Bronchoalveolar Lavage of Cranial and Caudal Lung Regions in Selected Normal Calves: Cellular, Microbiological, Immunoglobulin, Serological and Histological Variables

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ABSTRACT

Of a group of 30 clinically normal male Holstein calves two to eight weeks of age, six two week old and six four week old calves met various radiographical and clinicopathological criteria for normality. Bronchoalveolar lavage was performed by fiberoptic bronchoscopy on cranial and caudal lung regions in all 30 calves and samples analyzed for free cells, microorganisms, and immunoglobulins. Lateral chest radiographs and lung biopsies were also conducted on each calf. Calves were euthanized and necropsied ten days after bronchoalveolar lavage was conducted. Reported in this paper are results from the 12 normal calves.

Microorganisms were present in small numbers in the lower respiratory tract of some normal calves. There were no differences in the above parameters between cranial and caudal lobes. There were statistically significant changes in bronchoalveolar lavage cell proportions with age although there were no detectable differences in clinical signs. Four week old calves had a lower percentage of macrophages and a higher percentage of epithelial cells than two week old animals ($p < 0.05$). There was also a trend toward an increased percentage of neutrophils in older calves but this was not significant ($p > 0.05$). Total bronchoalveoolar lavage protein also appeared to increase with age ($p < 0.05$). In both groups a higher proportion of IgG_2 in bronchoalveolar lavage compared to serum was found, suggesting the presence of a local selective transfer mechanism into respiratory secretions.

RÉSUMÉ

Parmi un groupe de 30 veaux mâles Holstein, apparemment normaux et âgés de deux à huit semaines, six de deux semaines et six de quatre semaines rencontraient divers critères radiographiques et clinico-pathologiques de normalité. On effectua un lavage des régions pulmonaires crâniales et caudales des 30 veaux, par bronchoscopie fibreoptique, et on rechercha des cellules libres, des microorganismes et des immunoglobulines, dans l'eau de lavage. On prit aussi des radiographies latérales du thorax et des biopsies pulmonaires de chacun des veaux. Au bout de dix jours, on procéda à leur euthanasie et à leur nécropsie.

Cet article rapporte les résultats obtenus, chez 12 veaux normaux. Les voies respiratoires inférieures de certains d'entre eux recelaient un nombre plutôt restreint de microorganismes. On n'enregistra pas de différences relatives aux paramètres précités, entre les lobes pulmonaires crâniaux et caudaux, mais des changements significatifs du point de vue statistique, dans le nombre de cellules de l'eau de lavage, selon l'âge, en dépit de l'absence de différence détectable dans les signes cliniques. Les veaux âgés de quatre semaines affichaient moins de macrophages et plus de cellules épithéliales que leurs congénères âgés de seulement deux semaines ($p < 0,05$). On enregistra aussi une tendance non significative à l'augmentation des neutrophiles, chez les veaux âgés de quatre semaines ($p < 0,05$). La quantité de protéine sembla également proportionnelle à l'âge des veaux ($p < 0,05$). On constata aussi la présence d'une plus grande quantité d'IgG_2 que dans le sérum, indice d'un mécanisme de transfert sélectif, dans les sécrétions respiratoires.

INTRODUCTION

Bronchoalveolar lavage (BAL) is a potentially useful technique for assessing the health and disease state of the respiratory system of calves. Constituents of BAL include components of lower respiratory tract defense mechanisms as well as microbial inhabitants of the lower respiratory tract. However, until values for the various measurements which can be conducted on BAL from calves highly selected to fulfil the definition of normality are established, and lung state confirmed by necropsy, the baselines of this technique will not be available.

In the late seventies, Wilkie and Markham (1) introduced bronchoal-

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veolar lavage in live cattle as a safe and repeatable research tool which has subsequently been utilized in numerous other studies for investigation of cellular and noncellular immune events in the lower respiratory tract of cattle (2-5).

Normal cellular components of bovine BAL were initially reported to consist of approximately 60% alveolar macrophages with lymphocytes and neutrophils composing the remainder (6). Later reports, however, suggested that closer to 90% of recovered cells in normal bovine BAL were alveolar macrophages (4,7). The alveolar macrophage plays a central role in lower respiratory tract defense (8). Its function in this defense is intimately dependent on interactions with lymphocytes (9) that can change markedly with differing ratios of macrophages to lymphocytes (10). Hence, it is important that the actual normal cell constituents in the bovine BAL be confirmed.

The differences in results of differential cell counts obtained on BAL in previous studies may have been due to variation in the relative health status of the respiratory tracts of animals studied. Pulmonary normality in most earlier studies was primarily determined by clinical examination of the animals or visual evaluation of excised lungs, both of which have been shown to lack sensitivity in detecting respiratory disease in calves (11,12).

In most studies of BAL samples were obtained from whole lung, which pools samples from cranial and caudal areas, or from caudal lung areas whereas in bovine respiratory disease the majority of exudative lesions occur in the cranial portions of the lung (13). Studies in humans indicate regional heterogeneity of BAL constituents (14). In light of the high degree of anteroventral localization of bovine respiratory disease, a more detailed examination for lobar differences in bovine BAL constituents is warranted. It would be of interest as well to examine these parameters of respiratory defense at an early age which coincides with lowest immunity (15) and peak occurrence of pneumonia in Holstein calves (16).

Part of the challenge to lower respiratory tract defenses comes from inhalation of potential pathogens in nasopharyngeal flora, an aspect of respiratory disease well studied in cattle (17-19). According to prior studies (20) pathogenic microorganisms should not be present in the lower respiratory tract of normal animals. However, the results of recent studies conflict with previous work in the identification of potential pathogens in the lungs of normal calves (21,22). The presence of microorganisms in the lower respiratory tract can decrease the efficiency of pulmonary clearance mechanisms (23) and influence the resident lavageable cell population (5). Investigation of lung microflora in healthy calves is therefore necessary for comparison with BAL cell populations and lung disease states. In addition, should BAL in calves become a useful diagnostic tool it would be desirable to document normal airway microflora as well as the level of nasopharyngeal contamination anticipated.

The overall objective of this study was to compare various components of BAL from normal calves with findings at necropsy. The cellular, immunoglobulin and microbial components of BAL from the cranial and caudal lobes of the lungs of normal calves were examined for regional and age-related differences.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Over a one year time span 30 male Holstein calves were obtained at birth from selected farms after they had suckled colostrum. Total serum protein was measured in each calf by a refractometer with values above 50 g/L acceptable as evidence of adequate colostral transfer (24). Calves were housed in individual stalls. They were fed 2.5 L whole milk twice daily, given water *ad libitum*, bedded on wood shavings and monitored daily for clinical signs. Alfalfa hay was fed free choice from two weeks of age.

The calves were divided into the following age groups for study; Group I — two weeks old (n = 10), Group 2 — four weeks old (n = 10), Group 3 — eight weeks old (n = 10).

The criteria for accepting calves into the study were: (a) absence of detectable abnormalities on general clinical examination and on detailed physical examination of the respiratory tract (25); (b) absence of pulmonary pathology on a left lateral thoracic lung radiograph of the calf in lateral recumbency which included the cranial lung region; (c) a complete blood count (CBC) within normal acceptable limits; and (d) less than 10% neutrophils in any one lung wash.

This final criterion was necessary as high numbers of polymorphonuclear cells in the lung suggests pulmonary inflammation (26,27) and will reflect subclinical respiratory problems.

STUDY DESIGN

Table I outlines the procedures used. The calves were housed in isolation several days in advance of anticipated use. The day following initial clinical examination, chest radiography and blood sampling, the calves underwent bronchoalveolar lavage of a cranial and an ipsilateral caudal lung region. This BAL procedure coincided with the calf reaching an age of two, four or eight weeks. Percutaneous lung biopsies utilizing a biopsy needle (Tru-Cut® Biopsy Needle, Travenol Laboratories, Deerfield, Illinois) were obtained three days post-BAL (Day 4) from the lobes

TABLE I. Experimental Design

Day 0	Clinical examination
	Chest radiography
	Hematology, Serum for : Immunoglobulin analysis
	: Acute titers
Day 1	Bronchoalveolar lavage cranial and ipsilateral caudal lobe
Day 4	Percutaneous lung biopsy cranial and caudal lung lobes (as examined on day 1)
	Hematology
Day 10	Clinical examination
	Chest radiography
	Serum — convalescent titers
	Necropsy

undergoing BAL. This time delay from BAL was necessary to allow physiological responses due to lung washing to subside (28). Biopsy specimens were compared to BAL and necropsy findings.

On day 10, detailed respiratory examination (25), lateral chest radiography and serum collection were repeated. Calves were then euthanized with intravenous sodium pentobarbital (150 mg/kg) and necropsied.

BRONCHOALVEOLAR LAVAGE

Calves were tranquilized with xylazine (Rompun, Bayvet Division, Miles Laboratories Ltd., Rexdale, Ontario) at 0.25 mg/kg subcutaneously and restrained in sternal recumbency on a surgical table.

Following nasopharyngeal mucus sampling with 15 cm swabs (Culturette®, Canlab, American Hospital Supply Canada Inc., Mississauga, Ontario) the bronchoscopy proceeded as follows. A flexible fiberoptic bronchoscope (Olympus GIF Type XP, Olympus Optical Co. Ltd., Tokyo, Japan) which had been previously sterilized with a 2% glutaraldehyde solution (Sporocidin®, Sporocidin Company, Washington, D.C.) was used. The bronchoscope was passed through the nostril and visually guided to become wedged in the first or second bronchial division of the bronchus corresponding to the caudal segment of the right or left cranial lung lobe. A plastic sheathed cytology brush (Model B C-5/9, Olympus Corporation, Tokyo, Japan) with a sealed tip (Gelfoam®, Upjohn, Don Mills, Ontario), previously sterilized with ethylene oxide, was passed through the bronchoscope biopsy channel, the bronchial mucosa brushed under direct visualization and the brush retracted and retrieved from the bronchoscope. The brush was then placed in a sterile tube containing 2 mL isotonic saline and the sample submitted for bacterial, fungal and mycoplasmal culture.

The lung lobe was then lavaged with a total of 250 mL isotonic sterile saline held at 37°C. The fluid was administered slowly in three aliquots and aspirated immediately after each infusion. Collection was accomplished by an adjustable vacuum pump attached to the sidearm of the collecting flask with 5 mm Hg negative pressure. At completion of the cranial lobe lavage the bronchoscope was withdrawn from the airways, the biopsy channel and working length chemically disinfected, then rinsed with sterile distilled water.

Sampling of the caudal lung lobe was then performed similarly using a separate sterile protected biopsy brush and collecting flask. The distal end of the bronchoscope was guided to become wedged in the mainstem bronchus at approximately the sixth bronchial division of the caudal lung lobe.

Typically, lavage of each lung lobe was completed within 5 min.

The amount of fluid recovered per lavage was recorded and aliquots obtained for cytology, as well as bacterial, fungal and mycoplasmal culture. The remaining fluid was centrifuged at 400 x *g* for 10 min, the cell fraction discarded and the supernatant stored at -70°C.

CYTOLOGY

A 10 mL aliquot of lavage fluid was processed within 30 min of collection. The total cells were enumerated by an electronic cell counter (Coulter ZBI, Hialeah, Florida). The lower threshold was set at 13 dial divisions according to initial trials indicating bovine BAL cell counts showing a plateau at that level. The upper threshold was set at >100 dial divisions, the 1/Aperture current switch set at 0.354 and the 1/Amplification switch set at 0.1 according to calibration standards for cattle (Ontario Veterinary College, Guelph, Ontario). The cell count recorded was an average of two separate counts. The sample was then centrifuged at 400 x *g* for 10 min, the supernatant decanted and saved for protein determination. The cell pellet was reconstituted in 0.5 mL saline and used to prepare air dried slides which were stained with a modified Wright's stain by an automated stainer (Hema-Tek Slide Stainer, Miles Laboratories Inc. Elkhart, Indiana) and for nonspecific esterase as modified for ruminants by Osbaldiston *et al* (29). The cell differential for each lavage was determined by counting 500 cells on the Wright's stained slides with the reader blinded as to the source or location of the slides. Cell viability was determined by trypan blue exclusion.

PROTEIN, ALBUMIN AND IMMUNOGLOBULINS

The protein content of the supernatant from the cytology aliquot was determined by a colorimetric micromethod (30).

The stored lavage supernatant was processed in a manner similar to Wilkie and Markham (6) by initial dialysis against distilled water to desalt (Spectrapor® Membrane, Spectra/Por 4, Spectrum Medical Industries Inc., Los Angeles, California), then lyophilized and reconstituted to 30 g/L in saline. Albumin, IgG_1, IgG_2 and IgA in both lavage protein and day 0 serum samples were measured by radial immunodiffusion (31) using commercial standards, immunodiffusion plates and antisera (Miles Scientific, Miles Laboratories, Rexdale, Ontario). Specificity of each antiserum was confirmed by immunoelectrophoresis against bovine serum. The anti-IgA was produced by collection of sera from rabbits previously inoculated with bovine IgA and purified by insolubilization against appropriate Ig classes (32).

The immunodiffusion plates were read with an immunodiffusion reader (Bio-Rad Reader, Bio-Rad Laboratories (Canada), Mississauga, Ontario) after 24 h incubation at room temperature for albumin, IgG_1 and IgG_2 and after 48 h incubation for IgA.

BACTERIOLOGY, MYCOLOGY AND MYCOPLASMOLOGY

Nasopharyngeal samples and four separate samples per lung lobe (brush biopsy fluid and pellet of centrifuged fluid, BAL fluid and cell pellet of centrifuged BAL fluid) were plated on blood agar (5% citrated calf's blood), chocolate agar, MacConkey's agar and Sabouraud dextrose agar. Control samples of lavage saline passed through the bronchoscope biopsy channel before the initial lavage (cranial lobe) as well as after disinfection and rinsing prior to caudal lobe lavage were plated on blood agar and MacConkey's agar. Blood and chocolate agar plates were incubated for 48 h at 37°C in 10% CO_2, MacConkey's agar in air at 37°C for 48 h and Sabouraud dextrose agar at room temperature for 14 days.

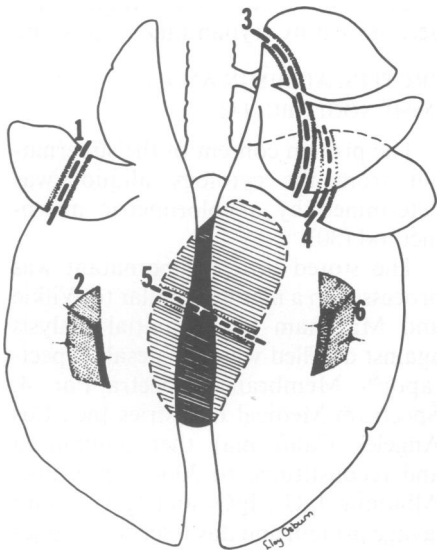

Fig. 1. Sites of lung lobe sections taken at necropsy for histological evaluation: 1 = caudal portion of left cranial lobe, 2 = centre of left caudal lobe, 3 = cranial and caudal portions of right cranial lobe, 4 = right middle lobe, 5 = accessory lobe, 6 = centre of right caudal lobe.

Bacterial and fungal colonies when present were identified (courtesy Dr. J. Prescott, University of Guelph, Guelph, Ontario) and quantified as scanty numbers (1-9 colonies), moderate numbers (10-30 colonies) or large numbers (> 30 colonies).

The same samples were cultured for mycoplasmas by Dr. S. Rosendal (University of Guelph, Guelph, Ontario) for 10-14 days and colonies were identified by immunofluorescence.

NECROPSY

Prior to opening the chest the trachea was sealed with a clamp. The lungs were carefully removed from the chest, photographed, then fixed by infusion of the pulmonary artery with 10% neutral buffered formalin solution at 32 cm of water pressure. They were held at 4°C and sectioned 24 h later after allowing partial fixation. Lung sections were obtained according to a predetermined protocol that sampled the central areas of all six lung lobes (Fig. 1). Specimens were also taken from biopsy regions and other areas of lung that appeared grossly abnormal which had not been included in previous samples. Additional samples for histology included midtrachea, thyroid, thymus, pulmonary artery, myocardium, bronchial lymph nodes, liver, spleen and kidney. All specimens were fixed in 10% formalin, embedded in paraffin, sectioned to 6 μm thickness and stained with hematoxylin and eosin.

Slides were examined blindly to prevent reader bias. Lung sections were graded subjectively on the basis of presence and severity of lesions with a value of 1 being normal ranging up to a value of 4 equalling the most severe changes. The grade was comprised of individual assessment of the lumen, the epithelium, presence of inflammatory cell cuffing, and smooth muscle thickness of bronchi and bronchioles; alveolar lumen and septa; pulmonary vasculature; and interlobular septa (Table II). This allowed calculation of a lung change index per lung lobe as well as entire lung grade by summation of individual grades.

SEROLOGY

Serum samples stored at -20°C were processed in pairs from the same calf on the same day by a diagnostic laboratory (Veterinary Laboratory Services, Ontario Ministry of Agriculture and Food, Guelph, Ontario) for viral antibody titers. The virus neutralization test was used to determine antibodies to infectious bovine rhinotracheitis (IBR) virus, bovine virus diarrhea (BVD) virus, and bovine respiratory syncytial (BRS) virus. The hemagglutination inhibition test was used for antibodies to bovine parainfluenza-3 (PI-3) virus and countercurrent immunoelectrophoresis for bovine adenoviruses 1 to 3. Titers to *Mycoplasma bovis* and *Mycoplasma dispar* were performed utilizing an indirect hemagglutination test (courtesy Dr. S. Rosendal, University of Guelph, Guelph, Ontario). A fourfold increase in titer was interpreted as positive evidence of a recent infection for all agents but adenovirus, for which positive evidence of recent infection was conversion from negative to positive on countercurrent immunoelectrophoresis.

STATISTICAL ANALYSIS

The data were evaluated using a computer based statistical program (SAS Institute Inco, Raleigh, North Carolina). Normality of the data distribution was determined by univariate statistics. Since data analysis showed an uneven distribution of variance in the immunoglobulin data, the $\log_{10}$ transformation of these data was utilized to achieve a normal distribution. Resultant means and standard deviations are reported as the antilogs of the results. The data were treated as a split plot design (33) with age as the whole plot and lung location as the subplot. Analysis of variance was used to test for differences with a level of $P < 0.05$ considered significant.

RESULTS

Based on our definition for entrance into the study only six calves in group 1 and six calves in group 2 fulfilled the requirements for acceptable normals. Thus, of the entire group of 30 calves 18 were rejected from this study. The rejected animals included 10/10 from the eight week old group as well as 4/10 of the calves in each of the two and

TABLE II. General Criteria for Grading[a] Lesions on Lung Histology

Structure		Type of Lesion
Alveolar	— septa	— congestion, cellular infiltrate, wall thickening, alveolar epithelialization
	— lumen	— presence of cellular or stainable contents
Vasculature		— intimal thickening, perivascular cuffing
Interlobular septa		— septal separation, cellular and stainable fluid infiltrate
Bronchioles and bronchi	— lumen	— presence of inflammatory cells, excess mucus
	— epithelium	— cell exfoliation, desquamation, ulceration, hyperplasia/metaplasia
	— cuffing	— thickness of cell layers and proportion airways affected
	— smooth muscle	— hypertrophy, hyperplasia

[a]Severity of grade based on average of grades given for the above structures with 1 = normal, 2 = mild focal changes, 3 = marked focal or multifocal changes, 4 = marked changes, locally extensive

four week old groups with the major factor for rejection being excess numbers of neutrophils in lung washings. It was necessary to exclude animals with elevated levels of neutrophils in any one lung wash as this was also felt to indicate inflammation in the lung (26,27). A maximum level of 10% neutrophils was chosen as our upper limit.

By the end of the test period (day 10) four of the twelve calves had clinical evidence of respiratory abnormalities (Table III). Significant hematological changes were restricted to the white blood cell parameters with 2/12 calves showing an increase in total leukocytes three days post-BAL. In neither case were immature neutrophils present. Other abnormalities noted were elevated platelet numbers (7/12) and mild anemia (2/12). These were not considered significant and did not vary over the course of assessment. Chest radiographs taken on day 0 were essentially normal although many radiographs showed a mild increase in airway thickness. Severity grades of chest radiographs taken on day 10, immediately prior to necropsy were higher than day 0 scores in 5/12 cases (Table III).

TABLE III. Results of Thoracic Radiography Taken Day 0 and Day 10 and Results of Clinical Examination and Necropsy on Day 10 of Twelve Calves Deemed Normal on Day 0[a]

				Grossly Detectable Lung Changes		
	Thoracic Radiography		Clinical Examination	Changes at Other Sites	Changes at Lavage Sites	Changes at Biopsy Sites
Day	0	10	10		10	
Group 1 Calf #						
254	0	0	0	0	0	0
256	0	+	0	+	+	0
263	0	+	+	+	+	+
265	0	0	0	+	0	+
267	0	+	+	+	0	0
133	+	+	+	+	0	+
Group 2 Calf #						
187	0	0	0	0	0	0
188	0	0	0	0	0	0
94	0	0	0	0	0	0
95	0	0	0	+	0	0
97	0	+	+	+	+	+
98	0	+	0	+	+	+
Total (+)	6/12		4/12	8/12	4/12	5/12

[a]Changes suggesting lung pathology : 0 = absent
+ = present

CYTOLOGY

Cytological findings according to area lavaged for each age group are shown in Table IV. Examples of some of the cell types obtained are shown in Fig. 2. Inter and intraobserver differences as well as Wright's stain vs nonspecific esterase stain differences were minimal (data not shown). No significant regional differences were found between cranial and caudal lung lobes of individual calves. There were, however, differences between age groups with group 2 having a lower percentage of total macrophages (89.2% vs 93.8%, $P < 0.05$) and a higher percentage of epithelial cells (3.2% vs 1.2%, $P < 0.05$) (Table V). The average proportion of fluid recovered from the cranial lobe lavage was slightly less than caudal lobe recovery (51% vs 62%). Cell viability was consistently high, with an overall mean of 90.8% of recovered cells excluding trypan blue dye.

MICROBIOLOGY

The culture results from nasal and lung regions are shown in Table VI. With one exception (calf number 256), the few bacterial and fungal isolations from the lower respiratory tract were in scanty numbers. The brush biopsy culturing of the lower respiratory tract was positive in only one instance from which *Mycoplasma bovirhinis* was obtained. There were three separate occasions on which lung isolates were not simultaneously found in the nasal flora. As isolations from the lung lobes were few in number, it was not possible to examine for age or regional differences.

PROTEIN, ALBUMIN AND IMMUNOGLOBULINS

The total protein, albumin and immunoglobulins showed large variations between individuals. When results from cranial to caudal lung lobes were compared, no significant differences were found (Table VII). On comparing group 1 to group 2 for age related changes only the total amount of protein obtained from lung washings showed a significant difference, with a twofold increase in total value from two weeks to four weeks of age (Table V). Normalization of immunoglobulin data with albumin concentration as a means of standardizing the dilution effects of the lung washing procedure on respiratory secretions yielded similar correlation coefficients (r) for

TABLE IV. Cytological Results of Bronchoalveolar Lavage from Regional Lung Lobes for 12 Calves (Mean ± SEM)

	Group 1 (n = 6)		Group 2 (n = 6)	
Age	2 weeks		4 weeks	
Lavage Location (lung lobe)	Cranial	Caudal	Cranial	Caudal
Total cells (x 10^7)	9.2 ± 1.0	8.3 ± 2.0	9.5 ± 1.9	8.4 ± 1.7
Macrophages (%)	93.3 ± 1.8	94.2 ± 1.7	88.6 ± 1.2	89.8 ± 1.8
Neutrophils (%)	2.9 ± 1.5	3.1 ± 1.1	4.5 ± 1.0	4.1 ± 0.8
Lymphocytes (%)	2.6 ± 1.2	1.8 ± 0.6	4.5 ± 1.3	2.5 ± 0.9
Epithelial and others (%)	1.2 ± 0.3	1.2 ± 0.3	2.5 ± 0.7	3.8 ± 1.2

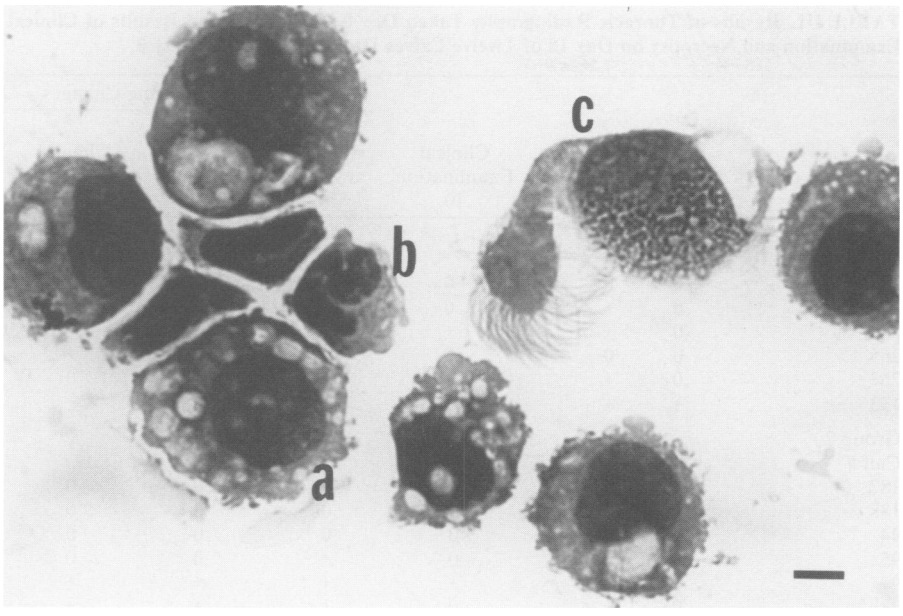

Fig. 2. Cells obtained from bronchoalveolar lavage: a = alveolar macrophage, b = neutrophil, c = epithelial cell. (Wright's stain. Scale bar = 6 μm).

individual Ig class ratios (i.e. IgG/Alb r = 0.91, IgG/IgA r = 0.87) and thus appeared of no additional benefit in data analysis. By convention data were presented for comparison in ratios of varying Ig classes. The study means of ratios of BAL IgG/IgA and IgG_1/IgG_2 were found to be 12:1 and 1.3:1 respectively (Table V).

NECROPSY

Examination of excised lungs revealed grossly detectable lesions in 8/12 calves as noted in Table III. Most were minor localized changes which appeared to be present at sites of lung lavage and/or biopsy. The detectable lesions of calves 95 and 267, also minor, were in areas not associated with either lung biopsy or lavage. Histological evaluation of sections from all lungs confirmed the localized minor changes with lavaged areas having mild inflammatory cell infiltration (Fig. 3) and biopsied areas having only mild focal hemorrhage and fibrosis (Fig. 4). The total lung gradings for each calf were low and in most cases similar to those given to individual lobes examined by BAL and lung biopsy (data not shown). Lung biopsy grades were low but were not considered suitable for comparative studies as the majority of samples contained mainly alveolar structures with relatively few bronchioli. As well, bronchial tissues were infrequently observed.

Two of the calves (#97, 263) had readily apparent lesions on gross and histological examination of cranial lung lobes. Both calves were acceptable for the study on day 1 for BAL and each developed clinical signs of respiratory disease following lung biopsy procedure on day 4. The clinical and radiographic findings of these two calves on day 10 corresponded to necropsy changes (Table III).

SEROLOGY

Except for one calf there was no evidence of seroconversion to any of the agents during the course of the study. Calf number 133 had an eight-fold rise in titer to *Mycoplasma bovis*.

DISCUSSION

One of the most significant findings of this study was that clinical criteria alone were insufficient to ensure absence of subclinical pneumonia. Thus, the criteria described for defining calf normality in this study eliminated over half (18/30) of the animals that may have been considered acceptable if clinical assessment was used as the sole prerequisite for determining normality. Elevation of neutrophils in lung washings above 10% of the total cells was the primary and in many cases sole cause for rejection from normality.

Within the 12 calves which fulfilled our normality criteria lung grades from necropsy indicated mild abnormalities in most of the animals in each

TABLE V. Mean Levels (Pooled Cranial and Caudal Lavages) of Cells and Protein from Bronchoalveolar Lavage of Calves Two and Four Weeks of Age and Combined Mean Values of the Two Groups (±SEM)

	Group 1 n = 6) (2 weeks)	Group 2 (n = 6) (4 weeks)	Combined Mean (n = 12)
Total Cells ($\times 10^7$)	8.8 ± 1.1	9.0 ± 1.7	8.9 ± 0.8
Macrophages (%)	93.8 ± 1.2[a]	89.2 ± 1.2	91.5 ± 1.0
Neutrophils (%)	3.0 ± 0.9	4.3 ± 0.7	3.7 ± 0.6
Lymphocytes	2.2 ± 0.7	3.5 ± 0.7	2.9 ± 0.5
Epithelial and others (%)	1.2 ± 0.2[a]	3.2 ± 0.7	2.2 ± 0.4
Total protein (g/L $\times 10^{-2}$)	0.94 ± 0.07[a]	2.04 ± 0.32	1.49 ± 0.20
IgG_1[b] ($\times 10^{-1}$)	2.0 ± 0.7	3.4 ± 0.9	2.8 ± 0.6
IgG_2[b] ($\times 10^{-1}$)	1.7 ± 0.5	2.5 ± 0.6	2.1 ± 0.4
IgA ($\times 10^{-1}$)	0.28 ± 0.06	0.47 ± 0.09	0.38 ± 0.06
$IgG_1 + IgG_2)/IgA$	11.5 ± 1.5	12.4 ± 1.6	12.0 ± 1.0
IgG_1/IgG_2	1.2 ± 0.2	1.4 ± 0.1	1.3 ± 0.1

[a]Values significantly different from four week old group ($p < 0.05$)
[b]All Ig values in g/L from samples concentrated to 30 g/L solids

TABLE VI. Results of Microbial Culture on the Upper and Lower Respiratory Tract in 12 Normal Calves (all Organisms Found in Small Numbers Unless Specified Otherwise)

Agent	Number of Animals With Positive Isolation		
	Nasal	Cranial Lobe	Caudal Lobe
Bacteria			
Pasteurella multocida	8 ([a])	0	0
Pasteurella haemolytica	1	0	0
Streptococcus (nonhemolytic)	2 ([a])	0	0
Corynebacterium spp.	1	0	0
E. coli	2 ([a])	1	2
Haemophilus spp.	0	0	1 ([a]) (1[c])
Neisseria spp.	1 ([b])	0	0
Actinobacter spp.	1	0	0
Diptheroids	2	0	0
Mycoplasmas			
M. bovirhinis	7	2	2 ([c])
M. bovis	3	2	2 ([c])
M. arginini	1	0	0
Fungi			
Aspergillus spp.	6	2	2
Rhizopus spp.	2	0	0
Mucor spp.	2	0	0

[a]Moderate number of colonies (10-30)
[b]Large number of colonies (> 30)
[c]Organism not concurrently in nasal flora

group. However, the sequential thoracic radiographs and sites of the lesions at necropsy suggested that these abnormalities were associated with the experimental interventions (Table III). Reported complications following BAL procedure are generally minor and temporary but have been shown to increase where large volumes of instillate are used (34) or multiple lung lobes are lavaged (35). The combination of multiple lobe lavage as well as lung biopsy was probably responsible for the complications encountered in the present study. The pathological lung changes noted would probably have resolved in time. Therefore, the calves in this study were considered to represent definable normal animals.

The study mean for cell numbers (Table V) was found to be higher than other studies (4,7). Additionally, we found a higher ratio of IgG:IgA when compared to other workers (6,36,37). It is possible some of the differences are related to variation in techniques; in particular the maximum diameter of airway from which a lavage sample was obtained. Those workers using smaller diameter instruments would have gained access to airways deeper in the bronchial tree and lavaged the free cells from a smaller volume of lung resulting in lower total cell collection. In relation to studies on immunoglobulins our technique would have excluded much of the large airway secretions included in samples obtained for prior studies of BAL immunoglobulins, two of which (36,37) involved whole lung lavage. It has been shown that proportionate levels of IgA decrease on moving deeper into the lung airways (38). Hence, our sampling depth alone would have contributed to proportionately lower IgA levels. Other variables shown to affect cell recovery, including temperature and pH of lavage instillate (35), lavage volume and number of aliquots (39,40) and fluid composition (41) were either not standardized or not mentioned in earlier studies.

The study results for cell differentials (Table V) are similar to other studies (4,7) with alveolar macrophages averaging 91.5% and the remaining cells consisting of neutrophils, lymphocytes and epithelial cells. The large numbers of neutrophils in the lung washings of Wilkie and Markham's calves (6) would have been cause for exclusion of these animals from the present study. Although neutrophils can play a protective role in the lung (42) they are also implicated as participants in, or causes of, lung injury (27,43) such that their presence in elevated numbers in lung washings as noted in the study of Wilkie and Markham (6) suggests pulmonary inflammation. It therefore appears that the description given for cell parameters for BAL from the calves of this and other studies must be interpreted in light of collection techniques and lung status of animals utilized. A need is apparent, however, for developing standardized techniques of BAL in cattle in order to compare and correlate future studies.

The microflora cultured from the lower respiratory tract of calves in this study substantiates prior reports of transient or resident microorganisms in the lungs of normal calves (21,22). Most organisms found were in very low numbers, with only one isolation of an organism in moderate numbers,

TABLE VII. Protein and Immunoglobulin Content from BAL of 12 Calves with Comparison of Age and Lung Location (Mean ± SEM)

Age	Group 1 (n = 6) 2 weeks		Group 2 (n = 6) 4 weeks	
Location	Cranial	Caudal	Cranial	Caudal
Total Protein ($g/L \times 10^{-2}$)	1.0 ± 0.14	0.88 ± 0.07	1.91 ± 0.42	2.17 ± 0.51
IgG_1[a] ($g/L \times 10^{-1}$)	1.9 ± 0.6	2.2 ± 1.3	4.5 ± 1.7	2.3 ± 0.5
IgG_2 ($g/L \times 10^{-1}$)	1.6 ± 0.6	1.8 ± 0.9	3.5 ± 1.1	1.6 ± 0.4
IgA ($g/L \times 10^{-1}$)	0.28 ± 0.07	0.29 ± 0.10	0.54 ± 0.16	0.40 ± 0.09
($IgG_1 + IgG_2$)/IgA	11.7 ± 1.8	11.4 ± 2.4	13.2 ± 2.4	11.6 ± 2.2
IgG_1/IgG_2	1.3 ± 0.3	1.1 ± 0.2	1.2 ± 0.1	1.7 ± 0.2

[a]All Ig from sample concentrated to 30 g/L of solids

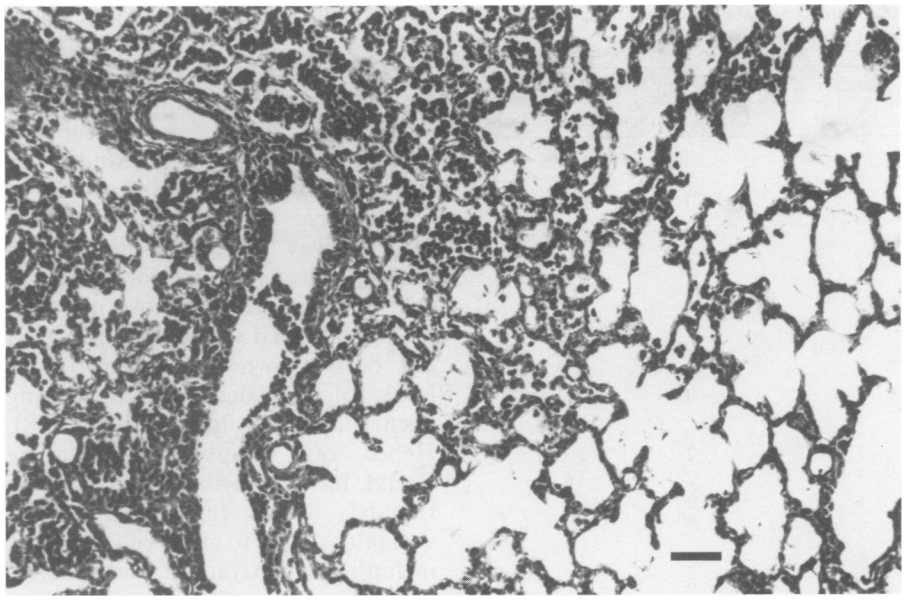

Fig. 3. Histological appearance of lung ten days postlavage showing mild focal inflammatory cell infiltration composed of neutrophils and alveolar macrophages (H & E. Scale bar = 63 μm).

which was a nonpathogenic *Haemophilus* species (Table VI). The presence of these microorganisms in the lower respiratory tracts did not appear to influence BAL cell types.

The types and levels of immunoglobulins (Ig) found in BAL indicate a much higher relative IgG component than found by other workers (6,36,37). This may imply that our calves had a decreased level of IgA, increased level of IgG or a combined effect of both when compared to prior studies. The elevated IgG in this study may have been due to our use of younger animals in which passively acquired antibodies from colostrum, predominantly IgG_1 (44), are secreted at mucosal surfaces (45).

The IgG_2 levels in our lung washes were similar to IgG_1 levels which is consistent with the findings of Butler *et al* (37). Unfortunately, their study did not also include simultaneous serum Ig determinations. The IgG_1/ IgG_2 serum values of our calves compare favourably with values previously published for calves of similar age (15). Since plasma cells containing IgG_2 do not appear in the respiratory tract of calves until after the second week of life (46) and even then only in low numbers (47) this suggests that the IgG_2 found in lung washings was produced by the calf at sites distant from the bronchus associated lymphoid tissue. When comparing these ratios of serum versus BAL IgG_1/ IgG_2 it appears that there may have been a fourfold selective transfer of IgG_2 into respiratory secretions sampled. Considering the importance of IgG_2 in the bovine immune system, particularly in the respiratory system (16) this may indicate a much greater role for IgG_2 at the lower respiratory tract mucosal surface than previously ascribed (36).

On assessing the cranial versus caudal lung regions of our calves there were no significant differences in any of the parameters measured (Tables IV and VII). However, the presence of large individual animal variations may have obscured actual regional differences present. As well, by imposing the restriction on maximum allowable neutrophil percentage in the cell differentials we were removing animals from the study that did have marked regional lung differences. In fact, many excluded calves had only one lobe lavage deemed cytologically abnormal. The neutrophil fraction appeared to exhibit marked fluctuations, often surprisingly elveated in the absence of microbial growth from the respective lung regions. Noninfectious mechanisms of lung neutrophil recruitment, such as complement activation (48), leukotriene B4 production by alveolar macrophages (49), lipopolysaccharide aerosol inhalation (5) or chemical damage may be implicated. It is unclear whether these cells were participating in lung defence or causing lung damage.

When examined for age related differences, the total protein values and cell differentials were found to vary significantly between groups. The rise in total lung lavage protein with age (Table V) conflicts with Fogarty *et al* (50) who found the total BAL protein in calves to fall over time when measured from the first week and eighth week of life. They also found marked elevations in lung lavage protein levels in calves with respiratory disease. Based on our criteria for acceptance of calves into the study this last point should not have influenced our findings. While it is tempting to postulate that the increase in lung protein content with age seen in our calves was the result of active Ig production, the data give only qualitative changes in Ig fractions and hence cannot supply the quantitative information necessary. The significant decrease in percentage of alveolar macrophages from two to four weeks of age appeared to be due to a significant increase in epithelial cells and a trend toward increased neutrophils, while the total cell numbers

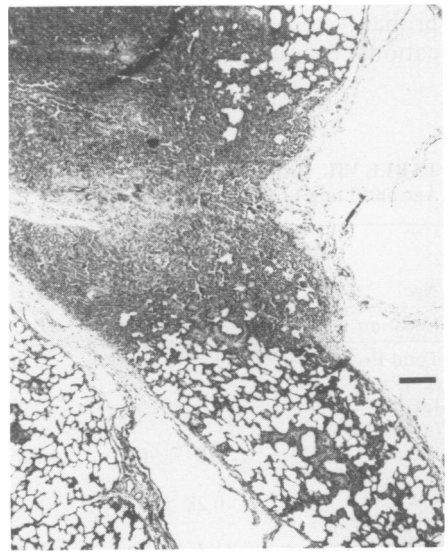

Fig. 4. Histological appearance of biopsy site six days postbiopsy showing mild hemorrhage and fibrosis(H & E. Scale bar = 250 μm).

remained essentially unchanged (Table V). The increased epithelial cells may have reflected a subtle disturbance in the lower respiratory tract epithelium, either the result of or causing the minor neutrophil influx. Although serological studies did not suggest concurrent respiratory infections this cannot be entirely ruled out as serum antibody levels do not always correlate well with local mucosal events (51) and a ten day span may have been insufficient for seroconversion to occur. However, the various cell changes noted were not felt to be clinically, cytologically or immunologically significant.

In conclusion, it appears that clinical examination alone is insufficient for the selection of normal calves for pulmonary studies. Thus, although regional lung differences are not found in our highly selected set of normal calves, they probably do exist in the general population and must be considered in pulmonary studies which rely solely on a clinical examination to select normal calves. As well, older animals may have markedly differing values as they can have higher environmental challenges. From the age of two to four weeks there appears to be a small, but statistically significant, decrease in the percentage of alveolar macrophages and minor increases in the percentages of epithelial cells and neutrophils obtained on bronchoalveolar lavage. Once calves reached eight weeks old there were none that met our criteria for normality. Normal calves were found to have in their lung washings approximately 90% alveolar macrophages, an IgG/IgA ratio of 12:1 and, in some, mycoplasmal organisms and small numbers of nonpathogenic bacteria and fungi. The structural changes induced by bronchoalveolar lavage and lung biopsy were mild local inflammatory cell infiltration in response to BAL and local hemorrhage and fibrosis in response to lung biopsy. Finally, in order to compare results of future studies utilizing bronchoalveolar lavage a need is apparent for the standardization of BAL collection techniques and a more detailed definition of pulmonary health.

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