T125
60

To the Graduate Council:

I am submitting herewith a thesis written by Scott Allen Wilson entitled "Investigation of Factors Involved in Bordetella bronchiseptica-Induced Ciliostasis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

David A. Bemis

David A. Bemis, Major Professor

We have read this thesis
and recommend its acceptance:

Robert N Moore

John R. Kennedy

Raymond W. Beck

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master of Science degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Scott Allen Wilson

Date 18 June 1986

INVESTIGATION OF FACTORS INVOLVED IN *BORDETELLA BRONCHISEPTICA*-INDUCED CILIOSTASIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Scott Allen Wilson

August 1986

ACKNOWLEDGEMENTS

The author would like to express his gratitude to Drs. Raymond Beck, John Kennedy, and Robert Moore for their critical review and timely guidance. This author has greatly appreciated the untiring support and patience of his mentor and chairman, Dr. David Bemis. His keen wit will be sorely missed. Electron microscopy for determination of pili and flagella was accomplished by Elizabeth Wilkinson-Singley. Douglas Dodd was indispensable in his help with computer-generated graphics. Monty Hatcher provided technical support on the PAGE and autoradiography procedures. Many thanks to Beth Goode for her quick smile and fast typing (the loss of either of those attributes would have made preparation of this thesis unspeakably horrendous).

This author also gratefully thanks all of his friends and fellow graduate students for their support during his tenure at the University of Tennessee.

ABSTRACT

Bordetella bronchiseptica strain 110H rapidly induced ciliostasis in canine tracheal outgrowth cultures. The induction of ciliostasis was both time and dose dependent. Eighteen strains of B. bronchiseptica were examined for their ability to induce ciliostasis. Fifteen strains grown on Brucella agar induced ciliostasis. The three strains that did not induce ciliostasis were morphologically stable, non-piliated, non-hemolytic, did not produce extracellular adenylate cyclase, and were not distinguishable from rough phase B. bronchiseptica on Brucella agar. All strains which were hemolytic on Bordet-Gengou agar produced extracellular adenylate cyclase and induced ciliostasis. Two non-hemolytic strains, one with rough phase colonial morphology on Brucella agar, induced ciliostasis. The only phenotypic characteristic studied which was positively associated with the ability to induce ciliostasis was the presence of pili. Formalin-killed and Chloramphenicol-inhibited B. bronchiseptica strain 110H organisms had detectable pili and attached to cilia, but did not induce ciliostasis. Protease-treated B. bronchiseptica strain 110H organisms did not have detectable pili and in the presence of Chloramphenicol did not induce ciliostasis, nor did they attach to cilia. Concentrated (100X) supernatants from 48 hour cultures of B. bronchiseptica strain 110H organisms grown in Waymouth's MB 752/1 medium did not induce ciliostasis. B. bronchiseptica strain 110H organisms which were separated from canine tracheal outgrowth cultures with a parabiotic

chamber were not able to induce ciliostasis. Filter sterilized supernatant fluids from ciliostatic B. bronchiseptica infected canine tracheal outgrowth cultures were not able to induce ciliostasis in non-infected tracheal outgrowth cultures. Filter sterilized supernatant fluids from sonicated B. bronchiseptica strain 110 organisms were not able to induce ciliostasis. Hyperimmune rabbit antisera produced against whole cell B. bronchiseptica strain 110H bacterins completely inhibited B. bronchiseptica induced ciliostasis in canine tracheal outgrowth cultures. Antisera against B. bronchiseptica did not inhibit its viability, nor cause the release of adherent organisms from cilia. Heat treatment of the antisera (56°C, 30 min) did not destroy the "protective" activity. Removal of antisera from the infected tracheal outgrowth culture resulted in complete ciliostasis ($\geq$98%) in four hours. Infected outgrowths which had been completely ciliostatic for as long as 2 hours regained approximately 65% of their normal activity within six hours after the addition of hyperimmune sera. The ability to protect cilia from B. bronchiseptica induced ciliostasis paralleled the B. bronchiseptica agglutinin titers of the sera. Protein A-Sepharose purified IgG fractions from hyperimmune rabbit sera also protected the cilia. Absorption of sera with Staphylococcus aureus strain Cowan I, Escherichia coli strain FDA Seattle 1946, Pseudomonas aeruginosa strain PAO-1, B. bronchiseptica strain 110NH and B. bronchiseptica strain 110H removed the protective activity of serum. Boiled (100°C, 60 min) E. coli, P. aeruginosa, and B. bronchiseptica strains 110H and 110NH also absorbed out the

protective activity of sera. Staphylococcus aureus strain Wood 46 and Staphylococcus epidermidis strain FDA PCI 1200 did not remove the protective activity of the hyperimmune sera. The detection of antibodies on the surfaces of boiled absorbants were determined by either ELISA, PAGE-autoradiography (with I^{125} labeled IgG fractions from rabbit anti-Bordetella hyperimmune serum) or immunofluorescence tests. Immunofluorescence was used to test all absorbants; ELISA and PAGE-autoradiography was used to test only boiled E. coli and boiled B. bronchiseptica. B. bronchiseptica antigens were positive in all tests. S. aureus strain Cowan I was positive with goat anti-rabbit IgG immunofluorescence, S. aureus strain Wood 46 and S. epidermidis were negative. Antibody to boiled E. coli antigen was detected by ELISA and PAGE-autoradiography but negative for immunofluorescence assays. These data suggest that attachment may be a requirement for the induction of ciliostasis, that ciliostasis can be reversed, and that antibodies may have a role in protecting cilia against induction of ciliostasis by B. bronchiseptica.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	5
	Bacterial Cultures	5
	Preparation of Bacterial Inocula and Extracts	5
	Bacterial Treatments	8
	Measurement of Extracellular Adenylate Cyclase	10
	Electron Microscopy	11
	Sulfonamide Susceptibility Tests	11
	Plasmid Detection	12
	Preparation of Canine Tracheal Outgrowth Cultures	12
	Measurement of Ciliostasis	13
	Determination of Tissue Associated Bacteria	13
	Parabiotic Chamber	14
	Preparation of Hyperimmune Antiserum	14
	Separation of Immunoglobulin G Fraction from Hyperimmune Antiserum and Normal Rabbit Serum	15
	Absorption of Hyperimmune Sera and Isolated IgG Fractions with Bacterial Antigens	17
	Bacterial Agglutination	17
	Immunofluorescence	18
	Enzyme Linked Immunosorbent Assay	19
	Determination of Cilia Protective Activity	20

CHAPTER PAGE

II. (Continued)

SDS-PAGE, Radioiodination of Immunoglobulins and Autoradiography 21

III. RESULTS 22

IV. DISCUSSION 51

LIST OF REFERENCES 60

VITA 67

LIST OF TABLES

TABLE		PAGE
1.	Bacterial strains used	6
2.	Effect of bacterial inoculum size on B. bronchiseptica attachment to cilia and the subsequent induction of ciliostasis	23
3.	Ciliotoxicity, extracellular adenylate cyclase (ECAC) activity and other bacteriological characteristics of selected B. bronchiseptica strains	26
4.	Effect of Formalin, Chloramphenicol, and Proteinase K, on the piliation, attachment, and the ability of B. bronchiseptica strain 110H to induce ciliostasis	29
5.	B. bronchiseptica agglutinating and cilia protective activity of various animal sera	36
6.	Removal of ciliary protective activity from serum by absorption with bacterial whole cell antigens	45
7.	Cilia protective activity of protein A-Sepharose derived serum fractions from rabbits before and after immunization with B. bronchiseptica	47

LIST OF FIGURES

FIGURE		PAGE
1.	Effect of Chloramphenicol and Polymyxin B on B. bronchiseptica induced ciliostasis	31
2.	Effect of serum on B. bronchiseptica induced cilostasis. . . .	34
3.	Association of ciliary protective effect of serum with B. bronchiseptica agglutination	37
4.	Rise in cilia protective activity and Bordetella agglutinating activity in rabbit serum following immunization with B. bronchiseptica	39
5.	Reversal of ciliostasis with rabbit anti-Bordetella hyperimmune serum	42
6.	Analysis of boiled E. coli after absorption of radiolabeled IgG fraction from rabbit anti-Bordetella hyperimmune serum .	49

INTRODUCTION

Bordetella bronchiseptica, a gram-negative coccobaccillus, has been recognized as a respiratory tract pathogen of animals since its isolation and identification by N. S. Ferry (1910, 1913). B. bronchiseptica has been associated with respiratory tract diseases in a variety of animals (Batey et al., 1976; Bemis et al., 1977a; Gallagher, 1965; Ganaway et al., 1965). Infection rates have been high, often approaching 100% in some populations of pigs, guinea pigs, dogs, and rabbits (Ferry, 1913; Jenkins et al., 1977; Thompson et al., 1976; Appel, et al., 1977; Ganaway et al., 1965).

An initial feature of B. bronchiseptica infection is the attachment of the organisms to cilia of the respiratory tract epithelium. Bacterial glycocalyx, pili, fimbrial hemagglutinin (FHA), and pertussis toxin (PT) have been implicated in the adherence of Bordetella species to mucosal epithelium (Bemis et al., 1977b; Beachey, 1981; Ashworth et al., 1982; Tuomanen et al., 1983a; Blom et al., 1983). Scanning and transmission electron micrographs of Bordetella infected epithelium revealed filamentous surface structures radiating from the bacteria and in very close association with the ciliary surface membranes (Bemis et al., 1977b; Yokomizu and Shimuzu, 1979; Sato et al., 1978; Tuomanen and Hendley, 1983). When similar tissue was stained with ruthenium red, heavy deposits of the stain filled the space between the cilia and adhered bacteria (Tuomanen and Hendley, 1983). This observation was consistent with the hypothesis that bacterial attachment to animal tissues often involves a matrix of

acidic polysaccharides referred to as a glycocalyx (Costerton, 1981). The glycocalyx may be produced by both the bacteria and animal tissues. Recent evidence has suggested a role of complex carbohydrates in adherence of Bordetella to cells. Monoclonal antibodies directed to fucosylated galactose-N-acetyl-D-glucosamine bound to human respiratory tract cilia and prevented attachment of B. pertussis (Tuomanen et al., Abst. Ann. Meet. Am. Soc. Microbiol., 1986, Abst. No. B6). Binding of N-acetyl-D-glucosamine to virulent strains of B. bronchiseptica prevented its adherence to hamster lung fibroblasts (Plotkin and Bemis, 1984).

After the attachment phase is completed, a wide variety of pathophysiological effects induced by Bordetella may be observed in vitro, the most pronounced of which is the destruction of ciliated epithelial cells. Both B. pertussis and B. bronchiseptica induce ciliostasis, with B. bronchiseptica inducing approximately 90% ciliostasis in three hours (Bemis and Kennedy, 1981). The mechanism of ciliostasis induced by either B. pertussis or B. bronchiseptica has yet to be determined. The ciliated cells become static (no ciliary beating), DNA and protein synthesis are halted, and the damaged ciliated cells are sloughed off from the epithelium (Collier et al., 1977; Muse et al., 1977; Bemis and Kennedy, 1981). B. pertussis secretes a tracheal cytotoxin (TCT) that induces marked ciliostasis in hamster tracheal epithelial cells (Goldman et al., 1982). No such extracellular tracheal cytotoxin has been isolated from B. bronchiseptica. An extracellular adenylate cyclase (ECAC) "toxin" has

been isolated from both B. bronchiseptica and B. pertussis (Hewlett el al., 1976; Endoh et al., 1980). A possible role in the virulence of Bordetella species by ECAC has been postulated since ECAC of B. pertussis has been demonstrated to enter target cells and convert endogenous (cellular) ATP into cAMP (Hewlett et al., 1976). Presence of ECAC has resulted in the dysfunction of lymphocytes, phagocytes and Chinese Hamster Ovary (CHO) cells (Hewlett et al., 1976); its effect on ciliated cells has not yet been demonstrated. Since high levels of ATP are required for ciliary motion, Bordetella species through the production of ECAC may be utilizing or destroying the cellular ATP supply and thus be acting as an energy parasite. The build-up of cAMP inside target cells may also have detrimental effects since cAMP is an inducer of a wide variety of biochemical pathways (Strewler and Orloff, 1977). When ECAC and hemolysin of B. pertussis were inactivated by transposon (TN 5) insertion, the virulence of B. pertussis drastically declined (Weiss et al., 1984). A 68,000 dalton molecule with adenylate cyclase activity has been isolated from B. bronchiseptica; furthermore, an immune response against this molecule has protected mice against B. bronchiseptica infection (Novotny et al., 1985). The exact role of ECAC in Bordetella pathogenesis is not known.

The purpose of this investigation was to study the interaction of B. bronchiseptica with ciliated epithelium. Different strains of B. bronchiseptica were examined to detect any phenotypic characteristics which correlated with their ability to induce ciliostasis. Since a hypothesis was formed that attachment and ciliostasis might be

separable events, attempts were made to isolate a soluble ciliotoxic factor and to identify the role attachment has in ciliostasis. A search was also made for substances which interfere with B. bronchiseptica induced ciliostasis, and which, therefore, would have potential applications in the control of B. bronchiseptica infection.

CHAPTER II

MATERIALS AND METHODS

Bacterial Cultures

Eighteen strains of Bordetella bronchiseptica, two strains of Staphylococcus aureus, one strain of Escherichia coli and one strain of Pseudomonas aeruginosa were utilized in this study (see Table 1). All of the bacteria were maintained on Brucella Agar (BA, Gibco Laboratories, Madison, WI). Bordetella strains were also cultured on Bordet-Gengou agar (BG, Gibco) with 15% horse blood and BA with 5% horse blood to screen for phase variation and hemolysis. The Bordetella strains were incubated aerobically at 37°C for 48 hours before use. The other bacteria used were incubated aerobically at 37°C for 24 hours prior to use.

Preparation of Bacterial Inocula and Extracts

Live bacterial inocula. Isolated colonies of each B. bronchiseptica strain were suspended in pre-warmed (37°C) Waymouth's MB 752/1 medium, pH 7.3 (Gibco), to an O.D. value which approximated 5 x 10^9 CFU/ml (O.D. 0.12 at 540 nm, Spectronic 20, Bausch and Lomb, Rochester, NY). One milliliter (ml) of this suspension was added to tracheal outgrowth cultures.

Bacterial sonicates. Inocula from B. bronchiseptica strains 110H and 110NH in 10 ml volumes were sonicated for six minutes at maximum power at room temperature in an ice water bath attached to an ultrasonicator (Heat Systems-Ultrasonicator Model W-220F, Heat

Table 1. Bacterial strains used.

Bacterial species	Strain(s)	Source
Bordetella bronchiseptica	110H, 110NH, 87, ILG, Rat 1, Rat 2, 501, Ft. Collins, B133, D-1, S. Madrid, D-1, CD-C47 SHGP-1, BTS, NYS #4, 19141, 17640, Columbus	D. Bemis, Univ. of Tenn. Knoxville, TN
Pseudomonas aeruginosa	PAO-1	J. Raulston, Univ. of Tenn. Knoxville, TN
Escherichia coli	FDA Seattle 1946	D. King, Univ. of Tenn. Knoxville, TN
Staphylococcus aureus	Cowan I (#12598) Wood 46 (#10832)	American Type Culture Collection Rockville, MD
Staphylococcus epidermidis	FDA PCI 1200 (ATCC #12228)	American Type Culture Collection Rockville, MD

Systems-Ultrasonics, Inc., Plainview, NY). The sonicated suspensions were pipetted into 1.5 ml polypropylene microcentrifuge tubes (Bio-Rad Laboratories, Richmond, CA) in 1.0 ml aliquots. The suspensions were centrifuged (Fisher microcentrifuge model 235A, Fisher Scientific Corporation, Springfield, NJ) for three minutes at 4°C. The supernatant fluids were decanted, pooled, and sterilized by filtration (0.22 um, Millex-GV, Millipore Corporation). The filtrate was stored at -80°C in 1.0 ml aliquots until testing in tracheal outgrowth cultures.

Concentration of culture supernatants. Ten ml inocula of B. bronchiseptica strain 110H were added to flasks containing 250 ml aliquots of Waymouth's medium. The cultures were stirred on a rotary mixer (New Brunswick Scientific model G2, New Brunswick Scientific Company, Inc., New Brunswick, NJ), at 100 revolutions per minute (RPM) during incubation at 37°C in 5% CO_2 for 48 hours. The cultures were decanted into centrifuge bottles (250 ml polycarbonate bottle, Oak Ridge style, DuPont Company, Wilmington, DE) and centrifuged at 10,000 RPM (Sorvall RC2-B, Ivan Sorvall, Inc., Norwalk, CT) at 5°C for 40 minutes. The supernatants were filter sterilized (Nalgene 0.22 um filters, Nalgene Corporation, Rochester, NY) and aliquots of 1 ml, 10 ml, 250 ml, and 1,000 ml were set aside for concentration to 1X, 10X, 20X, and 100X, respectively. Concentration of supernatants was accomplished with an Amicon ultrafiltration apparatus (Amicon Corporation, Danvers, MA) using a 1,000 molecular weight cut-off membrane (Amicon). Diafiltration was carried out at 4°C under 40 pounds pressure of gaseous N_2.

Bacterial Treatments

Heat treatment. Heavy suspensions of B. bronchiseptica strains 110H and 110NH, E. coli and P. aeruginosa were made in 10 ml of phosphate buffered saline (PBS) or Waymouth's medium. The suspensions were placed in a 100°C water bath for 60 minutes. The suspensions were then placed in 1.5 ml polypropylene microcentrifuge tubes (Bio-Rad) in 1 ml aliquots and centrifuged at 13,000 x g for 3.5 minutes at 4°C. The bacteria were washed four times with PBS before use.

Cold treatment. Heavy suspensions of B. bronchiseptica strain 110H were made in cold (4°C) Waymouth's medium. The suspensions were serially diluted 10-fold in cold Waymouth's medium before use. Subsequent experiments using cold treated organisms were carried out at 4°C with cold Waymouth's medium.

Formalin treatment. Heavy suspensions of B. bronchiseptica strains 110H and 110NH, E. coli and P. aeruginosa were made in 10 ml of PBS-containing 0.5% formalin (Fisher) and incubated overnight at 37°C. The suspensions were centrifuged as described above (in 1 ml aliquots), and washed six times with PBS to remove the formalin.

Antibiotic treatment. A standard inoculum of B. bronchiseptica strain 110H was prepared in Waymouth's medium containing a bacteriostatic concentration of Chloramphenicol (10 ug/ml, Sigma Chemical Company, St. Louis, MO). Prior to addition to tissue culture some inocula were incubated for six hours at 37°C. Inhibitory and bacteriocidal concentrations of Chloramphenicol and Polymixin B (Sigma)

were determined by the broth dilution method (Barry and Sabath, 1974) using Waymouth's MB 752/1 medium as the bacterial culture medium. Bacterial concentrations were calculated by standard serial dilution and plate count assays on Brucella Agar.

Proteinase K treatment. An inoculum of B. bronchiseptica strain 110H was incubated with a bacteriostatic dose of Chloramphenicol (10 ug/ml) for six hours at 37°C. The organisms were washed four times with Waymouth's medium and resuspended in Waymouth's medium plus proteinase K (20 ug/ml, Sigma) for 30 minutes at 37°C. The bacteria were washed twice with Waymouth's medium and suspended to their original concentration in Waymouth's medium with or without Chloramphenicol (10 ug/ml). Viability was determined after the 30 minute incubation period by a standard serial dilution and plate count assay.

N-acetyl-D-glucosamine (GlcNAc) treatment. Inocula of B. bronchiseptica strain 110H containing 0.5 M GlcNAc (Sigma) were incubated at 37°C for 30 minutes on a rocker (Labquake rocker, Labindustries, Inc., Berkeley, CA) and pelleted at 13,000 x g for 3 minutes. The organisms were washed twice with Waymouth's medium to remove unbound GlcNAc and suspended to the original concentration in Waymouth's medium.

Measurement of Extracellular Adenylate Cyclase

Extracellular adenylate cyclase (ECAC) activity of B. bronchiseptica strains was measured using a modification of the method described by Peppler (1982). Individual bacterial colonies were suspended in sterile isotonic PBS to an O.D. which approximated 5 x 10^9 CFU/ml. One ml of the bacterial suspension was added to microcentrifuge tubes (Fisherbrand 1.5 ml, Fisher) containing 100 ul each of ATP (Adenosine triphophate, 10mM, Sigma), $MgCl_2$ (Magnesium chloride, 10mM, Fisher), and freshly lysed horse red blood cells (1:1 osmotic lysis of packed washed cells in water). The latter was used as a source of calmodulin, a known activator of Bordetella ECAC (Goldhammer et al., 1981). Negative controls consisted of PBS substituted in place of the bacterial suspension or in place of the ATP. Positive controls were prepared by adding cAMP (cyclic 3', 5'-Adenosine monophosphate, Sigma) in place of the bacterial suspension. These mixtures were incubated for 60 minutes at 37°C. After incubation the suspensions were centrifuged for 10 minutes at 4°C in a microcentrifuge (Eppendorf microcentrifuge, Brinkmann Instruments, Westbury, NY). Duplicate 50 ul samples of the supernatant were assayed for cAMP content by the competitive binding method using a commercially available radiometric assay kit (Amersham Corporation, Arlington Heights, IL). By this method unlabeled cAMP in the unknown samples and positive controls competed with tritium labeled cAMP for binding to antibody. The cAMP-antibody complex was removed from solution by absorption with charcoal and centrifugation. Radioactive counts of 50 ul samples of supernatant fluids were measured in five ml of Aquassure

(New England Nuclear Corporation, Boston, MA) in polypropylene capped glass scintillation vials (Fisher) for five minutes in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Final values were based on a standard cAMP curve and corrected for and corrected for values obtained with PBS controls. Adenylate cyclase activity was expressed as picomoles cAMP per minute per 5 x 10^9 CFU.

Electron Microscopy

Individual colonies of B. bronchiseptica from 48 hour agar cultures were suspended in PBS and negatively stained with potassium phosphotungstate by the method described by Bemis et al., 1977. Grids were examined with a Hitachi model H600 electron microscope (Hitachi Ltd., Tokyo, Japan). After the entire grid was examined, a representative area was selected and at least 20 bacterial cells were examined for the presence of pili and flagella. Organisms were considered positive if they had 10 or more pili and one flagellum or more.

Sulfonamide Susceptibility Tests

Susceptibility to sulfonamides was determined by a standardized disk diffusion method as described by Bemis et al. (1977c). The compounds tested were triple sulfa (sulfamerazine, sulfadiazine, and sulfamethazine) and sulfa-methoxazole-trimethoprim.

Plasmid Detection

Plasmid DNA was extracted from well-isolated bacterial colonies by the alkaline whole cell lysis method of Kado and Liu (1981). Lysates were subjected to electrophoresis in 0.6% agarose gels, stained with ethidium bromide (0.5 ug/ml), and observed with transilluminated UV light.

Preparation of Canine Tracheal Outgrowth Cultures.

Canine tracheal outgrowth cultures were prepared by a modification of the method of Bemis and Kennedy (1981). Canine trachea were aseptically removed from freshly euthanized animals and placed in sterile 1X Waymouth's medium, pH 7.3 (Gibco) with 100 U/ml Penicillin-Streptomycin (Sigma) for transport. The trachea were dissected perpendicularly to the cartilage rings into strips 4 mm x 15 mm. Each strip was pinned mucosal side up on a plate of dental wax. The mounted material was viewed under a dissecting microscope (Unitron ZSB, Japan). The mucosal epithelium was separated from the cartilage by dissection with a scalpel; the epithelium was peeled back and removed with forceps. The mucosal strips were placed in petri dishes (100 x 15 mm, Miles Laboratories, Elkhart, IN) containing Waymouth's medium that was warmed to 37°C. The strips were then dissected into squares (1 mm x 1 mm) while in warm Waymouth's medium. Three squares (hereafter referred to as explants) were transferred to a tissue culture dish (10 x 35 mm, Falcon, Becton-Dickinson Corporation, Oxnard, CA). The explants were then covered with Waymouth's medium supplemented with 10% Nu serum (Collaborative Research Inc., Lexington, MA) and 1% Penicillin-Streptomycin solution (final concentration 100 U/ml, Sigma).

The culture medium was changed every 48 hours. The explant cultures were incubated at 37°C in 5% CO_2 for 4-7 days before use. The cultures were examined before use for the presence of ciliated epithelial outgrowths (hereafter referred to as outgrowths) with an inverted phase contrast microscope (American Optical Biostar, American Optical Scientific Instruments, Buffalo, NY).

Measurement of Ciliostasis

Ciliostasis was recorded as the percentage of ciliated outgrowth cells with no detectable motile cilia. Ciliary beating was classified over a range from 0 to +4 with 0 as no ciliary activity and +4 as beating no differently from negative controls. A minimum of 50 ciliated cells were counted in each outgrowth culture. Any physical changes in the outgrowths and explants were noted as well. With each tracheal outgrowth experiment and new batch of tracheal outgrowths, the following controls were used to check on reproducibility of the assay system: B. bronchiseptica strain 110H infected outgrowths (>90% ciliostasis in four hours), B. bronchiseptica strain 110NH infected outgrowths (<10% ciliostasis in four hours), and non-infected outgrowths (<10% ciliostasis in four hours).

Determination of Tissue Associated Bacteria

Infected tracheal outgrowth cultures were incubated for 20 minutes at 37°C in 5% CO_2. The infected outgrowth cultures were then washed three times with Waymouth's medium and trypsin was added (0.05%, Sigma). The explants and outgrowths were removed from the plastic tissue culture dish and suspended in 1 ml of Waymouth's medium plus

fetal bovine serum (final concentration 10%, Gibco). The tissues were ground in TenBroek-type tissue grinders (Bellco Glass Inc., Vineland, NJ). The bacterial concentrations were determined by a standard serial dilution and plate count method on Brucella agar.

Parabiotic Chamber

Separation of bacterial organisms from ciliated epithelium, in order to test for the presence of an extracellular toxin, was accomplished utilizing parabiotic chambers (Bellco #1944, Bellco Glass, Inc., Vineland, NJ). The chambers were separated by a membrane filter (0.2 um, Millipore Filter Corporation, Bedford, MA). Tracheal explants were placed on glass cover slips (Fisher). The cover slips were placed in one chamber and covered with plain Waymouth's medium (Gibco). The other chamber was filled with a suspension of B. bronchiseptica strain 110H (suspended in Waymouth's to a concentration of 5×10^9 CFU/ml). The chambers were placed in a 37°C, 5% CO_2 environment.

The ciliated epithelium was observed under an inverted phase contrast microscope at one hour intervals for a period of six hours, and every 12 hours thereafter over a five day period.

Preparation of Hyperimmune Antiserum

Two New Zealand white rabbits (female, 5-6 lbs.) were obtained from Clay Graves Rabbitry (Corryton, TN). Both rabbits were immunized with whole cell vaccines prepared from Bordetella bronchiseptica strain 110H. Both vaccines were administered by subcutaneous injection of 1

ml amounts which contained 5% aluminum hydroxide (Alhydrogel, Superfos Export Company, Denmark) and 1 x 10^{10} cfu of B. bronchiseptica strain 110H. Vaccine #1 (rabbit #2457) contained live organisms and vaccine #2 (rabbit #2456) contained formalin-killed organisms. A second inoculation of each type was given 10 days after the initial injection.

Blood samples were taken from ear vein bleedings prior to and at four day intervals following the first inoculation, until the 28th day post-inoculation when the rabbits were exsanguinated following CO_2 asphyxiation. The final blood sample was obtained by cardiac puncture. The blood samples were allowed to clot in the collection tube, reamed with wooden applicator sticks (Fisher) and the clot was then placed in a glass funnel (Corning 6180, Corning Glass Works) that had been lined with wooden applicator sticks. The serum was collected overnight at 4°C. The serum was clarified by centrifugation (2,000 rpm for 15 minutes at 25°C), aliquoted into screw cap tubes (Kimax 13 x 100 mm; Kimble 45066, Kimble Glass Corporation), and stored at -20°C. Samples were thawed in a 37°C water bath prior to use. All serum samples were heated at 56°C for 30 minutes to remove the activity of complement.

Separation of Immunoglobulin G Fraction from Hyperimmune Antiserum and Normal Rabbit Serum

Isolation of immunoglobulin G (IgG) from hyperimmune antiserum and normal rabbit serum was accomplished by affinity chromatography using a Protein A Sepharose CL-4B gel column (Pharmacia Fine Chemicals,

Uppsala, Sweden). The gel bed volume was five ml of packed sepharose beads, prepared according to the manufacturer's instructions. The column was washed with 50 ml of 0.5 M Tris, 0.15 M NaCl buffer (pH 8.64) to remove PBS-azide from the gel. The sample (3 ml of either hyperimmune antiserum or normal rabbit serum) was added to the column and allowed to run into the gel. Next, five ml of 0.5 M Tris 0.15 M NaCl buffer was added and run into the column. The collected fraction was re-added to the column at this point and allowed to completely enter the gel. The column was then washed with ten bed volumes (50 mls) of Tris/NaCl buffer through the use of a buffer reservoir with the flow rate maintained at 0.5 ml per minute. The first 20 ml fraction collected from the column was set aside. After washing, five milliliters of 0.1 M Acetate buffer (pH 3.5) was added and allowed to enter the column. The column was attached to a reservoir and eluted with 20 milliliters of the acetate buffer. The eluate was collected into a 50 ml beaker (Fisher) containing 10 ml of 0.5 M sodium phosphate buffer (pH 8.8) to neutralize the pH. The total neutralized eluate volume was 20 ml. After use, the column was washed with 50 ml of Tris/NaCl, and stored in PBS-azide buffer (0.1% azide) at 4°C.

The eluted fractions were concentrated and dialyzed against isotonic PBS with a Schleicher & Schuell collodion membrane apparatus (Schleicher & Schuell, Inc., Keene, NH) to approximately the original sample volume. Protein concentrations were determined using the Bio-Rad protein assay (BioRad). IgG samples were stored in PBS at -20°C. Samples were thawed in a 37°C water bath prior to use.

Absorption of Hyperimmune Sera and Isolated IgG Fractions with Bacterial Antigens

Hyperimmune sera and IgG fractions were absorbed with either live, formalin killed, or heat killed (100°C for 60 minutes) organisms. The organisms used were: B. bronchiseptica strains 110H and 110NH, Staphylococcus aureus strains Cowan I and Wood 46, Escherichia coli strain FDA Seattle 1946, and Pseudomonas aeruginosa strain PAO-1.

The bacterial whole cell antigens were suspended in PBS, placed in 1 ml aliquots in polypropylene microcentrifuge tubes, and centrifuged at 13,000 x g for 3.5 minutes. The process was repeated until 0.1 ml pellets were obtained. The PBS was then decanted and 1 ml volumes of the sera or fractions to be absorbed were added. After mixing, each suspension was gently rocked at 25°C for 60 minutes. The bacteria were then pelleted at 13,000 x g for 3.5 minutes at 4°C. The supernatant was carefully removed and used immediately for testing anti-bacterial and protective activities.

Bacterial Agglutination

A slide bacterial agglutination test was used to quickly monitor anti-Bordetella activity. The antigen consisted of formalin-killed B. bronchiseptica strain 110H suspended in PBS to a concentration of approximately 1 x 10^{12} CFU/ml. Sera or serum fractions were serially diluted in PBS and a 100 ul sample of each dilution was placed on a glass slide. An equal volume of the antigen was then added. The mixtures were rocked gently and observed for agglutinating activity over a period of five minutes. Agglutination was considered positive

if greater than 75% of the organisms were agglutinated. Agglutination titers were expressed as the reciprocals of the highest dilution at which positive agglutination was observed.

Immunofluorescence

Bacterial immunofluorescence was used to detect antibodies in serum samples or serum fractions. One-hundred ul of a suspension containing 1 x 10^9 CFU/ml of the bacteria being examined was placed on a glass slide, air dried, and fixed in acetone. The slide was flooded with the test serum or serum fraction for 60 minutes at room temperature. The slide was then gently washed with deionized water (dH_2O) for 30 seconds. The slide was flooded with either fluorescein conjugated goat anti-rabbit IgG (FC-GAR, final dilution 1:10, Cappel Laboratories, Westchester, PA) or fluorescein isothiocyanate conjugated protein A (FITC-PA, Pharmacia) diluted in isotonic PBS (final dilution 1:10) and incubated for 60 minutes at room temperature. The slide was again washed for 30 seconds with dH_2O and observed under episcopic UV, and phase contrast microscopy (Nikon Fluopholt microscope, Nikon Ltd., Tokyo, Japan). Immunofluorescence was also used to observe B. bronchiseptica adherence to cilia in tracheal outgrowths. The same procedure as described above using a 1:40 dilution of rabbit anti-B. bronchiseptica strain 110H serum was applied to infected tracheal outgrowths. Ciliated cells were located by phase contrast microscopy and then the fluorescence pattern of the same field was examined by episcopic UV microscopy.

Enzyme Linked Immunosorbent Assay

The ELISA was used to measure antibody activity to heat killed (100°C, 60 minutes) E. coli and B. bronchiseptica and to compare total IgG levels of hyperimmune and preimmune sera. Fifty ul of each bacterial antigen (1 x 10^7 CFU/ml suspended in carbonate buffer) or 100 ul of a serial dilution (in carbonate buffer) of serum or serum fraction were added to individual wells of a 96-well microtiter plate (Falcon #3912), covered with a lid and incubated overnight at 4°C. The plates were subsequently washed three times with a PBS-Tween solution (0.05% Tween 20 final dilution) (Tween 20, Bio-Rad).

Fifty ul of PBS-Tween with 3% fetal bovine serum (FBS) was added to each well (to block non-specific binding sites) and incubated for 60 minutes at 37°C. The plates were then washed three times with PBS-Tween. The bacterial antigen wells received 50 ul of a 1:10 dilution (in PBS-Tween) of either hyperimmune serum, pre-immune serum, or serum fractions (from hyperimmune or preimmune serum). The plates were incubated for 60 minutes at 37°C, followed by washing three times with PBS-Tween. Fifty ul of goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP) diluted 1:2000 in PBS-Tween was added to all wells (those containing bacterial antigen as well as those with sera or serum fractions as solid phase antigens) and incubated for 60 minutes at 37°C. All plates were washed six times with PBS-Tween. Fifty ul of substrate solution containing ortho-phenylenediamine (OPD, Sigma) and hydrogen peroxide (H_2O_2, Fisher) in Citrate buffer (pH 6.0) was added to each well. The plates were incubated in the dark

at room temperature for 30 minutes. Twenty-five ul of 2.5 M sulfuric acid (Fisher) was added to stop the OPD-HRP-H_2O_2 reaction. Numerical values of optical densities were obtained by an automated microtiter plate reader (Bio- Tek model EL 310, Bio-Tek, Burlington, VT). Reactions were considered positive if they developed to a level twice that of control wells. Controls used were: 3% FBS (as antigen) followed by antisera and GAR- HRP, bacterial antigen (no blocker and no antisera) followed by GAR- HRP, and blank wells (no antigen) with antiserum followed by GAR-HRP.

Determination of Cilia Protective Activity

The ability of sera or serum fractions to protect against B. bronchiseptica induced ciliostasis was determined by the addition of test sera to B. bronchiseptica infected outgrowths. Tracheal outgrowths were infected with standard inocula of B. bronchiseptica, incubated 20 minutes at 37°C in 5% CO_2. The outgrowths were washed three times with warm (37°C) Waymouth's medium and replenished with two-fold serial dilutions of sera or serum fractions (diluted in Waymouth's medium). The minimum dilution was 1:10. The cultures were observed at one hour intervals over a period of six hours post-addition of test sera. Test sera were also added, at the minimum dilution, to non-infected cultures to check for toxicity.

Another parameter of the testing of cilia protective activity that was examined was the ability of sera (hyperimmune or preimmune) to restore activity in infected, ciliostatic outgrowths. Tracheal outgrowths were infected with standard inocula of B. bronchiseptica

strain 110H, incubated 20 minutes, washed and replenished with non-supplemented Waymouth's medium. The outgrowths were incubated for six hours at 37°C in 5% CO_2, then washed three times with Waymouth's medium, and replenished with either Waymouth's medium plus hyperimmune serum (1:5 and 1:20 final dilutions) or plus preimmune serum (1:5 final dilution).

SDS-PAGE, Radioiodination of Immunoglobulins and Autoradiography

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with protein A-sepharose affinity chromatography purified fractions on a 12% polyacrylamide gel by the Laemmli system (Laemmli, 1970).

Protein A-sepharose binding (IgG) fractions were radioiodinated with enzymobeads (Bio-Rad) by the method of enzymatic iodination (Hubbard et al., 1972; Morrison et al., 1971). The radioiodinated IgG fractions were split into two aliquots: one aliquot to be absorbed with boiled E. coli, the other aliquot as non-absorbed control. The two aliquots and the absorbing antigen were analyzed by SDS-PAGE. Protein bands on the gels were visualized by Coomassie blue staining and autoradiography.

CHAPTER III

RESULTS

Relationship of Concentration of B. bronchiseptica to Attachment and Induction of Ciliostasis

The time required for B. bronchiseptica strain 110H to induce greater than 90% ciliostasis was directly proportional to the inoculating dose and the number of organisms which subsequently became tissue-associated (Table 2). The greater the number of organisms associated with the tissue the shorter the time to ciliostasis (50 hours for 1.6×10^3 CFU; 3 hours for 3×10^8 CFU). Greater than 90% of the ciliated cells consistently had B. bronchiseptica adhered to their cilia after 20 minutes of incubation with inocula containing greater than to 1×10^9 CFU. Fluorescent antibody labeled bacteria were seen only on cilia in the epithelial monolayers, although fluorescent labeled organisms were occasionally seen on fibroblasts in the periphery of the outgrowths. No fluorescence was observed in non-infected tracheal outgrowth cultures. Furthermore, the staining procedure did not in itself inhibit ciliary activity, since fluorescent bacteria were observed on beating cilia.

To determine if attachment alone was responsible for the induction of ciliostasis, formalin-treated strain 110H organisms were added to tracheal outgrowths at the same concentrations indicated in Table 2. The extent of bacterial attachment as observed by the fluorescence assay paralleled that seen in with live organisms, but at no concentration was there significant levels of ciliostasis above that

Table 2. Effect of bacterial inoculum size on B. bronchiseptica attachment to cilia and the subsequent induction of ciliostasis.[a]

Bacterial inoculum size	Number of viable tissue-associated bacteria	Bacterial attachment	Time to Ciliostasis
1.65×10^{10} ($\pm$ 5.0)[b]	$3.06 \times 10^{8} \pm$ (2.99)[c]	48/50[d]	3[e]
4.22×10^{9} ($\pm$ 3.85)	$2.14 \times 10^{7} \pm$ (1.34)	47/50	4
2.17×10^{9} ($\pm$ 6.69)	$2.08 \times 10^{7} \pm$ (1.82)	46/50	4
7.14×10^{8} ($\pm$ 0.76)	$2.69 \times 10^{6} \pm$ (2.48)	40/50	6
4.3×10^{6} ($\pm$ 3.82)	$2.08 \times 10^{6} \pm$ (1.04)	15/50	32
1.8×10^{3} ($\pm$ 0.98)	$1.65 \times 10^{3} \pm$ (0.49)	2/50	50

[a]Each inoculum of B. bronchiseptica strain 110H was incubated with canine tracheal outgrowths for 20 minutes. Outgrowths were washed and either examined immediately by counting or FA or replenished with tissue culture medium and reincubated.

[b]CFU per ml determined by plate count. Numbers in parentheses are $\pm$ SEM.

[c]CFU per total outgrowth culture (explant and outgrowth tissue).

[d]Ratio of the number of ciliated cells with adhered bacteria to total number of ciliated cells counted. Adhered bacteria were detected by a fluorescence assay with anti-Bordetella serum and FITC-Protein A. The ratio was determined immediately after the 20 minute incubation with each medium.

[e]Time (hrs) post-inoculation at which $\geq$ 90% of the ciliated cells counted were ciliostatic.

observed with live avirulent B. bronchiseptica strain 110NH infected and uninfected outgrowths (less than 2% ciliostasis with 5 x 10^9 CFU/ml strain 110NH organisms).

When live strain 110H organisms were allowed to adhere in the cold (4°C), the organisms adhered as described in Table 2. The cilia stopped their activity in the cold, but when moved from the cold into warm (37°C, 5% CO_2) incubation ciliary activity returned to normal and induction of ciliostasis in infected outgrowths proceeded in the time periods indicated in Table 2.

The induction of ciliostasis was dose and time dependent, but the physical act of attachment does not by itself cause the induction of ciliostasis.

Effect of Bacterial Culture Supernatants and Cell Free Extracts on Ciliary Activity

B. bronchiseptica strain 110H was grown in Waymouth's medium for 48 hours (from 2.18 x 10^7 CFU/ml to approximately 1.33 x 10^{10} CFU/ml), removed from the Waymouth's medium by sterile filtration, and the supernatant fluids were concentrated. Supernatant fluids which were concentrated as much as 100X by diafiltration had no discernable effects on tracheal outgrowth cultures. Sonicated cell free extracts from similarly grown organisms and filter-sterilized supernatant fluids from infected ciliostatic outgrowths likewise had no discernable effect on non-infected outgrowths. Although B. bronchiseptica strain 110H grew to concentrations of 1 x 10^{10} CFU/ml over 3 days in one side

of a parabiotic chamber, tracheal outgrowths in the other side of the chamber were unaffected.

Comparison of the Ability to Induce Ciliostasis, Produce ECAC and Other Phenotypic Characteristics Among Strains of B. bronchiseptica

Eighteen strains of B. bronchiseptica were examined for their ability to induce ciliostasis in canine tracheal outgrowths. Colonial morphology on BG and BA, hemolysis, piliation, flagellation, presence of plasmids, ECAC activity, and sulfonamide susceptibiltiy were also examined. The strains selected were representative of the phenotypic variation observed among strains of B. bronchiseptica (Bemis et al., 1977c). The results are listed in Table 3. Fifteen strains induced ciliostasis. The strains which did not induce ciliostasis, 110NH, ILG, and Ft. Collins, were non-hemolytic and non-piliated. They also did not produce ECAC and were of rough phase colonial morphology. Two strains, Columbus and 17640, did not produce ECAC but did induce ciliostasis. There was a positive association between hemolysis on BG and ECAC production. The level of cAMP produced by ECAC positive strains varied. The ECAC assay is very dependent on length of incubation, bacterial concentration, the physiologic state of the organisms and the concentration of ATP in the reaction mixture. None of the B. bronchiseptica strains tested produced measurable levels of cAMP in the absence of exogenous ATP. When the bacterial concentrations of the five ECAC negative strains were raised to 1 x 10^{10} CFU, the exogenous ATP concentration doubled or the incubation

Table 3. Ciliotoxicity, extracellular adenylate cyclase (ECAC) activity and other bacteriological characteristics of selected B. bronchiseptica strains.

Bacterial Strain	Colonial Morphology[a]	Hemolysis[b] BA	Hemolysis[b] BG	Pili[c] (%)	Flagella[d] (%)	Sulfonamide Susceptibility	Plasmids[e]	Adenylate Cyclase[f]	Ciliotoxicity[g]
110H	Phase I	+	+	90	0	S	0	>500	+
RAT 1	"	+	+	90	0	S	0	>500	+
RAT 2	"	+	+	80	0	S	0	>500	+
501	"	+	+	95	0	S	0	>500	+
110NH	Rough	-	-	0	80	S	0	<0.1	-
Columbus	"	-	-	100	70	S	0	<0.1	+
ILG	"	-	-	0	75	S	0	<0.1	-
Ft. Collins	"	-	-	0	75	S	0	<0.1	-
B133	Intermediate	-	+	50	40	S	0	408	+
S. madrid	"	-	+	55	60	S	0	>500	+
D-1	"	-	+	40	40	S	2(34.8, 12.4)	83	+
SHGR-1	"	-	+	70	50	S	0	>500	+
CD-C47	"	-	+	70	60	R	1(27.9)	7.0	+
BTS	"	-	+	65	75	R	1(24.5)	>500	+
NYS#4	"	-	+	50	70	R	0	>500	+
19141	"	-	+	55	60	R	1(19.3)	>500	+
87	"	-	+	60	60	R	1(26.2)	77	+
17640	"	-	-	40	60	R	0	<0.1	+

[a]Determined on Brucella agar as previously described (Bemis et al., 1977c).
[b]Brucella Agar (BA); Bordet-Gengou Agar (BG)
[c]Percentage of organisms with 10 pili or more; n = 20.
[d]Percentage of organisms with 1 flagellum or more; n = 20.
[e]Number of plasmids detected (estimated size, Kbp)
[f]Expressed as picomoles of cAMP generated/min/5 x 10^8 CFU.
[g]+, greater than 90% inhibition of ciliary activity after three hrs; -, less than 10% inhibition of ciliary activity after three hours.

period doubled to two hours, there was still no detectable levels of cAMP produced.

Plasmids were detected in five of the fifteen ciliostasis-inducing strains; none were detected in the three ciliostasis-negative strains. Four of the five plasmid-bearing strains possessed a single plasmid and were resistant to sulfonamides. Two of the sulfonamide-resistant strains did not have plasmids. One strain, D-1, possessed two plasmids and was sulfonamide susceptible.

Fourteen of the strains were flagellated. The four non-flagellated strains had pili, while three of the flagellated strains were non-piliated. All of the phase I and intermediate morphologic strains had pili, whereas only one rough strain was piliated.

The presence of hemolysis was detected with only phase I colonies on BA; on BG, however, most smooth strains produced hemolytic colonies and none of the rough phase organisms were hemolytic. One intermediate phase organism (17640) was non-hemolytic. Two colony types were observed on BG, small and large. Some strains produced only small colonies, some only large and some produced large and small. All of the small colony types on BG induced ciliostasis.

The presence of readily detectable pili was the only phenotypic characteristic which was positively associated with the ability to induce ciliostasis.

Effect of Chloramphenicol and Proteinase K on the Ability of B. bronchiseptica Strain 110H to Induce Ciliostasis

The effects of bacteriostatic concentrations of chloramphenicol and mild proteolysis on attachment and the subsequent induction of ciliostasis are presented in Table 4. The MIC of Chloramphenicol for B. bronchiseptica strain 110H was 10 ug/ml. Strain 110H stopped replicating at this dose after a lag period of approximately 4.5 hours and after 24 hours there was only a slight loss of viability (from 6.8 x 10^8 CFU/ml to 5.9 x 10^8 CFU/ml).

Strain 110H,, pretreated with Chloramphenicol for six hours, adhered to cilia in both the presence and absence of Chloramphenicol. When Chloramphenicol was removed from the outgrowth cultures after the 20-minute attachment period, ciliostasis occured in four hours. When washed outgrowth cultures with Chloramphenicol pretreated or untreated bacteria attached were replenished with medium containing Chloramphenicol, ciliostasis was not observed after four hours.

Proteinase K pretreatment of strain 110H resulted in the loss of attachment in the presence of Chloramphenicol. When the Chloramphenicol was removed from the suspending medium, the Proteinase K pretreated bacteria regained the ability to attach within 30 minutes, and were subsequently able to induce ciliostasis as well. Proteinase K treatment did not significantly reduce the viability of strain 110H.

Pili were abundant on strain 110H organisms and the pretreatment of these organisms with formalin or Chloramphenicol did not result in the loss of pili. Pili were not observed on Proteinase K treated organisms when maintained in the presence of Chloramphenicol. When the

Table 4. Effect of Formalin, Chloramphenicol, and Proteinase K, on piliation, attachment, and the ability of B. bronchiseptica strain 110H to induce ciliostasis.

Treatment[a]	Chloramphenicol[b]		Piliation[c] (%)	Attachment[d]	Ciliostasis[e]
	First medium change	Second medium change			
None (live)	-	-	90	+	+
None (live)	-	+	90	+	-
Formalin	-	-	80	+	-
Chloramphenicol	+	+	90	+	-
Chloramphenicol	+	-	85	+	+
Proteinase K	-	-	75	+	+
Proteinase K	+	-	0	-	-

[a]Treatments: Formalin, 0.5% in PBS at 37°C for $\geq$ 16 h; Chloramphenicol, 10 ug/ml at 37°C for 6 h; Proteinase K, 20 ug/ml at 37°C for 30 min. Each treatment was followed by two washes with Waymouth's medium.

[b]Bacteria were incubated in medium with or without Chloramphenicol (10 ug/ml) for 30 min at 37°C before evaluating piliation and attachment. Duplicate unstained cultures were replenished with medium (second change) with or without chloramphenicol and used to evaluate ciliostasis.

[c]Percentage of organisms with 10 pili or more, n = 20.

[d]Bacteria suspensions were incubated with canine tracheal outgrowth cultures for 15 min and washed. Bacteria were detected by a fluorescence assay: (+) bright fluorescence associated only with cilia, (-) no fluorescence observed.

[e](+) >90% or (-) <10% inhibition of ciliary activity.

Chloramphenicol was removed, and the organisms incubated in Waymouth's medium at 37°C for 30 minutes, pili were again observed.

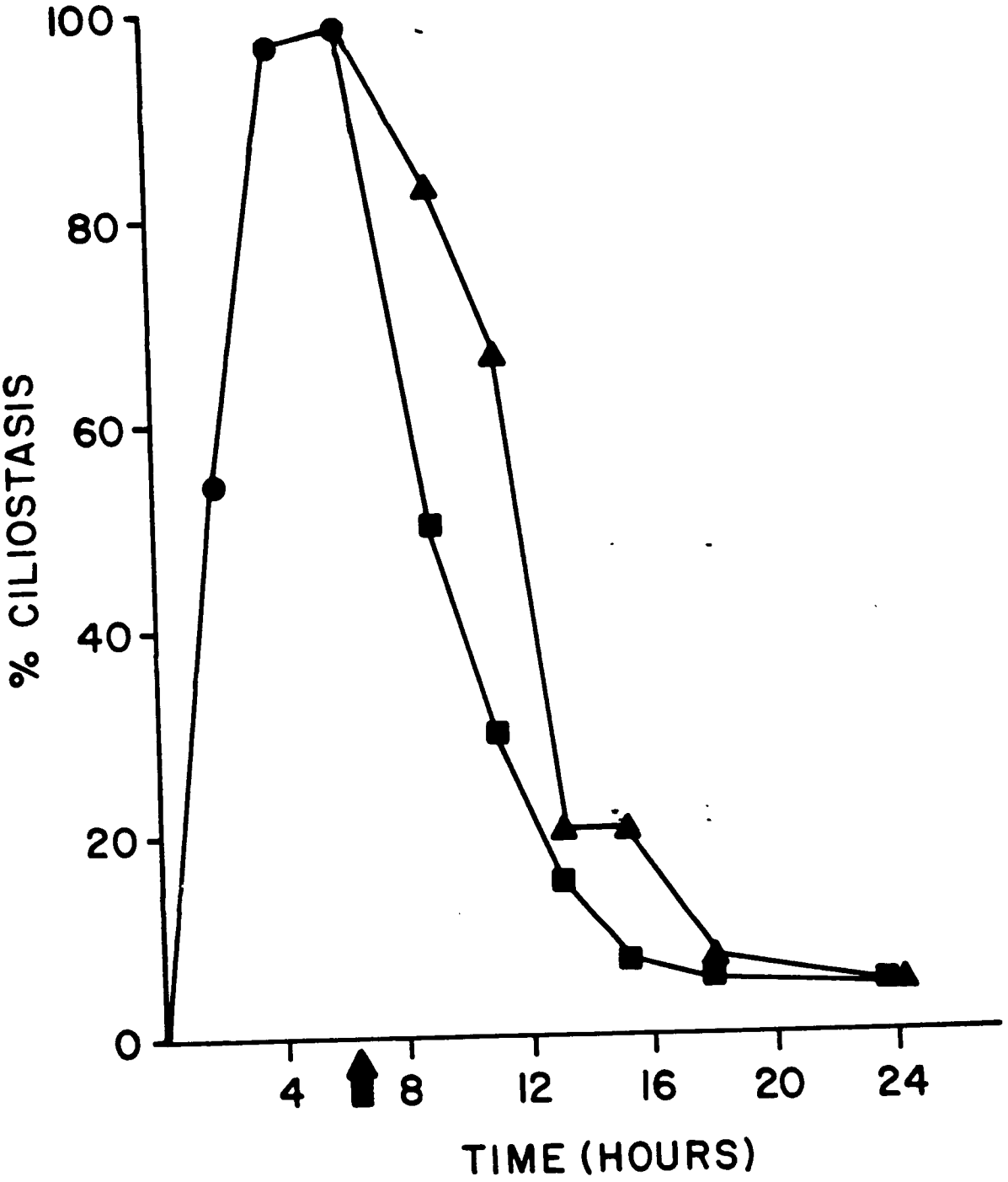
Reversal of B. bronchiseptica Induced Ciliostasis with Antibiotics

When 100% ciliostatic tracheal outgrowths (infected with B. bronchiseptica strain 110H) were washed and replenished with Waymouth's medium plus a bactericidal dose of Chloramphenicol (200 ug/ml) approximately 80% of the ciliostatic cells regained near normal activity after four hours incubation. By 18 hours post-addition of Chloramphenicol, 95% of the ciliated cells had regained ciliary activity (over a broad range of beat frequencies, but at least 75% were beating at +3 or +4). When the Chloramphenicol treated tracheal outgrowths were subsequently washed and replenished with plain Waymouth's medium, the ciliated cells did not become completely ciliostatic again, but remained at approximately 5% ciliostasis.

Similar results were obtained with a bacteriocidal dose of Polymyxin B (12.5 ug/ml) in the same experiment. When infected tracheal outgrowths were incubated for an additional two hours after becoming 100% ciliostatic, washed and replenished with antibiotics the same results were obtained (Figure 1). Neither Chloramphenicol nor Polymyxin B at the doses used had any toxic effects on tracheal outgrowth cultures, and they did not remove attached bacteria from cilia.

The reversal of ciliostasis by the addition of antibiotics indicates that metabolically active bacteria were required for the induction of ciliostasis and that ciliostasis (within the time frame

Figure 1. Effect of Chloramphenicol and Polymyxin B on B. bronchiseptica induced ciliostasis. Tracheal outgrowths were infected with 5×10^9 CFU/ml of B. bronchiseptica strain 110H, washed, and incubated until 100% ciliostasis was observed. The cultures were completely ciliostatic for two additional hours before the antibiotic compounds were applied (indicated by the arrow). The tracheal outgrowths were observed at hourly intervals post-addition of antibiotics. Chloramphenicol, 200 ug/ml (▲); Polymyxin B, 12.5 ug/ml (■).


100
80
60
40
20
0
% CILIOSTASIS
4
8
12
16
20
24
TIME (HOURS)

studied) was not the result of permanent structural damage to the cell.

Effect of Rabbit Anti-Bordetella Hyperimmune Serum on B. bronchiseptica Induced Ciliostasis

B. bronchiseptica infected outgrowths replenished with Waymouth's medium plus rabbit anti-Bordetella hyperimmune serum had less than 5% ciliostasis after four hours (Figure 2), whereas infected outgrowths replenished with Waymouth's medium alone had greater than 95% ciliostasis. Even after 12 hours the infected tracheal outgrowths with hyperimmune serum had only 15% ciliostasis. When the tracheal outgrowths plus hyperimmune serum were washed twice with Waymouth's medium at six hours post-addition of serum, greater than 98% ciliostasis occurred in four hours.

The anti-Bordetella serum inhibited the reduction of ciliostasis but not kill the bacteria.

Association of Ciliary Protective Activity of Animal Sera with Anti-Bordetella Agglutinating Antibodies

Several different animal sera which were tested for anti-Bordetella activity also exhibited the ability to protect cilia from B. bronchiseptica induced ciliostasis (Table 5). The highest levels of protective activity were associated with the ability of sera to agglutinate B. bronchiseptica (Figure 3). When two New Zealand white rabbits were vaccinated with live or formalin killed B. bronchiseptica, a rise in cilia protective activity as well as anti-Bordetella agglutinating activity was observed (Figure 4). The

Figure 2. Effect of serum on B. bronchiseptica induced ciliostasis. Canine tracheal outgrowth cultures were infected, washed, and replenished with either tissue culture medium (O) or tissue culture medium supplemented with anti-Bordetella hyperimmune rabbit serum (●) (final dilution 1:10). The cultures were examined by phase contrast microscopy. The arrow indicates the point at which serum treated tissue cultures were washed and replenished with tissue culture medium (non-supplemented). (Δ).

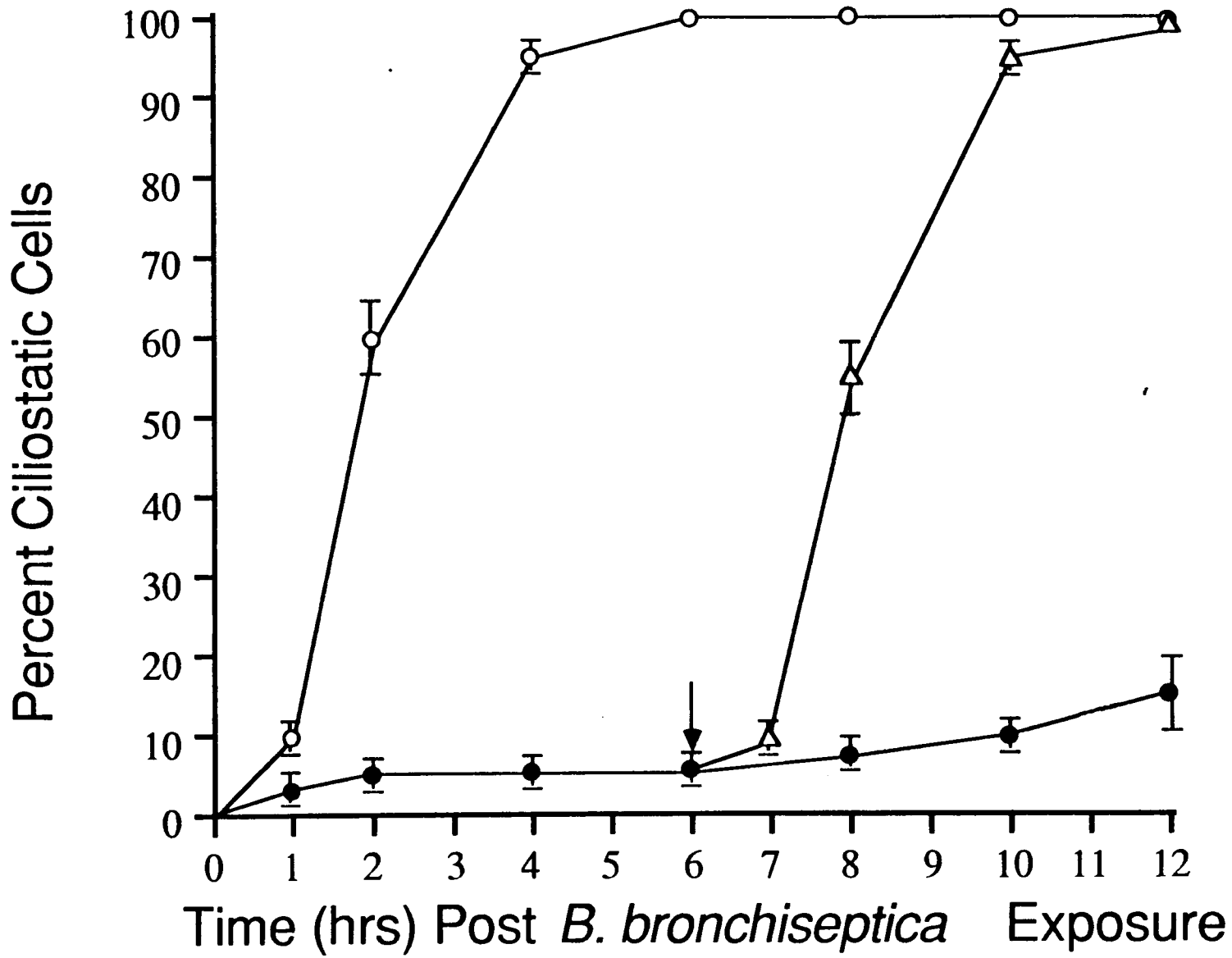

Percent Ciliostatic Cells
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hrs) Post B. bronchiseptica Exposure

Table 5. B. bronchiseptica agglutinating and cilia protective activity of various animal sera.

Animal Serum[a]	B. bronchiseptica Vaccination History	Percent Ciliostasis[b]	Agglutination[c]
SPF Canine (76-87)	vaccinated, strain 110H	0	+
SPF Canine Puppy (76-87)	unvaccinated	70	-
SPF Canine (J93)	vaccinated, strain 87	6	+
SPF Canine Puppy (#87)	unvaccinated	86	-
SPF Canine Adult (K-54)	unvaccinated	70	-
SPF Canine Puppy (C-53)	unvaccinated	90	-
Sterile Horse (Gibco)	unknown	71	-
SPF Swine	unknown	58	-
Normal Swine (Jenkins #2140)	unknown	63	-
Normal Mouse	vaccinated, strain 87	18	+
Fetal Bovine (Gibco)	unvaccinated	77	-
Normal Rabbit	unknown	55	-
Normal Rabbit	vaccinated, strain 110NH	9	+
Normal Rabbit (#2457)	unvaccinated	95	-
Normal Rabbit (#2457)	vaccinated, strain 110H	10	+

[a]Animal sera were diluted 1:10 in Waymouth's medium and applied to B. bronchiseptica strain 110H infected tracheal outgrowths in 1 ml volumes. Numerals in parentheses indicate animal identification number.

[b]Outgrowths were observed four hours after addition of serum. The number of ciliated cells with no cilia beating was compared to the number of ciliated cells with cilia beating.

[c]A 1:10 dilution of the animal serum was tested by the slide agglutination method. Reactions were considered positive if 75% (or greater) of the bacteria were agglutinated.

Figure 3. Association of ciliary protective effect of serum with B. bronchiseptica agglutination. All sera were tested at a 1:10 final dilution. Percent ciliostasis was determined four hours after addition of serum. Agglutination was considered positive if greater than 75% of the bacteria in suspension agglutinated. The sera tested were those presented in Table 5.

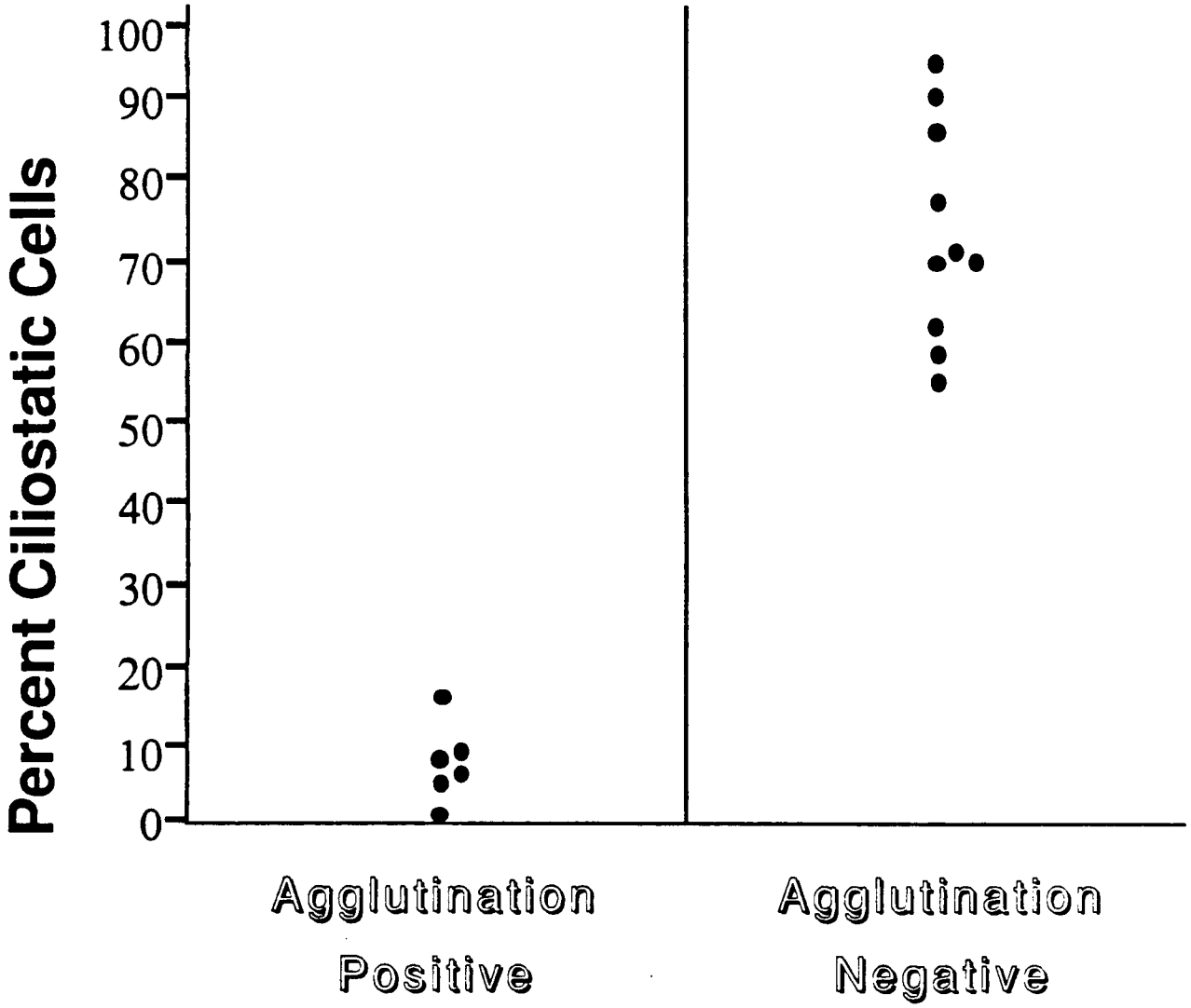
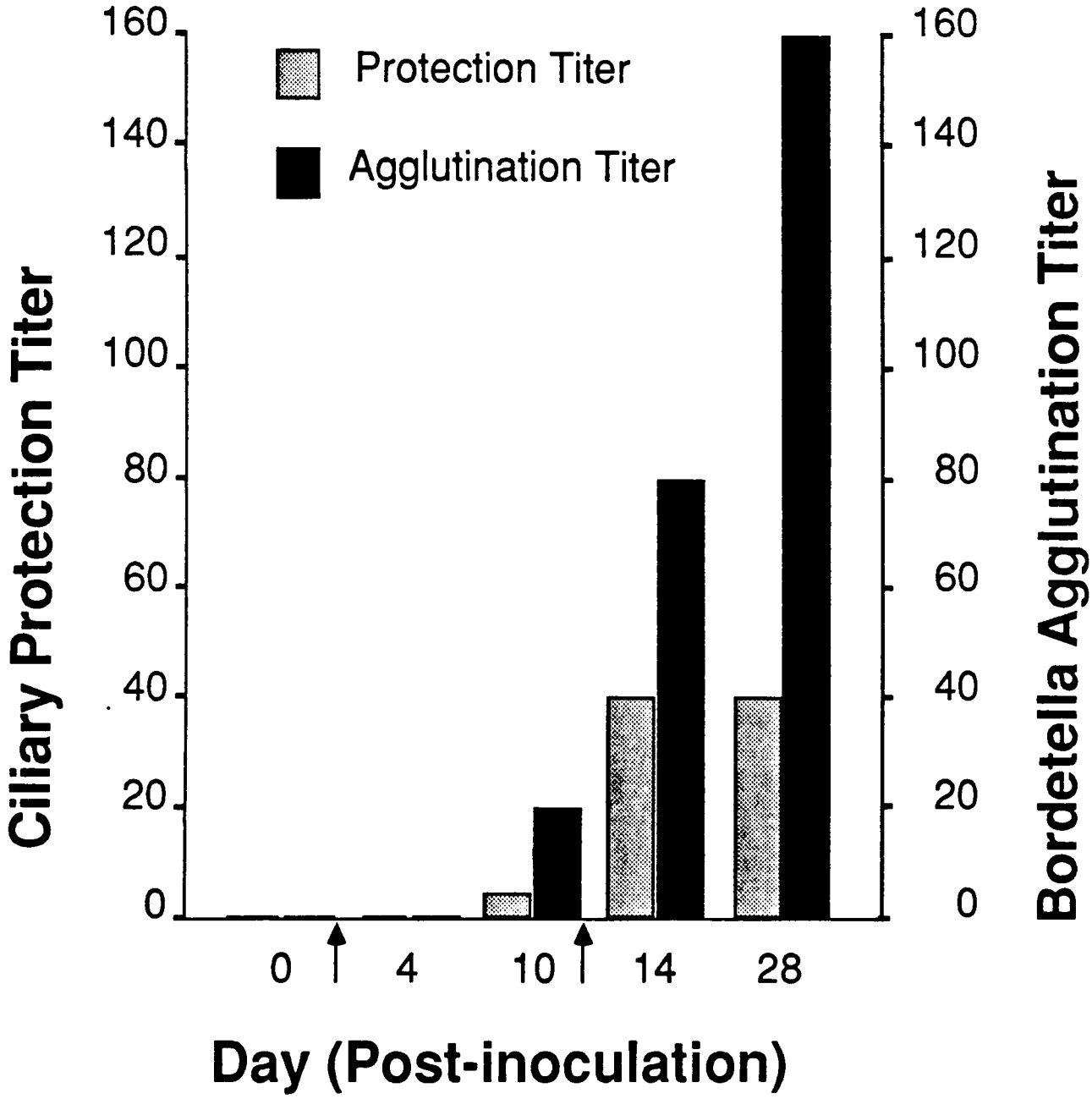

Percent Ciliostatic Cells
100
90
80
70
60
50
40
30
20
10
0
Agglutination Positive
Agglutination Negative

Figure 4. Rise in cilia protective activity and Bordetella agglutinating activity in rabbit serum following immunization with B. bronchiseptica. Arrows indicate inoculation time points. Vaccines consisted of live or formalin killed whole cells (1×10^{10} CFU) suspended in an aluminum hydroxide adjuvant and were administered by subcutaneous injection in 1 ml doses. The cilia protective titers are expressed as reciprocals of antiserum dilutions which protect greater than 75% of the ciliated cells from B. bronchiseptica induced ciliostasis. Agglutination titers are the reciprocals of antiserum dilutions which agglutinate greater than 75% of the bacteria in suspension.


Protection Titer
Agglutination Titer
Ciliary Protection Titer
Bordetella Agglutination Titer
160
140
120
100
80
60
40
20
0
0
4
10
14
28
Day (Post-inoculation)

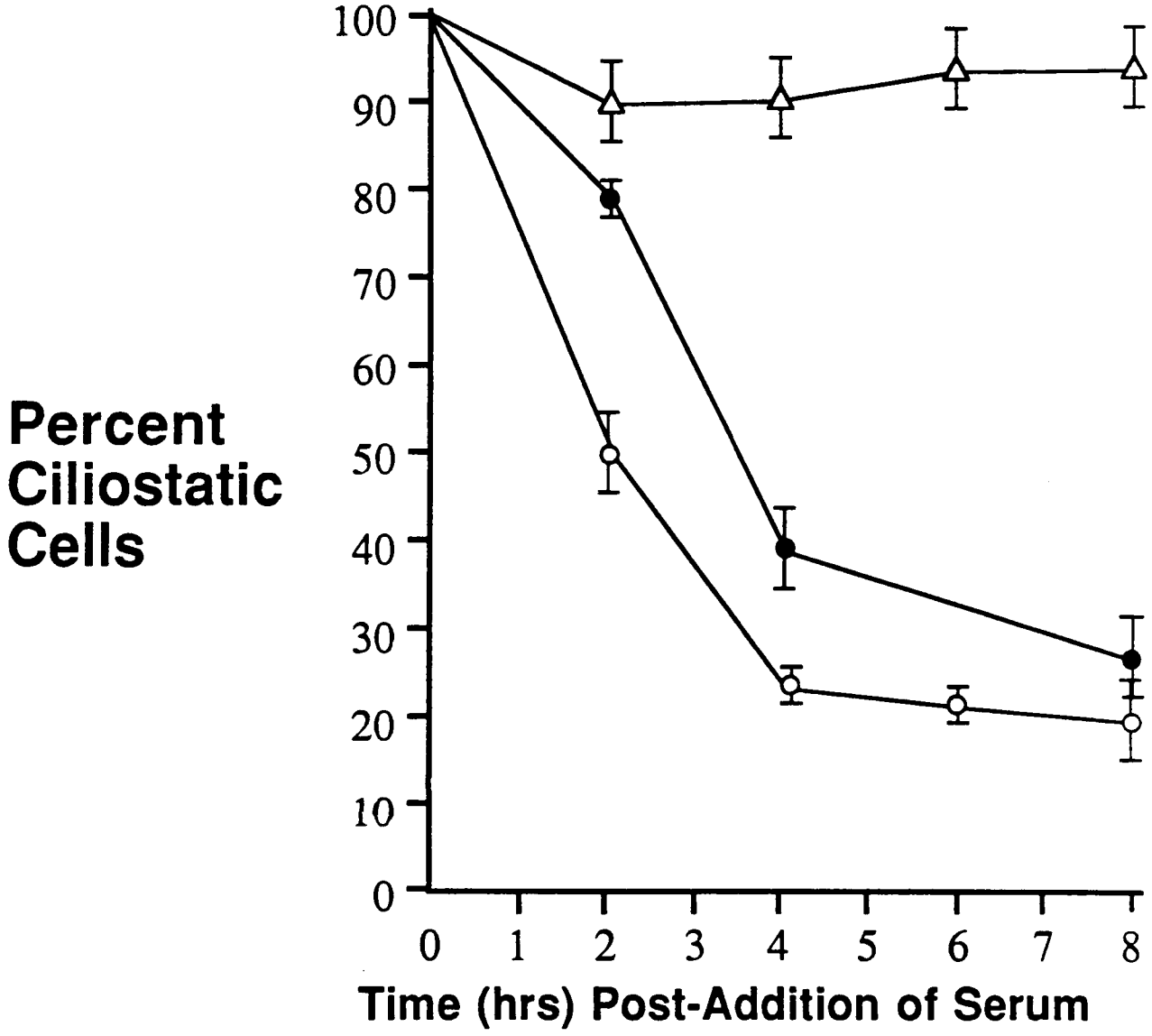
hyperimmune serum gathered on the 28th day after the initial vaccination had a cilia protective titer four-fold lower than its agglutinating titer (1:40 and 1:160, respectively). The maximum protective titer (1:40) was attained by day 14, whereas the agglutination titer did not reach its peak until day 28. Cilia protective activity was detectable by day 10 following a single inoculation with 1 x 10^{10} CFU of B. bronchiseptica strain 110H. Pre-immune sera from these rabbits had no detectable cilia protective or agglutinating activity.

The ability of animal serum to prevent the induction of ciliostasis by B. bronchiseptica was not a general property of the serum. There was a positive association between specific anti-Bordetella activity and the ability of serum to prevent the induction of ciliostasis.

Reversal of B. bronchiseptica Induced Ciliostasis with Anti-Bordetella Hyperimmune Serum

When 100% ciliostatic tracheal outgrowths (infected with B. bronchiseptica strain 110H) were washed and replenished with Waymouth's medium plus hyperimmune serum, the antiserum at a dilution of 1:20 reversed ciliostasis by approximately 60%, while the antisera at a dilution of 1:5 reversed ciliostasis by approximately 75% (Figure 5). Eight hours after the addition of antiserum, approximately 80% of the ciliated cells had active motility. The preimmune serum at a dilution of 1:5 did not significantly reverse ciliostasis at any time.

Figure 5. Reversal of ciliostasis with rabbit anti-Bordetella hyperimmune serum. Tracheal tissue cultures infected with B. bronchiseptica strain 110H which had become completely ciliostatic (approximately 6 hours post-infection) were washed and replenished with tissue culture medium supplemented with either pre-immune serum (Δ) or with hyperimmune serum at dilutions of 1:5 (○) or 1:20 (●). The cultures were re-incubated and examined at two hour intervals for ciliary activity.


Percent Ciliostatic Cells
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Time (hrs) Post-Addition of Serum

The reversal of ciliostasis by anti-Bordetella serum did not result in the removal of adherent bacteria from cilia.

Removal of Ciliary Protective Activity from Serum by Absorption with Bacterial Whole Cell Antigens

When rabbit anti-Bordetella hyperimmune serum was absorbed for 60 minutes at room temperature with B. bronchiseptica whole cell antigens, cilia protective activity was completely removed from the serum (Table 6). The avirulent, rough phase strain 110NH and boiled strain 110H also removed the protective activity. Furthermore, boiled whole cell E. coli and P. aeruginosa antigens also removed the protective activity. The protein A bearing strain of S. aureus (strain Cowan I) removed the protective activity but the non-protein A bearing strain (strain Wood 46) and S. epidermidis did not. When the unabsorbed serum was analyzed by immunofluorescence for activity against the bacterial absorbants, only B. bronchiseptica had positive surface fluorescence when tested with FITC-PA. But after testing with FC-GAR IgG, the S. aureus strain Cowan I was positive as were the B. bronchiseptica bacterins. The removal of protective activity by S. aureus strain Cowan I was due to absorption of antibodies from the serum.

The ability to remove ciliary protective activity from anti-Bordetella serum appeared to be a common property of gram negative bacteria.

Table 6. Removal of ciliary protective activity from serum by absorption with bacterial whole cell antigens.

Absorbant (Mode of Inactivation)	Anti-Absorbant Activity of Serum[a]	Protective Activity of Absorbed Serum[b]
None	NA	+
B. bronchiseptica strain 110H (0.05% Formalin)	+	-
B. bronchiseptica strain 110NH[c] (0.05% Formalin)	+	-
B. bronchiseptica strain 110H (100°C, 30 min)	+	-
Escherichia coli strain FDA Seattle 1946 (100°C, 30 min)	-	-
Pseudomonas aeruginosa strain PAO-1 (100°C, 30 min)	-	-
Staphylococcus aureus strain Cowan I (60°C, 1 hr)	- (FITC-PA) + (GAR-FC)	-
Staphylococcus aureus strain Wood 46 (60°C, 1 hr)	-	+
Staphylococcus epidermidis strain FDA PCI 1200 (60°C, 1 hr)	-	+

[a]Smears of the whole cell bacterins were reacted with unabsorbed, undiluted serum followed by either FITC-Protein A or fluorescein conjugated goat anti-rabbit IgG. Uniform bright fluorescence of the bacteria was considered positive.

[b]Absorbed serum was tested at a 1:10 dilution for its ability to prevent *B. bronchiseptica* induced ciliostasis. (+) <10% ciliostatic cells; (-) >90% ciliostatic cells.

[c]*B. bronchiseptica* strain 110NH does not induce ciliostasis.

Detection of Protective Activity in IgG Fractions from Rabbit Anti-Bordetella Hyperimmune Serum

A fraction obtained from hyperimmune serum by protein A-sepharose affinity chromatography protected tracheal outgrowths from B. bronchiseptica induced ciliostasis. This fraction had significant (>75%) protection to a dilution factor of 1:40 (Table 7). A similarly prepared fraction from pre-immune rabbit serum did not have a significant protective effect at any dilution, even though both fractions were tested at equivalent protein concentrations. Absorption of the protective fraction with boiled B. bronchiseptica or E. coli antigens resulted in loss of the protective activity.

Detection of IgG Fraction Binding to Boiled E. coli and B. bronchiseptica

When boiled B. bronchiseptica strain 110H was used as solid phase antigens in an ELISA, the hyperimmune serum IgG fraction (HI-IgG) had an ELISA titer of 10^8 and the preimmune serum IgG fraction (PI-IgG) had a titer of $10^{3.5}$. When the boiled E. coli was used as the solid phase antigen, the PI-IgG had a titer of 10^3, and the HI-IgG titer was $10^{5.5}$. Absorption of either the preimmune or hyperimmune fractions with boiled E. coli did not significantly reduce the HI-IgG or PI-IgG titers against boiled B. bronchiseptica strain 110H or boiled E. coli. Absorption of the hyperimmune fraction with boiled B. bronchiseptica strain 110H reduced reactivity of HI-IgG to boiled strain 110H from 10^8 to $10^{5.75}$. Reactivity of HI-IgG against boiled E. coli dropped from $10^{5.5}$ to $10^{4.5}$.

Table 7. Cilia protective activity of protein A-Sepharose derived serum fractions from rabbits before and after immunization with B. bronchiseptica.

	Dilutions				
Fractions from[a]	1/10	1/20	1/40	1/80	1/160
Pre-immune serum	90%[b]	95%	95%	100%	100%
Hyperimmune serum	10%	10%	25%	95%	100%

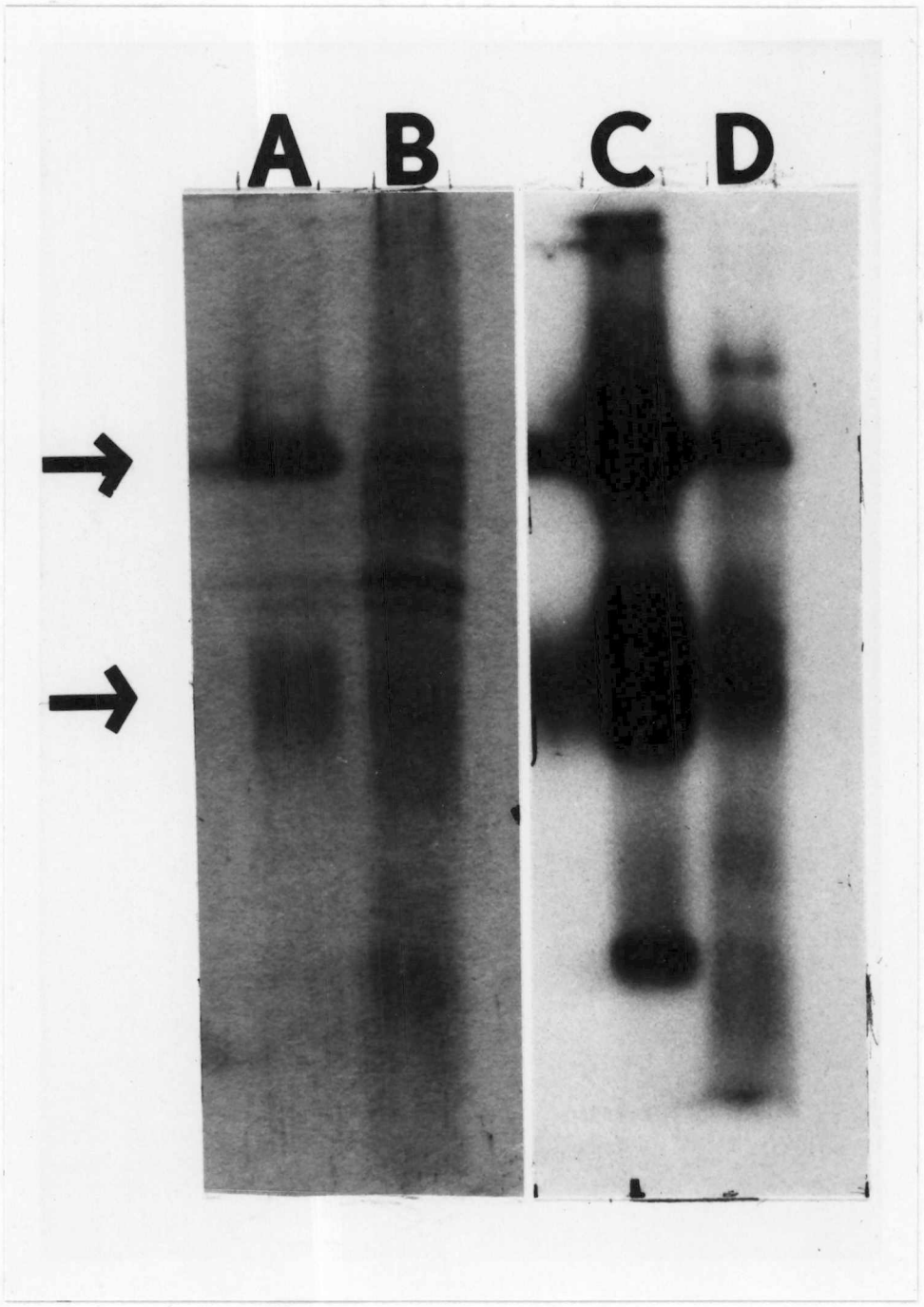
[a]The total protein concentrations (determined by Bio-Rad protein assays) were: pre-immune, 1.41 mg/ml; hyperimmune, 1.4 mg/ml.

[b]Percent ciliostatic cells four hours after addition of fraction to infected tracheal outgrowth culture.

Absorption of the preimmune fraction with boiled B. bronchiseptica strain 110H reduced the PI-IgG reactivity against boiled strain 110H from $10^{3.5}$ to $10^{2.5}$. The PI-IgG reactivity against boiled E. coli was not significantly reduced. When the PI-IgG and HI-IgG were used as antigens, each had dilution endpoints of $10^{6.9}$ which along with the total protein determinations confirmed that these fractions were being tested at equal immunoglobulin concentrations.

Analysis of boiled E. coli after absorption of radiolabeled IgG fraction from hyperimmune serum by PAGE and autoradiography indicated that labeled protein bands which corresponded to the approximate band migration of the radiolabeled IgG fraction were present on the boiled E. coli antigen (Figure 6). The coomassie blue stained gel of the IgG fraction had a dark band where one would expect the heavy chain of IgG to be (appropriately 50,000 daltons) and a more diffuse light chain band below it (appropriately 25,000 daltons). The boiled E. coli had a multitude of bands in all size ranges and IgG was not readily distinguishable in the coomassie blue stained gel. The ELISA and autoradiography confirmed that the hyperimmune sera and IgG fractions from this serum contained antibodies of the IgG class which reacted with boiled E. coli. Furthermore these antibodies were present in greater quantities in the anti-Bordetella hyperimmune serum and serum fractions than in the preimmune serum and serum fractions.

Figure 6. Analysis of boiled E. coli after absorption of radiolabeled IgG fraction from rabbit anti-Bordetella hyperimmune serum. The left photograph is an SDS-PAGE protein profile (coomassie blue stained). Lane A contains 100 ug of I^{125} labeled IgG fraction from rabbit anti-Bordetella hyperimmune serum; lane B contains 50 ug of boiled E. coli which had been used to absorb cilia protective activity from the radiolabeled IgG fraction. The right photograph is an autoradiograph of the SDS-polyacrylamide gel in the left photograph. Lanes C and D correspond to lanes A and B, respectively. Arrows indicate the locations of the IgG heavy and light chains.


A
B
C
D

CHAPTER IV

DISCUSSION

The diseases with which B. bronchiseptica have been associated are widespread and varied. They are often mild, self-limiting, chronic, and complicated with other bacterial or viral factors; thus, experimental comparison of B. bronchiseptica strains for virulence have been difficult. Specific virulence determinants of B. bronchiseptica have not been very well defined. Canine tracheal outgrowth cultures provided a convenient system for the study of B. bronchiseptica interaction with cilia. The primary emphasis of this study was the induction of ciliostasis by B. bronchiseptica.

Bemis and Kennedy (1981) observed that a dose of approximately 1 x 10^9 CFU/ml of B. bronchiseptica induced 80-90% ciliostasis in three hours. The present study indicated that the induction of ciliostasis by B. bronchiseptica in canine tracheal outgrowths was time and dose dependent. Significant levels (90% or greater) of ciliostasis did not occur in less than three hours even when high doses of B. bronchiseptica were used. Although the minimal infective dose was not established for this assay, the lowest dose tested in this study was lower than the minimal infective dose required to produce atrophic rhinitis in gnotobiotic piglets (Martineau et al., 1982). The time required for maxmimum binding of organisms to cilia was not addressed in this study; however, greater than 90% of the ciliated cells had numerous bacteria attached within 20 minutes for all but the lowest doses tested. The physical act of adherence does not appear to be

responsible for ciliostasis since high doses (10^{10} - 10^{12} CFU/ml) of formalin killed B. bronchiseptica adhered to cilia but did not induce ciliostasis. When the outgrowth cultures were infected at 4°C, the organisms appeared to attach to cilia as readily as at room temperature. After prolonged incubation (3 hours) at 4°C and return to 37°C, the cells resumed normal ciliary activity (with organisms adhered) within 60 minutes. With continued incubation at 37°C, ciliostasis was induced. This suggests that ciliostasis (but not attachment) is a temperature dependent process. The physical act of attachment, although not in itself causing ciliostasis may still be a required event in the induction of ciliostasis; physical damage or torsional strain of the ciliary membrane might allow bacterial products to pass through and interfere with movement.

The ability to induce ciliostasis appears to be common among strains of B. bronchiseptica. Strains that lacked the ability to induce ciliostasis produced colonies on BA that were identical in morphology to rough-phase variants of phase I organisms (Bemis et al., 1977c). ECAC, a suspected virulence factor present in phase I but not rough-phase variants (Ezzell et al., 1981; Wolff et al., 1984; Endoh et al., 1980) does not appear to be involved in the induction of ciliostasis since two strains of B. bronchiseptica (Columbus and 17640) did not generate cAMP in the presence of exogenous ATP but were able to induce ciliostasis. This does not necessarily mean, however, that no ECAC was produced. An enzymatically inactive but biologically active form of ECAC may have been produced, with the biological activity present in the binding component of the ECAC molecule. It has been

reported, for example, that some of the biological activities of pertussis toxin may be due to the binding portion of the molecule and not to the ADP-ribosylating enzymatic portion (Nogimori et al., 1984).

The presence of hemolysin was hypothesized to be another possible virulence factor because of observations with strain 110H (hemolytic, ciliostasis inducing), and strain 110NH (non-hemolytic, non-ciliostasis inducing). A hemolysin has also been isolated from Pseudomonas aeruginosa which induced ciliostasis in rabbit trachea (Reimer et al., 1980). Hemolytic activity of B. bronchiseptica was not positively associated with induction of ciliostasis since two ciliostasis inducing strains, 17640 and Columbus, were non-hemolytic. Bordetella hemolysin may still be a virulence factor in those strains which produce it, although not as an independent or primary ciliostasis inducer.

Plasmids do not appear to be involved in either colony variation or induction of ciliostasis by B. bronchiseptica. Even though all of the plasmid bearing strains induced ciliostasis, there were plasmid negative but strains which induced ciliostasis as well. No relationship appears to exist between possession of plasmids and production of ECAC, presence or absence of pili or flagella, or production of hemolysin.

The presence of pili was the only phenotypic trait that was positively associated with bacterial ability to induce ciliostasis. Pili have been associated with attachment by B. bronchiseptica (Yokomizu and Shimizu, 1979; Bemis et al., 1977c). Treatment of organisms with proteinase K removed pili. Organisms so treated were not able to attach to cilia, and no ciliostasis occured.

Chloramphenicol treated organisms were able to adhere to cilia, but were not able to induce ciliostasis. This suggests that metabolically active B. bronchiseptica must attach to induce ciliostasis. Ciliostasis has not yet been induced by concentrated culture supernatants or cell free extracts from metabolically active B. bronchiseptica.

There is evidence, however, that an extracellular toxin may be involved in B. bronchiseptica induced ciliostasis. The addition of Chloramphenicol or Polymyxin B to infected, ciliostatic tracheal outgrowths resulted in the reversal of ciliostasis. The doses of antibiotics used were killing doses, so in each case bacterial metabolism was completely shut down. In both cases the bacteria remained attached to the cilia. The antibiotics are killing the bacteria over time such that the slope of the curve of reversal suggests that a toxin or toxins must be continually synthesized and accumulated to a certain concentration before ciliostasis is induced. A small murein dipeptide-like fragment which causes ciliostasis in hamster tracheal ring cultures has been isolated from B. pertussis (Goldman et al., 1982). Although this tracheal cytotoxin could be isolated from both virulent and avirulent strains, only virulent strains induced ciliostasis, perhaps because of the need for attachment to target cells (W. E. Goldman, Abstr. Ann. Meet. Am. Soc. Microbiol. 1985, Abstr. No. S152). Variations in the abilities of different 1,000 molecular weight cut-off filters to retain pertussis tracheal cytotoxin (molecular weight 1,254) have been observed (W. E. Goldman, personal communication). This may explain previous failures to isolate

a similar toxin from B. bronchiseptica. Since ciliostasis was reversed after the cilia were static for a full two hours, the bacterial induction of ciliostasis does not irreversibly damage the cells within that time. If the infected outgrowths were incubated at 100% ciliostasis beyond two hours, considerable sloughing of cells from the outgrowths is observed. Destruction of ciliated cells may thus involve multiple virulence determinants.

Since antibiotic treatments were able to inhibit induction of ciliostasis, it was hypothesized that antisera against B. bronchiseptica would have the same effect. Sera from rabbits which had been vaccinated with B. bronchiseptica strain 110H were able to prevent induction of ciliostasis. When the antisera were removed, however, the tracheal outgrowths quickly became ciliostatic, thus demonstrating that the antisera were not killing the organisms. When strain 110H organisms were pre-incubated with anti-strain 110H sera, the bacteria were agglutinated and unable to induce ciliostasis, although clusters of agglutinated bacteria adhered to some (but not all) ciliated cells. Pretreatment of cilia with anti-strain 110H sera had no effect on attachment or the subsequent induction of ciliostasis. Further testing with other animal sera confirmed the association between the ability to agglutinate B. bronchiseptica and prevent induction of ciliostasis. A similar association of agglutinin titers and protection against B. bronchiseptica infection in animals has been demonstrated (Sakano et al., 1984; Bey et al., 1981; McCandlish et al., 1978).

Since antisera protected against induction of ciliostasis, much as the antibiotics had, it was hypothesized that the antisera may also

reverse ciliostasis. When the antiserum was added to ciliostatic, infected tracheal outgrowths, the ciliostasis was significantly reversed (80% reversal) within eight hours post addition of antiserum. Neither pre-immune rabbit sera nor fetal bovine serum was able to reverse ciliostasis at any time or diliution. The effects of antibiotics on B. bronchiseptica induced ciliostasis were no doubt due to inhibiton of protein synthesis or other alterations in bacterial metabolism. Since antisera did not inhibit viability of the bacteria, one explanation for the antisera inhibition and reversal of ciliostasis could be that the sera contained antibodies which were specific for the putative B. bronchiseptica ciliotoxin. Another explanation of this effect is that the binding of antisera to other surface antigens of B. bronchiseptica (perhaps outer membrane antigens shared with other gram-negative bacteria) may result in altered metabolism. The effects of hyperimmune antiserum on the growth of B. bronchiseptica were not determined in this study. Any alterations in bacterial metabolism could indirectly block the production of a specific ciliotoxin or inhibit its release from the bacterium. Additionally the ciliostasis induced by B. bronchiseptica could simply be due to non-specific effects of bacterial metabolism such as localized changes in pH or osmotic concentrations. Because serum agglutination of B. bronchiseptica was demonstrated to be associated with protective ability, a concomitant rise in ciliary protective activity and agglutination titer after B. bronchiseptica vaccination seemed to indicate that the protective effect of the antisera was due to antibody.

If antibody was the protective component then it was hypothesized that absorption with whole cell antigens should remove those protective antibodies. The whole cell B. bronchiseptica bacterins of both strains 110H and 110NH were able to remove the protective effect from the hyperimmune serum. When the bacterial cell surfaces were examined by FITC-PA and FC-GAR for adhered antibodies, both tests were positive. S. aureus strain Cowan I was used to absorb the hyperimmune serum because it has Protein A on its surface and Protein A has been demonstrated to be reactive with IgG antibodies (Sjoquist et al., 1967; Forsgren and Sjoquist, 1966). The S. aureus strain Cowan I did remove the protective activity and was positive with the FC-GAR but not FITC-PA. Neither S. aureus strain Wood 46 (non-Protein A bearing strain) nor S. epidermidis were able to remove the protective activity and neither was positive with either FITC-PA or FC-GAR. This gave a strong indication that anti-B. bronchiseptica IgG could be the protective component. This hypothesis was placed in doubt when boiled E. coli and P. aeruginosa bacterins were able to absorb out the protective activity of the hyperimmune sera but were both negative when tested with FITC-PA or FC-GAR. In order to clarify the issue, hyperimmune sera were fractionated by affinity chromatography with a Protein A-Sepharose column. The bound fraction from the hyperimmune sera was protective against B. bronchiseptica induced ciliostasis, but the preimmune fraction was not. Both fractions had bands consistent with IgG when analyzed by PAGE. Absorption of the hyperimmune IgG fraction by the same antigens as those used with the whole sera followed similar results, with boiled E. coli still negative when

tested by FITC-PA or FC-GAR. Boiled E. coli and B. bronchiseptica reacted with hyperimmune IgG by ELISA. When radiolabeled IgG from the hyperimmune fractions was reacted with boiled E. coli, the labeled bands on the autoradiograph also corresponded with those of the labeled IgG. It therefore seems most likely that the protective ability in serum was indeed attributable to IgG.

The question of anti-Bordetella specificity of such antibodies, however, is not so clear. As mentioned previously, the rise in agglutinating and cilia protective activity of serum suggests that B. bronchiseptica vaccination induces the production of protective antibody. Non-ciliotoxic rough phase B. bronchiseptica and two other gram negative organisms, however, removed the protective antibodies by absorption. None of the boiled bacteria nor endotoxin extracted from B. bronchiseptica induced cilostasis. The endotoxin also did not inhibit or neutralize the protective activity when mixed with serum. An interesting observation with boiled E. coli is that the ELISA data indicates an increase in antibody activity to boiled E. coli after rabbits had been vaccinated with B. bronchiseptica. This increase of ELISA titers to both E. coli and B. bronchiseptica is also associated with an increase in protective activity. The amount of reactivity of either the hyperimmune sera or HI-IgG to boiled E. coli was low, however, and the inability of immunofluorescence to detect antibodies against E. coli may indicate that either the reactive epitopes on boiled E. coli are very few in number or that the quantity of reactive antibodies in the sera tested was low or possibly both. The ELISA may be more sensitive than immunofluorescence due to the catalytic (and

amplifying) effect of the enzyme. The boiled gram negative bacteria must all have similar heat stable epitopes on their surfaces which are related to the actual ciliotoxin structure because although they do not induce ciliostasis by themselves, they do remove the protective antibodies. Questions which remain to be answered are: can an increase in protective activity be demonstrated after vaccination with boiled E. coli or vaccination with boiled B. bronchiseptica strains 110H or 110NH, and what is the relationship between heat stabile antigens (which are capable of removing cilia protective antibodies) and the active ciliotoxin?

The results of this investigation suggest that attachment may be a required event in the induction of ciliostasis by metabolically active B. bronchiseptica. Involvement of a bacterial surface associated or soluble protein toxin in ciliostasis is also suggested. Heat stabile antigens of B. bronchiseptica and other gram negative bacteria may either be part of the primary ciliostasis inducing mechanism or they may resemble the active portion(s) of the ciliotoxin(s) enough to remove protective antibodies from serum. The actual identities of the bacterial ciliotoxic component and antigens to which protective antibodies react are as yet not known.

LIST OF REFERENCES

LIST OF REFERENCES

Ankerst, J. 1974. A routine diagnostic test for IgA and IgM antibodies to rubella virus: absorption of IgG with Staphylococcus aureus. J. Inf. Dis. 130:268-273.

Appel, M. J. G., P. H. Pickerill, M. Menegus, D. H. Percy, I. M. Parsonson, and B. E. Sheffey. 1970. Current status of canine respiratory disease. In Gaines Veterinary Symposium, Kansas, pgs. 15-23.

Ashworth, L. A. E., L. I. Irons, and A. B. Dowsett. 1982. Antigenic relationship between serotype-specific agglutinogen and fimbrae of Bordetella pertussis. Infect. Immun. 37:178-1281.

Barry, A. L. and L. D. Sabath. "Special tests: Bactericidal activity and activity of antimicrobics in combination" in Manual of Clinical Microbiology, 2nd ed. E. H. Tennette, E. H. Spaulding, J. P. Truant, eds. 1974. Am. Soc. Microbiol., Washington, DC.

Batey, R. G. and A. F. Smits. 1976. The isolation of Bordetella bronchiseptica from an outbreak of canine pneumonia. Aust. Vet. J. 52:184-186.

Baum, S. G., M. S. Horwitz, and J. V. Maizel. 1972. Studies of the mechanism of enhancement of human Adenovirus infection in monkey cells by Simian Virus 40. J. Virol. 10(2):211-219.

Beachey, E. H. 1981. Bacterial adherence: adhesion-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Immun. 143:325-345.

Bemis, D. A., L. E. Carmichael, and M. J. G. Appel. 1977a. Naturally occurring respiratory disease in a kennel caused by Bordetella bronchiseptica. Cornell Vet. 67(2):282-293.

Bemis, D. A., H. A. Greisen, and M. J. G. Appel. 1977b. Pathogenesis of canine bordetellosis. J. Infect. Dis. 135:753-762.

Bemis, D. A., H. A. Greisen, and M. J. G. Appel. 1977c. Bacteriological variation among Bordetella bronchiseptica isolates from dogs and other species. J. Clin. Microbiol. 5:471-480.

Bemis, D. A. and J. R. Kennedy. 1981. An improved system for studying the effect of Bordetella bronchiseptica on the ciliary activity of canine tracheal epithelial cells. J. Infect. Dis. 144(4):349-357.

Blom, J., G. A. Hansen, and F. M. Poulsen. 1983. Morphology of cells and hemagglutinogens of Bordetella species: resolution of substructural units in fimbrae of Bordetella pertussis. Infect. Immun. 42:308-317.

Burek, J. D., G. C. Jersey, C. K. Whitehair, and G. R. Carter. 1972. The pathology and pathogenesis of Bordetella bronchiseptica and Pasteurella pneumotropica infection in conventional and germfree rats. Lab. An. Sci. 22(6):844-849.

Bjork, I., B.-A. Petersson, and J. Sjoquist. 1972. Some physiochemical properties of protein A from Staphylococcus aureus. Eur. J. Biochem. 29:579-584.

Collier, A. M., L. P. Peterson, and J. B. Baseman. 1977. Pathogenesis of infection with Bordetella pertussis in hamster tracheal organ culture. J. Infect. Dis. 136(supp):S196-S203.

Costerton, J. W., R. I. Irvin, and K.-J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 35:299-324.

Cowell, J. L., M. Oda, and D. G. Burstyn. 1984. Prospective protective antigens and animal models for pertussis. In Microbiology 1984. D. Schlesinger, ed. Am. Soc. Microbiol., Washington, D.C., pgs. 172-175.

Denny, F. W. 1974. Effect of a toxin produced by Hemophilus influenzae on ciliated respiatory epithelium. J. Infect. Dis. 129:2.

Endoh, M., T. Takezawa, and Y. Nakase. 1980. Adenylate cyclase activity of Bordetella organisms. I. Its production in liquid medium. Microbiol. Immunol. 24:95-104.

Ezzell, J. W., W. J. Dobrogosz, W. E. Koos, and C. R. Manclark. 1981. Phase shift markers in Bordetella: alterations in envelope proteins. J. Infect. Dis. 143:562-569.

Ferry, N. S. 1910. A preliminary report of the bacterial findings in canine distemper. Am. Vet. Rev. 37:499-504.

Ferry, N. S. 1913. Bacteriology and control of acute infections in laboratory animals. J. Pathol. 18:445-455.

Fetter, A. W., W. P. Switzer, and C. C. Capen. 1975. Electron microscopic evaluation of bone cells in pigs with experimentally induced Bordetella rhenitis (turbinate osteoporosis). Am. J. Vet. Res. 36:15-22.

Forsgren, A. and J. Sjoquist. 1966. Protein A from Staphylococcus aureus. 1. Pseudo-immune reaction with human gamma globulin. J. Immunol. 97:822-827.

Frank, D. W. and C. D. Parker. 1984. Interaction of monoclonal antibodies wth pertussis toxin and its subunits. Infect. Immun. 46(1):195-201.

Gallagher, G. L. 1965. Isolation of Bordetella bronchiseptica from horses. Vet. Rec. 77:632-633.

Ganaway, J. R., A. M. Allen, and C. W. McPherson. 1965. Prevention of acute Bordetella bronchiseptica pneumonia in a guinea pig colony. Lab. An. Care 15:156-162.

Ghetie, V., G. Mota, and J. Sjoquist. 1978. Separation of cells by affinity chromatography on SpA-Sepharose 6MB. J. Immunol. Meth. 21:133-141.

Goldman, W. E., D. G. Klapper, and J. B. Baseman. 1982. Detection, isolation, and analysis of a released Bordetella pertussis product toxic to cultured tracheal cells. Infect. Immun. 36(2):782-794.

Goodnow, R. A. 1980. Biology of Bordetella bronchiseptica. Microbiol. Rev. 44:722-738.

Goodnow, R. A., S. C. Causey, S. J. Geary, and W. S. Wren. 1983. Comparison of an infective avirulent and canine virulent Bordetella bronchiseptica. Am. J. Vet. Res. 44(2):207-211.

Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff. 1976. Extracytoplasmic adenylate cyclase of Bordetella pertussis. Proc. Natl. Acad. Sci. USA 73:1926-1930.

Hjelm, H., J. Sjodahl, and J. Sjoquist. 1975. Immunologically active and structurally similar fragments of protein A from Staphylococcus aureus. Eur. J. Biochem. 57:395-403.

Hu, P. C., A. M. Collier, and J. B. Baseman. 1976. Interaction of virulent Mycoplasma pneumoniae with hamster tracheal organ cultures. Infect. Immun. 14:217-224.

Hubbard, A. L. and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. J. Cell Biol. 55:390-405.

Jenkins, E. M., V. Anthony, R. T. Vance, J. Cleveland, and S. G. Gbadamosi. 1977. Prevalence of Bordetella bronchiseptica infections in swine in southeast Alabama. Am. J. Vet. Res. 38(12):2071.

Kato, C. I. and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Martineau, G. P., A. Broes, and B. Martineau-Doize. 1982. Atrophic rhinitis: appraisal of infection pressure on gnotobiotic piglets infected with Bordetella bronchiseptica. Can. J. Comp. Med. 46:376-381.

Morrison, M., G. S. Bayse, and R. G. Webster. 1971. Use of lactoperoxidase catalyzed iodination in immunochemical studies. Immunochem. 8:289-297.

Nakaoka, Y., H. Tanaka, and F. Oosawa. 1984. Ca^{2+}-dependent regulation of beat frequency of cilia in Paramecium. J. Cell. Sci. 65:223-231.

Nogimori, K., M. Tamura, M. Yajima, K. Ito, T. Nakamura, N. Kajikawa, Y. Maruyama, and M. Ui. 1984. Dual mechanisms involved in development of diverse biological activities of islet-activiating protein, pertussis toxin as revealed by chemical modification of lysine residues in the toxin molecule. Biochem. Biophy. Acta 801:232-243.

Pearce, H. G. and C. K. Roe. 1966. Infectious porcine atrophic rhenitis: a review. Can. Vet. J. 7:243-251.

Pennock, P. W. and J. Archibald. 1968. Disease of the lower respiratory tract. In Canine Medicine. E. J. Catcott, ed. Am. Vet. Pub., Inc., Wheaton, IL, pgs. 572-581.

Peppler, M. 1982. Isolation and characterization of isogenic pairs of domed hemolytic and flat nonhemolytic colony types of Bordetella pertussis. Infect. Immun. 35:840-851.

Pestka, S. 1971. Inhibitors of ribosome function. Ann. Rev. Microbiol. 25:487-562.

Pittman, M. 1984. The concept of pertussis as a toxin-mediated disease. Ped. Infect. Dis. 3(5):467-486.

Plotkin, B. J. and D. A. Bemis. 1984. Adherence of Bordetella bronchiseptica to hamster lung fibroblasts. Infect. Immun. 46(3):697-702.

Reimer, A., K. Klementsson, J. Ursing, and B. Wretlind. 1980. The mucociliary activity of the respiratory tract. 1. Inhibitory effects of products of Pseudomonas aeruginosa on rabbit trachea in vitro. Acta Otolaryngol. 90:462-469.

Rutter, J. M. 1983. Virulence of Pasteurella multocida in atrophic rhinitis of gnotobiotic pigs infected with Bordetella bronchisetpica. Res. Vet. Sci. 34(3):287-295.

Rutter, J. M. and X. Rojas. 1982. Atrophic rhinitis in gnotobiotic piglets: differences in the pathogenicity of Pasteurella multocida in combined infections with B. bronchiseptica. Vet. Res. 110:531-534.

Sato, Y., K. Izumiya, M.-A. Oda, and H. Sato. "Biological significance of Bordetella pertussis fimbrae or hemagglutinin: A possible role of the fimbrae or hemagglutinin for pathogenesis and antibacterial immunity" in International Symposium on Pertussis. C. R. Manclark and J. C. Hill, eds. 1978. U.S. Department of Health, Education, and Welfare, Washington, D.C.

Sekiya, K., M. Kawashira, and Y. Nakase. 1983. Protection against experimental Bordetella bronchiseptica infection in mice by active immunization with killed vaccine. Infect. Immun. 41(2):598-603.

Shade, F. J. and R. A. Goodnow. 1979. Intranasal immunization of dogs against Bordetella bronchiseptica induced tracheobronchitis (kennel cough) with modified live Bordetella bronchiseptica vaccine. Am. J. Vet. Res. 40:1241-1243.

Sjoquist, J., A. Forsgren, and G. T. Gustafson. 1967. Interaction between gamma globulins and protein A from Staphylococcus aureus. Nobel Symp. 3:341-348.

Strewler, B. J. and J. Orloff. 1977. Role of cyclic nucleotides in the transport of water and electrolytes. Adv. Cyc. Nuc. Res. 8:311-361.

Switzer, W. P. 1956. Studies on infectious rhinitis. V. Concept that several agents may cause turbinate atrophy. Am. J. Vet. Res. 17:478-484.

Switzer, W. P. and D. O. Farrington. 1975. Infectious atrophic rhinits. In Diseases of Swine. H. W. Dunne and A. D. Leman, eds. Iowa State Univ. Press. 4th Edition, pgs. 687-711.

Thompson, H., I. A. P. McCandlish, and N. G. Wright. 1976. Experimental respiratory disease in dogs due to Bordetella bronchiseptica. Res. Vet. Sci. 20:16-23.

Tuomanen, E. I. and J. O. Mendley. 1983a. Adherence of Bordetella pertussis to human respiratory cells. J. Infect. Dis. 148(1):125-130.

Tuomanen, E. I., J. Nedelman, J. O. Hendley, and E. L. Hewlett. 1983b. Species specificity of Bordetella adherence to human and animal ciliated respiratory epithelial cells. Infect. Immun. 42(2):692-695.

Tuomanen, E. I., L. A. Zapain, P. Galvan, and E. L. Hewlett. 1984. Characterization of antibody inhibitng adherence of Botdetella pertussis to human respiratory epithelial cells. J. Clin. Microbiol. 20(2):167-170.

Wagener, J. S., R. Sobonya, L. Minnich, and L. M. Taussig. 1984. Role of canine parainfluenza virus and Bordetella bronchiseptica in kennel cough. Am. J. Vet. Res. 45(9):1862-1866.

Wolff, J., G. H. Cook, A. R. Goldhammer, C. Londos, and E. L. Hewlett. 1984. Bordetella pertussis: Multiple attacks on host cell cyclic AMP regulation. Adv. Cyc. Nucl. Prot. Phosph. Res. 17:161-172.

Wright N. G., H. Thompson, D. Taylow, and H. J. C. Cornwell. 1973. Bordetella bronchiseptica: a reassessment of its role in canine respiratory disease. Vet. Rec. 93:486-487.

Yokomizu, Y. and T. Shimizu. 1979. Adherence of Bordetella bronchiseptica to swine nasal epithelial cells and its possible role in virulence. Res. Vet. Sci. 27:15-21.

Zhang, J. M., J. L. Cowell, A. C. Steven, P. H. Carter, P. P. McGrath, and C. R. Manclark. 1985. Purification and characterization of fimbrae isolated from Bordetella pertussis. Infect. Immun. 48:422-427.

VITA

Scott Allen Wilson was born on November 6, 1958 in Minneapolis, Minnesota. He was raised in the lakes and forests of northern Minnesota, but followed his family south to Jacksonville, Florida, where he received his high school diploma, with honors, from Trinity Christian Academy in June, 1977. He proceeded from there to Florida Junior College and received his Associate of Arts degree in December, 1979. He then enrolled at the University of North Florida as a Biology major and graduated in May, 1982, with a Bachelor of Arts in Biology. In the fall of 1982 he enrolled at the University of Tennessee and accepted a teaching assistantship. He was awarded the Master of Science degree in August, 1986.

He is an active member of the American Society for Microbiology, and an active officer in the United States Army.