The Pulmonary Clearance of *Pasteurella haemolytica* in Calves Given *Corynebacterium parvum* and Infected with Parainfluenza-3 Virus

S.A. Al-Izzi, M.G. Maxie and M. Savan*

ABSTRACT

Four control calves were aerosolized with parainfluenza-3 virus and one week later with *Pasteurella haemolytica*. Three calves were given *Corynebacterium parvum* at a dose of 15 mg/m² body surface area, infected with parainfluenza-3 virus one week later, and aerosolized with *P. haemolytica* two weeks after *C. parvum* injection. All calves were killed four hours after *P. haemolytica* exposure and the bacterial retention in the lung was determined. Parainfluenza-3 viral infection did not exert any suppressive effect on pulmonary clearance of *P. haemolytica* in six out of seven calves used. However, the bacterial colony counts in the lungs of control calves were higher ($P < 0.05$) than those in calves given *C. parvum*. Hence, *C. parvum* appeared to enhance bacterial clearance. Despite the marked influx of neutrophils into the lungs after the bacterial inoculation, the neutrophil:macrophage ratio in lavage samples was less in calves given *C. parvum* than in the control calves. The alveolar macrophages in *C. parvum* treated calves were generally larger but did not differ significantly ($P > 0.05$) from those in the controls. There was no significant ($P > 0.05$) correlation between the percentages of alveolar macrophages and the bacterial clearance. In calves given *C. parvum*, bacterial clearance was enhanced in those calves which had larger macrophages.

RÉSUMÉ

Cette expérience consistait à soumettre quatre veaux témoins à des aérosols porteurs du virus PI-3 et à répéter le même manège, une semaine plus tard, avec *Pasteurella haemolytica*. Trois autres veaux reçurent d'abord, par la voie intraveineuse, une injection équivalente à 15 mg/m² de surface corporelle d'une bactérine de *Corynebacterium parvum*; une semaine plus tard, on les soumit à des aérosols porteurs du virus PI-3 et, deux semaines après l'injection de la bactérine, à des aérosols porteurs de *P. haemolytica*. On sacrifia tous les veaux, quatre heures après les avoir exposés à *P. haemolytica*, et on détermina la rétention bactérienne au sein de leurs poumons. L'infection avec le virus PI-3 n'exerça aucun effet de suppression sur la clairance pulmonaire de *P. haemolytica*, chez six des sept veaux impliqués dans l'expérience. Le nombre de colonies bactériennes s'avéra toutefois plus élevé ($P < 0,05$) dans les poumons des témoins que dans ceux des veaux auxquels on avait injecté la bactérine de *C. parvum*; elle sembla par conséquent intensifier la clairance bactérienne. En dépit de l'arrivée massive de neutrophiles dans les poumons, après l'inoculation bactérienne, le rapport: neutrophiles/macrophages s'avéra plus faible dans la solution de lavage, chez les veaux qui avaient reçu l'injection de la bactérine de *C. parvum* que chez les autres. Les macrophages alvéolaires des veaux qui avaient reçu l'injection de cette bactérine s'avérènt sensiblement plus volumineux que ceux des témoins, sans toutefois présenter de différence appréciable ($P > 0,05$). On n'enregistra pas de relation appréciable ($P > 0,05$) entre le pourcentage de macrophages alvéolaires et celui de la clairance bactérienne. Cette dernière se trouva cependant intensifiée chez les veaux qui avaient reçu l'injection de la bactérine de *C. parvum* et qui présentaient les macrophages les plus volumineux.

INTRODUCTION

Pulmonary clearance is an *in vivo* technique used to measure the rate at which inhaled bacteria disappear from the lung. This technique has been used as a tool to study pulmonary defense mechanisms against viral and bacterial infection in laboratory animals

*Department of Pathology (Al-Izzi, Maxie) and Department of Veterinary Microbiology and Immunology (Savan), Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1.

Requests for reprints should be addressed to Dr. M.G. Maxie.

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(13, 16) and in calves (10, 17). Lopez *et al* (17) found that the pulmonary clearance of *Pasteurella haemolytica* was depressed in calves exposed to parainfluenza-3 (PI-3) virus seven and 11 days previously. In mice, Sendai viral infection interfered with the pulmonary clearance of *Staphylococcus aureus* seven days after viral aerosolization (14). This interference in mice was related primarily to defects in intracellular processing mechanisms of alveolar macrophages.

Corynebacterium parvum has been shown to be effective in increasing host resistance to viral (11, 15, 20, 21), bacterial (1, 9, 24), and protozoal infection (8, 22, 24) by activating mononuclear phagocytes. The present study was designed to investigate whether *C. parvum* injection, which causes nonspecific stimulation of the mononuclear phagocyte system, could modify the suppressive effect of PI-3 virus on pulmonary clearance of *P. haemolytica* in calves.

MATERIALS AND METHODS

Six male and two female Holstein-Friesian calves were selected from a group of 22 similar calves on the basis of having low serum antibody titers to PI-3 virus (1:16 or lower in a hemagglutination inhibition test). The ages of these calves ranged from six to eight weeks and their weights varied between 46 and 85 kg. Clinical, hematological and parasitological examinations were performed prior to the experiment to ensure that all calves were normal. Nasal swabs obtained from these calves were negative for PI-3 cytopathic effect (CPE) and hemadsorption in bovine embryo spleen cell cultures. These calves were divided into two groups (A and B). The first group (A) included two control calves (A1, A2) and two calves given *C. parvum* (A3, A4), and the second group (B) composed of two control calves (B1, B2) and one calf which was given *C. parvum* (B3). An additional calf (B4) was given *C. parvum*, but this calf was eliminated from the study due to the necropsy finding of extensive chronic suppurative bronchopneumonia. The studies on groups A and B were initiated two weeks apart. In addition, ten adult white mice[1] weighing 28-32 grams were used.

CORYNEBACTERIUM PARVUM TREATMENT

A suspension of formalin killed *C. parvum*[2] (strain CN 6134) was diluted 1:6 with sterile normal saline. At zero time three calves (A3, A4, B3) were each given an intravenous injection (into the jugular vein) of the diluted bacterial suspension at a dose of 15 mg/m^2 body surface area. The four control calves (A1, A2, B1, B2) were each given an intravenous injection of 35 mL of normal saline.

TITRATION OF SERUM FOR PI-3 VIRUS AND *P. HAEMOLYTICA* ANTIBODIES

A hemagglutination inhibition test (7) and an indirect hemagglutination test (6) were used to titrate serum antibodies for PI-3 virus and *P. haemolytica* respectively.

AEROSOLIZATION

The aerosol apparatus and the method of aerosolization were the same as previously used and described by Gilka *et al* (10) and Lopez *et al* (17).

The virus used in this experiment was the ninth tissue culture passage of a field isolate of PI-3 virus (4644-9, O.V.C.). One hundred mL of a viral suspension with a cell culture infective dose 50% (C.C.I.D. 50) of 10^4 per 0.2 mL were aerosolized for a time of approximately 30 minutes for each group (A and B) one week after *C. parvum* treatment.

Pasteurella haemolytica, serotype I, biotype A, was utilized in this experiment. The bacterial suspensions were prepared according to Lopez *et al* (17). Both groups were aerosolized with *P. haemolytica*, one group at a time, two weeks after *C. parvum* treatment. Five mice were aerosolized together with each group of calves. The mice and the calves were killed at zero and four hours, respectively, after bacterial exposure and the bacterial retention in their lungs was determined.

PULMONARY CLEARANCE

The euthanasia, sample collections and determination of number of bacteria per gram of lung in calves at four hours after bacterial inoculation were performed as described by Lopez *et al* (17). The determination of the initial deposition of *P. haemolytica* in calves lungs was calculated from the mice's lung deposition according to Lopez-Mayagoitia (18).

BRONCHOALVEOLAR LAVAGE

Immediately after four samples of lung were taken for the clearance study, the lungs were removed from the carcass, placed in a tray and the left apical lobe was separated by cutting the main bronchus. A plastic tube mounted on a 60 mL syringe was introduced into a secondary bronchus and the lung was lavaged with 250 mL of phosphate-buffered saline (PBS). The lavage fluid was transferred into a 250 mL polypropylene bottle. The pulmonary lavage sample was passed through sterile cotton gauze to remove mucus and debris, and then centrifuged at 400 g for 30 min. The resulting cell pellet was suspended in 20 mL of PBS, transferred into a 50 mL conical plastic tube and washed twice with PBS. The cells were resuspended in 10 mL of PBS, two smears were made from each sample and the remaining cells were cultured for virus isolation and detection of viral antigen.

The smears were stained with Wright's stain and 500 nucleated cells were differentiated in two smears from each animal. The areas and the perimeter lengths of 100 macrophages from each

[1]Connaught Laboratories, Toronto, Ontario, Canada.
[2]Burroughs Wellcome Ltd., Montreal, Quebec, Canada.

animal were calculated using an image analyzer[3] with a camera lucida attached to a microscope.[4]

ISOLATION OF VIRUS IN CELL CULTURES

Primary bovine embryo spleen cells were infected with 0.2 mL of 10% suspensions from organ specimens (lung, trachea and bronchial lymph node) and 0.2 mL of undiluted PBS in which nasal swabs had been placed. The cultures were incubated at 37°C for six to eight days and observed for evidence of CPE. The CPE-producing virus was identified by a hemadsorption (HAD) test.

The alveolar macrophages were cultured in Eagle's minimum essential medium[5] containing 5% fetal calf serum.[6] The cultures were incubated at 37°C for four to fourteen days and observed for evidence of CPE and tested for hemadsorption.

DEMONSTRATION OF VIRAL ANTIGEN BY IMMUNOFLUORESCENCE

Lung, trachea and bronchial lymph node samples and cultured alveolar macrophages were fixed in chilled-acetone and examined by direct immunofluorescence using commercial conjugates.[7]

STATISTICAL ANALYSIS

The number of *P. haemolytica* present in calves lungs at zero and four hours after bacterial aerosolization were transformed to common logarithms (Log_{10}). The ratios between log number of bacteria at four hours and log number of bacteria at zero hour in calves given *C. parvum* were compared with those in control calves using Student's t-test. The percentages and the sizes of the alveolar macrophages in *C. parvum* treated calves were compared with those in control calves using Student's t-test. Correlation of percentages and sizes of alveolar macrophages with pulmonary clearance were examined by use of simple linear regression analysis.

RESULTS

SERUM ANTIBODY TITERS

The aerosolization with PI-3 virus did not cause an increase in serum antibodies to this virus in calves given *C. parvum* or in control calves (Table I).

The serum antibody titer to *P. haemolytica* did not change in two calves given *C. parvum*, but the titer increased in the third calf (Table II). The calf with the highest titer had the lowest retention of

TABLE I. Serum Antibody Titers to PI-3 Virus in Control Calves and in Calves Given *C. parvum*

Type of Treatment	Calf number	Days after *C. parvum* injection			
		-6 or -7	7[a]	13	14[b]
Controls	A1	1:16	1:4	1:4	1:4
	A2	1:16	1:4	1:4	1:4
	B1	1:8	1:16	1:16	1:8
	B2	1:16	1:16	1:4	1:8
C. parvum	A3	1:16	1:4	1:4	1:8
	A4	1:8	1:8	1:4	1:4
	B3	1:16	1:8	1:8	1:8

[a]One hour before PI-3 inoculation
[b]Three hours after *P. haemolytica* inoculation

TABLE II. Serum Antibody Titers to *P. haemolytica* in Control Calves and in Calves Given *C. parvum*

Type of Treatment	Calf number	Days after *C. parvum* injection			
		-6 or -7	7[a]	13[b]	14[c]
Controls	A1	1:16	1:2	1:8	1:4
	A2	1:8	1:16	1:8	1:8
	B1	1:16	1:16	1:16	1:32
	B2	1:8	1:4	1:8	1:16
C. parvum	A3	1:8	1:8	1:4	1:4
	A4	1:8	1:8	1:4	1:4
	B3	negative	1:8	1:128	1:128

[a]One hour before PI-3 inoculation
[b]One day before *P. haemolytica* inoculation
[c]Three hours after *P. haemolytica* inoculation

TABLE III. Inoculum, Deposition and Retention of *P. haemolytica* in Calves Lungs

Type of Treatment	Calf Number	*P. haemolytica*[a] inoculum titer/mL ($\times 10^9$)	*P. haemolytica* in calves lungs at zero hour ($\times 10^5$/g)	*P. haemolytica* in calves lungs at four hours ($\times 10^3$/g)	Percent of *P. haemolytica* retained in lung
Controls	A1	2.5	4.6	2.12 ± 1.06[b]	0.46 ± 0.23
	A2	2.5	4.6	8.11 ± 5.68	1.77 ± 1.23
	B1	0.31	5.1	6.90 ± 4.35	1.35 ± 0.85
	B2	0.31	5.1	91.98 ± 172.3	18.04 ± 33.73
C. parvum	A3	2.5	4.6	3.68 ± 5.56	0.80 ± 1.21
	A4	2.5	4.6	4.78 ± 3.10	1.04 ± 0.67
	B3	0.31	5.1	0.92 ± 0.57	0.18 ± 0.12

[a]Time of aerosolization = 30 minutes. Volume = approximately 150 mL
[b]Mean ± standard deviation

[3]MOP-3, Carl Zeiss Inc., West Germany.
[4]Ultraphot II, Carl Zeiss Inc., West Germany.
[5]Flow Laboratories, Toronto, Ontario, Canada.
[6]Grand Island Biological Co., Burlington, Ontario, Canada.
[7]Colorado Serum Company, Denver, Colorado, U.S.A.

P. haemolytica. The contol calves had an erratic pattern in which the titer increased and decreased during the study.

PULMONARY CLEARANCE OF *P. HAEMOLYTICA*

The numbers of *P. haemolytica* in the inoculum, in calves lungs at zero and four hours after aerosolization, and the percentages of bacterial retention in calves lungs are given in Table III. There was significantly lower ($p < 0.05$) bacterial retention in calves given *C. parvum* than in control calves.

BRONCHOALVEOLAR LAVAGE

The distribution of the cells in the lavage fluid was almost the same in both groups with the exception of macrophages where the percentage in calves given *C. parvum* was generally higher than that of the controls (Table IV). However, there was no significant ($p > 0.05$) difference in the percentage of macrophages between the two groups.

The alveolar macrophages appeared to be larger in calves given *C. parvum* than in control calves (Table V), but there was no significant ($p > 0.05$) difference in the sizes of the alveolar macrophages between the two groups.

There were no significant ($p > 0.05$) correlations either between the percentages of the alveolar macrophages and the pulmonary clearance in control calves or in calves given *C. parvum*, nor were there significant ($p > 0.05$) correlations between the sizes of the alveolar macrophages and the pulmonary clearance in control calves. However, the size of the alveolar macrophages were correlated significantly ($p < 0.05$) with the pulmonary clearance in *C. parvum* treated calves, i.e. bacterial clearance was enhanced in calves with larger macrophages.

PARAINFLUENZA-3 VIRUS ISOLATION

Parainfluenza-3 virus was not recovered from either the nasal swabs or from the tissues (lung, bronchial lymph node, trachea and alveolar macrophages) collected at postmortem, except for one control calf in which cultured alveolar macrophages were positive in the hemadsorption test, but hemadsorption was not reproduced in subculture. Viral antigen was present in lung, bronchial lymph node, trachea and alveolar macrophages according to the direct fluorescent antibody technique used (Table VI).

TABLE IV. Percentage Distribution of Cells in the Bronchoalveolar Lavage Four Hours After *P. haemolytica* Aerosolization. A Total of Five Hundred Nucleated Cells Were Differentiated in Two Smears from Each Calf

Type of cell	% of each cell type in the lavage fluid: Controls (N[a] = 4)	C. parvum (N[a] = 3)
Macrophages	10.0 ± 6.68[b]	15.67 ± 13.32
Neutrophils	75.0 ± 12.82	74.0 ± 22.87
Lymphocytes	8.75 ± 4.92	6.0 ± 5.0
Plasma cells	0.50 ± 0.58	0.0
Epithelial cells	5.75 ± 2.50	4.33 ± 4.93

[a]N = number of calves
[b]Mean ± Standard deviation

TABLE V. The Sizes of the Alveolar Macrophages Four Hours After *P. haemolytica* Aerosolization. The Sizes of One Hundred Macrophages Were Measured in a Smear from Each Calf

Type of Treatment	Calf Number	Macrophages sizes Mean ± S.D.: Area μ^2	Perimeter Length μ
Control	A1	97.7 ± 26.6	36.4 ± 4.9
	A2	78.4 ± 19.1	32.7 ± 4.3
	B1	94.2 ± 20.1	35.4 ± 3.7
	B2	78.7 ± 15.4	32.6 ± 3.3
		Grand mean = 87.3 ± 10.1[a]	Grand mean = 34.3 ± 1.9
C. parvum	A3	98.3 ± 17.4	36.1 ± 4.3
	A4	96.2 ± 28.1	36.3 ± 5.4
	B3	115.2 ± 35.5	40.6 ± 5.7
		Grand mean = 103.2 ± 10.3	Grand mean = 37.7 ± 2.5

[a]± Standard deviation

TABLE VI. Immunofluorescent Examination for PI-3 Virus Antigen in Tissues Obtained from Control Calves and Calves Given *C. parvum*

Type of Treatment	Calf Number	Immunofluorescence: Lung	Trachea	Bronchial Lymph node	Alveolar Macrophages %[a]
Controls	A1	+++[b]	++	+	25
	A2	+	+	+	33
	B1	+	+	+	–[c]
	B2	+	+	+	–[c]
C. parvum	A3	+	+	negative	50
	A4	+	+	negative	50
	B3	+	+	+	–[c]

[a]Percentage of alveolar macrophages containing fluorescent particles
[b]Fluorescent particles appeared to be present in one (+), two (++), or three (+++) out of four fields examined at 160×
[c]= Not available

DISCUSSION

The well known protective effects associated with antibodies to PI-3 virus in experimental virus-bacteria combined infections in cattle (10, 17) were avoided by using calves that had 1:16 or less serum antibody titers. Calves given *C. parvum* and control calves did not seroconvert after PI-3 virus infection. These calves may not have had enough time (seven days) to express a detectable antibody response (3). Likewise, Allen *et al* (2) and Lopez *et al* (17) did not find any seroconversion in calves within seven days of PI-3 virus infection.

In the present study the serum

antibody titer to *P. haemolytica* was 1:16 or less initially and did not change dramatically during the experiment in calves given *C. parvum* or in control calves with the exception of one calf in which the titer increased to 1:128 one day before bacterial aerosolization. This calf had the lowest retention of *P. haemolytica* which might suggest that the increase in specific antibody titer could provide some protective immunity against this bacterium.

Parainfluenza-3 viral infection did not exert any suppressive effect on pulmonary clearance of *P. haemolytica* in six out of seven calves used in this study, contrary to what was expected (17). These findings can be explained in two ways; either these calves were resistant to PI-3 viral infection, or this strain of virus was not virulent enough to exert pathological changes in the lung and in turn cause depression of pulmonary clearance. The first explanation seems to be invalid since these calves had low titers to PI-3 virus and should not have been protected against viral infection (17). However nasal and bronchoalveolar lavage antibodies were not examined and if present they might have protected the animals against PI-3 viral infection. These calves did not show any of the expected clinical signs due to PI-3 viral infection, such as fever and polypnea. Parainfluenza-3 virus was not isolated either from nasal swabs or from trachea, bronchial lymph node, lung or alveolar macrophages. The presence of viral antigen in these tissues, as detected by direct fluorescent antibody technique, indicated that this virus was present but it was not virulent and had died within the cells. All the available information supports the second suggestion concerning the low virulence of this strain of PI-3 virus.

Although the PI-3 viral infection did not suppress the pulmonary clearance of *P. haemolytica* in six calves, the bacterial colony counts in the lungs of control calves were higher ($p < 0.05$) than those in calves given *C. parvum*. An increase in bacterial clearance was observed in the liver, spleen and blood in mice intravenously injected with *S. enteritidis* and previously treated with *C. parvum* (9). Resistance to bacterial infection has been attributed to the stimulation of mononuclear phagocytes by *C. parvum* (1, 24). Macrophage activation was reflected by an increase in lysosomal enzymes (4, 23), phagocytosis of sheep erythrocytes (4), or killing of bacteria (9) and protozoa (24). In addition, *C. parvum*-stimulated mice peritoneal macrophages were cytotoxic to tumor cells *in vitro* (5). There was a significant ($p < 0.05$) correlation between the sizes of the alveolar macrophages and the bacterial clearance in calves given *C. parvum*. These findings indicated that there was a relationship between cell size and antibacterial activity of alveolar macrophages. Positive correlation between cell size and antitumor activity of peritoneal macrophages obtained from rats given *C. parvum* was demonstrated by Miller *et al* (19). They showed that most of the antitumor activity in a population of peritoneal macrophages was localized in fractions containing large dense cells using a Ficoll gradient cell separation technique. Our results suggest that alveolar macrophages were activated by *C. parvum* which was reflected by the increases in their numbers, size, and their ability to clear bacteria from the lung.

The mechanisms whereby neutrophils were recruited into the airways four hours after *P. haemolytica* aerosolization have not been established in calves. However, this response could be explained on the basis of previous observations which showed that a potent neutrophilic chemotactic factor was released from guinea pig alveolar macrophages after *Staphylococcus aureus* phagocytosis (12).

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