Interaction of Bovine Respiratory Syncytial Virus with Bovine Alveolar Macrophages *in vivo*: Effects of Virus Infection upon Selected Cell Functions

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ABSTRACT

The effect of bovine respiratory syncytial virus (BRSV) upon alveolar macrophage (AM) function was investigated using an *in vivo* calf inoculation model. Alveolar macrophages were collected sequentially from live calves at multiple time points during the 14 day period following viral inoculation. Alveolar macrophages from bronchoalveolar lavage fluids were purified by density gradient centrifugation (> 95% AM) prior to *in vitro* evaluation of cell functions. There were significant but variable and inconsistent differences in the functions of AM from the BRSV inoculated calves compared to the control calves. Fc-receptor mediated phagocytosis was either increased or unchanged by BRSV inoculation. Nonopsonized phagocytosis was decreased during the early postinoculation period and later increased. There was a variable effect on AM phagosome lysosome fusion with increased fusion activity on postinoculation days 2 through 5, 7 and 12 but reduced activity on days 6 and 10. The AM respiratory burst, as measured by nitroblue tetrazolium dye reduction, was essentially unaffected with a reduction in activity on day 10 only. In this model, BRSV inoculation of calves primarily resulted in an alteration of the membrane associated phagocytic functions of the alveolar macrophages ($p < 0.05$).

RÉSUMÉ

L'effet du virus respiratoire syncitial bovin (VRSB) sur la fonction des macrophages alvéolaires (MA) a été étudié en utilisant comme modèle *in vivo* l'inoculation de veaux. Des MA ont été prélevés de façon séquentielle chez des veaux vivants à différents temps durant la période de 14 jours suivant l'inoculation virale. Les MA provenant des liquides de lavages broncho-alvéolaires étaient purifiés par centrifugation en gradient de densité avant de procéder à l'évaluation *in vitro* des fonctions cellulaires. Des différences significatives, mais variables et inconsistentes, étaient notées entre les fonctions des MA provenant des veaux inoculés avec le VRSB et ceux des veaux témoins. La phagocytose via l'intermédiaire des récepteurs Fc était soit augmentée ou inchangée suite à l'inoculation du VRSB. La phagocytose sans opsonisation était diminuée au début de la période post-inoculation mais augmentait par la suite. Un effet variable sur la fusion des phagosomes avec les lysosomes des MA était noté avec une augmentation de cette activité aux jours 2 à 5, 7 et 12 post-inoculation mais une réduction de l'activité aux jours 6 et 10. La poussée respiratoire des MA, telle que mesurée par la réduction du bleu de tétrazolium n'a pas semblé être affectée, une réduction d'activité étant notée au jour 10 seulement. Dans le modèle étudié, l'inoculation de VRSB à des veaux a entraîné un changement dans les fonctions de phagocytose principalement associées aux altérations des membranes des MA ($p < 0,05$). ***(Traduit par Dr Serge Messier)***

INTRODUCTION

Bovine respiratory disease is a significant problem in the cattle industry (1–4). An important component of bovine respiratory disease complex is the bovine respiratory syncytial virus (BRSV) (5–8).

A small number of experimental *in vivo* and *in vitro* BRSV studies have demonstrated variable alterations in the function of the alveolar macrophage (AM) (9–12). These studies used differing experimental conditions (calf age, BRSV source, dose of viral inoculum) making it difficult to compare the findings. The AM used as controls in previous *in vivo* experimental BRSV models were either obtained from different groups of calves sacrificed at varying times during the experiment or from the study calves prior to BRSV inoculation. The purpose of this study was to compare the function of AM collected concurrently from calves inoculated with a field strain of BRSV and from sham inoculated control calves.

MATERIALS AND METHODS

ANIMALS

Colostrum deprived Holstein calves were obtained at birth and immediately transported to an approved isolation facility at the College of

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Veterinary Medicine, University of Minnesota, St. Paul, Minnesota. An oral rotavirus/coronavirus vaccine (Calf Guard, Norden Laboratories Inc., Lincoln, Nebraska) and a K99 monoclonal antibody (Genecol 99, Schering-Plough Animal Health, Union, New Jersey) were administered to each calf at birth. All calves were fed a commercial milk replacer. Calves entered the experimental inoculation protocol at 6 to 7 wk of age in groups of four. Five different sets of calves were used and each set of calves was lavaged on different days postinoculation. Some data were collected at half day time points (i.e. day 5.5 and 6.5).

VIRUS

A low passage BRSV field isolate (a gift from Dr. J.C. Baker of the College of Veterinary Medicine, University of Michigan, East Lansing, Michigan) was propagated on bovine turbinate cells (National Veterinary Services Laboratory, Ames, Iowa). Virus was harvested at 96 hours by cell scraping. The suspended cells and virus were frozen (−70°C) and thawed once before they were inoculated into the calves. The concentration of infectious virus was estimated using the Reed and Muench method (13).

IN VIVO INOCULATIONS

Calves were inoculated by the combined intranasal and intratracheal route (14,15). Twenty mL of cell culture fluid containing $10^{6.5}$ $TCID_{50}$ of BRSV was administered once daily for five consecutive days. Control calves received the same volume of a virus free cell culture fluid.

Bovine respiratory syncytial virus infection was confirmed by identification of viral antigen in cells collected by nasal mucosal scraping (direct smear preparations) and bronchoalveolar lavage (cytospin preparations). Cell preparations were examined for the presence of BRSV antigen and the absence of bovine viral diarrhea virus (BVDV) antigen using immunocytochemistry and immunofluorescence. Serum samples were collected before and after inoculation and analyzed for virus neutralizing antibody to BRSV, bovine virus diarrhea virus, bovine herpes type 1 virus (BHV-1) and for antibody to parainfluenza type 3 (PI-3) virus.

VIRAL ANTIGEN DETECTION

Air dried cell preparations were fixed for 10 min in acetone (immunofluorescence) or 95% ethanol (immunocytochemistry). The slides were stored at −70°C until stained. A fluorescein conjugated anti-BVDV antibody (National Veterinary Services Laboratory, Ames, Iowa) was used to localize BVDV viral antigen in a direct immunofluorescence assay. Bovine respiratory syncytial virus was detected using immunofluorescence and an immunocytochemical technique (16).

ALVEOLAR MACROPHAGES

Alveolar macrophages (AM) were collected from both control and infected calves on the same day using bronchoalveolar lavage (BAL) (17). Calves were lightly sedated with an intramuscular injection of xylazine HCl (Rompun, Mobay Corporation, Shawnee, Kansas) at a dose rate of 0.05 mg/kg. A flexible fiberoptic endoscope (Olympus GIF P10 Fiberoptic Endoscope, Olympus Corporation, Lake Success, New York) was passed nasotracheally until it was wedged in a sub-segmental bronchus. Four 100 mL aliquots of the lavage solution [phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) with 0.01% EDTA and 5 mL/L of PSN antibiotic (Gibco Laboratories Life Technologies, Grand Island, New York)] were infused through the biopsy channel and gently aspirated at a vacuum pressure of 10 cm water.

The AM were purified from the lavage fluid using a ficoll-diatrizoate (Ficoll, Sigma Chemical Company, St. Louis, Missouri; Hypaque, Winthrop Pharmaceuticals, New York, New York) gradient with a specific gravity of 1.077 gm/dL (18,19). Macrophage identity was confirmed by demonstration of nonspecific esterase activity (α-Naphthyl acetate esterase. Sigma Diagnostics, St. Louis, Missouri). Cell viability was greater than 95% based on trypan blue dye exclusion. For the cell function assays alveolar macrophages were suspended in RPMI-1640 media (Sigma) supplemented with 10 mM L-glutamine, hepes buffer, pH 7.2 and 10% gamma-irradiated heat inactivated fetal bovine serum (Sigma).

MACROPHAGE FUNCTION ASSAYS

The phagocytosis of nonopsonized particles assay was based on established protocols (20). Over a two hour incubation period (37°C, 5% CO_2, humidified chamber) 1 mL of AM (10^6 cells/mL) were allowed to adhere to glass coverslips preplaced in each well of a 24-well tissue culture plate. The medium was replaced with heat killed *Candida albicans* suspended in 1 mL of PBS at a ratio of ten yeast per AM and the mixture reincubated for 30 min. The wells were washed with PBS. The coverslips were fixed with acetone-methanol (50:50), stained with 1:50 Giemsa for 10 min, mounted on a glass slide and examined using a light microscope. One hundred AM were examined and the percentage of AM with at least one phagocytosed yeast organism was recorded.

Fc-receptor mediated phagocytosis was performed using opsonized sheep red blood cells (sRBCs) as targets (19). One mL of AM (10^6 cells/mL) were placed in each well of a 24-well plate and allowed to adhere (60 min, 37°C, 5% CO_2). Sheep red blood cells were suspended in PBS to a concentration of 5% and opsonized with rabbit anti-sRBC IgG (Sigma) (60 min, 37°C, 5% CO_2). (Opsonization was verified by visually comparing the number of sRBC attached to macrophages with the number of IgG-sRBC attached to macrophages.) The sRBCs were washed in PBS, centrifuged and labeled with 100 uCi of Cr^{51} (Amersham Corporation, Arlington Heights, Illinois) per 24-well plate. After two PBS washes, the sRBCs were suspended at a concentration of 1.0% in PBS and 300 μL added to each well (60 min, 37°C, 5% CO_2). The nonphagocytosed sRBCs were lysed with hypotonic PBS. After a gentle PBS wash, the AM were disrupted with 0.1% Triton X-100 and the radioactivity (cpm) of the resulting solution was measured in a gamma counter (Beckman Instruments Inc., Brea, California).

The Fc-receptor binding assay was performed similarly to the Fc-receptor mediated phagocytosis assay except that there was no hypotonic lysis of

sRBCs. The wells were washed three times with PBS after the final incubation and then treated with 0.1% Triton X-100. The activity represented all the sRBC which had been bound to the macrophages during the 90 min incubation period.

In the phagosome lysosome fusion assay, 1 mL of AM (10^6/mL) was added to each well of a 24-well plates containing round preplaced coverslips (19,21). The AM were incubated (20 min, 37°C, 5% CO_2) in RPMI 1640 media containing acridine orange (10 μg per mL). Heat killed *Candida albicans* were added to each well at a ratio of ten yeast per AM and the incubation continued for an additional 60 min. The wells were washed with PBS, the coverslips removed and mounted inversely on glass slides for examination with an UV microscope (Carl Zeiss Instruments, Atlanta, Georgia). In each well the percentage of AM containing phagolysosomes (yellow-orange fluorescing granules) was determined.

Nitroblue tetrazolium (NBT) dye reduction was used as an indicator of the respiratory burst activity of the AM (19,22). The AM and reagents were suspended with Hank's balanced salt solution (HBSS) in 5 mL silicon coated glass tubes (Vacutainer, Beckton Dickinson and Company, Rutherford, New Jersey) to facilitate centrifugation of cells. In each tube, 1 mL of AM (10^6 cells/mL) were stimulated with 40 μL of opsonized (autologous calf serum) zymosan (10 mg/mL). The NBT solution (0.05% NBT in HBSS) was added to each tube at the same time and the tubes incubated (90 min, 37°C, 5% CO_2). Three milliliters of 0.5N HCl was added to stop AM activity, the tubes mixed gently and the AM separated by centrifugation (10 min, 1000 *g*). The pellet was resuspended in 2 mL of dimethylformamide and incubated in a boiling water bath for 10 min to extract the formazan formed by AM metabolism of NBT. Potassium hydroxide (0.5 mL 5N KOH) was added to optimize the light absorbance characteristics and the AM-dimethylformamide-KOH solution mixed four times (inversion) before centrifugation (10 min, 1000 *g*). The top phase of the solution was transferred to a spectrophotometric cuvette and the optical density measured at 710 nm. Results were recorded as fractional absorbance.

STATISTICAL ANALYSIS

The data were analyzed in a randomized block design using a general linear model program (SAS, SAS Institute, Cary, North Carolina). The level of significance was set at 5% ($p < 0.05$) for all of the F statistics calculated and tested.

RESULTS

CLINICAL DISEASE

Mild respiratory disease was induced in the experimentally infected calves. There was a very mild non-significant increase in rectal temperature of the BRSV inoculated calves. The BRSV inoculated calves coughed more frequently, had an increased respiratory rate and a more pronounced abdominal component to the respiratory pattern compared to the control calves. These differences were not consistent in all calves. The BRSV antigen was detected in the nasal mucosal cell scrapings and in the cells collected by BAL from the BRSV inoculated calves. The BVDV antigen was not detected in cells from any of the calves. Initially the inoculated calves had no BRSV specific serum antibody, but developed specific antibody with serum virus neutralizing antibody titers ranging from 1:2 to 1:16 after viral inoculation. The control calves did not develop BRSV serum antibody during the study and up to four weeks later. There was no detectable antibody production to BVDV, PI-3 or BHV-1 in any of the calves.

BRONCHOALVEOLAR LAVAGE

The BAL technique was easily performed in these calves. Recovered fluid volume was greater than 60% of the infused lavage solution volume. Calves had mildly increased lung sounds for 12 to 24 hours after the BAL procedure. These increased lung sounds were associated with the anatomical lung region which had been lavaged. There was no difference in lung sounds or the percentage of fluid collected between the control and the BRSV inoculated calves. In some cases, calves were lavaged in the evening rather than the morning. Those BALs were considered to be performed on the postinoculation day plus one-half (i.e PID 5.5). The cell differential counts from the recovered lavage fluids had increased neutrophil percentages at several time points during the experimental protocol. This observation did not appear to be significant as there was no statistically significant difference in lavage cell differential counts between BRSV inoculated and control calves on any certain day postinoculation (data not shown).

ALVEOLAR MACROPHAGE FUNCTION

There was considerable difference in AM function between the different postinoculation days. This could not be clearly attributed to BRSV inoculation because of significant interactions among the variables which were present in the statistical model. Comparisons were not made between different postinoculation days of the study.

The nonopsonized phagocytic activity of the AM of the BRSV inoculated calves was significantly decreased on PID 2, 3, 5, 5.5 and 6 and day 9. On PID 7 and 11 the activity was increased relative to AM collected from control calves (Table I). The Fc-receptor binding activity of AM collected from the BRSV inoculated calves was increased on PIDs 4, 12 and 13 (Table II). The Fc-receptor mediated phagocytosis activity of the AM collected from the BRSV inoculated calves was significantly increased on postinoculation days (PID) 1, 4, 5.5, 8 and 12 compared to the control calves (Table III). The formation of phagolysosomes from the fusion of phagosomes and lysosomes was significantly decreased in the AM collected from calves inoculated with BRSV on PID 2, 3, 5, 5.5, 7 and 10. On PID 12 the BRSV inoculated calves had a higher percentage of AM exhibiting fusion of phagosomes with lysosomes than did the control calves (Table IV). The reduction of nitroblue tetrazolium dye by the AM collected from the BRSV inoculated calves was not significantly different from the control calves except for a decrease in NBT reduction on PID 10 (Table V).

DISCUSSION

The severity of clinical BRSV disease produced in this and in other experimental models has been less than the naturally occurring disease (9,23,24). The inoculation method may influence the clinical signs. Calves inoculated by an intranasal route had less severe signs than those inoculated by the combined intranasal and intratracheal routes (23). The source of virus influences the severity of clinical disease. Calves challenged with wild (field strain) virus became infected whereas calves challenged with the same dose and strain following three tissue culture passages did not become infected (25). This variation in the severity of experimental disease may explain some variation between studies. The mild experimental disease produced may be associated with alterations in AM function that are less significant than the changes in AM which occur in natural BRSV infection. In our *in vivo* BRSV inoculation model we have demonstrated alterations in the functional activity of the alveolar macrophage subsequent to BRSV challenge. It is difficult to be absolutely sure that variability due to the individual calf response did not influence the results especially on a day to day basis.

In our study Fc-receptor mediated phagocytic activity of the AM was increased in the BRSV inoculated calves. In contrast, nonopsonized phagocytic activity of the AM from the same calves was depressed in the period immediately following BRSV inoculation. Other AM studies using different viral agents have documented a reduction in AM phagocytosis of yeast organisms at approximately PID 7 (20,26). Our results are somewhat similar, but we did not observe a decrease in Fc-receptor mediated activity simultaneously with the reduced phagocytosis (20,26). However, there was no increased Fc-receptor mediated phagocytosis and there was a reduction in nonopsonized phagocytosis up to PID 7. This period of reduced phagocytosis in our model may be consistent with observations in feedlot calves. Maximum respiratory disease developed seven to fourteen days after arrival (27). This same time period corresponded to the onset of respiratory disease in weaned calves (28).

TABLE I. Nonopsonized phagocytosis of *Candida albicans* by alveolar macrophages recorded as percentage of AM containing at least one phagocytosed yeast. Several different groups of calves were used as indicated. [a]Values represent mean ± standard deviation. [b]Indicates significant difference between BRSV infected (treated) calves and control calves ($p < 0.05$). PID indicates the postinoculation day. Treated calves were inoculated with BRSV

PID	Calf group	Treated calves	Control calves
2	B	59.4 ± 6.7[a]	78.3 ± 5.9[b]
3	C	18.1 ± 3.9	28.2 ± 3.6[b]
4	D	53.8 ± 10.9	49.4 ± 3.4
5	A	42.0 ± 2.0	72.6 ± 1.2[b]
5.5	B	28.2 ± 7.5	87.0 ± 2.2[b]
6	E	43.5 ± 2.3	69.7 ± 1.8[b]
6.5	C	16.5 ± 5.0	24.2 ± 10.0
7	D	54.7 ± 4.5	28.0 ± 3.4[b]
8	E	78.6 ± 8.4	82.8 ± 5.1
9	A	64.9 ± 7.7	78.2 ± 4.9
10	C	54.0 ± 7.8	43.7 ± 16.6
11	D	54.5 ± 3.2	40.6 ± 2.0[b]
12	B	66.7 ± 6.6	62.5 ± 3.0
13	A	66.6 ± 5.2	67.3 ± 9.7
14	D	63.5 ± 4.5	72.5 ± 11.4

Table II. Fc-receptor mediated binding to alveolar macrophages recorded as counts per minute of Cr^{51} activity. See Table I for legend

PID	Calf group	Treated calves	Control calves
1	A	5520 ± 788[a]	7254 ± 1540
4	D	12190 ± 3463	6913 ± 7473[b]
5	A	11860 ± 2728	14100 ± 7242
6	E	22670 ± 2611	19870 ± 1129
7	D	8076 ± 1900	8191 ± 739
8	E	4716 ± 625	4729 ± 620
9	A	18650 ± 6276	16110 ± 7407
10	C	4786 ± 749	5490 ± 986
11	D	9546 ± 2725	8775 ± 931
12	B	7572 ± 81	4388 ± 1150[b]
13	A	7566 ± 799	4791 ± 2095[b]

Table III. Fc-receptor mediated phagocytosis of alveolar macrophages recorded as counts per minute of Cr^{51} activity. See Table I for legend

PID	Calf group	Treated calves	Control calves
1	A	1594 ± 1386[a]	1802 ± 432[b]
2	B	844 ± 28	902 ± 63
3	C	1470 ± 564	715 ± 215
4	D	3298 ± 2019	354 ± 107[b]
5	A	4184 ± 3423	7563 ± 6499
5.5	B	3733 ± 534	1324 ± 150[b]
6	E	1761 ± 441	2466 ± 1179
6.5	C	6308 ± 2369	7988 ± 2864
7	D	3161 ± 573	3555 ± 773
8	E	3042 ± 657	1666 ± 129[b]
9	A	5189 ± 3318	5915 ± 4551
10	C	1975 ± 289	1912 ± 165
11	D	2668 ± 74	2934 ± 738
12	B	10450 ± 177	6223 ± 512[b]
14	D	1975 ± 640	1385 ± 192

The reduction in the phagocytosis of nonopsonized particles by AM overlapped a period of low Fc-receptor phagocytosis. The BRS virus may have affected the ability of the AM plasma membrane to undergo the structural alteration(s) needed for the engulfment of attached foreign substances. It has been demonstrated that viral infection enhances arachidonate metabolism of bovine alveolar macrophages (29). The postulated mechanisms include an alteration of the transmembrane signalling events, enhanced phospholipase activity, and virus-induced disorganization of the phospholipid bilayer (29). These changes could affect the membrane events required for phago-

Table IV. Phagosome lysosome fusion by alveolar macrophages recorded as percentage of AM containing at least one formed phagolysosome. See Table I for legend

PID	Calf group	Treated calves	Control calves
1	A	73.2 ± 12.6[a]	41.1 ± 11.1
2	B	2.5 ± 2.5	59.4 ± 0.1[b]
3	C	2.5 ± 2.6	13.7 ± 7.3[b]
4	D	72.0 ± 12.0	56.0 ± 3.2
5	A	22.8 ± 0.8	80.1 ± 2.6[b]
5.5	B	3.5 ± 1.5	25.0 ± 0.1[b]
6	E	60.0 ± 5.9	67.7 ± 17.0
6.5	C	16.7 ± 13.4	1.8 ± 1.3
7	D	37.8 ± 21.2	68.6 ± 2.0[b]
8	E	55.4 ± 5.4	51.0 ± 3.1
9	A	1.0 ± 0.0	1.5 ± 1.0
10	C	65.7 ± 10.2	88.2 ± 2.7[b]
12	B	76.1 ± 0.0	25.4 ± 0.2[b]
14	D	93.4 ± 4.2	95.4 ± 1.6

TABLE V. Reduction of nitroblue tetrazolium dye by alveolar macrophages recorded as the optical density of the extracted formazan solution. See Table I for legend

PID	Calf group	Treated calves	Control calves
1	A	0.29 ± 0.07[a]	0.27 ± 0.04
4	D	1.60 ± 0.42	1.57 ± 0.15
5	A	1.12 ± 0.39	0.86 ± 0.15
5.5	B	0.30 ± 0.03	0.24 ± 0.01
6	E	1.07 ± 0.06	0.70 ± 0.08
6.5	C	0.09 ± 0.11	0.04 ± 0.003
7	D	0.90 ± 0.35	1.23 ± 0.42
8	E	0.73 ± 0.04	1.06 ± 0.06
9	A	0.63 ± 0.18	0.60 ± 0.07
10	C	0.75 ± 0.01	1.08 ± 0.09[b]
11	D	0.96 ± 0.06	1.02 ± 0.08
12	B	1.06 ± 0.07	1.05 ± 0.09
13	A	1.41 ± 0.03	1.34 ± 0.17
14	D	0.74 ± 0.13	1.16 ± 0.11

cytosis of particulate material and be observed as changes in the specific AM membrane associated functions evaluated in our model.

The fusion of phagosomes and lysosomes was depressed on day 2 and 3 after BRSV inoculation and apart from two time points was never significantly elevated over the control calves. The fusion of phagosomes and lysosomes has been altered in other viral infections (19,20,30,31). Inhibition of the fusion of phagosomes with lysosomes may be an important factor in the pathogenesis of the bovine respiratory disease. This *in vitro* observed AM function defect could be an indication of an inability of AM to kill phagocytosed pathogens *in vivo* which would facilitate the establishment of a bacterial infection. The mechanism of the alteration of this AM function is not known but may be related to altered arachidonic acid metabolism (29,31).

The reduction of nitroblue tetrazolium dye was essentially unaffected by BRSV infection. Since the reduction of NBT is indicative of the level of superoxide anion release (32,33) and this intermediate oxygen metabolite is a product of the respiratory burst (34), the degree of NBT reduction is an indicator of microbial killing capacity of the cell. Therefore, in our model of BRSV infection there was no indication that the metabolic processes of the AM responsible for killing microorganisms were more than transiently affected by BRSV infection. Other models have documented that an increase in intracellular acid phosphatase and superoxide anion occurs concurrently with a transient bactericidal impairment (12). The AM concentration of acid phosphatase is indicative of a general activation of the cell whereas superoxide anions were believed to be involved with organism killing (35).

The increased concentrations of acid phosphatase and superoxide anions (12) in the presence of reduced bacterial killing may be compatible with the results of our phagosome-lysosome fusion assay. It could be hypothesized that if there is an overall failure of phagosome lysosome fusion and microorganisms do not come in direct contact with the lysosomal degradative substances (superoxide anions and other intermediate oxygen metabolites), then no increase in bactericidal killing is likely to occur even if an increased concentration of potential microbicidal products is present within the cell. An increased rate of phagosome and lysosome fusion would be required.

Previous studies examining the effect of BRSV upon AM function have been conducted using a variety of experimental conditions that could influence the results (9-11). In an *in vivo* inoculation model, AM that were collected from conventionally raised, one month old calves had increased nonopsonized phagocytosis of latex beads. The control calves were not tested on the same days and the AM were collected from the calves immediately after euthanasia (9). An *in vivo* BRSV model using AM collected from awake conventional calves four months old demonstrated increased numbers of AM per mL of BAL fluid with a shift in the AM/PMN ratio due to increased numbers of PMN. There were increased levels of acid phosphatase, increased levels of superoxide anion and impaired bacterial killing on PID 6 (12). An *in vitro* model using AM collected from BRSV seronegative conventional cattle 14 to 20 months old had reduced Fc receptor mediated phagocytosis, reduced phagocytic efficiency and increased lysosomal enzyme activity at 72 hours. Fc-receptor function, cell viability and adherence were unaffected (11). In contrast to our study, calves raised in conventional housing are often exposed to factors such as dusts, gases (ammonia) and infectious agents of respiratory disease from older cattle. There may be no clinical signs of disease in the calves but there is the potential for alterations in the airways which could have a direct impact on alveolar macrophage functions examined *in vitro*. There is certainly an alteration in the cytological cell types recovered by BAL which can occur in both inoculated and control animals leading to potential increased assay variability.

It is important to evaluate not one but several AM functions and to consider the choice of day postinoculation because apparent defects in AM function may depend on the function examined and the specific sampling period postinoculation. Although our model did not allow us to rigorously examine the effect of postinoculation day, it does illustrate the variability that can occur and the importance of considering multiple time points when evaluating AM functions in *in vivo* models. This type of variability has also been observed in models of murine AM function using Sendai virus (26). The phagocytosis of bacteria by murine AM was not affected but intracellular killing was impaired (26). The intracellular dysfunction in the processing of phagocytosed material was in part a failure of phagosome and lysosome fusion (20). In another study, phagocytic activity was initially enhanced followed by a depression and then a stimulation of activity (20). This pattern of alteration in AM function could facilitate bacterial infection if the bacteria become established during the period of suppressed AM function.

In any *in vitro* model system variables exist that may not accurately reflect the natural (*in vivo*) disease process. An apparent overall augmentation of AM activity may be misleading if a single specific and important function is impaired. *In vivo* BHV-1 models have demonstrated increases in certain AM functions and decreases in others (36–38). The role of these changes in AM function in the bovine respiratory disease complex is difficult to ascertain.

The variability in AM function between the BRSV inoculated and the control calves may be influenced by the presence of PMN in the lung lavage fluid. However, in this study there was no statistical evidence for this as a possible cause of the variability among AM collected on the same day. It is obvious from examining the data that considerable variation exists between days. The intent of our model of concurrent lavage of control and treated calves was to try to minimize the variability between control and inoculated samples that may occur if control samples are collected and assayed on different days than treated samples. Our *in vivo* BRSV inoculation model did not appear to be as successful as hoped as there is too much calf associated day to day variation. A different modeling system will need to be considered in the future.

The AM used in any assay may bias the experiment. The AM population is a diverse group ranging from monocyte-like cells to highly differentiated large phagocytic cells with different functional capabilities (39). Variation between calves and the effect of pathogenic organisms on the distribution of the AM subpopulations may affect assay results (40). Noninfectious environmental irritants (dusts, noxious gases) could be a potential source of subpopulation variability. Bronchoalveolar lavage may not randomly sample the underlying AM population in the alveolar compartment of the lung (41) and the effect of repeated lung lavage upon the AM subpopulations has not been investigated. Our same day lavage of the control and challenge calves may not have fully compensated for the potential variations in technique and cell types. Assays which use fluorescence activated cell sorter (FACS) technology may reduce experimenter associated variability and should allow an increased number of different assays to be performed on a single sample thereby improving the characterization of AM the populations and functions (41,42). A significant ($p < 0.05$) calf-treatment interaction occurred in a few cases which may have been a function of small numbers of calves. Most clinical disease due to BRSV is very mild suggesting that other factors, such as genotype, affect the response of the individual calf to BRSV infection. We used calves of a single breed but genetic variability could have existed as the pedigrees of the calves were not examined.

The interpretation and extrapolation of data from different studies must allow for possible differences in environmental conditions, calf ages, previous exposure to pathogens and other factors that could affect AM function. *In vitro* assessment of AM function is influenced by culture conditions, AM density and whether adherent or suspended cells are being used (43).

The impact of BRSV upon the function of the alveolar macrophage was primarily upon the uptake of foreign particulates and the fusion of phagosomes (containing these particulates) with lysosomes. The most significant impact appears to occur in the early days of infection which is similar to field observations of feedlot calf pneumonia (27).

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