D and 3D counts across the inner airway wall on biopsies.

In comparing 2D with 3D counts from the same reference area on histological sections, the ratio of (cells mm^{-2})/(cells mm^{-3}) provides a rough estimate of the mean cell height, perpendicular to the plane of the section [25]. Averaging this ratio for different count areas across cases gave an estimated mean cell 'height' for mast cells of ~ 11μm in biopsies and ~ 12μm in transverse sections. These figures are consistent with reported values for the diameters of mast cells in human lung tissue fixed in formalin [46]. The physical height of the relatively rounded, discrete mast cell thus accounts for the difference in order of magnitude between 2D and 3D mast cell densities.

Some issues regarding generalisability of the findings: the type of inflammatory cell used for investigation, disease states and proximal versus peripheral airways

The present study focussed on different methods for estimating inflammatory cell density in proximal airways. It used only mast cells for comparing these methods. Mast cells were chosen for initial study for three reasons: (i) they

are potent and constitutive inflammatory cells long associated with asthma and now the subject of renewed interest [47-49], (ii) they have been shown to stain clearly with the marker for mast cell tryptase (AA1) employed in the formalin fixed tissues used in our laboratory, (iii) we have found their counts to be highly reproducible and (iv) in proximal airways, they are distributed relatively widely across the airway wall, including the smooth muscle layer and mucous glands [23, 44], although their absolute density and level of activation may vary in different compartments and disease states [23].

Unlike many other studies reporting mast cell density in proximal airways, this study did not compare subjects with known disease (e.g. asthma) and control subjects. It could be argued that cases grouped on the basis of disease are likely to have a much greater range of data than that observed in this study, and it is therefore possible that fewer biopsies than the average of four suggested here would be sufficient to discriminate case groups. Due to differences in methods of measuring and/or reporting mast cell densities, it is difficult to compare the data from this study directly with the data from other studies examining mast cell density in proximal airways in disease states. However, in the few instances where a comparison seems possible, the order of magnitude of mast cell densities is similar, while the spread of data values in this study appears slightly greater than in some studies [12], and slightly less than in others [8, 23].

The results of this study were based data from only ten subjects. Had it been possible to use lung tissue from much larger numbers of subjects it is more

likely that case means based on fewer than four biopsies would have been correlated significantly with case means over the inner airway wall in all transverse sections; adding weight to the argument that two or three biopsies could be sufficiently representative of the inner airway wall generally in such circumstances. However, in biopsy studies of airway inflammation in asthma, the size of case groups representing disease states is typically of the order of ten subjects [3, 4, 7, 8, 11, 12, 15, 16, 19], rarely much more [6], and in the case of control subjects, often much less [4, 7-9, 11, 12]. On the other hand, increasing the number of subjects is unlikely to substantially alter '*r*' values, so that while four or more biopsies will probably reflect more than 50% of the variance over the inner airway wall generally, fewer than four biopsies will probably reflect much less.

The results we obtained will not necessarily be generalisable to other inflammatory cells, since their distributions vary from that of the mast cell. For example, the density of neutrophils and activated eosinophils in the submucosa is not only much less than that of mast cells [6, 8], but their density relative to that of mast cells appears to vary according to sub-compartments such as mucous glands [34] and airway smooth muscle [50]. Lymphocyte numbers are much higher in the airway wall [6, 24] and their density is subject to much greater variability [51]. Furthermore, the mast cell appears to be by far the predominant inflammatory cell within the layer of smooth muscle [52]. Therefore, when counting other inflammatory cells, the inclusion or exclusion of smooth muscle in the measurement area may have a significant effect on overall cell density.

Finally, the density and distribution of inflammatory cells in the small peripheral airways may differ from that observed in the larger proximal airways [23, 24]. Therefore the relationship between inflammatory cell density in endobronchial biopsies and that in the remainder of the bronchial tree and throughout the lung requires further study.

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Table 1. Intracase correlations of mast cell densities.

Biopsies	Count type	*vs*	Transverse sections	Count type	r values * mean (range)
WAi	2D	*vs*	WAi	2D	-0.08 (-0.85 to 0.91)
WAi	2D	*vs*	WAt	2D	0.09 (-0.38 to 0.45)
100μ deep	2D	*vs*	WAi	2D	-0.02 (-0.58 to 0.54)
100μ deep	2D	*vs*	WAt	2D	0.07 (-0.42 to 0.92)
WAi	3D	*vs*	WAi	3D	0.23 (-0.95 to 0.99)
WAi	3D	*vs*	WAt	3D	-0.33 (-0.93 to 0.48)
100μ deep	3D	*vs*	WAi	3D	0.32 (-0.39 to 0.99)
100μ deep	3D	*vs*	WAt	3D	0.37 (-0.97 to 0.78)
WAi	2D	*vs*	WAi	3D	0.15 (-0.54 to 0.65)
WAi	2D	*vs*	WAt	3D	-0.03 (-0.79 to 0.62)
100μ deep	2D	*vs*	WAi	3D	-0.03 (-0.79 to 0.89)
100μ deep	2D	*vs*	WAt	3D	-0.06 (-0.85 to 0.93)
WAi	3D	*vs*	WAi	2D	0.22 (-0.72 to 0.92)
WAi	3D	*vs*	WAt	2D	-0.25 (-0.90 to 0.98)
100μ deep	3D	*vs*	WAi	2D	0.18 (-0.47 to 0.70)
100μ deep	3D	*vs*	WAt	2D	0.14 (-0.42 to 0.99)

Abbreviations: WAi = inner wall area, WAt = total wall area, 100μ deep = to a depth of 100μm below basement membrane, 2D = two dimensional (cells/mm^2), 3D = three dimensional (cells/mm^3), r = Pearson correlation co-efficient

***** Fisher Z weighted mean

Table 2. Intercase correlations of mean mast cell densities using all biopsies per case and all transverse sections per case

Biopsies		*vs*	Transverse sections					
Count Area	Count type		Count area	Count type	r	p_r	ICC	p_{ICC}
WAi	2D	*vs*	WAi	2D	0.73	< 0.05	0.75	< 0.005
WAi	2D	*vs*	WAi	3D	0.70	< 0.05		
WAi	3D	*vs*	WAi	2D	0.83	<0.005		
WAi	3D	*vs*	WAi	3D	0.72	< 0.05	0.68	< 0.01
WAi	2D	*vs*	WAt	2D	0.43	ns		
WAi	2D	*vs*	WAt	3D	0.61	ns		
WAi	3D	*vs*	WAt	2D	0.65	< 0.05		
WAi	3D	*vs*	WAt	3D	0.60	ns		
100μ deep	2D	*vs*	WAi	2D	0.74	< 0.05		
100μ deep	2D	*vs*	WAi	3D	0.31	ns		
100μ deep	3D	*vs*	WAi	2D	0.63	ns		
100μ deep	3D	*vs*	WAi	3D	0.49	ns		
100μ deep	2D	*vs*	WAt	2D	0.48	ns		
100μ deep	2D	*vs*	WAt	3D	0.37	ns		
100μ deep	3D	*vs*	WAt	2D	0.23	ns		
100μ deep	3D	*vs*	WAt	3D	0.17	ns		

Abbreviations: ICC = intraclass correlation coefficient, p_r = probability of Pearson r, p_{ICC} = probability of intraclass correlation co-efficient, ns = not significant. Other abbreviations as for Table 1.

Table 3. Intercase correlations of mast cell densities within the *inner airway wall* using the count of 1 randomly selected biopsy per case, or the mean of 2, 3 or 4 randomly selected biopsies per case

Biopsies			*vs*	Transverse sections					
Number used	Count Area	Count type		Count area	Count type	*r	p_r	ICC	p_{ICC}
1	WAi	2D	*vs*	WAi	2D	0.50	ns	0.45	ns
1	WAi	2D	*vs*	WAi	3D	0.55	ns		
1	WAi	3D	*vs*	WAi	2D	0.35	ns		
1	WAi	3D	*vs*	WAi	3D	0.13	ns	0.12	ns
2	WAi	2D	*vs*	WAi	2D	0.65	< 0.05	0.58	< 0.05
2	WAi	2D	*vs*	WAi	3D	0.60	ns		
2	WAi	3D	*vs*	WAi	2D	0.61	ns		
2	WAi	3D	*vs*	WAi	3D	0.49	ns	0.47	ns
3	WAi	2D	*vs*	WAi	2D	0.67	< 0.05	0.55	< 0.05
3	WAi	2D	*vs*	WAi	3D	0.70	< 0.05		
3	WAi	3D	*vs*	WAi	2D	0.60	ns		
3	WAi	3D	*vs*	WAi	3D	0.57	ns	0.60	< 0.05
4	WAi	2D	*vs*	WAi	2D	0.73	< 0.05	0.73	< 0.005
4	WAi	2D	*vs*	WAi	3D	0.65	< 0.05		
4	WAi	3D	*vs*	WAi	2D	0.82	< 0.005		
4	WAi	3D	*vs*	WAi	3D	0.68	< 0.05	0.68	< 0.01

Abbreviations as for Table 2.

* Correlations were with case means over the inner airway wall on all transverse sections.

Table 4. Comparison of grouped case data* using mean mast cell densities in transverse sections and in biopsies

Count Area	Count type	Lower 4 cases (mean cell density)	Upper 6 cases (mean cell density)	t	p
X – WAi	2D	65	108	4.252	< 0.005
X – WAi	3D	5038	10779	5.057	< 0.001
B – WAi	2D	73	106	2.447	< 0.05
B – WAi	3D	6839	11632	3.225	< 0.05
B - 100μ	2D	92	118	1.549	ns
B - 100μ	3D	8309	13137	1.871	ns

Abbreviations: X-WAi = Inner wall area on airway transverse sections, X-WAt = total wall area on airway transverse sections, B-100μ = to a depth of 100μm on biopsies, B-WAi = inner wall area on biopsies

* Data grouped by mean mast cell densities in WAi on transverse sections. Grouping by 2D counts produced same grouping as by 3D counts: Lower 4 cases *vs* Upper 6 cases. 2D values are cells/mm^2; 3D values are cells/mm^3.

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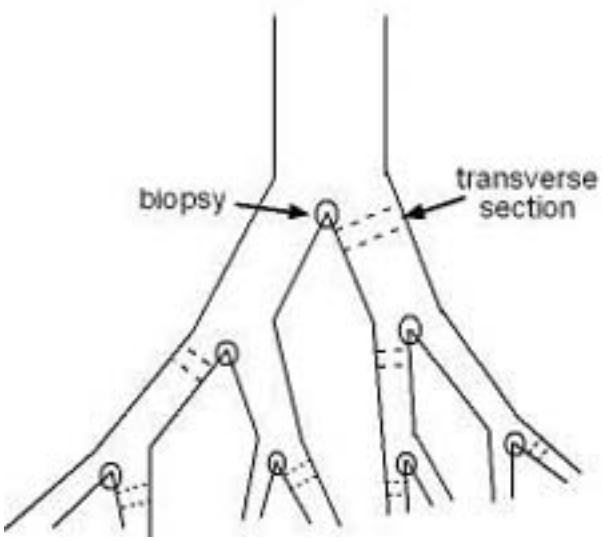

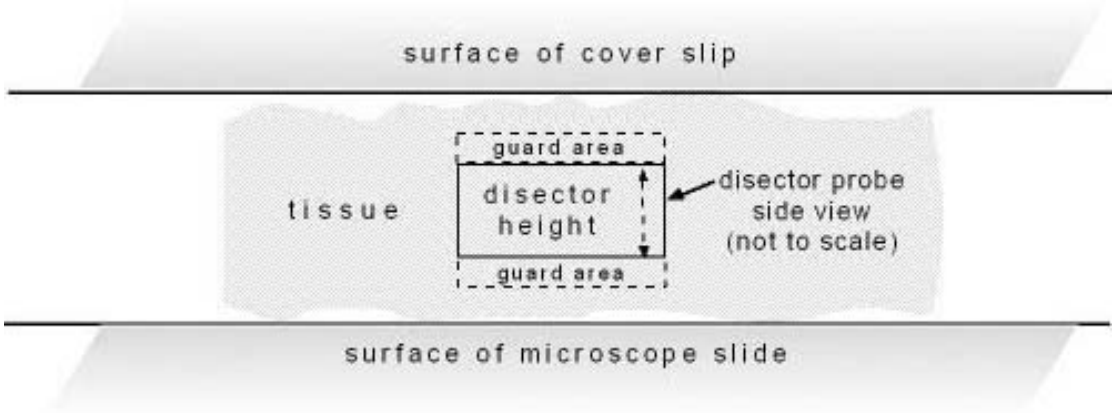

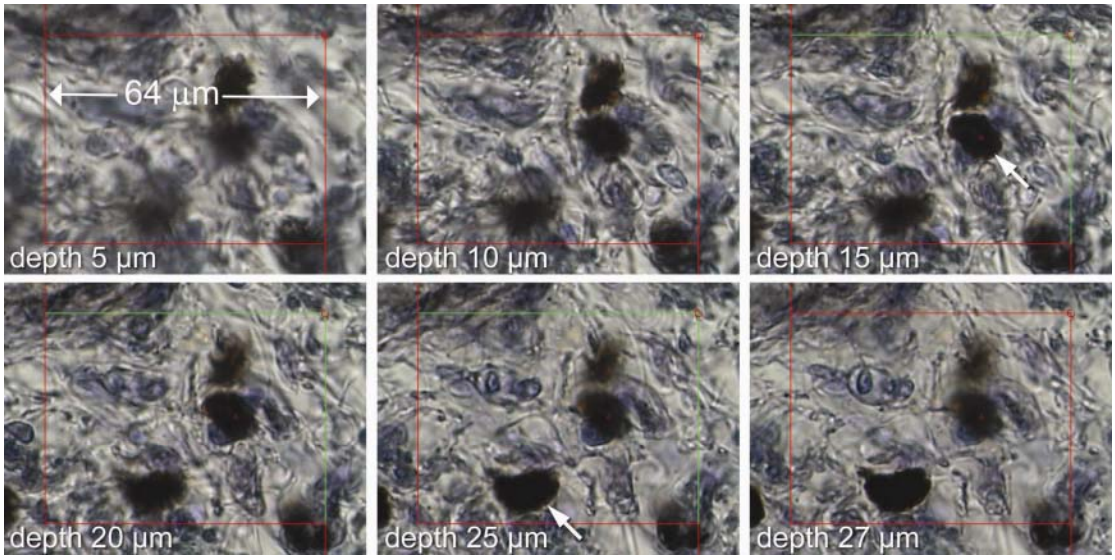
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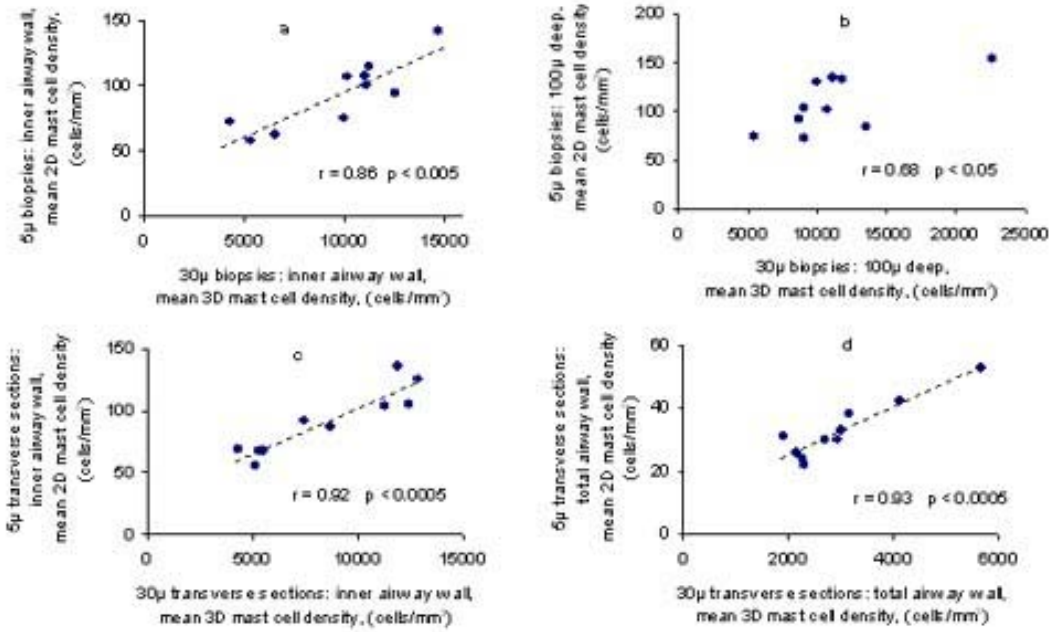
a
5µ biopsies: inner airway wall, mean 2D mast cell density, (cells/mm²)
r = 0.86 p < 0.005
30µ biopsies: inner airway wall, mean 3D mast cell density, (cells/mm³)
b
5µ biopsies: 100µ deep, mean 2D mast cell density (cells/mm²)
r = 0.68 p < 0.05
30µ biopsies: 100µ deep, mean 3D mast cell density, (cells/mm³)
c
5µ transverse sections: inner airway wall, mean 2D mast cell density (cells/mm²)
r = 0.92 p < 0.0005
30µ transverse sections: inner airway wall, mean 3D mast cell density, (cells/mm³)
d
5µ transverse sections: total airway wall, mean 2D mast cell density (cells/mm²)
r = 0.93 p < 0.0005
30µ transverse sections: total airway wall, mean 3D mast cell density, (cells/mm³)