Effect of Viral Dose on Experimental Pneumonia Caused by Aerosol Exposure of Calves to Bovine Herpesvirus 1 and *Pasteurella haemolytica*

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ABSTRACT

The effect of various aerosol doses of bovine herpesvirus 1, followed four days later by aerosol exposure to a constant level of *Pasteurella haemolytica*, was studied in 16 crossbred Hereford range calves. A Collison nebulizer was used to generate aerosols from virus suspensions with concentrations of $10^{8.2}$ (high), $10^{5.2}$ (moderate) or $10^{2.2}$ (low) $TCID_{50}$/mL. The bacterial suspension contained 10^7 colony forming units/mL.

Control calves exposed only to *P. haemolytica* developed no pulmonary lesions. Calves in the low, moderate and high virus exposure groups developed lobular areas of atelectasis; in addition, one calf in the moderate and all four in the high virus exposure group developed fibrinous pneumonia. One of the latter calves died.

The 50% effective dose for fibrinous pneumonia under these experimental conditions was $10^{6.0}$ $TCID_{50}$ bovine herpesvirus 1/mL of suspension in the nebulizer reservoir, and approximately $10^{4.0}$ infectious units inhaled per calf.

RÉSUMÉ

Cette expérience consistait à étudier, chez 16 veaux de race Hereford croisée, l'effet de diverses doses d'herpèsvirus bovin #1, en aérosols, suivies, quatre jours plus tard, d'une exposition à des aérosols d'une concentration constante de *Pasteurella haemolytica*. Les auteurs utilisèrent un nébuliseur "Collision" pour produire les aérosols viraux, dont la teneur en doses infectieuses $_{50}$ de culture tissulaire/mL était élevée ($10^{8.2}$), modérée ($10^{5.2}$) ou faible ($10^{2.2}$). La suspension bactérienne contenait par ailleurs 10^7 unités formatrices de colonies/mL.

Les veaux témoins exposés seulement à *P. haemolytica* ne développèrent pas de lésions pulmonaires. Ceux qui appartenaient aux groupes soumis à des aérosols viraux de concentration élevée, moyenne ou faible, développèrent des zones lobulaires d'atélectasie; de plus, un des veaux soumis à des aérosols viraux de concentration modérée, tout comme les quatre soumis à ceux de concentration élevée, développa une pneumonie fibrineuse et l'un de ces derniers en mourut.

La dose de virus qui permit de provoquer une pneumonie fibrineuse, dans la proportion de 50%, correspondait à 10^6 doses infectieuses $_{50}$ de culture tissulaire de l'herpèsvirus bovin #1/mL de la suspension versée dans le réservoir du nébuliseur et à environ 10^4 unités infectieuses inhalées par les veaux.

INTRODUCTION

Experimental pneumonia has been reproduced consistently by aerosol exposure of calves to bovine herpesvirus 1 (BHV1), isolate 108, followed in four or more days by *Pasteurella haemolytica*, biotype A, serotype 1 (1, 2). However, the pathogenesis of this disease and its relationship to pneumonic pasteurellosis and "shipping fever" are poorly understood at present. The work reported here examines the dose response relationship between BHV1 and disease production in this experimental model.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Sixteen bull calves were obtained from the Animal Diseases Research Institute herd, which is managed under range conditions typical for western Canada. All were born in April and May, and the experimental studies were done in August. On day 0 of the experiment the calves ranged in age from 85 to 126 days and in weight from 87 to 166 kg.

The calves were selected on the basis of negative results to the following tests: standard serum neu-

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tralization test for antibodies to BHV1; BHV1 virus isolation from nasal swabs placed in minimal essential medium[1] (MEM) with bacterial inhibitors[2] and then grown on primary bovine fetal kidney tissue cultures; complement fixation tests for antibodies to *P. haemolytica* or *P. multocida*; standard cultural and biochemical tests for the presence of *Pasteurella* spp. in nasal swabs placed in transport medium[3] immediately following sampling; and standard cultural and fluorescent antibody procedures for *Mycoplasma* spp. from swabs placed in noninhibitor horse serum broth immediately after sampling. Hemagglutination-inhibition and serum neutralization tests were performed for parainfluenza type 3 (PI-3) and bovine virus diarrhea (BVD) viruses, respectively, but were not considered in selecting calves.

AEROSOL EXPOSURES

A suspension of BHV1, isolate 108, was prepared and titrated as described previously (3). Two 1000-fold dilutions of this suspension were then made in MEM and the three resulting concentrations were designated arbitrarily as high, moderate or low levels of BHV1. The concentrations of these three suspensions were: $10^{8.2}$, $10^{5.2}$, and $10^{2.2}$ $TCID_{50}$/mL, respectively, after freezing for one week at -62°C.

Pasteurella haemolytica cultures were also prepared as described previously (1, 4) and stored at -62°C. The titer was in excess of 10^8 colony forming units (cfu) per mL two weeks prior to the start of this experiment. A separate aliquot was thawed immediately prior to exposure of each group of four calves.

The Collison nebulizer (5) was used to generate the viral and bacterial aerosols into a metal face mask which had a vinyl fringe and drawstring to secure it around each calf's neck. The median diameter of particles generated by this nebulizer with the media employed is less than 1 μm (6). The calves received individual five minute exposures.

The approximate volume of viral or bacterial suspension expended per calf was determined from the difference in volume within the nebulizer reservoir before and after each group of four calves was exposed. Estimations of viable organisms in the aerosol cloud within the hood were made with a multistage liquid impinger (7) containing MEM and bacterial inhibitors in the case of the virus, or 0.5% gelatin in physiological saline for the bacterium. Virus samples were taken for five minutes before and after exposure of each group with the hood sealed where the calf's neck would be during exposures. Pre- and post-exposure reservoir samples were also titrated.

EXPERIMENTAL DESIGN

The 16 calves were weaned and sampled for preexposure status on day 0. They were then randomly divided into groups of four and placed in separate outdoor pens with free access to alfalfa hay and water. The control group was left unexposed, whereas the others were exposed to one of the three levels of BHV1 aerosol.

All calves were examined individually on each day throughout the experiment. Serum samples were obtained on days 4 and 8, as were nasal swabs to be tested for BHV1, pasteurella and mycoplasma. Each calf, including the controls, was exposed to an aerosol of *P. haemolytica* on day 4.

The calves were killed on day 8 by the use of a captive bolt pistol, except in the case of one that died earlier of pneumonia. Tissues were collected and fixed in 10% neutral buffered formalin and Bouin's solution for pathological studies, as well as aseptically for microbiological tests. The processing of tissues for histological sections was done by conventional methods.

CALCULATION OF 50% EFFECTIVE DOSE (ED_{50}) FOR FIBRINOUS PNEUMONIA

Calculation of the number of infectious units in the face mask air to which the high level group was exposed was made from the multistage sampler titers by the conversion method of Rhodes and van Rooyen (8).

An adaptation of previously reported formulae (9, 10) was then used to calculate inhaled dose as shown below:

The principles of Reed and Muench (11) were then used to calculate the ED_{50} for production of fibrinous pneumonia in terms of the concentration of BHV1 in the nebulizer reservoir at the time of exposure and the approximate number of infectious units inhaled per calf.

RESULTS

AEROSOL EXPOSURES

Approximately 2 mL of virus suspension was expended in the five minute exposure given to each calf. Titrations of samples from the nebulizer reservoir for each of the three principal groups are pre-

Inhaled infectious units = concentration in aerosol cloud × minute respiratory volume of calf × exposure time

where concentration = $\frac{\text{total infectious units collected in 5 min}}{\text{volume of air sampled in 5 min}}$

minute respiratory volume of calf = 30 L

and inhaled infectious units = the number of infectious BHV1 particles taken into the respiratory tract at the level of the nostrils.

[1]Grand Island Biological Co. of Canada Ltd., Burlington, Ontario.
[2]Sodium penicillin G 200 IU/mL, streptomycin sulphate 200 μg/mL, neomycin 50 μg/mL.
[3]Precision Culture Cats (Collection and Transport System) PDC-100 AMIE (Modified) Medium, Precision Dynamic Corp., Burbank, California.

TABLE I. Concentrations of BHV1 Used to Expose Calves by Aerosol

Group	Concentration ($TCID_{50}$/mL in Collison reservoir) Before aerosol	After aerosol
High level virus exposure	$10^{8.2}$	$10^{7.7}$
Moderate level virus exposure	$10^{5.2}$	$10^{5.0}$
Low level virus exposure	$10^{2.2}$	$10^{2.2}$

sented in Table I. Virus was not detected in face mask air in the low exposure samples by the method used, and only one sample was positive in the moderate level exposure. In the high level virus exposure, 2.1×10^6 infectious units of BHV1 were collected from the multistage liquid impinger over a five minute sampling period at 55 L of air/min (7.6×10^3 infectious units/L of air). By extrapolation, the moderate level exposure would be expected to have contained approximately 2.1×10^3 infectious units and the low level $2.1 \times 10^0 = 2.1$ infectious units.

The calculated inhaled virus dose for the high level exposure group was, therefore:

7.6×10^3 infectious units/L $\times$ 30 L $\times$ 5 min
$= 1.1 \times 10^6$ infectious units of BHV1 per calf.

By extrapolation, calves in the moderate and low level exposure groups would be expected to have inhaled approximately 1.1×10^3 and 1.1×10^0 infectious units, respectively, all other factors being equal.

The mean concentration of *P. haemolytica* in the nebulizer reservoir before and after exposure of each group of four calves was $3.7 \pm 0.6 \times 10^7$/mL of suspension on nine titrations. Impinger samples taken from the closed hood without a calf averaged $1.1 \pm 1.3 \times 10^4$/mL on two samplings. Using calculations similar to those applied to the virus exposures, it was estimated that the inhaled dose for each calf would be approximately 1.1×10^6 *P. haemolytica*, at the level of the nostrils, during the five minute exposure.

CLINICAL SIGNS

Most calves in all groups produced mucoid nasal exudates within several days after the start of the experiment. There were no other remarkable clinical signs in the controls (exposed to *P. haemolytica* but not to BHV1) or calves exposed to the low viral dose.

Two animals in the moderate exposure group had a rectal temperature of 40°C by day 4 and a third by day 5. One of these maintained this fever through day 7, one through day 6, and the other was normal after day 5. Nasal exudates in three of the four calves became mucopurulent during the latter days of the experiment. Lung sounds were normal on auscultation.

Three of the calves in the high exposure group were depressed by day 5. One of these became moribund and died late on day 6. The fourth calf became depressed on that day. The three survivors remained depressed but ate sparingly. All four had rectal temperatures of 40.0°C or more by day 2, and maintained this level through day 6. The highest temperature was 42.1°C reached by one calf on day 6. On that day, increased lung sounds, suggestive of consolidation, were present. There were occasional râles, and one calf had pleuritic friction rubs. The calf that died naturally had a marked expiratory grunt on the evening of day 6. On day 8, prior to necropsy, the rectal temperatures of the three surviving calves were 40.3, 39.5 and 39.1°C. All had mucopurulent nasal exudates, often quite copious in amount, during the last few days of the experiment.

The rectal temperatures for the four groups are outlined in Fig. 1.

In summary, the control and low

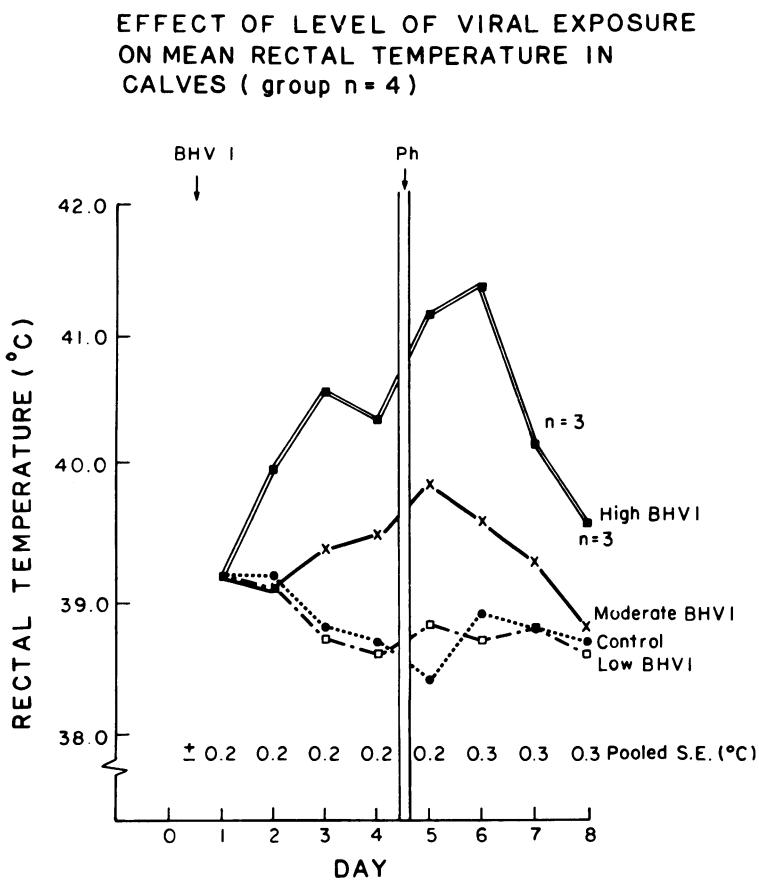

Fig. 1. Group means for rectal temperatures. The moderate exposure group became marginally febrile but returned to normal by the end of the experiment. In contrast, those exposed to high level BHV1 were markedly febrile by day 3 and became increasingly so after *P. haemolytica* (Ph) exposure, until the most severely affected calf died.

TABLE II. Isolations of BHV1 at Necropsy[a]

Group	Tissue	
	Pharynx	Lung
High level virus exposure	4	3
Moderate level virus exposure	2	2
Low level virus exposure	0	0

[a]Number of calves positive in each group of four

TABLE III. Isolations of *P. haemolytica* at Necropsy[a]

Group	Tissue		
	Pharynx	Bronchial lymph node	Lung
High level virus exposure	3	4	4
Moderate level virus exposure	4	1	1
Low level virus exposure	3	0[b]	0
Control (No virus exposure)	4	2	0

[a]Number of calves positive in each group of four
[b]Only three of the four calves in this group were sampled

viral exposure groups remained normal, the moderate viral exposure group became febrile and the high exposure group became severely ill with one death.

MICROBIOLOGY AND SEROLOGY

Serum neutralization (SN) tests for BHV1 were negative for all calves on days 0 and 4. On day 8 one calf in the moderate virus exposure group had a titer of 1:4; the calf that died in the high exposure group was not tested serologically after day 4. Necropsy tissue isolations of BHV1 are presented in Table II.

Serum neutralization tests on day 6 for BVD were positive at ¼ 1:400 in all except one calf, which was, however, positive at 1:200. All calves were negative by hemagglutination inhibition for antibodies to PI-3 virus on day 0, but, when tested on days 4 and 8, some calves in each group had low positive PI-3 titers.

All calves were negative for complement fixing antibodies to *P. multocida* and *P. haemolytica* except for the moderate level exposure calf that will be described below as having fibrinous pneumonia. This calf had a low titer to *P. haemolytica* (1:8) on the day of necropsy.

All nasal swabs cultured for *P. multocida* and *P. haemolytica* were negative on days 0 and 4. Bacteriological results at necropsy are presented in Table III.

Nasal swabs taken on days 0, 4 and 8 of the experiment showed a variable pattern of *Mycoplasma* spp. isolations which included *Acholeplasma laidlawii* (by far the most common isolate), *Mycoplasma bovirhinis* and *Ureaplasma* sp. At necropsy, all lungs were negative for *Mycoplasma* spp. including *M. dispar*.

GROSS NECROPSY LESIONS

As in previous studies with this model (1,2), two types of lung lesion were seen and these will be used to classify dose response effects of the viral-bacterial interaction: (a) collapsed, firm, dark red to black areas of atelectasis. These could involve part or all of a lobule, or several confluent lobules. Such changes will be referred to as infectious bovine rhinotracheitis ("IBR (108)") lesions for the work presented here; (b) firm swollen areas, lobar and anteroventral in distribution, and usually covered by fibrinous pleuritis. Interlobular septa were distended with edema and fibrin. These changes will be referred to here as "fibrinous pneumonia". The airways associated with both types of lesion usually contained plugs of white, viscid exudate and debris.

The control calves, exposed only to *P. haemolytica*, had no lung lesions. One animal had a small amount of mucopurulent exudate on its vocal cords, and another had copious amounts of very mucoid material in its palatine sinus. The nasal passages, pharynx and trachea were normal in each calf.

In the low level virus exposure group one calf had diphtheritic laryngitis, but this was the only upper respiratory tract lesion seen in any of the four. All had "IBR (108)" lung lesions. These were scattered over the anteroventral regions in three calves, and over all areas in the fourth. The lesions were not numerous and none were greater than one lobule in size. There was no fibrinous pneumonia.

Two of the four calves exposed to the moderate level of virus had diphtheritic laryngitis. These were the only upper respiratory tract lesions. Lobular-sized "IBR (108)" lesions were scattered over most lobes of each lung, but were predominant in the anteroventral area. These lesions were in general larger than those in the low exposure group, but were not more numerous. In addition to viral lesions in several lobes, one calf had fibrinous pneumonia throughout the dependent portion of the cranial part of the right cranial lobe.

In the high level virus exposure group, all four calves had diphtheritic rhinitis and pharyngitis, three had focal, diphtheritic tracheitis, and two had laryngitis. The "IBR (108)" and fibrinous lung lesions were present in all calves of this group. In three of the four, the viral lesions were far more extensive than in the previous groups. Many confluent lobules and, in one case, almost one-half the right middle lobe, were affected. In general the distribution was anteroventral. Fibrinous pneumonia, involving large areas of lung, was present in each calf (Fig. 2) and was always distributed over the anteroventral two-thirds.

HISTOPATHOLOGICAL FINDINGS

The four control calves had no histological lesions in the respiratory tract.

The "IBR (108)" lesions seen grossly in the low and moderate exposure groups consisted of collapsed lobules but no exudative pneumonia. Focal, necrotizing bronchitis and bronchiolitis were seen occasionally.

The fibrinous pneumonias seen in one moderate level and all four high level exposure calves (Figs. 3 and 4) consisted of fibrinous pleuritis and

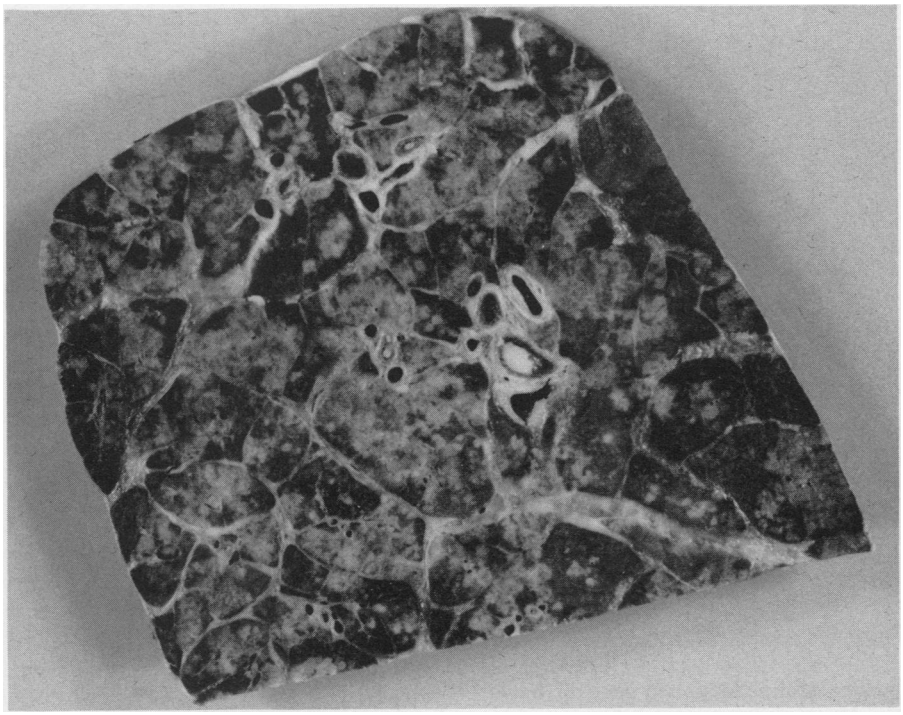

Fig. 2. Fibrinous pneumonia in a cross-section of formalin-fixed lung from calf in high virus exposure group. Note the "marbled" appearance of the entire lobe and the fibrinous distension of interlobular septa.

interlobular septal distension, areas of parenchymal necrosis bordered by pyknotic, "streaming" leukocytes, and alveolar exudate composed of edema, fibrin, macrophages and some neutrophils. Clumps of Gram-negative bacteria were seen sporadically in alveoli of pneumonic areas.

The pathological changes are summarized in Table IV.

FIFTY PERCENT EFFECTIVE DOSE (ED_{50}) FOR FIBRINOUS PNEUMONIA

The ED_{50} of BHV1 for the production of fibrinous pneumonia in this experiment was a concentration of $10^{6.0}$ $TCID_{50}$/mL of BHV1 in the nebulizer reservoir and the inhalation of $10^{4.0}$ infectious units of BHV1 per calf.

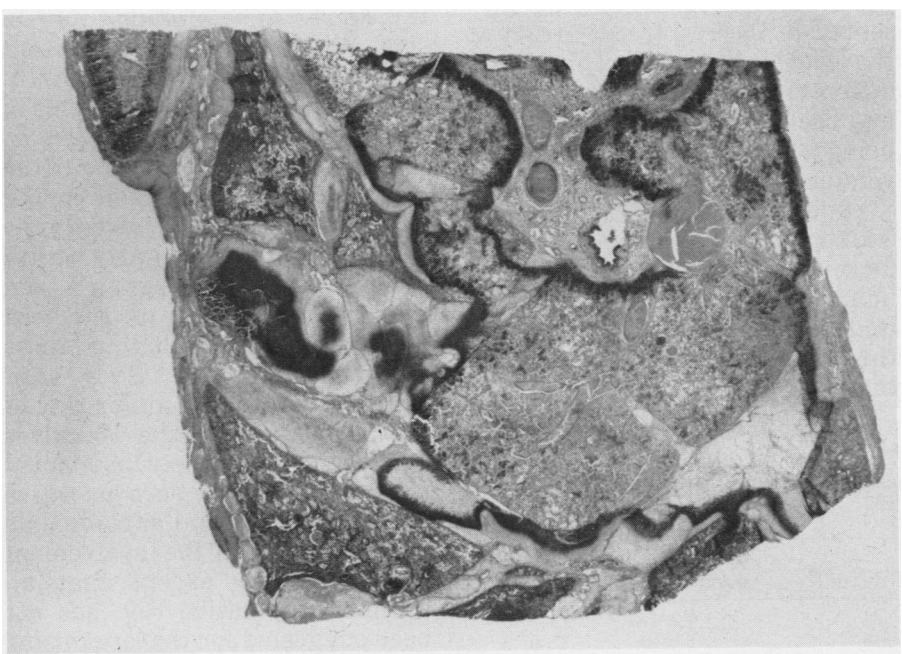

Fig. 3. Macrophotograph of a histological section from the high exposure group. Note fibrinous pleuritis and septal distension, and the demarcation of affected areas by dense margins of leukocytes. Masson's trichrome X2.

DISCUSSION

The results demonstrated a positive relationship between the dose of BHV1 to which calves were exposed prior to *P. haemolytica*, and the presence and severity of fibrinous pneumonia that was produced subsequently. The moderate level of virus (10^5 $TCID_{50}$/mL in nebulizer reservoir) was the minimum exposure necessary to stimulate a febrile clinical response and to produce fibrinous pulmonary lesions; these disease manifestations were markedly more consistent and severe in the high level exposure group.

There was considerable individual variation within groups. Only one of four calves in the moderate exposure group had fibrinous pneumonia and, although all four calves in the high exposure group had such lesions, only one died. Such variability in response to combined infection was not unexpected in light of similar findings after pure virus exposures. Gaskell and Povey (12), working with feline herpesvirus 1, found that with increasing dose there was a shorter incubation period before the onset of clinical signs and virus excretion, but that individual variation in the response was quite marked. If response to virus is variable, then viral bacterial interactions, as yet poorly understood, may be subject to similar or even more complex influences.

The calculation that $10^{4.0}$ infectious units of BHV1 was the 50% endpoint for fibrinous pneumonia in this experiment required several assumptions and qualifications. 1) The calculations applied to virus levels followed in four days by exposure to *P. haemolytica* at a concentration of 10^7/mL in the nebulizer reservoir. 2) The conversion of $TCID_{50}$ to infectious units necessitated the use of mathematical formulae based on

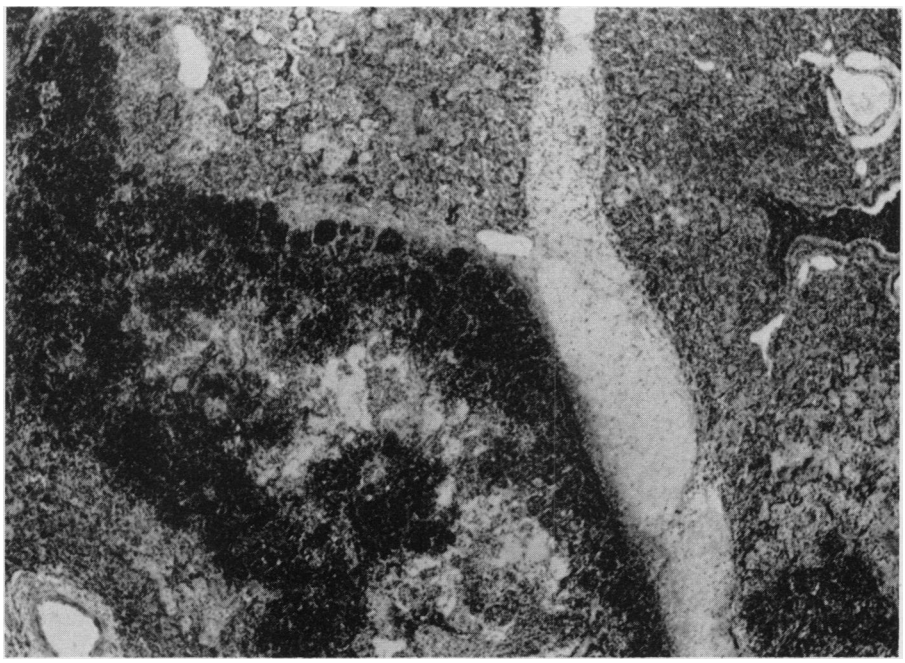

Fig. 4. Fibrinous pneumonia in the high exposure group. Section of necrotic parenchyma surrounded by a margin of leukocytes. Masson's trichrome X20.

probabilities. 3) The minute respiratory volume of calves is subject to considerable variation according to size, demeanor, etc., so that selection of the 30 L figure was done arbitrarily based on calves of similar age described in the work of Hales and Findlay (13). 4) Sampling and titration systems are never entirely accurate. Frank and Marshall (14) stated that, for their apparatus, the differences in minute volumes of individual calves were overshadowed by variation in impinger sample assays of the aerosols. Our selection of the multistage liquid sampler was an attempt to increase virus recovery by a gentler sampling method. 5) Finally, the presence of a calf's head within the mask, and the animal's respirations during exposure, might affect the actual inhaled dose when compared with calculations made with an empty hood. The calf would draw in additional fresh air beyond that supplied by the nebulizer, so that the true dose necessary for viral-bacterial interaction might be somewhat lower than the calculated dose.

It should also be pointed out that the calculations given here refer to the numbers of organisms presented to the respiratory tract at the level of the nostril. This is very different from retention, which has been calculated by others as being 50% of the product of minute volume × concentration in aerosol × exposure time (14). Brain and Valberg (15) have pointed to the lack of an accurate means of measuring the dose of a pathogen deposited in the respiratory tract due to the "constellation of variables" that affect it.

In view of the qualifications that must be made on inhaled dose calculations, emphasis might more appropriately be placed on the concentration of BHV1 in the nebulizer reservoir at the time of exposure, for which the ED_{50} for fibrinous pneumonia was $10^{6.0}$ $TCID_{50}$/mL. Support for this is found in the work of Laurenzi *et al* (9), who found a linear relationship among bacterial concentration in: a) the nebulizer reservoir, b) the exposure air and c) retained in the lungs of mice exposed to staphylococci.

The health status of calves used in respiratory disease studies is of great importance. Calves used in the present experiment were negative for BHV1, *P. haemolytica* and *P. multocida*. The *Mycoplasma* spp. isolated from nasal swabs appeared to represent an ever-changing population. However, negative cultures from all lungs at necropsy and the absence of lesions in the controls suggested that, in this experiment, mycoplasma did not play a role in producing the pulmonary lesions. High BVD titers posed more of an interpretive problem. Other workers (16), faced with similar titers, have commented that the specific effects caused by the presence of BVD virus are unknown, but that it is known to be immunosuppressive. With regard to the present study, several observations indicated that BVD was not a factor in the results. Firstly, there were no clinical signs nor any lesions in the control calves, despite their having BVD titers similar to calves in the other groups. Secondly, mild mucous nasal discharges are routinely seen in calves of this herd after weaning, including those with no detectable BVD antibodies. Thirdly, the uniformity of the BVD titers in the 16 calves would suggest that any undetected effects should have been operative over all groups. Similar comments could be made for the involvement of PI-3 virus, except that an immunosuppressive role has not been suggested for this organism.

Microbiological results permit some speculation on the pathogenesis of the lesions produced.

TABLE IV. Pathological Effects in Calves Exposed to Various Levels of BHV1 Followed by *P. haemolytica*[a]

Group	IBR (108)	Fibrinous pneumonia	Death
High level virus exposure	4	4	1
Moderate level virus exposure	4	1	0
Low level virus exposure	4	0	0
Control (No virus exposure)	0	0	0

[a]Number of calves positive in each group of four

There appeared to be a threshold for BHV1 infection in that the virus was only isolated from lung tissue in the moderate and high level exposure groups. Smith (17) commented that, as for bacteria, the ability of a virus to cause disease depends on host defenses, the microbe's capacity to counteract them, and also on the "number of invaders". The pharyngeal tonsil was a good site for postmortem isolation of *P. haemolytica*, which was present there in most calves of all four groups. However, only calves exposed to moderate or high levels of virus had *P. haemolytica* in their lungs. Most calves, therefore, became infected with the bacterium, but its persistence within the lung was correlated with increasing virus dose.

The mechanism whereby BHV1 affects the ability of *P. haemolytica* to remain and proliferate in the lung is not clear. Jakab (18) has shown that, in a mouse system using Sendai virus (parainfluenza type 1), there is a spectrum of viral dose-dependent suppression of lung antibacterial activity. It is possible that, if increasing virus dose caused greater numbers of viral atelectatic lesions, subsequent aerosolization with *P. haemolytica* could result in more intense exposure of uncollapsed areas of lung, which in turn could overwhelm local defenses. Such an altered deposition pattern has been shown experimentally in mice (19). Viral-bacterial interactions in respiratory disease are under intensive study at present, and numerous mechanisms have been suggested (20).

Bovine respiratory disease is a complex problem in which any of numerous pathogens may be involved, but the numbers of each that are required to produce pneumonia under field conditions are generally unknown. The work presented here indicates that, in a BHV1-*P. haemolytica* model system, the degree of virus exposure has a profound effect on the type and severity of pneumonia produced. As management systems become more refined, such information may be of importance in selecting density ratios and transportation methods and in setting other standards for humane and economical cattle production.

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