The Effect of Edema, Hydrocortisone Acetate, Concurrent Viral Infection and Immunization on the Clearance of Pasteurella hemolytica from the Bovine Lung

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ABSTRACT

The influence of pulmonary edema, hydrocortisone, immunization against Pasteurella hemolytica and concurrent infection with parainfluenza-3 virus upon pulmonary clearance of aerosolized P. hemolytica was studied in 31 calves. Following the various treatments calves were challenged with an aerosol of P. hemolytica. One control calf was killed immediately after the aerosol and the numbers of bacteria in the lung taken as 100%. Two calves were killed four hours after challenge and the numbers of bacteria in the lungs were compared to the 100% of the control calf. The result was the percentage clearance of bacteria at four hours.

Pulmonary edema was induced by three different methods: by an aerosol of histamine, by intravenous injection of endotoxin and by intravenous injection of croton oil emulsion. The edema impaired the clearance of P. hemolytica, which was reflected in high numbers of P. hemolytica present in the lungs at four hours after challenge: 260% after histamine, 300% and 400% after endotoxin and 92% after croton oil.

Six days of treatment of four calves with high doses of hydrocortisone acetate produced inconsistent results: two calves treated with a higher daily dose (36 mg/kg) had normal clearance whereas two calves treated with a lower dose had pulmonary edema and displayed lowered clearance with 111% and 31% respectively of P. hemolytica retained in the lungs four hours after challenge.

Immunization of calves by three different methods, a subcutaneously injected bacterin of P. hemolytica (2 calves), single aerosol (2 calves) and four aerosols (4 calves) of live P. hemolytica was reflected in an accelerated pulmonary clearance of P. hemolytica (with a mean of 1.55% of bacteria retained at four hours).

Concurrent infection with parainfluenza-3 virus did not lower the clearance of P. hemolytica in the lungs of 12 calves over 15 days except on the first day following the exposure to parainfluenza-3 virus. These calves had hemagglutinating antibodies against P. hemolytica before exposure.

RÉSUMÉ

Cette expérience visait à étudier l'influence de l'oedème pulmonaire, de l'hydrocortisome, de l'immunisation contre Pasteurella hemolytica et d'une infection simultanée avec le virus para-influenza 3, sur l'élimination de P. hemolytica des poumons de 31 veaux soumis à une infection au moyen d'aérosols. Après les différentes opérations énumérées ci-haut, on assujettit les veaux à un aérosol de P. hemolytica. Immédiatement après, on en abattit un

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que l'on considéra comme témoin, et dont le nombre de bactéries qu'on isola de ses poumons compta pour 100%. On en sacrifia deux autres, quatres heures plus tard, et on compara le nombre de bactéries isolées de leurs poumons avec celui du veau témoin. Le résultat de cette comparaison constitua le pourcentage d'élimination des bactéries, au bout de quatre heures après l'infection.

On provoqua l'oedème pulmonaire de trois façons différentes, à savoir: inhalation d'histamine en aérosol, injection intra-veineuse d'endotoxine et injection intra-veineuse d'huile de croton. Cet oedème fit obstacle à l'élimination de P. hemolytica et résulta en la présence d'un plus grand nombre de ces bactéries dans les poumons, quatre heures après l'infection, dans les proportions suivantes: 260% après l'inhalation d'histamine, 300% et 400% après l'injection d'endotoxine et 92% après celle d'huile de croton.

L'injection de fortes doses d'acétate d'hydrocortisone à quatre veaux, pendant six jours, donna des résultats irréguliers. Les deux ayant reçu la plus forte dose quotidienne (36 mg/kg) éliminèrent les microbes de façon normale; les deux autres, assujettis à une plus faible dose, développèrent de l'oedème pulmonaire et éliminèrent moins bien (111% et 31% respectivement) les P. hemolytica retenues dans leurs poumons, quatre heures après l'infection.

L'immunisation de quelques veaux, de trois façons différentes: une injection sous-cutanée d'une bactérine de P. hemolytica (deux veaux), un seul aérosol de P. hemolytica vivantes (deux veaux) et quatre aérosols (quatre veaux), se traduisit par une accélération de l'élimination de ces microbes (avec une moyenne de rétention de seulement 1.55%, au bout de quatre heures).

Une infection simultanée avec le virus parainfluenza 3 ne diminua pas l'élimination de P. hemolytica des poumons de 12 veaux, sur une période de 15 jours, sauf le premier jour après l'infection virale. Ces veaux possédaient des anticorps hémagglutinants contre P. hemolytica, avant qu'on les infecte expérimentalement.

INTRODUCTION

Lillie and Thomson (12) found that the pulmonary clearance of *Pasteurella hemolytica* in normal calves up to eight hours after aerosolization was rapid and comparable with the results of normal clearance studies in other animals (6, 9, 10, 11, 16). This paper is an application of these base-lines to investigate clearance under abnormal conditions in the bovine lung. The experimental procedures were intended to create conditions which may be significant in the pathogenesis of pneumonic pasteurellosis in cattle. The effect of pulmonary edema, hydrocortisone acetate, immunization with *P. hemolytica* and concurrent viral infection with parainfluenza-3 (Pl-3) on the clearance of inhaled *P. hemolytica* were tested in different groups of experimental calves. Histological and cytological responses to the treatments will be published separately.

MATERIALS AND METHODS

A total of fifty-three calves aged one to four months were used in the experiments; 22 were control or zero hour calves and 31 were treated. The calves were mostly Holsteins purchased at local sales and all were raised in small groups in semi-isolation facilities. Dosages of drugs used were roughly established from preliminary experiments.

Clearance was assessed by comparing the numbers of bacteria which should be cultured from the lung immediately after aerosol challenge (called zero hour) with the numbers of bacteria cultured from the lungs at various time intervals after aerosol challenge (in this case four hours). Zero hour was taken as 100% and the number of bacteria cultured above or below this level was taken as the percentage clearance at the given time interval. If the bacteria multiplied *in vivo* after the aerosol challenge, the clearance at four hours would be over 100% but if the bacteria were cleared the clearance would be less than 100%.

The experimental procedures and numbers of animals used are outlined in Table I. It was intended that three calves be available for each treatment, one control or zero hour calf and two calves killed at four hours after exposure to an aerosol of *P. hemolytica*. The four hour period was selected for logistical reasons and also because previous results (12) indicated that

the effects on clearance should have been evident by that time.

The aerosol chamber used by Lillie and Thomson (12) was enlarged in order to allow four calves to be aerosolized at the same time but basic air flow patterns were similar. The methods of handling animals, collection and processing of lung tissue and the calculation of the percentage clearance of bacteria were conducted as outlined previously (12). Three areas in each lung from the middle of the apical, cardiac and anterodorsal diaphragmatic lobes were sampled. Previous study (12) indicated constant colonization of these areas by aerosolized *P. hemolytica.*

Pasteurella hemolytica serotype 1 was employed in these experiments. The suspension for aerosolization was prepared according to Lillie and Thomson (12). Eight litres of the brain-heart infusion broth were inoculated for aerosolization of 30 minutes duration and cultured in a shaker bath (85 oscillations per minute) at 38°C for ten to 12 hours.

PI-3 virus strain 4644, isolated from a nasal swab of a calf, was cultured in bovine kidney cells and had been passed three times in fetal bovine kidney tissue culture for purification by the end point dilution. The virus stock in the form of neat infected cell culture fluids had a titer of 10^{-6} per 0.2 ml based on hemadsorption in embryo bovine spleen (EBS) cell cultures. The culture fluid with virus was aerosolized for 30 minutes.

Experimental Procedures

I. Pulmonary edema was induced by three different methods.

1. *Histamine aerosol* — Five calves in two groups were aerosolized with a freshly prepared solution (0.15 M) of histamine dihydrochloride in sterile physiological saline. The treatment caused dyspnea and the aerosolization had to be stopped for several three to five minute intervals. The first group was treated for 15 minutes and the second group was treated for 30 minutes of total exposure time. Immediately after the histamine aerosol the calves were challenged by an aerosol of *P. hemolytica*

TABLE I. Treatments Used to Study Clearance of *P. hemolytica*

Experimental Procedure	Time After Treatment When Clearance was Determined		Numbers of Calves Challenged 0 hr.	4 hr.
Pulmonary Edema				
Aerosol of histamine	immediately		2	3
Endotoxin of *E. coli* 0:111 intravenously	immediately		1 1	1 1
Croton oil emulsion intravenously	immediately		1	2
Hydrocortisone				
Subcutaneously for 6 days	on 7th day after first hydrocortisone injection		3	4
Immunization by *P. hemolytica*				
Bacterin of *P. hemolytica* subcutaneously	on the 21st day after injection		2	2
Single aerosol of *P. hemolytica* for 30 minutes	on the 22nd day after aerosol treatment		2	2
Aerosol of *P. hemolytica* four times for 30 minutes	on the 21st day after last aerosol		2	2
Aerosol of *P. hemolytica* four times for 30 minutes	on the 62nd day after last aerosol treatment		2	2
Concurrent Infection with PI-3 Virus				
PI-3 virus aerosol for 30 minutes	on the following days after virus aerosol treatment	1st	1	2
		3rd	1	2
		6th	1	2
		9th	1	2
		12th	1	2
		15th	1	2

and the "zero hour" calves were killed. The experimental calves in the second group were then treated with an additional histamine aerosol for 20 minutes beginning one hour after the challenge with *P. hemolytica* (making a total of 50 minutes) and were killed four hours after the bacterial aerosol.

2. *Endotoxin* — Lipopolysaccharide[1] *Escherichia coli* (O111:B4) was injected intravenously into four calves. Endotoxin (1 mg/ml in sterile physiological saline) was injected into two calves at a dosage of 50 ug/kg and into two other calves at 35 ug/kg. Samples for total white blood cell count and differential white cell count were collected into 10 ml test tubes containing heparin[2] before the administration of endotoxin and at the time of killing. Challenge with an aerosol of *P. hemolytica* followed within five minutes after the treatment.

3. *Croton oil emulsion* — Three calves were injected with 0.1% emulsion of croton oil[3] intravenously at a dosage of 0.1 ml/kg. The emulsion of croton oil was in a finely dispersed state and was stable for 12 hours. It was freshly prepared by dissolving 0.1 ml of croton oil in 10 ml of absolute ethanol and by adding distilled water to 100 ml. The emulsion was administered slowly into the jugular vein. Challenge with an aerosol of *P. hemolytica* followed within five minutes after the treatment.

II. Hydrocortisone acetate was used to examine the influence of cortisol upon defense against *P. hemolytica.*

Four calves were treated subcutaneously with 36 mg/kg of hydrocortisone acetate[4] and later in a similar experiment three calves were treated with 30 mg/kg of hydrocortisone acetate per day. The calves were treated for six days and on the seventh day challenged with an aerosol of *P. hemolytica.* Hydrocortisone acetate is insoluble in water, therefore the dose was dispersed in physiological saline and injected subcutaneously into various sites of the prescapular and cervical regions.

III. Immunization was carried out using injected and aerosolized antigens.

1. *Injection with a bacterin of P. hemolytica* — Four calves were injected at four day intervals with four subcutaneous doses of bacterin which was prepared as described previously (3). One ml contained 1.6 x 10^9 organisms of *P. hemolytica.* Five ml were injected for the first two doses and 10 ml for the last two doses. Calves were challenged seven days after the last injection. Serum and nasal secretions were collected prior to and during the experiment and indirect hemagglutinating antibodies (IHA) against *P. hemolytica* type 1 were estimated according to Duncan and Thomson (3).

2. *A single aerosol of P. hemolytica* — Four calves were treated for 30 minutes with an aerosol of *P. hemolytica.* Serum and nasal secretions were collected prior to and during the experiment for estimation of IHA antibody against *P. hemolytica* as described previously (2).

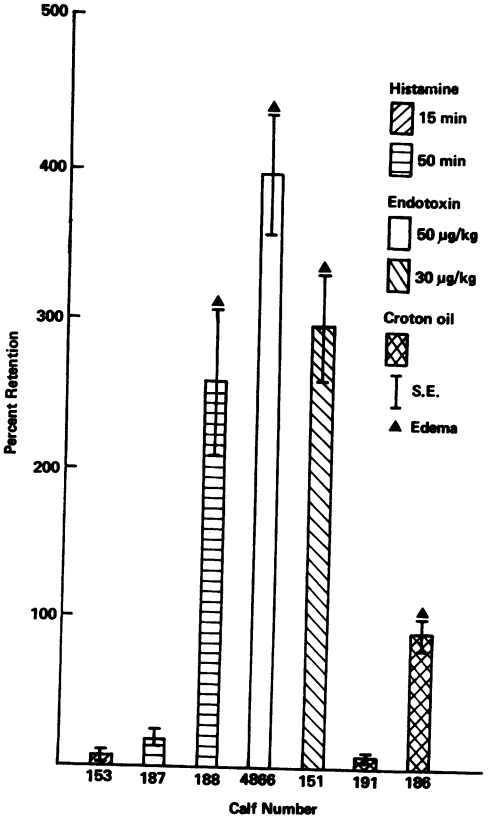

Fig. 1. Percent of P. hemolytica retained in lungs of calves treated for induction of pulmonary edema. S.E. standard error of the sampling. ▲ pulmonary edema.

[1]Lipopolysaccharide W., Difco, Detroit, Michigan.

[2]Vacutainer, Becton & Co., Clarkson, Ontario.

[3]Croton Oil, Sigma, St. Louis, Missouri.

[4]Hydrocortisone acetate, Sigma, St. Louis, Missouri.

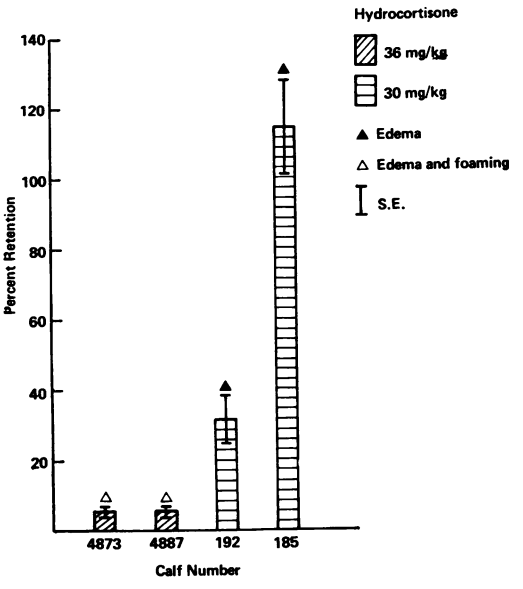

Fig. 2. Percent of P. hemolytica retained in lungs of calves treated with hydrocortisone acetate.

▲ pulmonary edema.

△ pulmonary edema with alveolar foaming.

S.E. standard error of the sampling.

3. *Four aerosols of P. hemolytica and challenge with P. hemolytica after one month* — Four calves were treated four times with an aerosol (30 minutes each time) of *P. hemolytica* at four day intervals. Calves were challenged on the 21st day after the last treatment. Blood samples and nasal secretion samples were collected prior to and during the experiment for serology.

4. *Four aerosols of P. hemolytica and challenge with P. hemolytica after two months* — Four calves were treated as described in the former experiment (III-3). Samples for estimation of serum and secretion IHA antibodies were collected as described previously (3).

In all aerosol experiments five mice were placed in the exposure chamber and received the same exposure as the calves. The mice were killed immediately after exposure and the number of *P. hemolytica* in their lungs determined. This check indicated that exposures to *P. hemolytica* in all experiments were uniform.

IV. Infection with parainfluenza-3 (PI-3) virus

All calves received viral aerosol on the same day and with the same stock suspension of virus. Groups of three calves were

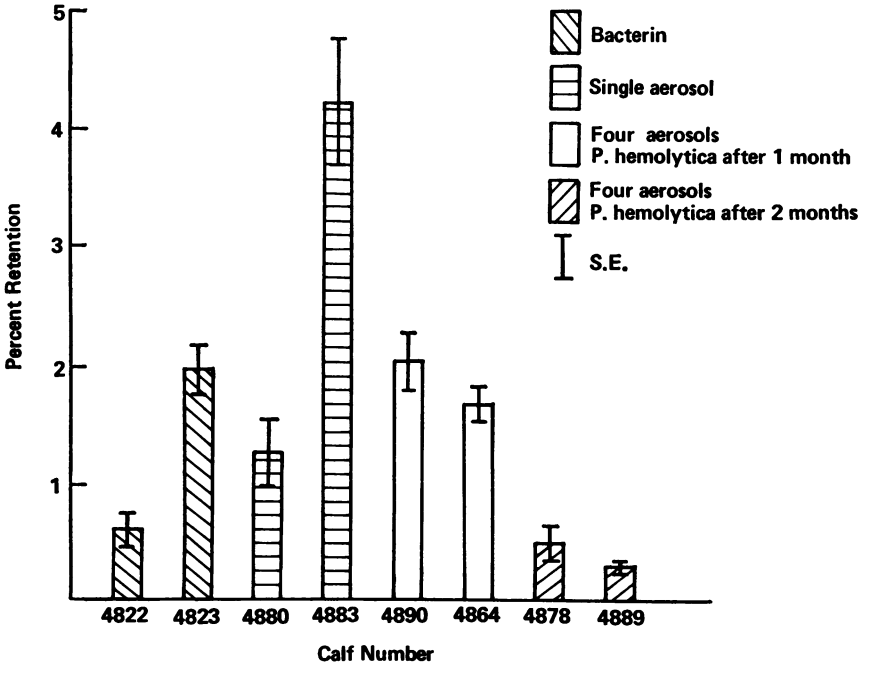

Fig. 3. Percent of P. hemolytica retained in the lungs of calves immunized against P. hemolytica. S. E. standard error of the sampling.

subsequently challenged with *P. hemolytica* on the first, third, sixth, ninth, 12th and 15th day after the aerosol infection with PI-3 virus. The amount of virus inhaled by the calves was not determined. Samples of nasal secretion and serum were collected for estimation of the serum and secretory IHA antibodies against *P. hemolytica* and for estimation of hemagglutination inhibition antibody titres against PI-3 virus from all calves before the treatment with PI-3 virus and before killing each group.

TABLE II. IHA Antibodies to *P. hemolytica* in Serum and Nasal Secretions in Immunized Calves

Treatment	Animal	Reciprocal IHA titres on days					
		0	14	21	31	36	64
Bacterin							
	4822	8/–[a]	16/0[b]	32/4			
	4824	0/–	32/0	32/4			
Single Aerosol							
	4880	4/8	4/0	16/16			
	4883	0/0	64/128	64/256			
Four Aerosols Challenged One Month Later							
	4890	0/0			128/256	128/32	
	4864	8/–			64/128	64/128	
Four Aerosols Challenged Two Months Later							
	4878	8/0			16/256		16/0
	4889	4/0			64/128		32/256

[a]not determined
[b]serum on left side, nasal on right side

TABLE III. Reciprocal Status of the Hemagglutination Inhibition Titers Against PI-3 Virus in Sera and Nasal Secretions in Calves

Days of Challenge	Calf No.	Hour After Challenge	Days Before Treatment with PI-3		PI-3 Aerosol	Days After Treatment with PI-3 Virus				
			30	2	0	2	5	8	12	14
1	172	4	16/–[a]	10/0[b]						
	173	4	16/–	20/0						
	184	0	–/–	20/0						
3	164	4	8/–	10/0		8/0				
	165	4	64/–	80/0		16/2				
	162	0	8/256	8/128		8/32				
6	181	4	16/–	0/0		4/0	10/2			
	182	4	16/–	10/0		8/0	40/0			
	174	0	16/–	0/0		40/0	10/0			
9	169	4	8/–	10/0		4/0	10/0	80/0		
	170	4	8/–	10/0		2/0	0/0	40/4		
	178	0	8/–	10/0		40/0	10/4	40/0		
12	160	4	8/–	10/0		4/4	10/4	10/2	40/4	
	166	4	8/–	20/0		4/0	10/0	10/4	80/4	
	163	0	10/–	10/–		20/128	0/0	10/2	40/0	
15	168	4	8/–	10/4		8/4	10/2	20/8	40/8	40/8
	176	4	8/–	10/–		8/0	10/0	10/0	10/4	20/2
	183	0	–/–	20/0		40/0	20/0	20/2	20/2	20/2

[a]not determined
[b]serum on left side, nasal on right side

Isolation of virus from the lungs was either by culture of washed out alveolar macrophages or by inoculation of lung homogenates into fetal bovine kidney cell culture. Hemadsorption and cytopathic changes were used as criteria for positive isolation.

RESULTS

Pulmonary edema resulted from all three treatments (endotoxin, histamine and croton oil) and markedly reduced the clearance of *P. hemolytica* from the lungs of four calves. Endotoxin had the greatest effect on the number of retained bacteria (400% and 300%) followed by prolonged histamine exposure (260%) and croton oil (92%). All calves did not react in a similar manner to similar treatments, for example one calf which received histamine for 50 minutes and one which received croton oil did not have impaired clearance and did not develop edema (Fig. 1).

Pulmonary edema was evident grossly as typical edematous infiltration between lobules, moist cut surface and fluid and froth in the trachea and bronchioles.

Histamine caused listlessness, coughing, moderate dyspnea and salivation in all calves treated in addition to severe dyspnea in calf 188. Endotoxin caused a similar clinical response to histamine but more severe and calves 4866 and 151 became prostrate. Marked leukopenia and a shift to the right was present at four hours in the calves treated with endotoxin. Croton oil also caused severe respiratory distress.

The effect of treatment with hydrocortisone acetate on the clearance of *P. hemolytica* was quite variable among four calves (Fig. 2). Although calves 4873 and 4887 had normal clearance at four hours (95%) they developed foam in alveoli and exhibited more respiratory distress than calves 192 and 185 during the four hour post exposure period. The latter calves had grossly visible pulmonary edema and displayed lowered clearance of *P. hemolytica* — 31% and 111% respectively of retained bacteria.

Immunization with *P. hemolytica* increased the clearance rate in all calves (Fig. 3) compared to the normal rate of 10% at four hours (12). All calves had serum antibody titres to *P. hemolytica* at the time of challenge and most had nasal antibody (Table II).

Concurrent infection with PI-3 virus reduced the clearance of *P. hemolytica* only on the first day following PI-3 infection (Fig. 4). The only clinical sign of PI-3 viral infection was a slight elevation of body temperature one to four days after infection. Most of the calves had serum antibody levels to PI-3 virus but not nasal antibody prior to PI-3 viral infection (Table III). Also most calves had serum and nasal antibody titres to *P. hemolytica* before and during the experiment (Table IV). PI-3 virus was isolated from the lungs of all infected calves.

DISCUSSION

Pulmonary edema reduced the rate of clearance of *P. hemolytica* from the lungs of most of the calves. Immunization with *P. hemolytica* increased the rate of clearance and concurrent PI-3 virus infection affected clearance to a slight degree but only one day after infection.

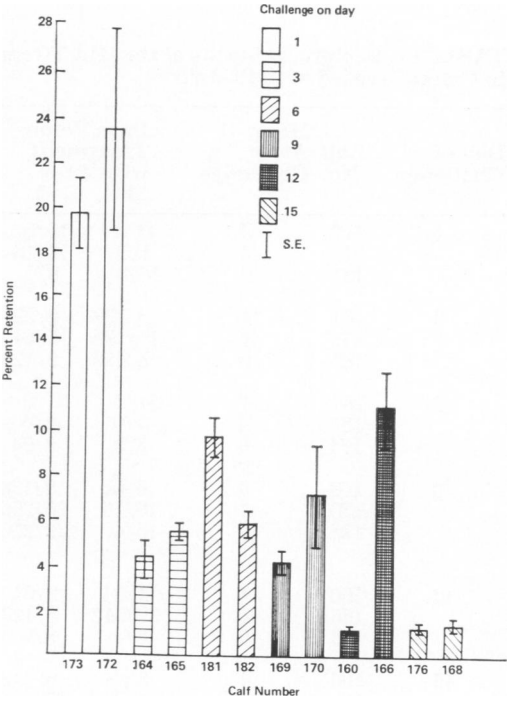

Fig. 4. Percent of P. hemolytica retained in lungs of calves treated with aerosol of PI-3 virus. Calves were challenged with P. hemolytica on the first, third, sixth, ninth, 12th and 15th day after PI-3 virus aerosolization. S. E. standard error of the sampling.

Pulmonary edema was produced by histamine. endotoxin and croton oil. However, an individual variation of calves existed to each kind of treatment.

The administration of histamine via aerosol was advantageous since the doses could be applied according to the immediate reactivity of calves. Calf 153 (treated for 15 minutes) and calf 187 (treated for 50 minutes) had normal clearance of *P. hemolytica* (Fig. 1). Both calves reacted with respiratory distress only at the time of treatment, recovered rapidly after histamine inhalation and did not develop macroscopic pulmonary edema. Histamine exposure of calf 188 evoked pulmonary edema and impaired clearance probably by a greater individual sensitivity of pulmonary smooth muscles, mainly veins, to histamine (4). The difference in clearance is not likely due to the direct action of inhaled histamine on the pulmonary phagocytes since the same treatment of two calves lasting 50 minutes resulted in different clearance rates. Northover observed that phagocytosis of *Staphylococcus* spp. *in vitro* by polymorphonuclear leukocytes was unaffected by histamine and phagocytosis by macrophages was stimulated by histamine (15).

Endotoxin produced edema consistently. There was a general correlation between the amount of edema and the number of bacteria retained. Injection of endotoxin in 19 calves resulted in pulmonary vasoconstriction and increased pulmonary vascular resistance which in turn led to pulmonary edema (17). Endotoxin activates C'3-C'9 components of complement and this may cause changes in the clotting system, vascular permeability, smooth muscle reactivity and chemotaxis of neutrophils. Endotoxin also causes release of histamine, serotoxin and adrenalin (14).

The reason why only one of two calves given croton oil developed pulmonary edema is not clear. The croton oil contains highly irritating toxalbumin-crotin which causes, when topically applied, a vesiculation of the skin (1) and after injection has a necrotizing and suppurative effect upon tissues (5). Croton oil is believed to impair permeability of the pulmonary capillaries. Fibrinous strands and erythrocytes in the alveolar and interstitial edematous fluid in the lungs of calves is a morphological sign of highly disturbed permeability after treatment with croton oil.

The effect of treatment with hydrocortisone upon clearance was inconsistent.

TABLE IV. Reciprocal Status of the IHA Titers Against *P. hemolytica* in Sera and Nasal Secretions in Calves Treated with PI-3 Virus

Day of Challenge	Calf No.	Hour After Challenge	Days Before Treatment with PI-3		PI-3 Aerosol	Days after Treatment with PI-3				
			30	2	0	2	5	8	12	14
1	172	4	8/0[a]	16/0						
	173	4	8/8	16/16						
	184	0	16/4	8/0						
3	164	4	8/128	8/32		8/64				
	165	4	16/128	16/64		16/64				
	162	0	8/256	8/128		8/32				
6	181	4	64/0	64/0		32/0	32/0			
	182	4	4/0	2/0		4/0	8/0			
	174	0	8/0	8/64		8/32	8/16			
9	169	4	8/64	8/128		8/512	8/256	8/256		
	170	4	16/16	16/256		16/256	16/32	16/64		
	178	0	16/4	32/256		16/512	32/512	32/512		
12	160	4	4/64	2/64		2/64	4/32	2/64	4/64	
	166	4	32/512	8/128		8/256	8/64	8/128	8/256	
	163	0	8/–[b]	8/–		8/256	8/64	8/256	8/128	
15	168	4	8/64	8/512		8/256	8/256	8/256	8/256	8/256
	176	4	4/0	16/–		16/256	32/256	64/128	32/256	64/256
	183	0	4/0	4/0		4/0	4/0	4/0	4/0	4/0

[a]serum on left side, nasal on right side
[b]not determined

Gross edema and impaired clearance occurred in the calves only at the lower dosage level, whereas calves at the slightly higher dosage level had normal clearance. Again pulmonary edema did impair clearance. The reason for this paradoxical difference is not clear.

From the various experiments involving agents which caused edema it may be concluded that the extent and duration of pulmonary edema is a factor which influences the rate of clearance of *P. hemolytica* in the lungs. Wood and associates (18) discovered that freely floating phagocytes in pulmonary edematous fluid were unable without tissue support or opsonization to engulf *Klebsiella pneumoniae* and pneumococci in rats.

All of the animals immunized with *P. hemolytica* had increased rates of clearance. Correlation did not exist between the clearance rates and levels of individual IHA titres against *P. hemolytica*. The IHA has been shown by Cameron (2) to be a poor indicator of immunity against *P. hemolytica* in individual sheep. He concluded that the IHA test could be used for detection of immunity of a group but was not reliable for individual animals.

It is not known if secretory antibodies act in the pulmonary alveoli. Circulating specific opsonins may have reached the alveoli from the capillaries as focal microscopical areas of edema were present in all the challenged calves. The presence of possible cellular immunity was not evaluated.

The positive effect of immunization with *P. hemolytica* upon clearance warrants investigation of the use of immunization, particularly via aerosol, for reducing the economic losses from pneumonic pasteurellosis in cattle.

Concurrent virus infection may decrease bacterial clearance in the lung between six and ten days after virus infection (7, 13). This effect on clearance of *P. hemolytica* was not evident in calves with concurrent PI-3 virus infection. Green (8) observed that only immunization against the bacterial species used for challenge protected against the adverse influence of viral infection upon clearance in guinea pigs. The presence of nasal antibodies against *P. hemolytica* in calves indicated that an immune mechanism might protect the calves from impaired clearance due to virus infection. We had hoped to determine whether or not PI-3 virus impaired the clearance of *P. hemolytica* because such an association has been suspected for some time. However our results in addition to Jakab and Green's work (8) indicates that further experiments on calves free of immunity to *P. hemolytica* and PI-3 virus must be conducted to determine whether or not PI-3 virus infection will impair the clearance of *P. hemolytica* in calves.

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