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Lung immunopathology in cases of sudden asthma death

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Lung immunopathology in cases of sudden asthma death. J.L. Faul, V.J. Tormey, C. Leonard, C.M. Burke, J. Farmer, S.J. Horne, L.W. Poulter. ©ERS Journals Ltd 1997.

ABSTRACT: The histopathology of airway inflammation in rare cases of sudden asphyxic asthma death (SAAD) is unclear. This study examines, for the first time, the relative disposition of lymphocyte and macrophage subsets and eosinophils in proximal and distal tissues of such cases.

Multiple resection specimens from five cases of SAAD were studied. Tissue blocks were obtained at necroscopy and immediately frozen in liquid nitrogen within 18 hours of death (death occurring within 1 h of the onset of an unprovoked asphyxic asthma attack). After immunohistological staining, frozen sections underwent semi-quantitative analysis (cell counts per unit area) for T-cells, macrophages and eosinophils using computerized imaging systems. Subsets of T-cells and macrophages were estimated using double immunofluorescence techniques. Variability within samples, between samples and between cases was compared.

These cases of fatal asthma showed infiltrates of T-cells, macrophages and eosinophils within peribronchial tissues. Distinct from stable asthma, a CD8+ T-cell dominance was found. A high proportion of eosinophils were activated (EG2+), whereas the relative proportion of antigen-presenting cells (RFD1+) did not seem to be abnormal, although numbers of these cells were high. These features were seen both in proximal and distal tissues. The variability of these parameters within an individual was 9.4–15.2%, however, the variability between individual cases was greater.

Sudden asphyxic asthma is associated with inflammatory infiltrates both of proximal and distal lung tissues. In contrast to stable asthma, this infiltrate contains large numbers of CD8+ T-cells, suggesting distinct qualitative as well as quantitative characteristics in the immunopathology of sudden asthma death.
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The postmortem appearance of the lung in asthma death is characterized by bronchial epithelial shedding, mucosal oedema and mucous plugging [1, 2]. Chronic peribronchial inflammation is a consistent finding; however, the histological appearance of the bronchial mucosa may be inflamed, oedematous or even normal in different areas [3–6]. Such variation may reflect the heterogeneity within populations of patients with airway obstruction, rather than true variation of airway inflammation [1, 2]. Previous immunohistological studies of paraffin-embedded tissues have compared slow onset fatal asthma and the clinically distinct sudden asphyxic asthma, and have shown significant differences in terms of neutrophil and eosinophil infiltration [7].

The immunopathology of the bronchial wall of asthmatics has been shown to have the features of a chronic T-cell mediated immunological reaction [8–10]. These observations were made using frozen sections of bronchial biopsies taken from proximal airways under direct vision through the bronchoscope. Other studies of asthma using bronchoalveolar lavage (thought to obtain material which reflects the situation in distal airway) have either shown no evidence of heightened cell-mediated immunity [11], or a modest lymphocytosis [12]. Little is known, therefore, of the state of the distal tissues in asthmatics or the distribution of inflammation in this condition, nor is it known whether the small endobronchial biopsies being used to investigate this inflammatory process are truly representative of the overall state of the endobronchial tissues within the lung.

Distribution of inflammation throughout asthmatic airways can only be extensively investigated postmortem, in those rare cases of asthma death. It is from such investigations that the original descriptions of chronic inflammation associated with this disease were made [1, 2]. These observations, and indeed more recent studies [13, 14], have been restricted mainly to histopathological stains, with which it was not possible to fully dissect the subsets of immunocompetent cells involved, due to the limited number of monoclonal antibodies (MoAbs) available for use on formalin-fixed tissue [13]. Where MoAbs have been used [15], increased numbers of T-cells and eosinophils have been reported. To use a full range of such techniques postmortem, fresh frozen material needs to be obtained rapidly after death to prevent postmortem changes or fixation possibly compromising the localization of antigens identified by immunocytochemical or immunofluorescence staining. In the current study, multiple resection specimens were frozen at necroscopy from five cases of sudden asthma

death (sudden asphyxic asthma death (SAAD); death within 1 h of symptoms). All samples were obtained within 18 h of death.

The aims of this study were to describe the immunopathology associated with strictly defined cases of acute asphyxic fatal asthma, and to compare the inflammatory processes in proximal and distal airway at postmortem. Furthermore, the variability of inflammatory parameters recorded from this semiquantitative approach was tested in individual and multiple samples from defined areas of the lung.

Subjects and methods

Using a 24 h telephone and fax alert system, all cases (aged 20–55 yrs) of asthma death were prospectively collected from 1992 to 1994 in the areas of Co. Dublin, Co. Kildare and Co. Meath, Ireland. Details of cases studied are presented in table 1.

Only cases that met the following rigorous criteria were studied: history of pre-existing asthma (defined by clinical records of prior asthma attacks, or treatment for chronic asthma); clinical history attributing death to an attack of asthma (defined as complaint of and observed shortness of breath during the terminal episode); autopsy lung pathology and coroner's report consistent with asthma as the cause of death; sample collection within 18 h of death; duration of final asthma attack less than 1 h; absence of an identifiable precipitating factor (aeroallergen, mental or physical stress, recent infection, drug ingestion); and death outside of hospital. Cases who had a recent viral infection (within 3 months); a recent asthma exacerbation (apart from the final attack, within 3 months); aspirin, betablocker or isocyanate-induced asthma; cardiac disease or other respiratory disease; or a recent change in asthma medication (within 3 months), were excluded.

Tissue samples were collected postmortem, all within 18 h of death, following consent from the coroner and relatives. Multiple samples (0.5 cm^3) of proximal airways were obtained from second generation right upper lobe, right middle lobe and right lower lobe bronchi. Samples of distal airway were obtained by resecting multiple blocks of lung tissue approaching from the pleura. Tissue samples of peribronchial lymph nodes were also resected, to be used as control sections for the immunohistological techniques.

Table 1. – Details of cases studied

Case No.	Sex	Age yrs	Medication	Case summary
1	F	43	T, H, TN, TN, SM, NS, IS, OS	"Catastrophic" asthma Multiple severe attacks requiring mechanical ventilation. Nonsmoker.
2	F	20	S (*p.r.n.*)	Mild asthma. One previous near fatal attack requiring mechanical ventilation. Nonsmoker.
3	M	20	S (*p.r.n.*)	Mild asthma. Nonsmoker.
4	M	45	OS, IS, T, I&F	Two previous severe asthma attacks. Nonsmoker.
5	F	51	IS, S	"Brittle" asthma. Previous smoker (5 cigarettes·day^{-1} for 7 yrs).

F: female; M: male; S: salbutamol; OS: oral steroids; IS: inhaled steroids; T: theophylline; H: antihistamine; IN: nebulized ipratropium; TN: nebulized terbutaline; SM: salmeterol; NS: nasal steroid; I&F: ipratropium and feneterol; *p.r.n*: as needed basis.

Tissue preparation

Samples were placed in "cryoembed" medium (Brights Inst. Co., Huntingdon, UK) on small cork discs and snap frozen in isopentane cooled in a bath of liquid nitrogen. These were stored in liquid nitrogen until used. Sections, 6 μm thick, were cut on a cryostat and collected onto poly-L-lysine coated slides. These were air-dried for at least 1 h, fixed in chloroform acetone (1:1) for 5 min, wrapped in cling film, and stored at -20°C until used. To document the histopathology, some samples were fixed in formal saline and paraffin wax sections were prepared. Sections of human palatine tonsil were prepared as above and used for MoAb reagent controls.

Histology

Sections from paraffin-embedded tissue were stained with haematoxylin and eosin to reveal the histopathology and confirm that proximal and distal samples were collected. Distal tissue was defined as the areas surrounding small airways (<1 mm internal diameter), and alveoli.

Immunohistology

The indirect immunoperoxidase method [14] was used to identify the presence of T-lymphocytes (using a mixture of CD2+, CD5+, CD7+ and CD8+ MoAbs), B-lymphocytes, (using a mixture of CD19+ CD20+ MoAbs), monocytes and macrophages (using CD68+ MoAb), eosinophils (using EG1+ MoAb) and activated eosinophils (using EG2+ MoAb). A peroxidase conjugated goat anti-mouse immunoglobulin was used as the second layer and development included the use of 3,3,diaminobenzidine tetrahydrochloride (DAB) as the chromogen. All sections were counterstained with Harris's haematoxylin. Negative controls were employed, omitting the first layer of monoclonal antibody, and positive controls were employed, using sections of human palatine tonsil where specificity and appropriate dilution of the monoclonal antibody was determined. Control staining, using irrelevant monoclonal antibodies of the same isotype, was also employed to test the specificity of the staining on the lung tissues. Parallel staining of sections from peribronchial lymph nodes obtained at necroscopy was also performed, to confirm that no postmortem changes were causing artefactual results.

To determine the CD4:CD8 ratio of T-cell subpopulations and the proportions of macrophage subsets, combinations of monoclonal antibodies were used in double

immunofluorescence methods [16]. For the T-cell subsets, the primary antibody employed was a combination of CD4+ (mouse immunoglobulin G (IgG)) and CD8+ (mouse immunoglobulin M (IgM)) MoAbs, followed by a second layer containing a mixture of affinity purified goat anti-mouse IgM conjugated to fluorescein isothiocyanate (FITC), and goat anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC), (both from Southern Biological Associates, Alabama, USA). To determine the distribution of macrophage subsets, a combination of monoclonal antibodies RFD1 (mouse IgM) and RFD7 (mouse IgG) were employed, with subsequent use of affinity purified anti-IgM and anti-IgG second layers used as described above. These latter two reagents have been demonstrated, both in this laboratory and others, to identify antigen-presenting cells (RFD1+, RFD7-), mature phagocytes (RFD7+, RFD1-), and suppressive macrophages (RFD1+, RFD7+) [17–22].

Quantification

For each immunohistological stain, duplicate sections from each block were examined using semiquantitative methods (numbers of cells per unit area). Areas with evidence of infiltration were identified and random high power fields observed. Positive cells were point counted in frame defined areas using image analysis systems [20]. As the proximal and distal samples from each of the lung lobes showed different histology, comparison was made using semiquantitative methods by estimating numbers of immunocompetent cells related to area of tissue [20]. In the case of the proximal samples, computerized image analysis equipment was used to draw frames around the subepithelial lamina propria of the tissue where inflammatory infiltrates were evident, but avoiding airway lumen, muscle, cartilage and clearly identifiable areas of collagen/elastin. In the case of the distal tissue (tissue surrounding small airways <1 mm diameter) areas of lung parenchyma exhibiting inflammation were framed on the computer, avoiding the airway lumen, and vessels (see Results). The lumens of many small airways were noted to be packed with mucous plugs and cellular debris, and the airway lumen was excluded from the semiquantitative analysis in all cases. Immunofluorescence staining was quantified using a Zeiss fluorescence microscope, with epi-illumination and appropriate barrier filters for FITC and TRITC. In the case of CD4: CD8 ratios, counts of positive lymphocytes (FITC+ and TRITC+) were made visually over multiple high power fields (not less than five) where T-cells were seen.

To determine relative proportions of macrophage subsets, more than five high power fields were observed on each section. Using the FITC and TRITC barrier filters, individual cells were scored as: RFD1+ (+ve on green); RFD7+ (+ve on red); and RFD1+RFD7+ (positive on red and green). Relative proportions were then calculated with the formula:

$$\frac{\#\ \text{Specific subset}}{(\text{RFD1+})+(\text{RFD7+})+(\text{RFD1+RFD7+})} \times 100$$

Statistics

The numbers of T-cells identified by immunoperoxidase staining was the parameter selected as a model of distribution of immunocompetent cells. T-cells per unit area were counted in multiple sections from a single sample, multiple samples from a single subject and separate samples from the five different subjects investigated. The results from these studies are presented to show the full range of results.

The results quantifying cell distribution between proximal and distal tissue and different lung lobes is presented as mean and standard deviation. When comparing these results, significance was determined using Student's t-test. A p-value equal to or less than 0.05 was considered significant.

Results

Histopathology

Haematoxylin and eosin and toluidine blue staining revealed mononuclear cell infiltrates in all samples examined. These were evident within the lamina propria of the proximal tissues and within the interstitium around the terminal airways in the distal samples. Eosinophils were also consistently seen, particularly in the proximal samples (fig. 1a). Mucous plugs were observed in many airways, together with collections of cells. Disruption of the epithelium was evident in the majority of proximal samples, with epithelial "shedding" also observed. In the majority of samples, extensive thickening below the epithelial basement membrane was also seen.

Immunohistology

Immunopathology in proximal and distal airways. The availability of postmortem material offers the opportunity of comparing the disposition of immunocompetent cells both in proximal and distal tissues of the lung. The major population of cells identified both in proximal and distal tissue were T-lymphocytes and macrophages (figs. 1b and c and fig. 2). Greater concentrations of both cell types were seen in the proximal tissue (T-cells 10.8 cells·unit area^{-1}, macrophages 9.8 cells·unit area^{-1}, compared to distal tissue (T-cells 7.5 cells·unit area^{-1}, macrophages 3.5 cells·unit area^{-1}) (fig. 2). The difference in T-cell numbers was not statistically significant, whilst the macrophage numbers were significantly greater in the proximal samples ($p<0.01$) compared to distal samples. The combination of CD19 and CD20 monoclonal antibodies staining B-cells failed to identify any significant numbers of these cells in any sample investigated (data not shown).

Although a minor population compared to T-cells and macrophages, eosinophils (MoAb EG1+) were present in significantly greater numbers in proximal compared to distal tissue (fig. 2). In the mucosa of proximal tissue, they appear diffusely distributed throughout the lamina propria, with small accumulations being noticeable adjacent to the epithelial basement membrane. The

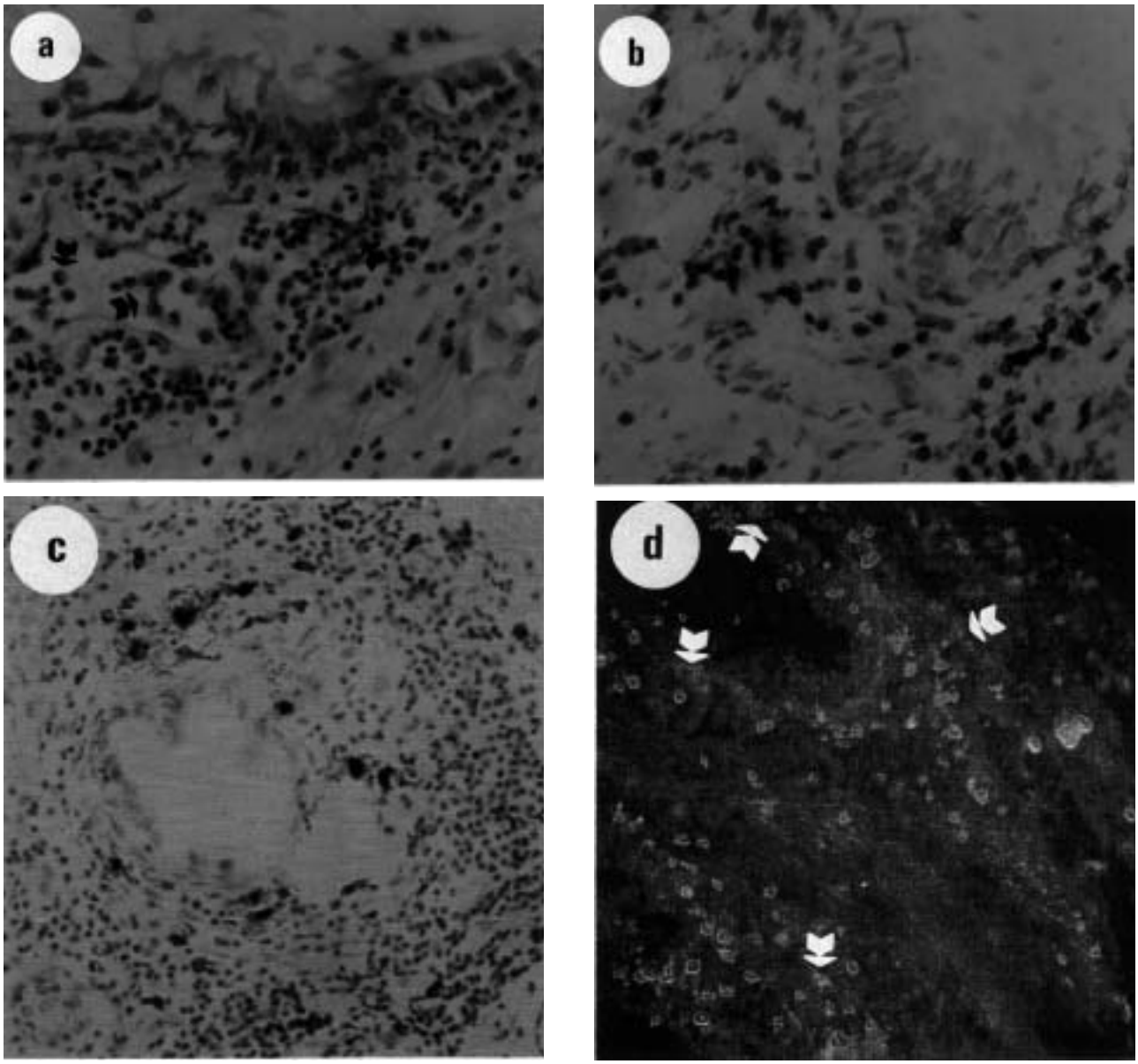

Fig. 1. – Examples of all staining/cell analysis used in this study of the immunopathology of asphyxic asthma. All samples postmortem. a) Proximal airway sample showing accumulation of inflammatory cells in the lamina propria, epithelial shedding, and thickening below the epithelial membrane, with several eosinophils visible (⮝). (Haematoxylin and eosin staining; scale bar = 25 μm). b) T-cells (mixture of CD2+, CD5+, CD7+ and CD8+) in proximal lung tissue (Indirect immunoperoxidase staining; scale bar = 25 μm). c) Macrophages (CD68+) in distal lung tissue. Macrophages are seen around a terminal airway amidst an accumulation of lymphocytes. (Indirect immunoperoxidase staining; scale bar = 40 μm). d) Proximal tissue, double immunofluorescence using monoclonal antibodies (MoAbs) CD4+ and CD8+ (CD8+ cells red, CD4+ cells green). A clear dominance of CD8+ cells is visible. Only small numbers of CD4+ cells are present (⮝). (Scale bar = 40 μm).

majority appeared activated, as evidenced by positive staining with EG2 monoclonal antibody: 60% of total eosinophils in proximal tissues and 75% in distal samples (fig. 2).

Subset analysis using double immunofluorescence techniques revealed that the majority of T-cells both in proximal and distal tissue were of the CD8+ subset, creating an inverse CD4:CD8 ratio (table 2 and fig. 1d). This was most marked in the proximal tissues (median CD4:CD8 ratio 1:6), but was also present in the interstitium of 4 out of 5 of the distal samples investigated (median CD4:CD8 ratio 1:2).

Distribution of macrophage subsets showed a dominance of RFD1+ RFD7- antigen-presenting cells in proximal tissues (mean 50% of all positive macrophages) with minor populations of RFD1- RFD7= tissue phagocytes and RFD1+ RFD7+ suppressive macrophages (mean 19 and 31% of total stained macrophages, respectively) (fig. 3). This distribution was slightly different in the distal tissues, where a higher proportion of RFD1- RFD7+ tissue macrophages was recorded (mean 24%). This increase being at the expense, proportionately, of the RFD1+ RFD7+ subset, which exhibited a mean value of 21% of all stained macrophages (fig. 3). Care was taken when framing distal areas for image analysis not to include the lumen of terminal airways, which were frequently seen to contain clusters of RFD1- RFD7+ macrophages within what appeared to be mucous plugs.

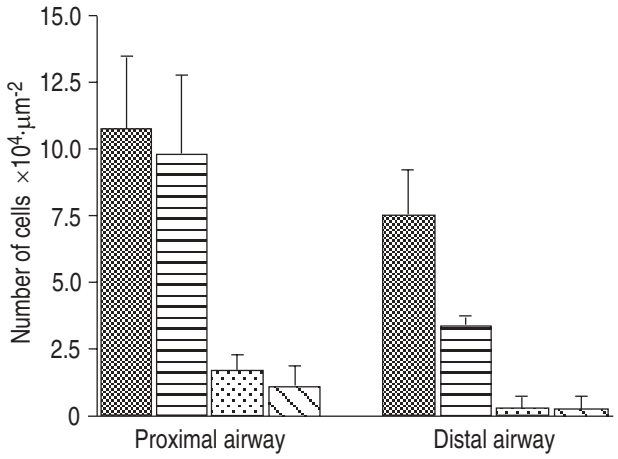

Fig. 2. – The numbers of T-cells ( ), macrophages ( ), eosinophils ( ) and activated eosinophils ( ) in proximal (second generation right middle lobe bronchus) and distal (small airway diameter of <1 mm) lung tissue. Cells were identified using the indirect immunoperoxidase method. Values are presented as mean±SD.

Table 2. – CD4:CD8 ratios+ in sudden asphyxic asthma death (SAAD)

Case No.	Proximal airway	Distal airway
1	1:8	1:10
2	1:5	1:3
3	1:3	1:2
4	1:5*	3:1
5	1:8	1:3

Counts were performed of multiple fields in sites of inflammation. +: correct to nearest whole numbers; *: "follicle" of CD4+ cells seen but not recorded in the ratio.

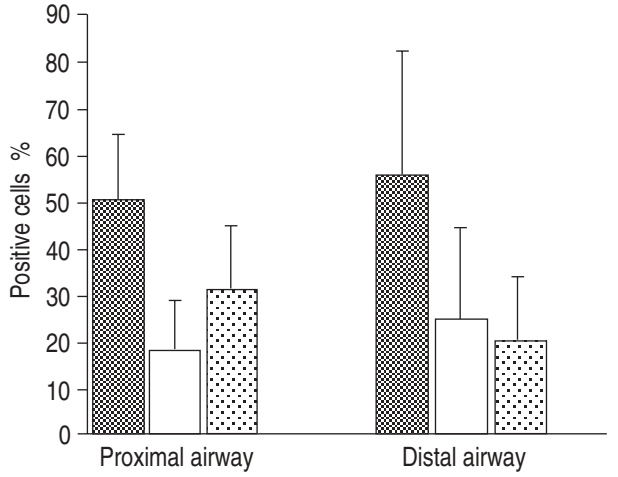

Fig. 3. – The relative proportions of monoclonal antibodies (MoAbs). RFD1+ inducer ( ); RFD7+ effector [( );] and RFD1+/RFD7+ suppressor ( ) macrophages in proximal (second generation right middle lobe bronchus) and distal (small airway diameter of <1 mm) lung tissue taken postmortem. Cells were identified using double indirect immunofluorescence. Values are presented as mean±SD.

Frozen sections of peribronchial lymph node taken simultaneously at postmortem were also analysed. Without exception, these lymph node sections revealed a normal distribution of T-cells in paracortical areas and B-cells in follicular areas. The CD4:CD8 ratio was estimated at 2:1. No evidence of "diffusion" of reaction product or any apparent postmortem associated artefact was seen.

Variability. In order to determine sample variability, the individual data for each patient is presented in table 3. Using immunoperoxidase staining of T-cells as a "model" of immunohistological technique the variability of cell distribution in the samples used was investigated. Initially, one section from one frozen block of proximal tissue was prepared and five separate areas framed with the image analyser, all exhibiting a typical inflammatory cell infiltrate as determined by haematoxylin counter-staining. Numbers of T-cells per unit area in each of these five areas was calculated (fig. 4). These five areas showed a median T-cell incidence of 9.7 cells·unit area^{-1}, with a range of 8.1–10.6 cells·unit area^{-1}. Subsequently, one sample of proximal tissue was taken and 40 sections were prepared. This is the normal number of sections cut from frozen samples of lung tissue for routine analysis in this laboratory. From this series, eight sections were selected at intervals of five sections throughout the series, and T-cell immunoperoxidase staining repeated. The numbers of T-cells per unit area was quantified on each section and results compared. This study revealed a remarkable consistency throughout the sections, with less than a 10% variability overall. Results ranged from

Table 3. – A comparison of the incidence of immunocompetent cells in the bronchial mucosa of proximal airway after sudden asphyxic asthma death (right middle lobe bronchus)

Case No.	Cells ×10⁴·µm⁻²*				Relative proportions#		
	T-cells	CD68+	EG1+	EG2+	D1+	D7+	D1+& D7+
1	14.5	9.7	2.3	1.83	51	28	18
2	9.6	10.8	2.2	1.9	62	10	31
3	12.6	14.2	1.9	1..2	48	13	35
4	7.8	6.4	0.7	0.1	54	23	24
5	9.3	8.02	1.6	0.6	35	20	45

*: semiquantitative image analysis of inflammatory infiltrates; #: relative proportions of macrophage subsets discriminated using double immunofluorescence methods.

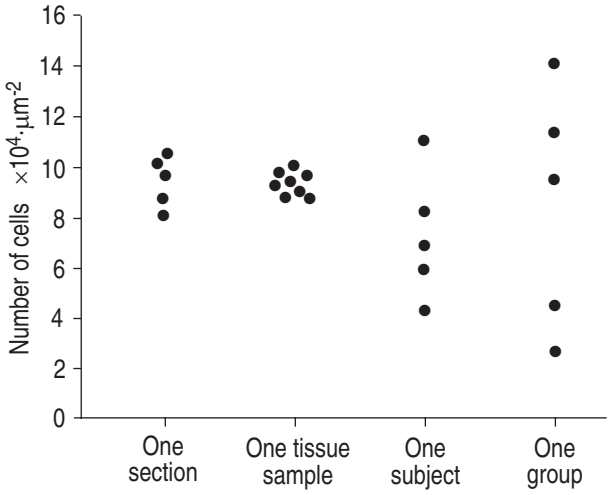

Fig. 4. – Variability in T-cell counting between areas of a section ("One section"), sections of a sample ("One tissue sample"), samples from a subject ("One subject"), or between subjects in the group ("One group"). Cells were point counted in framed areas using image analysis systems (see Methods).

a median of 8.8 cells·unit area^{-1} in one section to 10.1 cells·unit area^{-1}, the median being 9.4 cells·unit area^{-1}.

Sections were then anaylsed in an identical manner taken from five tissue samples of the same subject. When these were compared, a greater variability was seen: the median number of cells per tissue sample in this analysis was 6.9, and the results ranged 4.4–11.1. The range of these results, thus, fell outside that of the selected sections from a single block, showing that variability between individual biopsies, even from the same subject, was greater than that within any given sample. Tissue samples of proximal areas (right middle lobe) from each of the 5 different cases of asthma death were then compared. This analysis showed the greatest variation of all (range 2.7–14.2 cells·unit area^{-1}). Within the five samples, the median T-cells per unit area was calculated as 9.6. This investigation, thus, shows that analysis of groups of samples from different individuals encompasses the inherent variability within and between samples from any one subject, thus the documentation of results (median and range from the five subjects investigated) was taken to reflect the overall variability within the techniques of the investigation.

Discussion

This study of postmortem tissue from cases of sudden asthma death confirms the observations of DUNNIL [1] that evidence of inflammation characterized by mononuclear cell infiltration is present throughout the tissues of the lung. Quantification of T-cells macrophages and eosinophils within proximal and distal tissue in such cases demonstrates a significant accumulation of these cells that is greater (in terms of mean figures) than that previously reported from biopsies of proximal tissue from cases of stable asthma [8, 23], yet consistent with other studies of sudden asthma death [7]. The presence of a significant inflammatory infiltrate in the distal tissues of these cases demonstrates that the accumulation of inflammatory cells in asthma is not restricted to the bronchial wall of major airways. It is possible that distal inflammation might explain those cases where BAL has identified a T-cell lymphocytosis in asthma [12]; although in the majority of asthmatics lavage findings are relatively normal [24]. The absence of consistent reports of a restrictive defect in stable asthmatics would suggest that any inflammation in distal areas is minimal. It would seem unlikely, however, that the degree of cellular infiltration seen in distal tissues in the present study only occurred acutely prior to these catastrophic episodes. Thus, the presence of some inflammation around terminal airways in stable asthmatics would seem likely.

The observation that T-cells, antigen-presenting cells and eosinophils appear to represent the major cell types within this inflammatory reaction is consistent with the observations made on stable asthmatics [8, 9]. However, the inverse CD4:CD8 ratios identified in the current study, the relatively normal proportions of macrophages with a suppressive phenotype and the significant number of activated eosinophils identified, all indicate that the immune mechanisms at play associated with asthma death are not identical to those seen in biopsies taken from patients with stable asthma. In particular, the increased accumulation of CD8+ T-cells is quite distinct from the situation seen previously in endobronchial biopsies from asthmatic patients [8–10, 22, 23]. This observation has led to a more extensive analysis of the CD8+ T-cells. Furthermore, the distribution of macrophage subsets in the proximal tissue does not reflect that seen in stable asthma [25], where a significant reduction in normal levels of suppressive macrophages has been identified as a possible key factor in pathogenesis [26]. Thus, on immunopathological grounds, asphyxic asthma is not simply an exaggeration of an existing immune abnormality. Clinical studies suggest that some asthmatics may suffer catastrophic symptoms, even while on optimal therapy [27, 28]. This study describes inflammation in asphyxic asthma death, which is distinct from stable asthma and which occurs in all cases of SAAD, irrespective of corticosteroid therapy.

A previous report described eosinophils and neutrophils in sudden asthma death [7]. The present results are consistent with the data on eosinophils (described as lower than in slow onset attacks), yet we saw no significant number of neutrophils in the current investigations. These cells are described as "raised" in the submucosa of the three subjects investigated previously [7]. However these three cases all involved intubation and attempts at cardiopulmonary resuscitation (CPR) prior to death, which, unlike the current cases, occurred in hospital.

The fact that no inverse CD4:CD8 ratio was seen in the samples of lymph node taken from the cases would discount the possibility that changes to cell surface antigen expression were a postmortem effect or a result of a more rapid decay of the CD4-associated antigen following death. The investigation of variability within different sections taken from the tissue block, different blocks from the same subject, and, indeed, between subjects demonstrates that the variability is much greater between subjects than the variability either between blocks of tissue or between separate sections from within a single sample. These results indicate that, even in an extremely select group of asthmatic patients, considerable variation exists between subjects, therefore subject variabi lity carries more importance than sample variability. Furthermore, it is reassuring that good consistency is seen between sections from a single sample. However, these results clearly demonstrate that it is important to take into account the variability demonstrated here between tissue blocks from the same area and sections from the same tissue in studies when repeat biopsies are undertaken from the same individual. Furthermore, the variability seen here reveals that although median values for the incidence of specific cells within this group of samples is greater than that previously seen in stable asthmatics (see above) the range recorded is consistent. The variability may be explained by the widespread distribution of inflammation in proximal and distal tissues; quantitative evidence for which has not previously been reported.

Together, the results described in these five cases of sudden asthma death, with no obvious acute provocating factor, indicate that the mechanisms within the lung's immune system are different to those seen in stable asthma and may be associated with these catastrophic events. This possibility is being investigated further.

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