Experimental Pneumonia in Rabbits Inoculated with Strains of *Pasteurella multocida*

D.H. Percy, J.L. Bhasin and S. Rosendal*

ABSTRACT

Domestic rabbits were inoculated with either a 3:A or 3:D serotype of *Pasteurella multocida* by aerosol, intravenous, or intratracheal inoculation. Different colony forming units of *P. multocida* were used. Animals which died or were killed after the 14 day observation period were examined macroscopically and microscopically for lesions in the lower respiratory tract. Pneumonic lesions were most consistently produced in rabbits inoculated intratracheally with serotype 3:A. Pulmonary and pleural lesions were observed in some animals inoculated intravenously with serotype 3:A. Lesions were minimal in rabbits inoculated with serotype 3:D. Of the three routes of inoculation evaluated, the intratracheal route appeared to be the best method to produce *Pasteurella*-associated lesions in the lower respiratory tract.

Key words: *Pasteurella multocida*, experimental bronchopneumonia, serotypes 3:A and 3:D.

RÉSUMÉ

Cette expérience consistait à inoculer un nombre variable d'unités formatrices de colonies des sérotypes 3:A et 3:D de *Pasteurella multocida* à des lapins domestiques, au moyen d'aérosols, ainsi que par les voies intraveineuse ou intratrachéale. On procéda à la recherche de lésions macroscopiques et microscopiques dans les voies respiratoires inférieures des lapins qui moururent et de ceux qu'on sacrifia au bout de la période d'observation de 14 jours. Les lapins auxquels on avait injecté le sérotype 3:A, par la voie intratrachéale, arborèrent le plus souvent des lésions de pneumonie; certains de ceux qui avaient reçu ce sérotype, par la voie intraveineuse, développèrent des lésions pulmonaires et pleurales. Les lapins inoculés avec le sérotype 3:D ne développèrent que très peu de lésions. Des trois voies expérimentales d'inoculation, l'intratrachéale sembla la meilleure pour produire des lésions imputables à *Pasteurella*, dans les voies respiratoires inférieures.

Mots clés: *Pasteurella multocida*, broncho-pneumonie expérimentale, sérotypes 3:A et 3:D.

INTRODUCTION

Pasteurella multocida infection is an important disease problem in the domestic rabbit. Rhinitis, conjunctivitis, otitis media, abscessation, chronic bronchopneumonia, acute fibrinous bronchopneumonia and septicemia are all patterns of disease associated with *Pasteurella* infections (1). Commercial rabbitries suffer economic losses due to pasteurellosis and concomitant infections of laboratory rabbits complicate research projects (1,2). Methods used to control pasteurellosis in rabbits have included the establishment of *Pasteurella*-free colonies (3), and the removal of carriers identified by repeated culture of nasal samplings for *P. multocida* (1). Different immunization procedures have been evaluated as a means of control, with varying degrees of success (4,5,6,7). In two vaccination trials, the intranasal challenge with *P. multocida* produced lesions in the upper and lower respiratory tract (4,5). However, not all challenge procedures have consistently produced lesions in the lower respiratory tract (4,8). In one study, intranasal inoculation of rabbits with a 12:A serotype resulted in pneumonia in only 20% of nonimmunosuppressed rabbits, but in 80% of hydrocortisone-treated animals (9). It is evident that a reliable challenge procedure is essential in order to evaluate the efficacy of vaccinations.

Other routes of inoculation used to produce *Pasteurella* infections in the rabbit have included the subcutaneous, intravenous or intratesticular routes (10), the intraperitoneal route (11), aerosols (7), and the intratracheal route (10,12). The purpose of this study was as follows: 1) To determine the most effective route of inoculation of *P. multocida* to produce pneumonic lesions in the rabbit and 2) To compare the optimum number of organisms required to produce disease by *P. multocida* serotypes 3:A and 3:D selected for inoculation studies.

MATERIALS AND METHODS

Young male New Zealand White rabbits weighing 2.5-3.5 kg were obtained from a commercial rabbitry (Rieman's Fur Ranches, St. Agatha, Ontario). Deep nasal swabs were collected at 24 hour intervals for three consecutive days. Swabs were streaked on blood agar plates contain-

*Department of Pathology (Percy), Department of Veterinary Microbiology and Immunology (Rosendal), Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1 and the Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6 (Bhasin).

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ing 2 μg/mL of clindamycin (13), a medium selective for *P. multocida* and *Bordetella bronchiseptica*. Only animals with three consecutive negative nasal cultures for *P. multocida* were used for the inoculation studies. On arrival at the research facility, animals were held for one week prior to inoculation. Rabbits were housed singly in stainless steel cages in an isolation room at a temperature of 20-22°C with approximately ten air changes per hour and a light:dark cycle of approximately ten hours of light and 14 hours of darkness. Rabbits were fed pellets (Purina Rabbit Chow, St. Louis, Missouri) *ad libidum* throughout the study and had free access to water from individual water bottles supplied with sipper tubes. Rabbits were chosen for the inoculation procedures on a random basis. A total of 22 rabbits were used per experiment, including 18 inoculated and four noninoculated control rabbits.

SELECTION OF STRAINS OF *P. MULTOCIDA*

Isolates were identified by methods previously described (14,15,16,17). Strain C_1 was isolated from a rabbit with pneumonia obtained from a commercial rabbitry which had an enzootic of pasteurellosis characterized by acute fibrinous pneumonia and metritis. This isolate was identified as serotype 3:A (14,15,16,17). Strain R53 of serotype 3:D was recovered from a fatal case of acute fibrinous pneumonia in a domestic rabbit. Both strains were grown on trypticase soy and dextrose agar in Roux flasks for 24 hours at 37°C. Bacteria were collected by flooding the agar surface with phosphate buffered saline (PBS) and adding glass beads, then the suspensions were stored in 2 mL aliquots at -70°C prior to use. Both strains produced fatal systemic pasteurellosis following intraperitoneal inoculation of adult mice. The number of colony-forming units (CFU) in the stock inoculum was determined by standard plate counting techniques in thawed aliquots of both strains.

INOCULATION PROCEDURES

Aerosol Exposure — Rabbits were placed in a fiberglass chamber approximately 1.6 x 1.0 x 0.7 meters high, then exposed to a fine mist of organisms generated from the stock suspension of organisms suspended in saline by a nebulizer (Devilbiss Nebulizer, Model 880). Animals were exposed to *P. multocida* for 15, 30 or 60 minutes, then returned to their cages. In the 3:A inoculated rabbits, approximately 10^{10} CFU were delivered into the chamber by aerosol every 15 minutes. With the 3:D strain, 3.6 x 10^{10} CFU were delivered every 15 minutes. A blood agar plate was suspended in the chamber to ascertain the viability of *P. multocida* delivered in the aerosol.

Intratracheal Inoculation — Rabbits were anesthetized by the intravenous administration of sodium pentothal (Parke Davis, Detroit, Michigan), and placed in dorsal recumbency. A midline incision was made to expose the anterior third of the trachea. A 16 gauge 1″ hypodermic needle was inserted between the cartilaginous rings, then a polyethylene catheter (Intramedic Polyethylene tubing (#7415), Clay Adams, Parsippany, New Jersey) was passed to the bifurcation of the trachea. The inoculum (0.5 mL) was then injected through the tubing at the level of the tracheal bifurcation, the catheter was withdrawn, and the skin was closed with simple interrupted sutures using nonabsorbable suture material.

Intravenous Inoculation — Using a 25 gauge 3/4″ hypodermic needle, 0.5 mL of innoculum was delivered via the marginal ear vein.

NECROPSY AND MICROBIOLOGY TECHNIQUES

All moribund animals were killed *in extremis*; others were killed by sodium pentobarbital given intravenously after the 14 day observation period. Gross findings were recorded, and tissues were fixed in 10% buffered formalin for histological examination. Samples for culture were collected on swabs from the lung and heart blood from inoculated and control animals. Swabs were streaked on blood agar containing clindamycin (13), incubated for 24-48 hours at 37°C and examined for *Pasteurella* or *Bordetella* colonies. Biochemical and serological testing was performed in order to make a positive identification. Suspect colonies were Gram stained, examined for cytochrome oxidase, streaked onto MacConkey's agar, inoculated into Hugh and Leifson's oxidative-fermentative basal medium with 1% dextrose, used for the identification of certain Gram negative bacteria, and examined for the ability to produce indole and urease. Representative isolates of *P. multocida* from the lower respiratory tract were serotyped in order to confirm that they were the same serotype as the challenge strain.

SEROLOGY

Antibodies to *P. multocida* in the pre and post inoculated serum specimens from rabbits were examined by a microagglutination test procedure of Gaultney *et al* (18) with minor modifications. The antigen, a heat inactivated (100°C 1 h), decapsulated (1 Normal HCl overnight) cell suspension was prepared as per Namioka and Murata (17), and adjusted to an optical density of 0.75 on a Spectronic 20 at 600 nm and 0.05 mL volumes of the antigen and serum were used in microtiter plastic trays. The serum was diluted serially from 10 to 2560 times. The diluent for serum was PBS pH 7.2 containing 0.005% basic fuchsin. The end point was the highest dilution of serum which lacked a clear definite button of stained antigen at the bottom of the V-shaped well, after 24 h incubation at 37°C followed by 2 h incubation at 6°C.

RESULTS

CLINICAL AND MACROSCOPIC FINDINGS

No fatalities occurred in those inoculated intravenously or by aerosol with the 3:A strain during the 14 day observation period. One rabbit inoculated intravenously with 0.5 x 10^8 CFU and killed at 14 days postinoculation (pi) was thin, with depletion of fat reserves. There was consolidation of the middle lobe of the right lung, with pleural adhesions. The entire left lung consisted of thick purulent material surrounded by a thick fibrous sac. Several intratracheally inoculated

animals exhibited dyspnea, depression and anorexia for 24 hours or more prior to death or euthanasia. Five of six rabbits inoculated with the 3:A strain of *P. multocida* died or were euthanized at five to nine days pi. At necropsy, there was marked consolidation of the anteroventral portions of lung, frequently with hemorrhage and fibrinopurulent exudation into affected areas. Localized or diffuse fibrinopurulent pleuritis and pericarditis was observed in four of six rabbits inoculated intrachacheally with this strain. Extensive empyema involving the left lung was present in one animal, inoculated intratracheally with 0.5 x 10^9 organisms. Localized areas of consolidation were observed in the one animal inoculated intratracheally that survived and was killed at 14 days pi (Table I). In rabbits inoculated with the 3:D strain, two animals died with 18 hours following intravenous inoculation of 3.6 x 10^9 CFU. At necropsy, marked pulmonary congestion was observed in both animals. The remaining rabbits were essentially asymptomatic throughout the 14 day observation period. At necropsy, focal to confluent areas of consolidation were observed in two of four animals inoculated intratracheally with the largest numbers of organisms (Table II).

HISTOPATHOLOGY

Focal peribronchial and perivascular lymphocytic infiltration were observed in both inoculated and control animals. In three of six animals inoculated intravenously with 3:A, there were focal to confluent pneumonic lesions. Infiltrating cells in alveoli consisted of heterophils and macrophages, and there was a prominent mononuclear cell infiltrate in the interstitial regions (Fig. 1). Larger airways were relatively free of inflammatory exudate. In one intravenously-inoculated rabbit, there was massive polymorphonuclear cell infiltration, with obliteration of the normal architecture and pleural fibrosis. In four of five rabbits which died following intratracheal inoculation of the 3:A strain, there was a localized to extensive fibrinopurulent bronchopneumonia, with concurrent fibrinopurulent pleuritis and pericarditis (Fig. 2). The fifth rabbit which died at nine days pi had extensive congestion of alveolar capillaries and alveolar flooding with proteinaceous material. Death was attributed to systemic pasteurellosis and *P. multocida* was isolated from the heart blood and lung. Minimal pulmonary lesions were observed histologically in animals exposed to the 3:A strain of *P. multocida* via aerosol (Table I).

In rabbits inoculated with the 3:D

TABLE I. Experimental Pasteurellosis (3:A Strain)

Route of Inoculation	No. of Organisms	Animal Number	Examination Day Post-inoculation	Pulmonary Lesions[a]	Bacteriology: *Pasteurella multocida* (lung)	Bacteriology: *Bordetella bronchiseptica* (lung)	Bacteriology: *Pasteurella multocida* (heart blood)	Serology: Agglutinating Antibody Titers to 3:A[b] Day 0 Preinoculation	Serology: Agglutinating Antibody Titers to 3:A[b] Day 14 Postinoculation
Control	—	17	0	–	–	–	NT	NT	
Control	—	26	0	–	NT[c]	NT	NT	NT	
Aerosol	15 min	15	14[d]	–	+	+	+	<20	640
		6	14	–	+	+	–	<20	<20
Aerosol	30 min	16	14	–	+	+	–	—	—
		39	14	–	+	+	–	<20	<20
Aerosol	60 min	37	14	+	+	+	–	—	—
		40	14	–	+	+	–	<20	<20
Intratracheal	Control	5	14	–	–	+	–	—	—
	(Saline)	9	14	–	–	+	–	—	—
Intratracheal	0.5 x 10^8	12	14	+	–	–	–	NT	NT
		33	7 (E)	+++	+	–	+	—	NT
Intratracheal	0.5 x 10^9	4	6 (E)	+++	+	+	+	—	NT
		20	8 (E)	++	+	–	NT	—	NT
Intratracheal	0.5 x 10^{10}	7	5 (D)	+	+	–	+	—	NT
		21	9 (E)	+	+	–	+	—	NT
Intravenous	0.5 x 10^7	8	14	+	+	+	–	<20	160
		14	14	–	+	+	+	—	640
Intravenous	0.5 x 10^8	13	14	–	+	+	–	<20	320
		19	14 (E)	+++	+	–	–	—	160
Intravenous	0.5 x 10^9	27	14	–	+	+	–	—	80
		28	14	++	+	–	+	—	160
Totals (Inoculated Animals)				9/18	17/18	11/18	7/17		

[a]Based on gross and microscopic evaluation
[b]Titers expressed as reciprocal of serum dilution
[c]Not tested
[d]All remaining animals killed at 14 days postinoculation
(E) Euthanized
(D) Died
(+) Focal lesions (++) Multifocal to confluent lesions (+++) Extensive lesions

TABLE II. Experimental Pasteurellosis (3:D Strain)

Route of Inoculation	No. of Organisms	Animal Number	Examination Day Post-inoculation	Pulmonary Lesions[a]	Bacteriology			Serology
					Pasteurella multocida (lung)	*Bordetella bronchiseptica* (lung)	*Pasteurella multocida* (heart blood)	Agglutinating Antibody Titers to 3:D[b]
Control		1	0	–	–	NT[c]	NT	NT
		2	0	–	–	NT	NT	NT
Aerosol		16	14[d]	–	–	+	–	10
(Saline)		18	14	–	–	–	–	0
Aerosol	15 min	35	14	–	–	+	–	<20
		38	14	–	–	+	–	<10
Aerosol	30 min	22	14	–	+	+	–	10
		60	14	–	–	+	–	<40
Aerosol	60 min	24	14	–	–	–	–	-0
		37	14	–	–	+	–	<40
Intratracheal	1.4×10^7	5	14	–	–	–	–	10
		15	14	–	–	+	–	80
Intratracheal	3.6×10^8	9	14	+	–	+	–	10
		11	14	–	–	–	–	40
Intratracheal	3.6×10^9	8	14 (E)	+++	–	+	–	160
		44	14	–	–	+	–	20
Intravenous	0.5×10^7	23	14	–	–	+	–	10
		34	14	–	–	+	–	80
Intravenous	0.5×10^8	12	14	–	–	+	–	10
		27	14	–	+	+	–	10
Intravenous	0.5×10^9	14	1 (D)	+	+	–	–	NT
		32	1 (D)	+	+	+	+	NT
Totals (Inoculated Animals)				4/18	3/18	14/18	1/18	

[a]Based on gross and microscopic evaluation
[b]Antibody titers identical in pre and postinoculation sera
[c]Not tested
[d]All remaining animals killed at 14 days postinoculation
(E) Euthanized
(D) Died
(+) Focal lesions (++) Multifocal to confluent lesions (+++) Extensive lesions

strain, congestion of alveolar capillaries and intraalveolar edema was observed in the two rabbits that died within 18 hours following intravenous inoculation. Focal to confluent subacute bronchoalveolitis was observed in two of six rabbits inoculated by the intratracheal route (Table II).

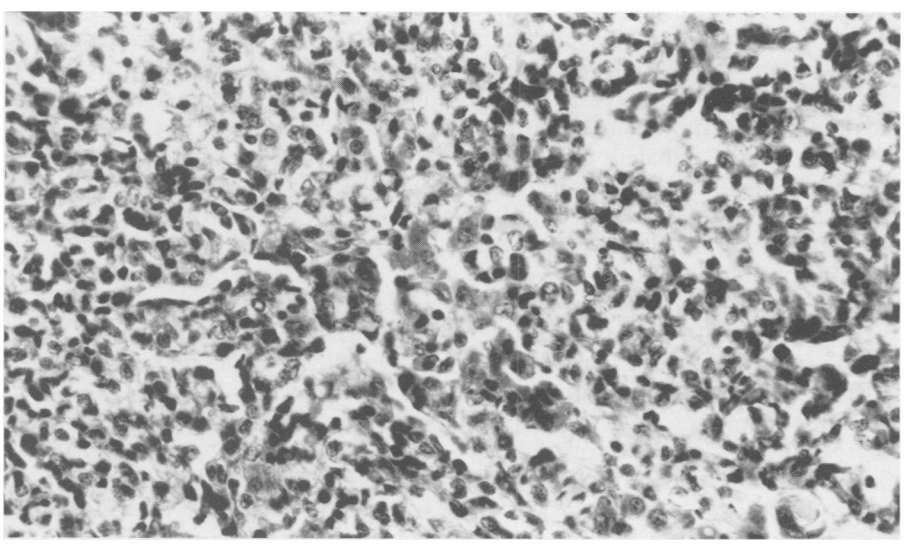

Fig. 1. Section of lung from rabbit (#28) inoculated intravenously with approximately 0.5×10^9 colony forming units of *P. multocida* (serotype 3:A) and killed at 14 days postinoculation. There is a diffuse alveolitis, with proliferation of pneumocytes, leukocytic infiltration and obliteration of the normal architecture. H & E. X214.

BACTERIOLOGY

Pasteurella multocida was recovered from the lung in all animals inoculated with strain 3:A by the aerosol or intravenous route, and in five of six rabbits inoculated intratracheally with the organism. In one surviving animal inoculated intratracheally with 0.5×10^8 *P. multocida*, the organism was not recovered, although focal pneumonic lesions were observed histologically at 14 days pi (Table I). *Pasteurella multocida* was not recovered from the lung of the four control rabbits used in this study (Table I). *Bordetella bronchiseptica* was isolated from the lung of 11 of the 18 rabbits inoculated with 3:A and from two of four control rabbits (Table I). *Pasteurella multocida* was recovered from the lung of only three of the 18 rabbits inoculated with serotype 3:D, including one animal

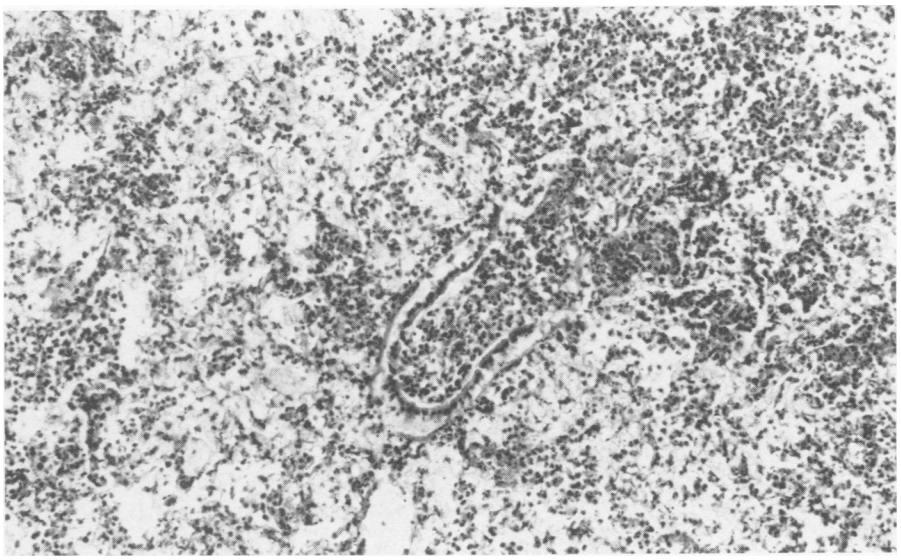

Fig. 2. Lung from rabbit (#33) inoculated intratracheally with approximately 0.5 x 10^8 colony forming units and killed *in extremis* at seven days postinoculation. There is an acute necrotizing fibrinopurulent bronchopneumonia with alveolar flooding and leukocytic infiltration. H & E. X85.

which died within 18 hours after intravenous inoculation. *Bordetella bronchiseptica* was isolated from the lung of most of the 3:D inoculated animals (Table II).

AGGLUTINATING ANTIBODIES TO *P. MULTOCIDA*

The sera of noninfected rabbits were either negative or contained low levels of agglutinins to *P. multocida* 3:A or 3:D. Antibody to serotype 3:D was present in sera from a few rabbits collected prior to inoculation with this serotype. Most animals exposed to an aerosol of 3:A or 3:D serotypes did not develop antibodies by day 14. On the other hand, intravenous administration of 10^8–10^{10} cells of 3:A elicited an appreciable antibody response in all animals by day 14 (Table I). Infection of rabbits with 3:D via aerosol, or by intravenous and intratracheal routes failed to elicit any appreciable rise in antibody titer by day 14 of the experiment (Table II).

DISCUSSION

In a study of different routes of inoculation of *P. multocida* performed over 50 years ago, Webster concluded that the intravenous route of inoculation is a relatively reliable method of producing acute pneumonic lesions in the rabbit (10). We did produce focal to confluent alveolar and pleural lesions in some rabbits inoculated intravenously with the 3:A strain of *P. multocida*. These lesions were similar to those seen histologically in some cases of acute to subacute pasteurellosis in this species (1). However, in rabbits inoculated with the 3:A strain, the intratracheal route of inoculation most consistently produced pulmonary lesions. In our studies, delivery of the organism by aerosolization was not an effective means of producing pneumonic lesions. It is likely that the upper respiratory tract served as an effective barrier for the trapping and expulsion of most of the inhaled organisms. Although the intratracheal route of inoculation is a contrived method of exposure, investigators have used this route to produce lesions in the lower respiratory tract in other species. Intratracheael inoculation of *P. hemolytica* has proven to be an effective means of producing acute bronchopneumonia in calves (19), whereas other routes and procedures have frequently met with limited success (20).

Based on these studies, it is evident that the strain of 3:A used was more virulent than the 3:D strain under study. Numbers of organisms, inoculation procedures, and the source of animals was comparable in both studies. Of particular interest was the ability of the 3:A strain to persist in the lower respiratory tract for two or more weeks postinoculation, including animals that were free of obvious pneumonic lesions. Presumably the organism was able to persist due to the ability to avoid elimination by the phagocytic cell-mucociliary system responsible for the clearance of microorganisms from the lung. The ability of the organisms to survive in the lung should enhance the opportunity to proliferate and produce a pneumonic process.

In a study to compare the adhesive properties of different capsular serotypes of *P. multocida* to epithelial cells, strains with serotype A capsules were approximately ten times more adherent to rabbit pharyngeal epithelial cells *in vivo* and to HeLa cells *in vitro* than were most strains of capsular type D evaluated. Adherence was attributed to the interaction of bacterial fimbriae with N-acetyl-D-glucosamine receptors on the host cell. Glorioso *et al* also concluded that the adhesive strains of *P. multocida* were more pathogenic for rabbits than nonadherent strains when inoculated by either the intranasal or intravenous route (21). Immunologically compromised animals have been shown to be more susceptible to clinical pasteurellosis (2). Thus environmental change or experimental manipulation could provide the opportunity for a virulent strain of *P. multocida* resident in the lower respiratory tract to produce detectable disease. The reduced number of rabbits with pulmonary lesions and positive cultures of *P. multocida* following inoculation with serotype 3:D was an unexpected finding. The 3:D strain had been isolated from a fatal case of acute fibrinous pneumonia, and mice were readily killed due to acute pasteurellosis following intraperitoneal inoculation with this strain. The 3:A strain is considered to be more virulent than the more prevalent 12:A strain (5,9,22). In one study, serotype 3:A or 3:D were the most frequent isolates from spontaneous fatal cases of acute pasteurella pneumonia (22). However, there may be other unrecognized host/microbial factors that

played a role in the outcome of the 3:D inoculation studies.

Of interest was the presence of detectable agglutinating antibody to *P. multocida* in the preinoculation serum samples, particularly to serotype 3:D. In a previous survey (22), serotype 12:A was isolated from rabbits sampled from our supplier's rabbitry. Thus prior exposure to this serotype may have produced a detectable antibody response to a heterologous strain. Cross-reacting antigens have been detected between *P. multocida* and certain other Gram negative organisms (23). However, it seems unlikely that titers of this magnitude would be produced other than through prior exposure to a strain of *P. multocida*. One rabbit (#8) had a relatively high antibody titer to serotype 3:D present in the preinoculation serum sample. This animal developed extensive pneumonia following intratracheal inoculation with this serotype. In one immunization study using *P. haemolytica* in calves, pulmonary lesions were most extensive in animals vaccinated subcutaneously, then challenged by intratracheal inoculation with the organism. It was suggested that anti-*P. haemolytica* titers enhanced phagocytosis of the organism in the lung, resulting in macrophage destruction, and a stimulus for pulmonary inflammation (24). Whether similar mechanisms may be involved in *P. multocida* infections of the lung has not been determined.

The frequent isolation of *B. bronchiseptica* from the lung in both inoculated and control rabbits has been reported previously. In one survey in rabbits, *B. bronchiseptica* was isolated from 97% of macroscopically normal lungs sampled (12). Similarly, the organism was a frequent isolate from the lower respiratory tract of specific pathogen free rabbits used in a *P. multocida* vaccination-challenge study (8). *Bordetella* is not considered to be an important respiratory pathogen in rabbits by most observers (1). However, it is possible that the prominent peribronchial lymphoid hyperplasia seen in both inoculated and control rabbits may be primarily in response to a persistent *B. bronchiseptica* infection. *Bordetella* has been shown to be a surface dweller in the respiratory tract. For example, *B. bronchiseptica* may specifically attach to the cilia of epithelial cells of the canine respiratory tract (25). These bacteria may also reside in the layer of mucus overlying the ciliated epithelial cell (26). Peribronchial lymphoid hyperplasia has been associated with canine cases of *B. bronchiseptica* infections (25) and in human infections with *Bordetella pertussis* (27).

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