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Diagnostic value of cytology of bronchoalveolar fluid for lung diseases of sheep

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SUMMARY

The diagnostic utility of bronchoalveolar lavage (BAL) fluid cytology for common ovine lung diseases was examined. BAL fluid was collected at necropsy from 113 ovine lung specimens and total and differential cell counts analysed in relation to the presence of concomitant lung pathology identified at the gross and histologic level. The diffuse lung diseases, maedi and sheep pulmonary adenomatosis, were both characterised by an increase in overall cellularity with increased percentages of lymphocytes and neutrophils respectively. Focal parasitic lung disease was characterised by an increase in the percentage of eosinophils and mast cells. Consolidated lung lesions were characterised by a slight increase in cellularity but no change in the differential cell profile. Non-lesional areas of parasitic and consolidated lungs were also assessed. In both instances the differential cell profile was consistent with that identified in association with focal lung pathology although the slight increase in cellularity noted for consolidated areas was not reflected in non-lesional areas. A decision tree was developed to facilitate interpretation and indicate the likely predictive capacity of ovine BAL fluid differential cytology. The results highlight the uncertainty associated with predicting presence or absence of lung disease on the basis of BAL fluid differential cytology. A 'normal' profile can exist in the presence of lung pathology and likewise, an 'abnormal' profile can exist in the absence of gross or histopathological evidence of lung pathology.

INTRODUCTION

Bronchoalveolar lavage (BAL) provides a means to collect the cellular and acellular constituents of the airway surface liquid in the lung. Although the assessment of differential cell profiles has potential diagnostic utility in clinical veterinary medicine there is currently a dearth of information in relation to interpreting abnormal cell profiles in small ruminants.

This study was undertaken in order to characterise bronchoalveolar fluid cytology associated with various naturally occurring ovine lung diseases. The presence of disease was confirmed at post-mortem examination and pathological entities comprised sheep pulmonary adenomatosis (SPA), maedi, parasitism and pneumonic consolidation.

A concomitant aim was to determine whether, in diseases characterised by areas of discrete pathology, any identified cytopathological abnormality extended throughout the lung. This latter aim directly addresses the uncertainty surrounding the interpretation of bronchoalveolar cytology derived in a 'blind' fashion, wherein the specific location of the sampled region and/or its relationship to areas involving pulmonary pathology is unknown.

MATERIALS & METHODS

POST MORTEM MATERIAL

Lungs were sourced from sheep presented for necropsy examination at this institution, and from Sandyford Abattoir, Paisley, UK. The sheep varied in breed, age and sex, and were derived from farms representing a cross-section of the husbandry conditions present in

Scotland and the North of England at the present time. Only animals that had been euthanased immediately prior to necropsy examination were used in the project. The BAL fluid and tissue samples were collected within 20 minutes of death. Lungs with no gross or histological evidence of pathology were used as controls. Gross lung pathologies were identified on a preliminary basis and classified under the following headings - SPA, maedi, parasitism and pneumonic consolidation.

LUNG LAVAGE PROCEDURE

Following identification of gross pathologies the trachea and major airways were carefully dissected to reveal the airway opening of the 2nd generation bronchus serving the affected area. 50ml Hanks balanced salt solution (HBSS)(Gibco BRL, Paisley, UK) diluted 1 in 10 with purite water was instilled and recovered following gentle massage of the lavaged lung tissue. Each BAL fluid sample was filtered through 4 layers of gauze and centrifuged at 500g for 5 minutes. The supernatant was discarded and the pellet resuspended in 5ml of diluted HBSS. Where possible, a further sample was derived from an area of lung with no discernible gross pathology – henceforth referred to as ‘non-lesional’. In addition lavage fluid was separately collected from randomly selected areas of normal lungs for comparison.

TISSUE SAMPLING

Following collection of BAL fluid, tissue samples were derived from the same lung segments and fixed in 10% buffered formalin. Following fixation, tissue blocks were routinely processed and embedded in paraffin wax. Serial 4-5 µm sections cut from the embedded tissue and stained with haematoxylin and eosin for routine histological assessment served to confirm, or otherwise, the preliminary gross pathological classification.

DIFFERENTIAL CYTOLOGY

Cells were counted using a Neubauer haemocytometer and values expressed per millilitre BALF. Cytocentrifuge slides were prepared and stained using Leishman's stain. Differential counts were carried out on 400 cells. Cells were classified as neutrophils, macrophages, eosinophils, lymphocytes or mast cells according to standard morphological criteria. Cells not classified in the aforementioned groups were recorded as 'miscellaneous' cells.

STATISTICAL ANALYSIS

Between-lung comparisons were made using the Mann Whitney non-parametric test for two independent groups. Within-lung comparisons (lesional vs non-lesional) were made using the Wilcoxon two-sample paired signed rank test. A value of $P < 0.05$ was accepted as indicating statistical significance. Statistical calculations were made using Minitab 13 software (Minitab Ltd., Coventry, UK). Decision tree analysis was used to develop a classification tree for interpreting ovine BAL fluid differential cytology. The classification assigned on the basis of histopathological examination represented the 'target' variable, the value of which was modelled and predicted as a function of the total and differential cell count data, the 'predictor' variables. An entropy splitting algorithm was used to split nodes in the tree and the tree was pruned to the number of nodes that produced the minimal error in a 10-fold cross-validation process. No *a priori* values were assumed in relation to the proportion of each classification for the population from which the samples were drawn.

RESULTS

Overall, 113 sets of lungs were examined. Of these, 44 were considered grossly and histologically normal, 12 had pathology consistent with maedi, 9 with SPA, 27 with parasitism and 21 with pneumonic consolidation.

Lungs with pathological features of maedi were characterized grossly by the presence at necropsy of large, heavy lungs which did not collapse fully when opening the chest cavity. Histologically the lesion was widespread with no distinction between affected and non-affected lobes. The microscopic lesion consisted of extensive lymphofollicular proliferation of perivascular and peribronchiolar lymphoid tissue. Some of the proliferative lymphoid follicles contained germinal centres. There were often less prominent secondary features such as interstitial inflammation and bronchiolitis.

Lungs with SPA were characterized grossly by the presence at necropsy of large, solid, heavy lungs which, in contrast to maedi, contained a large quantity of mucus in the airways. Histologically there was papillary proliferation of cuboidal or columnar alveolar epithelial cells forming an adenomatous-type pattern. The affected cells consisted of type-II alveolar epithelial cells and non-ciliated bronchiolar epithelial cells. There was often accompanying secondary bronchiolitis and/or bronchopneumonia.

Lungs with evidence of parasitism were characterized grossly by the presence at necropsy of multifocal subplueral grey/brown nodules throughout the dorsal and caudal lungs. Histologically this corresponded to multifocal granulomatous lesions with a predominance of eosinophils and macrophages. At the centre of each lesion there were sections of parasites or accumulations of effete eosinophils and neutrophils or, in more chronic cases, foci of calcification. Although a more specific diagnosis was not made in each instance it was recognised that most lesions were consistent with the presence of *Meullerius capillaris*.

Lungs with pneumonic consolidation were characterized grossly by the presence at necropsy of dark red or purple areas of collapse of the lung parenchyma. There was often a sharp line of demarcation between the affected and unaffected lung. Histologically this consisted typically of areas of consolidation, involving alveolar collapse, alveolar and bronchiolar inflammation and interstitial fibrosis. Whilst this lesion was distinct from the other disease entities in this study it was accepted that the aetiology of this lesion was multifactorial and therefore the non-specific classification of pneumonic consolidation applied.

Within the categories where lesions were identified, 14 lungs with evidence of parasitism and 8 with pneumonic consolidation had 'non-lesional' areas of lung from which additional samples were taken.

Total and differential cell count data, and the results of statistical comparisons are shown in table 1.

The diffuse lung diseases, maedi and SPA, were characterised by a striking and significant increase in cellularity with the increase being more pronounced for maedi than for SPA. In the case of SPA, the differential profile was characterised by an increase in neutrophils and decrease in macrophages, whereas maedi was characterised by a slight but significant increase in the percentage of lymphocytes.

The principal changes in the bronchoalveolar cell population that characterised parasitic lung disease, namely an increase in the proportion of eosinophils and/or mast cells, extended throughout the lung and were not simply confined to areas of the lung considered abnormal at

the gross and histological level. A significant decrease in the percentage of alveolar macrophages was detected in areas with lung pathology with a similar but non-significant trend apparent in non-lesional areas ($P=0.084$).

Analysis of total cell count data in consolidated lungs revealed an increase in cellularity relative to normal lungs with no abnormality in the differential cell profile. However, although within-lung analysis demonstrated that the differential profile was consistent throughout the lung, comparison of non-lesional segment data with normal lung data failed to demonstrate any difference in overall cellularity. This anomaly probably reflects the limited numbers of consolidated lungs used in this study. Regardless of this discrepancy it is clear that there was no significant abnormality in the differential cell profile of consolidated lungs, whether sampling directly from the lesion or not.

The predictive decision tree is shown in figure 1. The prediction success for the original dataset is shown in table 2. No features of differential cytology were identified that would serve as predictors of lung consolidation. Cross-validation, a technique that averages the performance of the tree against a series of randomly derived independent 'test' datasets, indicates the likely performance of the original model on a future independent set of ovine BAL fluids derived under similar conditions. The cross-validation procedure indicates that using the tree to offer predictive diagnoses of parasitism, SPA, maedi and the presence of no abnormality would be associated with misclassification rates of 37, 57, 10 and 30% respectively (Figure 1).

DISCUSSION

As with human clinical medicine (Pickles and others 2003), collection of BAL fluid from small ruminants in the UK generally involves sedation or anaesthesia, procedures that would offer an inherent risk to animals with a degree of respiratory compromise. As such its clinical utility is quite limited. In contrast, a perusal of recent literature indicates that this procedure is currently widely used in small ruminant lung research (Abraham and others 2000, Collie and others 2001, Ahmed and others 2000, Dunphy and others 2002, Fujimoto and others 2002, Bischof and others 2003). Indeed a considerable body of information exists in relation to ovine lung physiology and immunology, the comparative aspects of which are of ongoing relevance to human lung disease research.

With this background we considered it appropriate to collate information in relation to defining the BAL cytology associated with the common ovine lung diseases. This information will serve as an aid to clinicians or researchers in interpreting such cytology wherein the BAL procedure is conducted in a broadly similar fashion in the living animal. The related aim of determining whether, in diseases characterised by discrete pathology, the BAL cell profile extends throughout the lung is of wider relevance to all those interested in the pathophysiology of lung disease.

As will be appreciated lungs were categorized on the available best evidence of the predominant pathology at gross and histopathological levels. Such categorization is necessarily simplistic in that the possible presence of minor concurrent disease processes is ignored. Whilst it is acknowledged that this compromise may have confounded the accuracy of the classification it was regarded as the most practical way

of relating bronchoalveolar lavage cytology to the principal pathologic entity likely to feature on an independent veterinary pathologists report.

We demonstrated that although both maedi and SPA were characterised by increased cellularity, these diseases could be broadly distinguished on the basis of their differential cell profile, particularly in relation to the greater proportion of neutrophils present in SPA. It should be noted however that previous studies have demonstrated that natural infection with MVV is also associated with a neutrophilic response (Cordier and others 1992, Legastelois and others 1997). The reasons behind such a discrepancy are not clear but may relate to differences at the level of the target population or in the methodology employed in the collection and/or analysis of BAL fluid.

Parasitic lung disease was definitively characterised by changes that affected the whole lung implying that it is unlikely that the presence of disease would be missed in the event that bronchoalveolar lavage was not optimally targeted to areas with pathology. In contrast bronchoalveolar lavage cytology would be of negligible use in detecting the presence of consolidated lungs. Indications were that any increase in cellularity was likely confined to areas of pathology and was not associated with any shift in the differential cell profile.

Interest in classification and regression tree analysis techniques has intensified over the last decade with its tree-building ability being of direct relevance to those involved in clinical research (Selker and others 1995, Barriga and others 1996, Rainer and others 1999, Thoenner and others 2003, Markey, Tourassi, and Floyd, Jr. 2003, Gerger and Smolle 2003). The analysis, involving binary recursive partitioning, results in a regression tree that is relatively

simple and logical to interpret, in contrast to multivariate regression models which yield regression coefficients that can be awkward to use in clinical practice.

From the point of view of decision-making one fundamental question, frequently asked, is “whether, on an individual basis, BAL fluid cytological analysis can reliably predict the presence of the common ovine lung diseases?” The data indicate that even in the presence of a cytological profile that would suggest the absence of disease, 30% of such cases will have lung pathology i.e. there is considerable overlap between normal BAL profiles and those associated with disease. In the case of profiles suggestive of parasitism, 37% of these will be derived from lungs with no evidence of parasitism. More certainty surrounds a profile suggestive of maedi with only 10% of these being misclassified. In the case of profiles suggestive of SPA, 57% will be derived from lungs with other pathologies. Thus there is considerable uncertainty associated with any prediction made on the basis of BAL fluid differential cytology.

Naturally the severity of the disease will presumably influence the extent of overlap between normal and diseased lung profiles and future studies will attempt to stage these disease processes in order to further define the predictive limits of ovine BAL fluid cytological analysis. Further, recent advances in bioinformatic and proteomic technology will in future likely provide the means to more clearly differentiate such diseases on the basis of BAL fluid constituent analysis.

In conclusion, this data should serve to indicate that ovine BAL fluid cytology should be interpreted with caution. A ‘normal’ profile can exist in the presence of lung pathology and

an 'abnormal' profile can exist in the absence of gross or histopathological evidence of lung pathology.

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