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The Host Defense Peptide Beta-Defensin 1 Confers Protection against *Bordetella pertussis* in Newborn Piglets

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Innate immunity plays an important role in protection against respiratory infections in humans and animals. Host defense peptides such as beta-defensins represent major components of innate immunity. We recently developed a novel porcine model of pertussis, an important respiratory disease of young children and infants worldwide. Here, we investigated the role of porcine beta-defensin 1 (pBD-1), a porcine defensin homologue of human beta-defensin 2, in conferring protection against respiratory infection with *Bordetella pertussis*. In this model, newborn piglets were fully susceptible to infection and developed severe bronchopneumonia. In contrast, piglets older than 4 weeks of age were protected against infection with *B. pertussis*. Protection was associated with the expression of pBD-1 in the upper respiratory tract. In fact, pBD-1 expression was developmentally regulated, and the absence of pBD-1 was thought to contribute to the increased susceptibility of newborn piglets to infection with *B. pertussis*. Bronchoalveolar lavage specimens collected from older animals as well as chemically synthesized pBD-1 displayed strong antimicrobial activity against *B. pertussis* in vitro. Furthermore, in vivo treatment of newborn piglets with only 500 μg pBD-1 at the time of challenge conferred protection against infection with *B. pertussis*. Interestingly, pBD-1 displayed no bactericidal activity in vitro against *Bordetella bronchiseptica*, a closely related natural pathogen of pigs. Our results demonstrate that host defense peptides play an important role in protection against pertussis and are essential in modulating innate immune responses against respiratory infections.

Pertussis, an acute respiratory tract infection caused by *Bordetella pertussis*, remains an epidemic disease responsible for significant infant and child morbidity and mortality. The disease is still perceived as a serious global health problem, with more than 45 million cases and more than 400,000 deaths every year (13). Immunization with either whole-cell or acellular vaccines has significantly reduced the disease load in developed and developing countries. However, the mechanisms of disease protection, in particular, the role of innate immunity in protection, are poorly understood. We recently developed a novel model of pertussis in newborn piglets and demonstrated that in contrast to newborn piglets, older piglets (>4 weeks of age) were fully resistant to infection with *B. pertussis* (15). Their response was both rapid and independent of previous exposure, indicating that innate immunity was crucial for disease protection.

Epithelia of skin and mucosa both serve as a physical barrier against pathogens and produce a vast array of endogenous substances including antimicrobial agents that can inhibit or neutralize invading pathogens (56). Many of these elements are the components of the innate immune response. The effector branch of innate immunity consists of two major mechanisms: the recruitment and activation of cellular components including macrophages, neutrophils, natural killer cells, and dendritic cells (DCs) and the release of a broad spectrum of extracellular mediators such as cytokines, chemokines, complement, and antimicrobial peptides (AMPs) (4, 56).

AMPs, also called host defense peptides (HDPs), include a wide range of proteins that can be classified into defensins and cathelicidins. HDPs have both direct, broad-spectrum antimicrobial activity and the ability to modulate immune responses against bacteria, fungi, parasites, and even viruses in a wide variety of species (24, 29, 35). Direct killing of bacteria by HDPs is mediated mainly through permeabilization of bacterial membranes (31, 58–60). Immunostimulatory functions include the ability to induce chemotaxis of immature DCs and T cells, activation of antigen-presenting cells, and induction of glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and interleukin-8 (IL-8) production by epithelial cells (7, 36, 57). Thus, HDPs appear to represent an important link between innate and acquired immunity and are potent immune modulators and adjuvants for vaccines (7). Beta-defensins, cationic peptides with broad-spectrum antimicrobial activity, are widely expressed in the skin and the mucosal surfaces of airways, the digestive tract, and the urogenital tract (16, 26, 27, 53, 54). They are either constitutively expressed or induced by bacterial products or proinflammatory cytokines. As part of the innate immune system, defensins are thought to play a significant role in protection against respiratory infections (14, 19, 44, 55, 57).

Pigs, like many other mammals, produce an impressive array of HDPs, such as cathelicidins PR-39 and protegrin 1 (PG-1), and defensins, which are synthesized predominantly by host phagocytes and mucosal epithelial cells. Although other beta-defensin genes have been identified (GenBank accession number AY460575) (42), porcine beta-defensin 1 (pBD-1) is the only well-studied beta-defensin with significant homology to human beta-defensins in pigs. pBD-1 is expressed throughout

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the epithelia of the tongue, the respiratory and gastrointestinal tracts, and a variety of other organs. Interestingly, expression has not been detected in peripheral blood mononuclear cells (PBMCs) (46, 61). pBD-1 has direct antimicrobial activity against a variety of porcine pathogens including *Listeria monocytogenes*, *Candida albicans*, *Escherichia coli*, *Streptococcus suis*, and *Actinobacillus pleuropneumoniae* (46). Furthermore, it also has immunomodulatory activity in porcine PBMCs and lymph node cells (data not shown).

Here, we demonstrate that resistance against respiratory infection with *B. pertussis* in older piglets was associated with the presence of pBD-1 in the upper respiratory tract. Furthermore, treatment with pBD-1 conferred complete protection of newborn piglets from intrapulmonary infection with *B. pertussis*. Thus, using a relevant model for a human disease of great importance, we show here that beta-defensins are involved in disease protection against respiratory infection. Our results not only further advance the understanding of the immunopathogenesis of pertussis but also show that treatment with defensins may represent an alternative strategy for treating pertussis in human patients.

MATERIALS AND METHODS

Bacterial cultures. *B. pertussis* suspensions of strain Tohama I were stored at −70°C in Casamino Acids plus 10% glycerol. Organisms were initially grown on Bordet-Gengou (BG) agar (Becton Dickinson and Company) containing 15% (vol/vol) defibrinated sheep blood and 40 μg/ml of cephalexin (Sigma-Aldrich) at 37°C for 48 h. Heavy inocula of bacteria were resuspended in Stainer-Scholte (SS) medium and grown aerobically at 37°C for 48 h either as a liquid culture at 250 rpm in a Thermo Forma shaker or on BG plate cultures. Bacteria were harvested from BG plates by scraping off and resuspending bacteria in SS medium. After centrifugation at 2,500 × *g* for 10 min, pellets were resuspended in Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBSA) (pH 7.2, 20 mM) and adjusted to a specified bacterial titer (3×10^9 to 4×10^9 CFU) by determining the optical density at 600 nm (Ultrospec 3000; Pharmacia Biotech, United Kingdom). The bacterial suspension (adjusted to 50% from liquid culture and 50% from BG plates) was kept on ice until it was used for challenge infection. Corresponding viable counts were determined by plating serial dilutions onto BG agar plates and subsequent incubation at 37°C for 4 to 5 days. For in vitro inhibition assays, bacteria were grown on BG agar, scraped off, washed in PBSA, and resuspended in SS medium at 5×10^8 to 7×10^8 CFU/ml. *Bordetella bronchiseptica* rabbit isolate RB50 (12, 43) (kindly provided by Andrew Preston, University of Guelph, Canada) was stored at −70°C in Casamino Acids plus 10% glycerol. Organisms were grown on BG agar plates (supplemented with 15% defibrinated sheep blood and cephalexin) at 37°C for 24 h. Bacteria were resuspended in SS medium at 5×10^8 to 7×10^8 CFU/ml of SS medium for in vitro inhibition assays.

Animals. Pregnant Landrace sows were purchased from the Saskatoon Prairie Swine Centre, University of Saskatchewan. Sows were induced to farrow by intramuscular injection of prostaglandin (Planate) (Schering, Quebec, Canada) at day 113 of gestation. Piglets were born at day 114 to 115 of gestation. Nursing piglets were kept within the same room but in separate pens and monitored very closely. The piglets were challenged at 3 to 5 days of age. All experiments were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

Respiratory infection and postmortem analysis. Newborn piglets were anesthetized with isoflurane and intubated using a laryngoscope. Infection was initiated by delivering 1.5 ml of PBSA (20 mM) containing 5×10^9 CFU of *B. pertussis* strain Tohama I intrapulmonarily (craniodorsal of the bifurcation) through a Micro-Renathane tube (size 0.95; Braintree Scientific Inc.), which was inserted through an endotracheal tube (3 mm; Jorgensen Laboratories Inc., Loveland, CO). The bottom end of the Micro-Renathane tube was sealed, and minute holes were made for the equal distribution of bacteria. Piglets were monitored twice daily for clinical symptoms including fever and respiratory symptoms such as nasal discharge, nonparoxysmal cough, and breathing difficulties. Piglets were euthanized by intraperitoneal injection of 5 ml of sodium barbiturate (Euthanyl; Bimeda-MTC, Ontario, Canada) at different time points over a 10-day period after challenge. The thoracic and abdominal cavities and the lungs were examined for any lesions, and abnormalities such as pleuritis or local collections of blood and fluids in the thorax were noted.

Isolation of bacteria. The number of bacteria in the bronchoalveolar lavage (BAL) specimens and lung lesions was examined over a 10-day period. To allow optimal recovery, because *B. pertussis* is a fastidious bacterium, SS medium was used to lavage the lungs. The BAL fluid was obtained by filling the lungs with 15 ml of SS medium and withdrawing as much fluid as possible. To quantify the presence of *B. pertussis* in the BAL fluid, samples were centrifuged to remove debris and host cells; supernatant and dilutions thereof were plated onto BG plates and incubated at 37°C for up to a week. To determine the number of bacteria within the tissues, lesions were excised, weighed, homogenized, and plated onto BG agar plates.

In vivo treatment with pBD-1. Prior to challenge infection with *B. pertussis*, 500 μg of pBD-1 in 1.5 ml of PBSA (20 mM) was delivered through the Micro-Penthane tube into the lungs of anesthetized piglets. In the early experiments, we used the same Micro-Penthane tube to deliver the peptide and bacteria. However, we cleared the tube of any remaining peptide by passing 30 ml of air through the tube before the bacteria were delivered. In further animal experiments, to avoid possible direct contact of bacteria and the peptide, the Micro-Penthane tube was changed and a new tube was inserted for delivering the bacteria. Either method resulted in protection, and there was no significant difference in the outcome of challenge. Control groups received 1.5 ml of PBSA. Following treatment, animals from both groups were infected with 5×10^9 CFU bacteria in 1.5 ml of PBSA (20 mM) as described above.

Reverse transcriptase PCR. Total RNA was extracted from porcine tissues by the acid guanidium thiocyanate/phenol-chloroform method using a total RNA isolation reagent (TRIzol reagent; GIBCO BRL). RNA was reverse transcribed to cDNA using the oligo(dT) primer (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcriptase products were amplified using a PCR kit (RED Taq, Ready Mix PCR Mix; Sigma-Aldrich) and a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems, Singapore). The primers used to detect a 287-bp cDNA sequence of pBD-1 in porcine tissues were sense primer 5′-TCCCATGAGACTCCACCGCCTCCT-3′ and antisense primer 5′-TTCGAGCAGCTTCTGAGCCATATCTGTAC-3′; primers used to detect a 100-bp cDNA sequence of PG-1 were sense primer 5′-TGGATCAGATCAAGGACCTA-3′ and antisense primer 5′-ACACAGACGCAGAACCTAC-3′; primers used to detect a 262-bp cDNA sequence of PR-39 were sense primer 5′-ACCCATCCATTCACTCAC-3′ and antisense primer 5′-AGCCACAACAATAAGATCC-3′; primers used to detect a 452-bp cDNA sequence of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were sense primer 5′-ACCACAGTCCATGCCATCAC-3′ and antisense primer 5′-TCCACCACCCTGTTGCTG-3′. The PCR conditions were a 1-min denaturation step at 95°C followed by 24 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by a final extension step at 72°C for 7 min. The PCR products were visualized by electrophoresis on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide.

In vitro growth inhibition assays. Noninfected piglets, either 4 to 5 weeks old or newborn (6-h-old colostrum-deprived or colostrum-fed piglets), were euthanized, and BAL specimens were collected in 20 mM PBSA, which was used to facilitate optimal conditions for the AMPs. The BAL fluid was obtained by filling the lungs with 10 ml of PBSA and withdrawing as much fluid as possible (this procedure was performed once). Alveolar macrophages and other cells were removed by centrifugation at 500 × *g* for 10 min. BAL specimens (290 μl) were cocultured in microtiter plates with 10 μl of a bacterial suspension containing 5×10^6 to 7×10^6 CFU of *B. pertussis* at 37°C. Supernatants were plated onto BG agar plates at different time points to evaluate the number of viable bacteria. The sensitivity of *B. pertussis* to human lysozyme (Sigma), human lactoferrin (Sigma), synthetically derived pBD-1, human beta-defensin 2 (hBD-2), and PG-1 was assayed by coculturing appropriate concentrations of lysozyme, lactoferrin, pBD-1, hBD-2, and PG-1 in 20 mM PBSA (280 μl) with 5×10^6 to 7×10^6 CFU (10 μl) bacteria. Plates were incubated at 37°C for 1, 2, and 4 h. The same procedures were used to investigate the inhibitory effects of BAL fluid, pBD-1, hBD-2, and PG-1 against *B. bronchiseptica* in vitro.

Synthesis of the pBD-1, hBD-2, and PG-1. pBD-1, hBD-2, and PG-1 were chemically synthesized on a Pioneer solid-phase peptide synthesizer (PerSeptive Biosystems, Foster City, CA) using Fmoc (9-fluorenylmethoxy carbonyl) chemistry. The peptide chains were synthesized from the carboxyl terminus to the amino terminus onto [5-(4-Fmoc-aminomethyl-3,5-dimethyloxyphenoxy) valeric acid]–polyethylene glycol–polystyrene (PAL-PEG-PS) resin. Both Fmoc-protecting groups at the amino terminus were deprotected with piperidine. The peptides were cleaved from the resin with concurrent deprotection of the side chain-protecting groups by treating the resin-bound peptide with

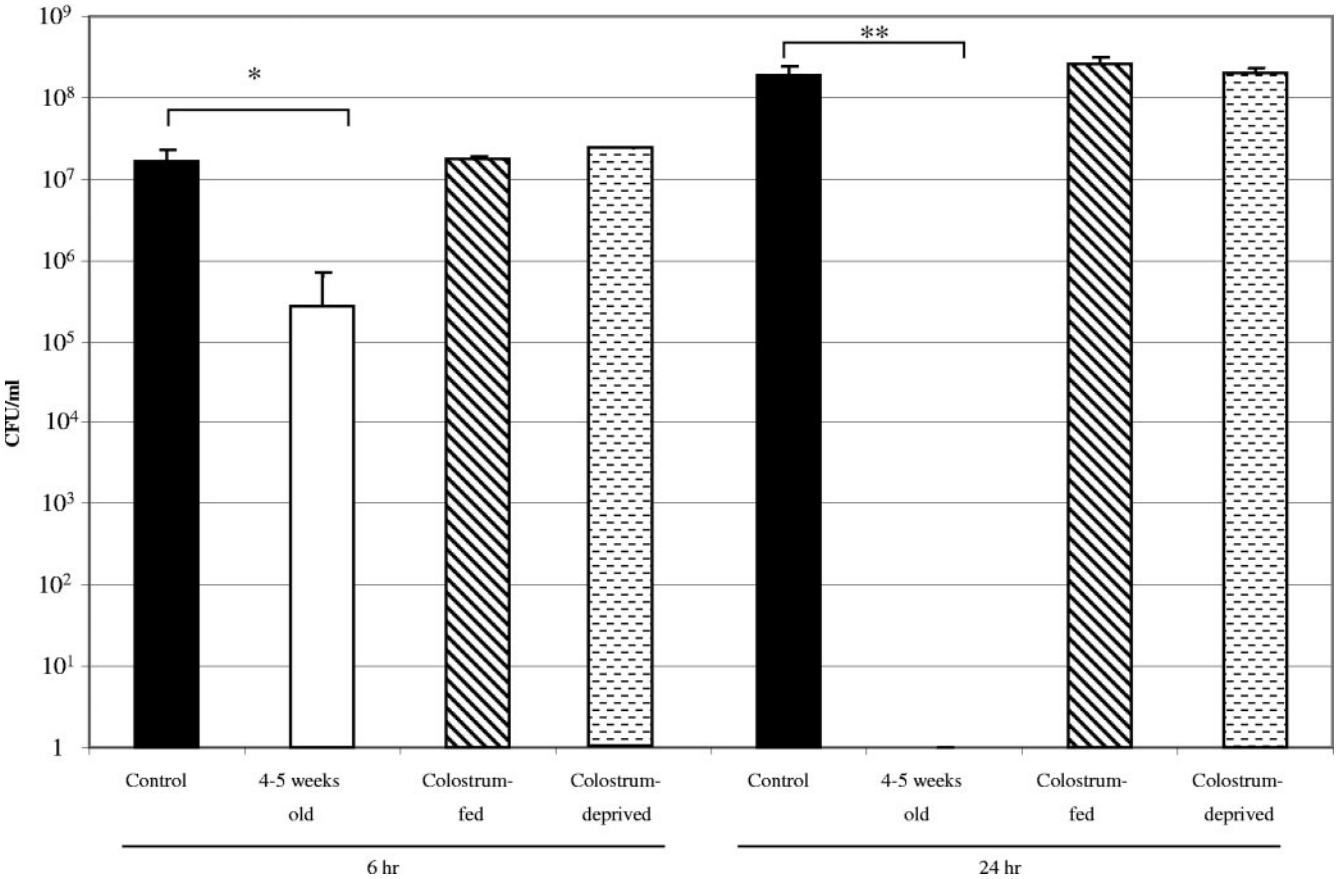

FIG. 1. Inhibitory effect of BAL specimens from either newborn or 4- to 5-week-old piglets. Approximately 5×10^6 to 7×10^6 CFU *B. pertussis* were cocultured in duplicate with SS medium (control) or BAL specimens obtained from newborn colostrum-fed, newborn colostrum-deprived, or 4- to 5-week-old piglets for 6 or 24 h. Supernatants were plated out onto BG agar plates to quantify the number of viable bacteria. Results are expressed as the means ± standard errors of the means (SEM) from two experiments with six animals per group. *, $P = 0.004$; **, $P = 0.002$.

trifluoroacetic acid (TFA) (9.3 parts) in the presence of scavengers (anisole–ethyl-methyl sulfide–1,2-ethanedithiol [3:3:1]), for 7 h. The crude peptides were filtered from the resin, and the TFA was evaporated. Diethyl ether was added to the residues to precipitate the crude peptide. The peptides were isolated and purified by high-performance liquid chromatography (HPLC) on Vydac protein C_4 columns (1.0 by 25 cm) eluting with a linear gradient of 35% buffer A (H_2O–0.1% TFA)–90% buffer B (acetonitrile–H_2O [90/10]–0.01% TFA) for 40 min at a flow rate of 3 ml/minute. The purity and molecular weight of the respective peptides were confirmed by matrix-assisted laser desorption ionization (MALDI)–time of flight mass spectrometry on a PE Biosystems Voyager system 4068 (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada) and by amino acid analysis.

The cysteine residues were oxidized in 0.1 M ammonium acetate buffer (pH 7.7) in the presence of reduced and oxidized glutathione at a ratio of 1/20/2 (peptide/reduced glutathione/oxidized glutathione) as described previously by Hiratsuka et al. (30). The oxidized peptides were purified by high-performance liquid chromatography on a Vydac protein C_4 column (1.0 by 25 cm) eluting with a linear gradient of 35% buffer A (H_2O–0.1% TFA)–90% buffer B (acetonitrile–H_2O [90/10]–0.01% TFA) for 40 min at a flow rate of 3 ml/minute. Since we were unable to perform two-dimensional nuclear magnetic resonance on the synthesized peptides to determine the correct pairing of the intermolecular disulfide bonds, we compared the bactericidal activity of synthetic pBD-1, hBD-2, and PG-1 against *E. coli* to the previously reported activity of a recombinant baculovirus-expressed peptide (30, 46, 48). Furthermore, BAL analysis provided strong evidence for synthetic pBD-1 and PG-1 being correctly folded, as both migrated similarly on reverse-phase HPLC (RP-HPLC) to their corresponding natural peptides. Also, MALDI data for pBD-1 confirmed the loss of 6 mass units, indicating that three disulfide bridges were formed.

Production of anti-pBD-1 antibody. Rabbit anti-pBD-1 serum was produced by immunization with synthetic pBD-1. The peptide was conjugated to either ovalbumin or keyhole limpet hemocyanin by using a single-step cross-linking technique with glutaraldehyde (28). New Zealand White rabbits were immunized with conjugated pBD-1 (300 μg) in complete Freund's adjuvant in a total volume of 1.25 ml subcutaneously at multiple sites. Booster immunizations were performed at 2 and 4 weeks after priming, blood was drawn 10 days after the last booster, and serum was analyzed by Western blot and stored at −20°C.

Transmission electron microscopy. Transmission electron microscopy was employed to evaluate the effect of pBD-1 on the bacterial membranes of *B. pertussis* and *B. bronchiseptica*. pBD-1 was added at a final concentration of 20 μg/ml to 5×10^9 to 7×10^9 CFU bacteria and incubated for 20 to 60 min at 37°C. Bacteria were washed with PBS, fixed with an equal volume of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and centrifuged at 5,000 × *g* for 5 min. Cells were resuspended in 1% agarose and pelleted. The bacterial pellets were stored in the fixative (2.5% glutaraldehyde) at 4°C for 2 h. The samples were dehydrated and en bloc stained with uranyl acetate in a series of ethanol concentrations and then embedded in Epon/Araldite. Thin sections were cut using an ultramicrotome with a diamond knife, mounted on specimen grids, and stained with uranyl acetate and lead citrate before being examined on a transmission electron microscope (Philips 410LS) operating at 80 kV and at magnifications ranging from ×24,000 to ×55,000.

Statistical analysis. All outcome data from this study followed nonnormal distributions. To account for this, all outcome data were ranked, and analysis of variance (ANOVA) or Student's *t* test was then used to detect differences among the experimental groups. The distributions of the ranked data and the residuals from each ANOVA were consistent with the assumptions of procedure. If there were more than two experimental groups in the analysis and the ANOVA was

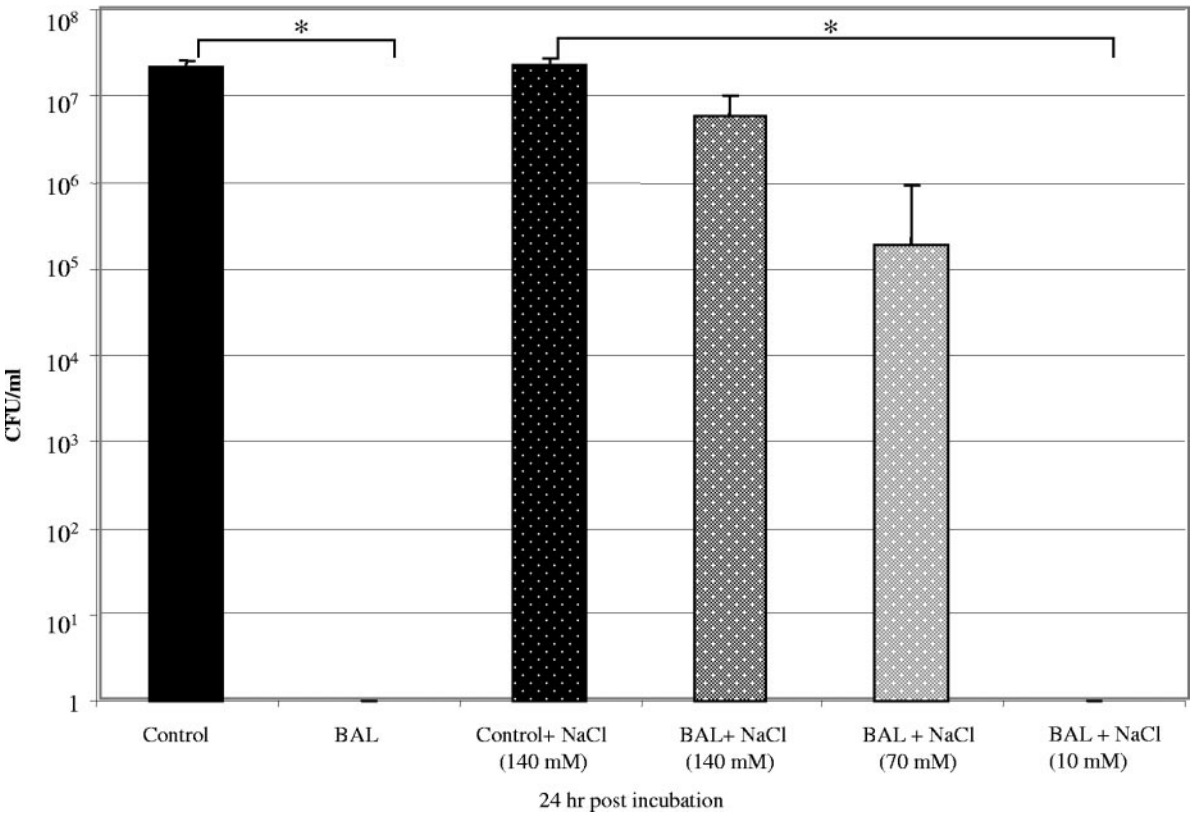

FIG. 2. Salt dependency of antimicrobial activity of BAL specimens. A total of 5×10^6 to 7×10^6 CFU *B. pertussis* were cocultured in duplicate with SS medium (Control) or BAL specimens obtained from three 4- to 5-week-old piglets (BAL). Various concentrations of NaCl (140 mM, 70 mM, and 10 mM) were added to the SS medium (Control + NaCl) or BAL specimens (BAL +NaCl) and incubated for 24 h. Supernatants were plated onto BG agar plates to quantify the number of viable bacteria. Results are expressed as the means ± SEM. $*P \leq 0.004$.

significant, the means of the ranks were compared using Tukey's test. Probabilities less than or equal to 0.05 were considered significant.

RESULTS

Inhibitory effect of BAL fluid against *B. pertussis*. We recently reported the development of a new model for pertussis in pigs (15). In this model, older piglets (4 to 5 weeks old) were fully protected against infection, whereas newborn animals (3 to 5 days old) were fully susceptible to the disease. To determine the mechanisms underlying protection, we investigated the bactericidal activity of BAL specimens obtained from noninfected piglets against *B. pertussis* in vitro. BAL specimens from either newborn (colostrum-fed/colostrum-deprived) or 4- to 5-week-old piglets were cocultured (total volume of 290 μl) with 5×10^6 to 7×10^6 CFU *B. pertussis* in SS medium (Fig. 1). Compared to the medium control, there was a significant reduction in the number of viable bacteria in BAL specimens from 4- to 5-week-old piglets. At 6 h of incubation, bacterial numbers were reduced by 1.5 logs ($P = 0.004$), and after 24 h, no viable bacteria were isolated from wells cocultured with BAL specimens obtained from 4- to 5-week-old piglets ($P = 0.002$). In contrast, regardless of whether the BAL specimens were obtained from colostrum-fed or colostrum-deprived newborn piglets, growth inhibition of *B. pertussis* was not observed (Fig. 1). Furthermore, these results confirmed that the presence of colostrum-derived secretory immunoglobulin A in BAL specimens did not have any effect on bacterial growth or interfered with our assays. We hypothesized that BAL specimens obtained from older animals contained antimicrobial components such as antimicrobial peptides and proteins that could be associated with in vivo protection against *B. pertussis*.

The antibacterial activity of BAL fluid against *B. pertussis* was salt dependent. The antimicrobial activity of many antimicrobial proteins and cationic peptides including beta-defensins is greatly affected by higher salt concentrations (3). For example, it has previously been reported that an elevated concentration of NaCl in the airway surface fluid of cystic fibrosis patients neutralizes the antimicrobial activity of defensins and predisposes the host to a wide range of infections (3). In order to determine whether the observed bactericidal activity of BAL specimens in our study was due to the presence of antimicrobial peptides, 10 to 140 mM NaCl was added to the BAL specimens. As shown in Fig. 2, 140 mM salt completely blocked and 70 mM salt partially blocked the growth-inhibitory effect of BAL specimens against *B. pertussis*. However, 10 mM NaCl did not inhibit the antibacterial activity of BAL specimens ($P \leq 0.004$) (Fig. 2). Thus, these results demonstrated that the observed antimicrobial effect was reversed by the addition of a high salt concentration to the culture and therefore further supported our hypothesis that the antimicrobial activity of

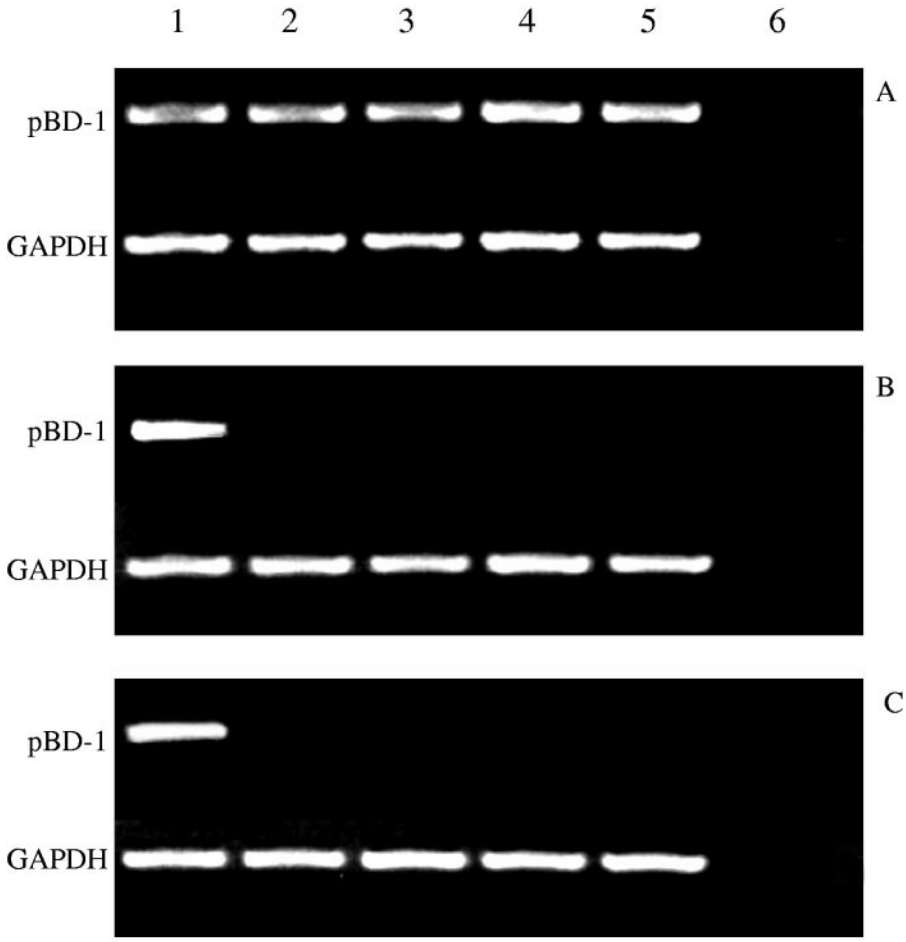

FIG. 3. Tissue expression of pBD-1 mRNA in either newborn (colostrum-fed/colostrum-deprived) or 4- to 5-week-old piglets. Tissue samples collected from (A) 4- to 5-week-old animals, (B) newborn colostrum-fed animals, or (C) newborn colostrum-deprived animals were analyzed for PCR products of 452 bp (GAPDH) and 287 bp (pBD-1) in length. Tissues included tongue (lane 1), nasal mucosa (lane 2), trachea (lane 3), lung (lane 4), intestine (lane 5), and water as a negative control (lane 6). Expression was found only in the tongue of newborn piglets but in all investigated tissues of 4- to 5-week-old piglets. Six animals per age group were analyzed.

BAL specimens obtained from older piglets could be due to the presence of antimicrobial peptides, such as lysozyme, lactoferrin, PR-39, PG-1, and pBD-1, in lung secretions.

Detection of pBD-1, PG-1, and PR-39 gene expression at the respiratory surfaces. Beta-defensins and cathelicidins are the most dominant antimicrobial peptides that are found in neutrophils and epithelia as components of the early host defenses of mammals against infection. Beta-defensins are the predominant cationic peptides in the lung (6, 47). In fact, lower levels of defensins may contribute to the increased susceptibility of premature infants to pulmonary infections (47). To determine differences in expression levels of pBD-1, PG-1, and PR-39 between older and newborn animals, we analyzed the gene expression of pBD-1, PG-1, and PR-39 in respiratory tissues obtained from newborn and older piglets. Expression of pBD-1 was detected in all analyzed tissues in 4- to 5-week-old piglets (Fig. 3A). In contrast, expression of pBD-1 in tissues obtained from newborn piglets was detected only in the tongue epithelium (Fig. 3B and C). Expression of PG-1 and PR-39 was detected in all lung tissues obtained from either newborn or older piglets (Fig. 4A). These results indicated that the expression of pBD-1 but not PG-1 or PR-39 developed with age. Moreover, infection with *B. pertussis* induced up-regulation of pBD-1 in the lung of newborn piglets as early as 2 days postchallenge, which demonstrated that pBD-1 might be an inducible beta-defensin in pigs (Fig. 4B). These results were confirmed by RP-HPLC, which revealed the presence of pBD-1 in the BAL specimens collected from older piglets but not from newborn animals (Fig. 5). In addition, RP-HPLC analysis indicated the presence of PG-1, a highly antimicrobial peptide, in BAL specimens obtained from both newborn and older animals (data not shown). Thus, these results supported our hypothesis that expression of pBD-1 in older piglets may contribute to resistance against infection with *B. pertussis*.

In vitro susceptibility of *B. pertussis* to synthetically derived pBD-1. We synthesized pBD-1 using Fmoc synthesis, and in order to analyze the functional activity of this peptide, we compared its inhibitory activity against *E. coli* strain JG280 to the inhibitory activity of a recombinant baculovirus-expressed pBD-1 described previously by Shi et al. (46). Similar results were obtained, which demonstrated that synthetic pBD-1 was biologically functional. We then analyzed the inhibitory effect

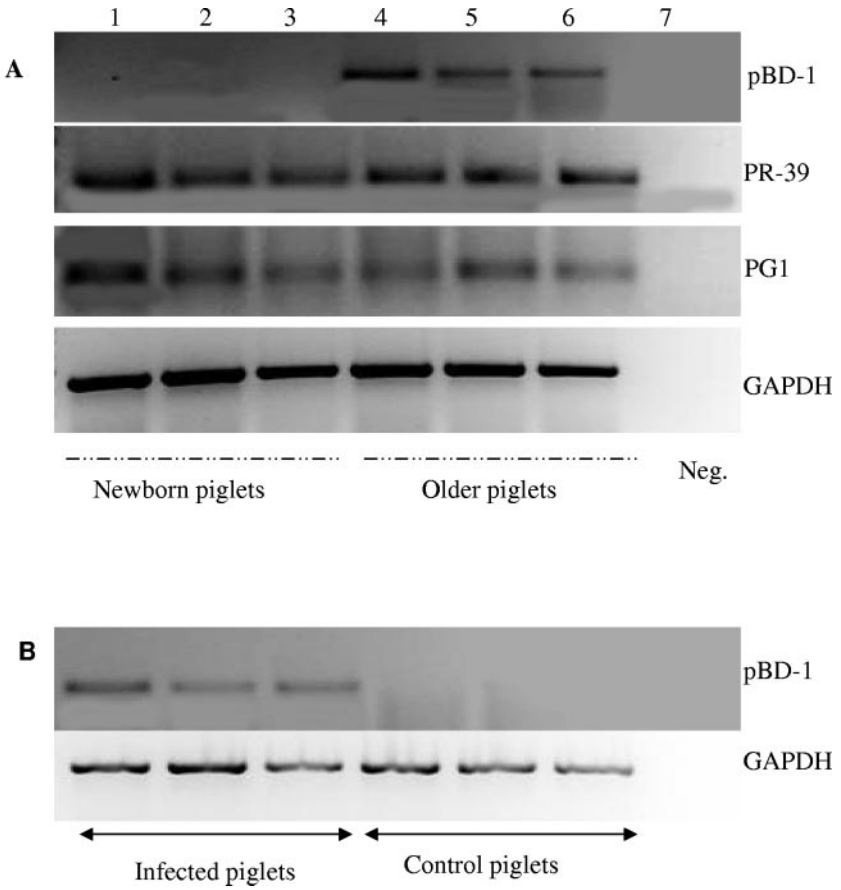

FIG. 4. Expression of pBD-1, PG-1, and PR-39 mRNA in the lungs of either newborn or 4- to 5-week-old piglets. (A) Tissue samples collected from newborn or 4- to 5-week-old animals were analyzed for PCR products of 452 bp (GAPDH), 100 bp (PG-1), 262 bp (PR-39), and 287 bp (pBD-1) in length. Tissues were collected from three newborn piglets (lanes 1 to 3) and three older piglets (lanes 4 to 6), and water was used as a negative control (Neg.) (lane 7). The expression patterns of PG-1 and PR-39 were similar in both groups, but in contrast, expression of PBD-1 was only found in the lungs of older piglets. (B) Expression of pBD-1 was noted in the lungs of piglets infected with *B. pertussis* 2 days after challenge but not in control animals (PBSA-challenged piglets). Results from 10 animals per age group are shown.

of pBD-1 against *B. pertussis* by inhibition assays using different concentrations of chemically synthesized pBD-1 in different cultural conditions such as SS medium, PBSA (140 mM NaCl), and 20 mM PBSA (2.8 mM NaCl). First, we tested the antibacterial activity of pBD-1 against *B. pertussis* (20, 40, and 80 μg/ml) in SS medium. As shown in Fig. 6A, at concentrations of 20 μg/ml of pBD-1, the growth of *B. pertussis* was inhibited by at least 2 logs after 6 h of incubation in comparison with control SS medium ($P < 0.0001$). Higher concentrations of pBD-1 and prolonged incubation resulted in significantly increased inhibitory activity. Indeed, at 80 μg/ml of pBD-1 and 18 h of incubation, 5×10^6 to 7×10^6 CFU of *B. pertussis* were completely neutralized ($P < 0.0001$). However, these assays were performed under in vitro conditions that were optimized for growing the bacteria. It is possible that in vivo concentrations of ions such as Ca^{2+}, Mg^{2+}, and K^+ as well as tissue proteins may interfere with the observed antimicrobial activity (8, 9). Although SS medium has concentrations of Ca^{2+}, Mg^{2+}, and K^+ comparable to those found in human lung secretions, we tested the effect of the medium itself by diluting the SS medium in PBSA (1/10 dilution). Interestingly, pBD-1 in diluted medium exhibited an even stronger bactericidal activity against *B. pertussis* in a shorter period of time ($P < 0.0001$) (Fig. 6B). Thus, pBD-1 displayed strong antimicrobial activity against *B. pertussis* at concentrations of Ca^{2+}, Mg^{2+}, and K^+ that were comparable to concentrations found in human lung secretions (which contain about 2 mM divalent and lesser monovalents [80 mM], mostly Na^+). Moreover, the inhibitory effect of pBD-1 against *B. pertussis* was also tested under conditions (20 mM PBS) typically used for the characterization of AMPs. As shown in Fig. 6C, at concentrations of 1 μg/ml of pBD-1, the growth of *B. pertussis* was inhibited by almost 3 logs after 1 h of incubation ($P < 0.0001$). Higher concentrations of pBD-1 (2.5 and 5 μg/ml) completely neutralized 5×10^5 to 8×10^5 CFU of *B. pertussis* within 1 h ($P < 0.0001$) (Fig. 6C), and prolonged incubation resulted in the absolute neutralization of bacteria even at 1 μg/ml of pBD-1 ($P < 0.0001$) (Fig. 6C). These results demonstrated that chemically synthesized pBD-1 displayed strong bactericidal activity against *B. pertussis* in vitro in both a time- and dose-dependent manner.

We also addressed the effect of various serum conditions in vitro by adding 2 to 10% human, porcine, or rabbit serum to the medium in order to simulate physiological conditions. In-

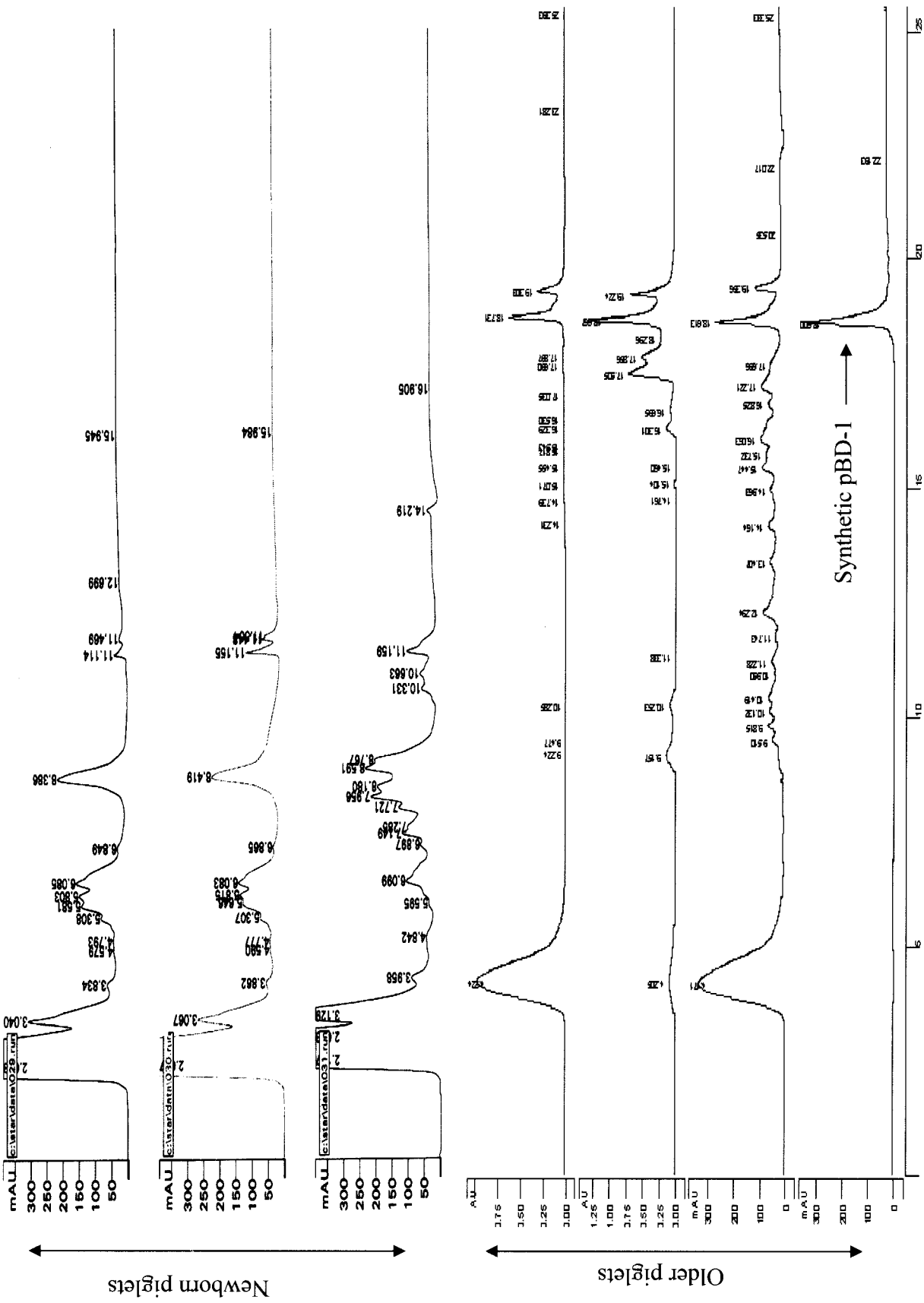

FIG. 5. RP-HPLC chromatograms of BAL fluid obtained from newborn or older piglets. BAL specimens were collected in 10% acidic acid and tested for the presence of pBD-1. BAL specimens from older piglets indicated the presence of a fraction of the same mass size of synthetic pBD-1. In contrast, this peak was absent in BAL specimens obtained from newborn piglets. To confirm the presence of pBD-1, fractions were verified by MALDI mass spectrometry. mAU, milli-Absorbance Unit.

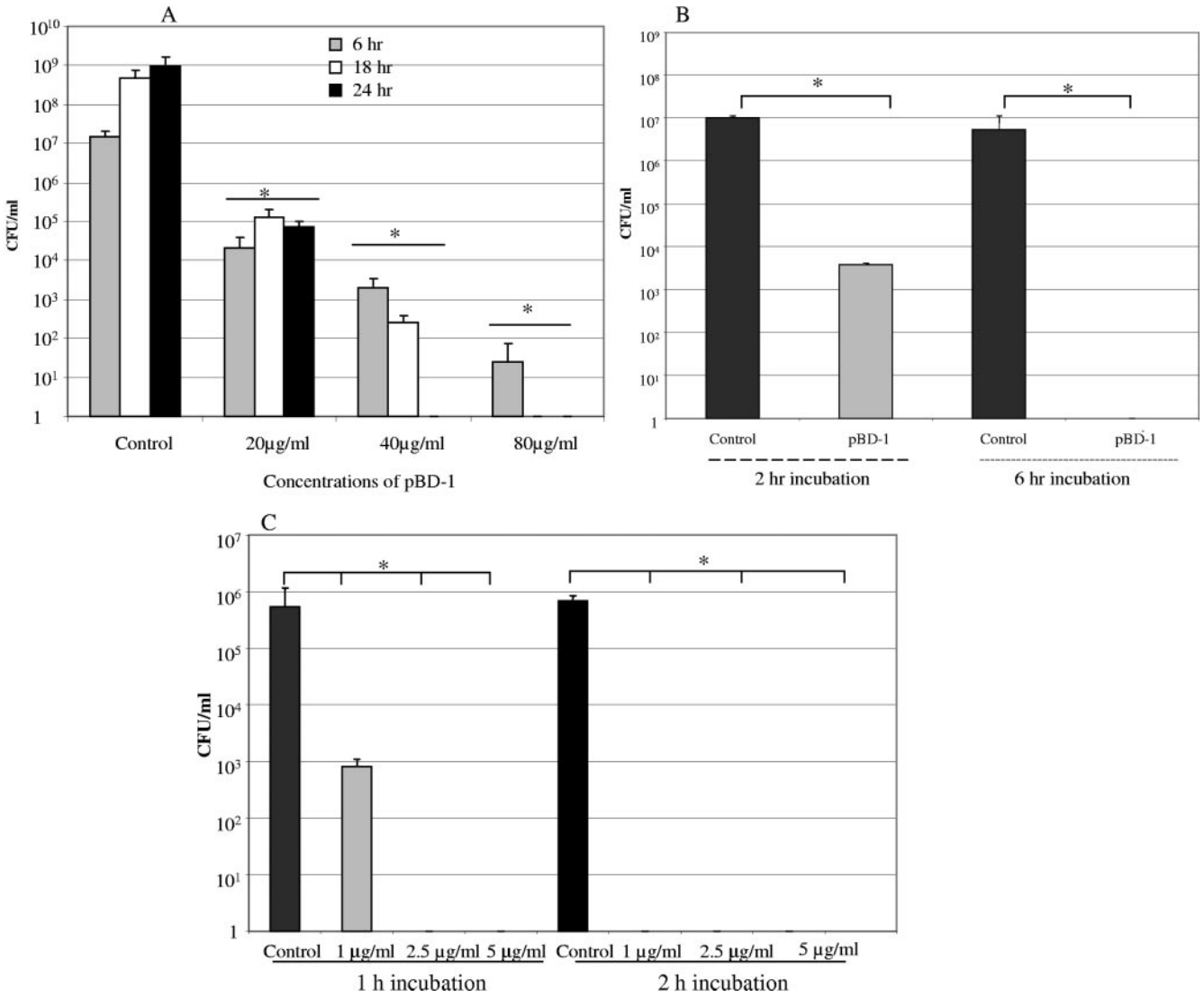

FIG. 6. Sensitivity of *B. pertussis* to synthetically derived pBD-1. (A) A total of 5×10^6 to 7×10^6 CFU *B. pertussis* were cocultured in duplicate with different concentrations of pBD-1 (20 μg/ml, 40 μg/ml, and 80 μg/ml) in SS medium (Control) for 6, 18, and 24 h. Bacterial numbers were determined by plate counts. (B) A total of 5×10^6 to 7×10^6 CFU *B. pertussis* were cocultured in duplicate with 10 μg/ml of pBD-1 in 1/10-diluted SS medium in PBSA for 6 or 24 h, and the number of bacteria were determined by plate counts. (C) A total of 5×10^6 to 7×10^6 CFU *B. pertussis* were cocultured in duplicate with different concentrations of pBD-1 (1, 2.5, and 5 μg/ml) in 20 mM PBSA. *, all values within each time point were significantly different ($P \leq 0.0001$) from their respective controls. Results are expressed as the means ± SEM.

terestingly, after 1 h of incubation, even very high serum concentrations (10%) did not completely inhibit the antimicrobial activity of pBD-1 (data not shown). Thus, these results indicated that pBD-1 displayed biological activity in the presence of lung secretions and serum and provided additional support for the hypothesis that the expression of pBD-1 at the respiratory surfaces was crucial for providing protection against infection with *B. pertussis*.

In vitro anti-*B. pertussis* activity of lysozyme, lactoferrin, and PG-1. Lysozyme and lactoferrin are the most abundant antimicrobial factors present on mucosal surfaces of the respiratory tract (50, 52). They contribute to airway defense by exhibiting strong antimicrobial activity against a wide range of gram-negative and gram-positive bacteria (2, 25). The antimicrobial activity of these peptides against *B. pertussis* was tested by inhibition assays using different concentrations of human lysozyme (100 and 1,000 mg/ml) and human lactoferrin (100 and 500 mg/ml). *B. pertussis* was completely resistant to both peptides after 2 h of incubation. Higher concentrations of these peptides and prolonged incubation did not result in any inhibitory effect (data not shown). In contrast, PG-1 displayed strong anti-*B. pertussis* activity in vitro (complete neutralization of 5×10^6 to 7×10^6 CFU at a concentration of 1 μg/ml for 1 h). Despite strong antimicrobial activity of PG-1 against *B. pertussis*, it was present in the BAL specimens obtained from both groups of animals. These results support our hypothesis that the observed antimicrobial activity of BAL fluid against *B. pertussis* was mainly due to the presence of pBD-1.

Synthetically derived pBD-1 restores in vitro activity of BAL specimens from newborn piglets against *B. pertussis*. To fur-

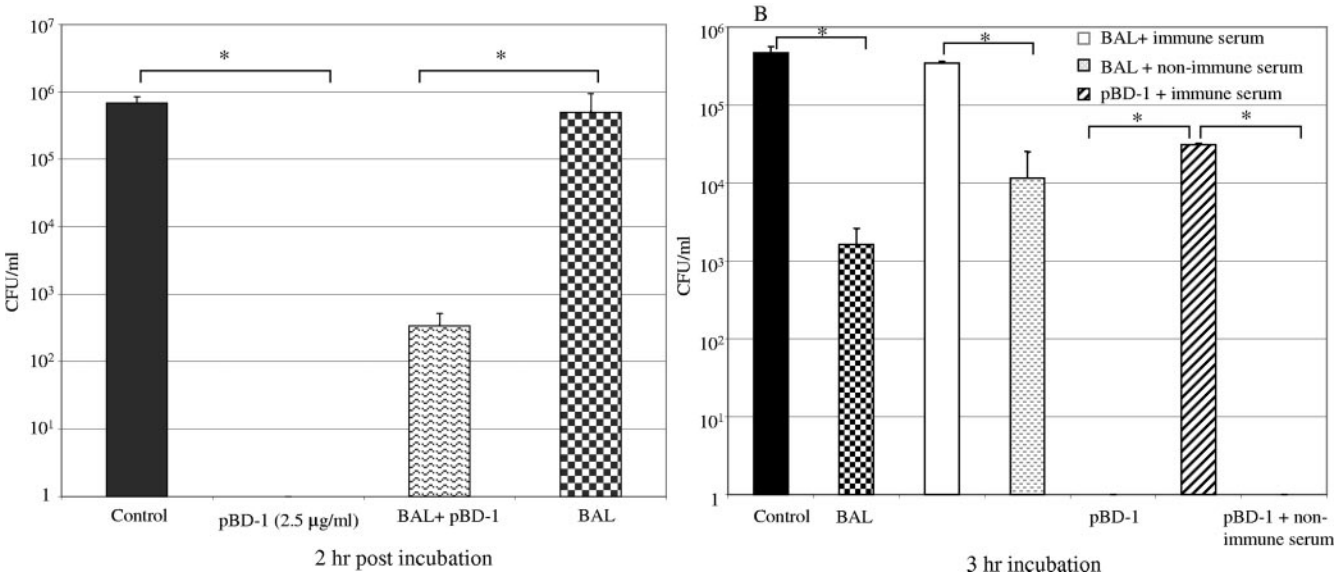

FIG. 7. Restoration of the antimicrobial activity of BAL specimens obtained from newborn piglets by synthetic pBD-1 and neutralizing effects of anti-pBD-1 antibody. (A) A total of 1×10^6 CFU *B. pertussis* were cocultured with BAL specimens obtained from newborn piglets with (BAL + pBD-1) (2.5 μg/ml) or without (BAL) synthetic pBD-1 for 2 h. Controls include SS medium (Control) and synthetic pBD-1 (2.5 μg/ml). (B) A total of 1×10^6 CFU *B. pertussis* were cocultured with BAL specimens or synthetic pBD-1 plus rabbit immune (<1%) or nonimmune (<1%) serum for 3 h. Columns include SS medium (Control), BAL specimens obtained from older animals (BAL), BAL specimens obtained from older animals plus immune serum (BAL + immune serum) or nonimmune serum (BAL + non-immune serum), synthetic pBD-1 plus immune serum (pBD-1 + immune serum), and synthetic pBD-1 plus nonimmune serum (pBD-1 + non-immune serum). Bacterial numbers were determined by plate counts. Data are presented as the means ± SEM. $*P < 0.001$.

ther support our hypothesis, 2.5 μg/ml of synthesized pBD-1 was added to the BAL specimens obtained from newborn piglets (Fig. 7A). The addition of synthetic pBD-1 restored the inhibitory activity of BAL specimens from newborn animals to levels that were comparable to those of BAL specimens from older animals ($P < 0.001$). Moreover, the addition of neutralizing antibody (rabbit anti-pBD-1 polyclonal antibody obtained from rabbits immunized with synthetic pBD-1 conjugated to ovalbumin) to the BAL specimens of older piglets reduced the activity to that of BAL specimens from newborn piglets or control medium ($P < 0.001$) (Fig. 7B). In contrast, serum from nonimmunized rabbits did not neutralize the anti-*B. pertussis* activity of BAL specimens obtained from older piglets ($P < 0.001$). Similar findings were found with synthetic peptide. While the immune serum inhibited the effects of synthetic pBD-1 against *B. pertussis*, the nonimmune rabbit serum had no effect on the biological activity of this peptide ($P < 0.0001$) (Fig. 7B). These results supported our hypothesis that the inhibitory effect of BAL fluid was specifically due to the presence of pBD-1 in the BAL specimens of older piglets.

Ultrastructural analysis of *B. pertussis* and *B. bronchiseptica* after treatment with pBD-1. To determine the potential mechanism of action, we studied the interaction between pBD-1 and the bacterial cell membrane using electron microscopy. The exposure of *B. pertussis* in SS medium to two different concentrations (20 and 40 μg/ml) of pBD-1 resulted in substantial morphological damage to the cell surface of bacteria as shown by scanning electron microscopy. Untreated bacteria displayed a rough, bright surface with no apparent perforation or cellular debris, as shown in Fig. 8. In contrast, *B. pertussis* exposed to pBD-1 exhibited a wide range of morphological abnormalities, as shown in Fig. 8. These included disruption of the cell surface, disappearance of the cell membrane, perforation and breakage in the cell membrane, and lysis of bacteria. In contrast, no cell structural effects were found on *B. bronchiseptica* even after incubation with significantly greater concentrations of pBD-1 (80 μg/ml).

Taken together, our in vitro studies clearly demonstrated a significant role for pBD-1 in protection against infection with *B. pertussis*. To further investigate the role of pBD-1, we decided to test its in vivo effects against *B. pertussis* in newborn piglets.

In vivo protection following treatment with pBD-1. pBD-1 was delivered into the lungs of newborn piglets prior to challenge with *B. pertussis*. We made sure that pBD-1 was completely administered into the lung before the bacteria were delivered. Indeed, treatment with only 500 μg of pBD-1 at the time of challenge resulted in complete protection of infected piglets, as demonstrated by the total absence of clinical symptoms and of pathological alterations at 2, 4, 7, and 10 days postinfection (Fig. 9A and B). In contrast, severe subacute hemorrhagic and necrotizing pneumonia was found in PBSA-treated piglets (Fig. 9C and D). Lesions were characterized by severe cellular infiltrations (neutrophils and macrophages) in the alveolar spaces, around the bronchioles, and in the walls of blood vessels with severe alveolar hemorrhagic, congestion, edema, and focal bronchiolar necrosis causing suppurative and histiocytic pleuropneumonia and bronchointerstitial pneumonia (data not shown). In addition, numbers of isolated bacteria in BAL specimens collected from pBD-1-treated animals were significantly reduced at 2, 4, and 7 days postinfection ($P < 0.0001$) (Fig. 10A). In addition, significantly lower numbers of

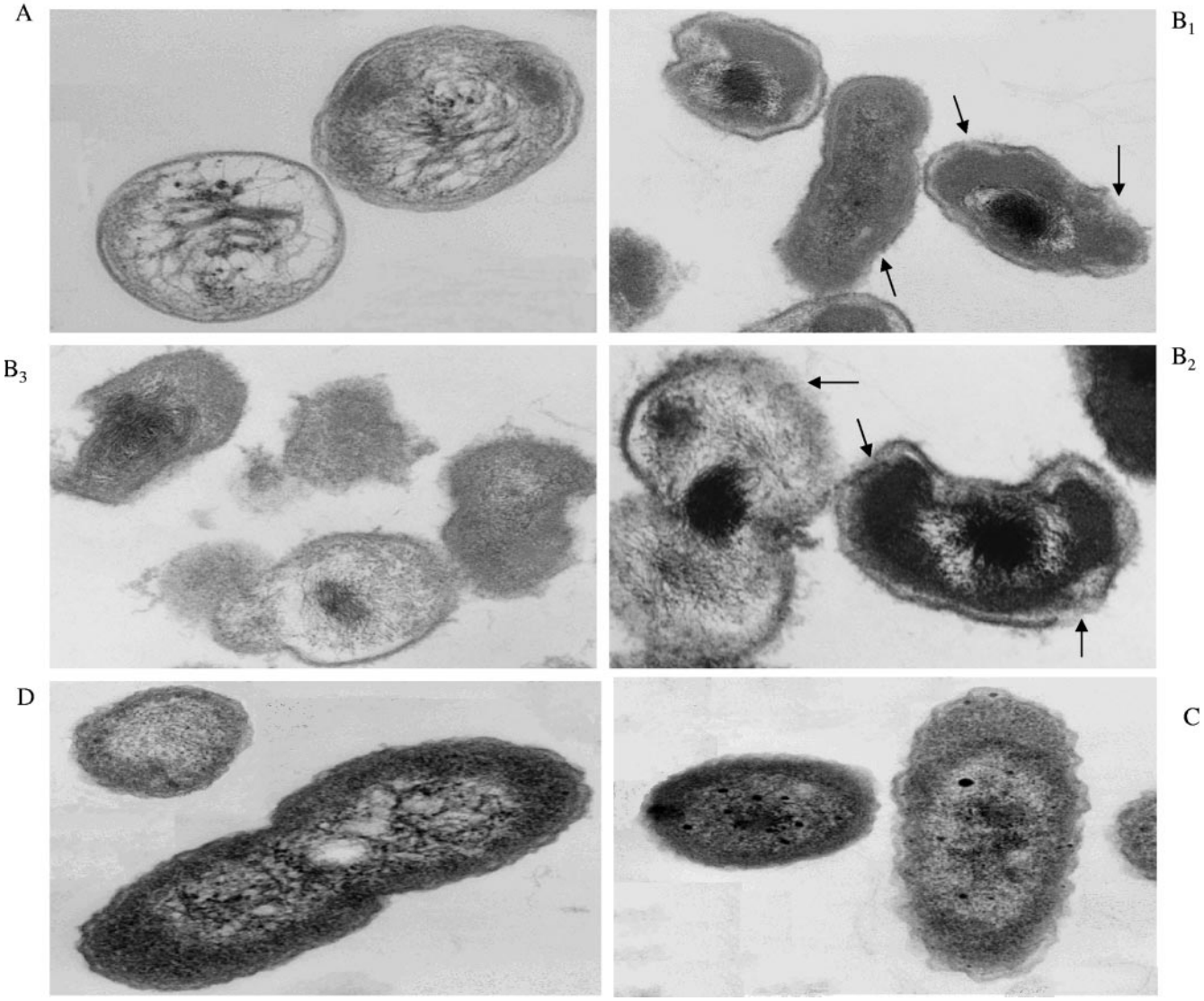

FIG. 8. Microscopical alterations after exposure to pBD-1. A total of 5×10^9 to 7×10^9 CFU of *B. pertussis* and *B. bronchiseptica* were exposed to pBD-1. (A) *B. pertussis* cells left untreated; (B) *B. pertussis* cells treated with 20 μg/ml of pBD-1 (B1, B2, and B3 represent different time points including 20, 40, and 60 min, respectively); (C) *B. bronchiseptica* left untreated; (D) *B. bronchiseptica* treated with 80 μg/ml of pBD-1. Micrographs are representative of at least 10 examined microscopic fields per sample. Magnification, ×40,500.

bacteria were found in homogenized lung tissues of treated animals at 2, 4, 7, and 10 days postinfection ($P < 0.005$) (Fig. 10B) compared to BAL specimens and tissues from control animals, which contained between 10^5 and 10^7 CFU/ml and 10^5 and 10^8 CFU/g, respectively, at these days. Thus, our results demonstrated that defensins played an essential role in mediating protection against pertussis. Furthermore, our results clearly demonstrate that treatment with chemically synthesized HDPs may represent a possible means of treating this important disease in young children and infants.

pBD-1 enhances innate immunity in vivo. To further investigate whether the observed protection was mediated primarily by direct antimicrobial activity or stimulation of innate immunity, we deposited 500 μg of pBD-1 in 1.5 ml PBSA (20 mM) into lungs of newborn piglets 4 h prior to challenge. Control animals received the same volume of PBSA instead. Both groups of piglets were challenged 4 h later with 5×10^9 CFU bacteria. Treatment with pBD-1 4 h prior to challenge resulted in a reduction in lesion size and bacterial load compared to control animals (data not shown). Furthermore, between 1.2×10^2 and 9.5×10^2 CFU of *B. pertussis*/ml BAL fluid and 1.6×10^2 to 8.8×10^2 CFU of *B. pertussis* /g tissue were isolated from treated piglets at days 2 and 4 postchallenge, whereas between 2.5×10^2 and 3.5×10^4 CFU/ml BAL fluid and 1.6×10^3 and 4.4×10^3 CFU/g tissue were isolated from control animals on these days, respectively (data not shown). These differences were highly significant for the lesions ($P < 0.0001$). Thus, these results indicate that protection was mediated, at least in part, through stimulation of the innate immune response.

Specificity of antimicrobial peptides. HDPs represent potential new drug candidates for innate immune treatments in humans and animals. However, resistance against HDPs has

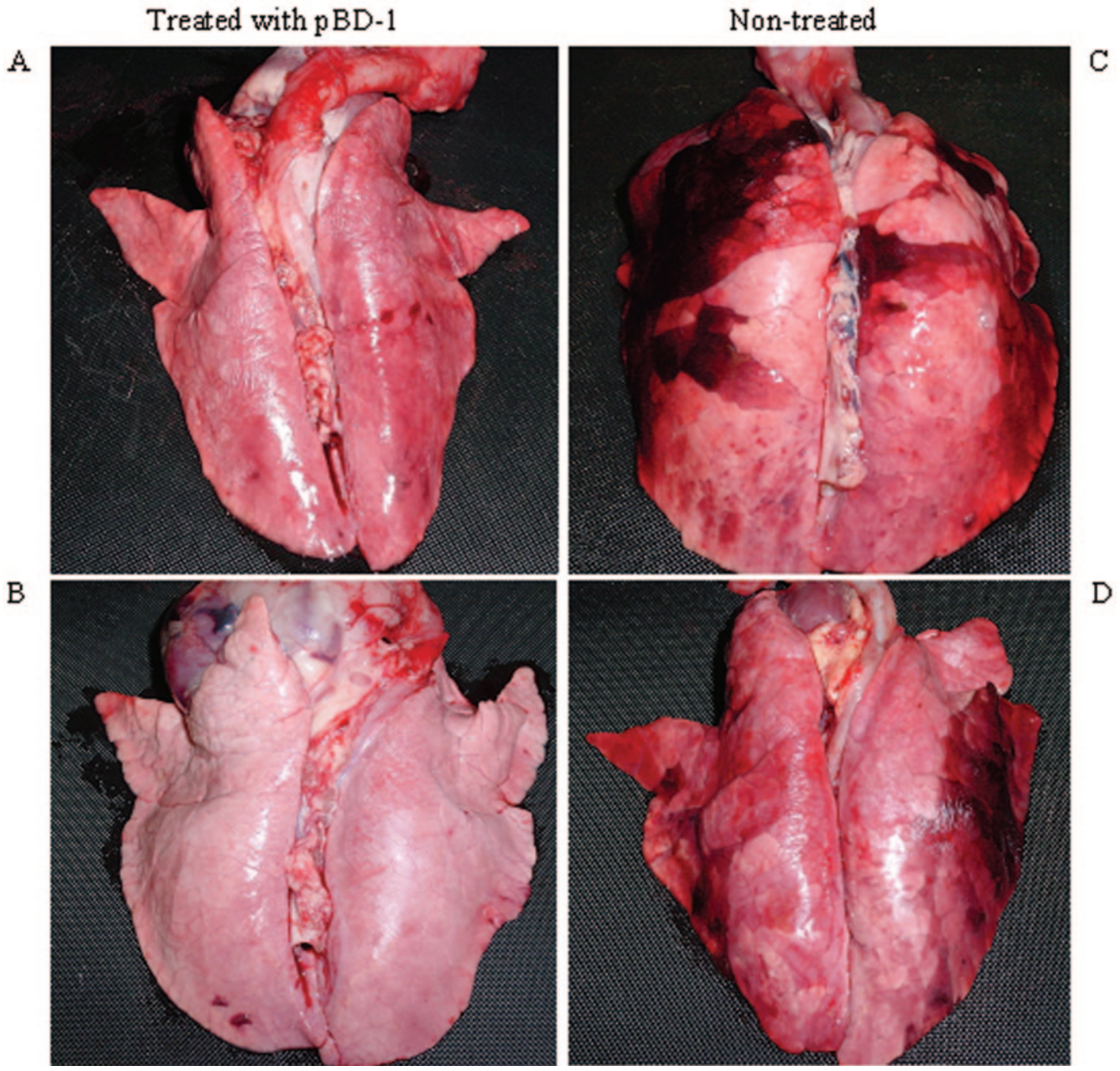

FIG. 9. Gross pathology of infected and treated lungs. Shown are lungs from piglets infected with 5×10^9 CFU of *B. pertussis* and treated with 500 μg pBD-1 at (A) 2 days and (B) 4 days postchallenge or untreated infected control (saline-treated) piglets at (C) 2 and (D) 4 days after challenge. Results shown are representative of three experiments, with at least three piglets per time point.

already been described for a number of bacteria (11, 18, 22, 33, 37, 39, 49). The present disease model allows us to compare a natural pathogen of pigs (*B. bronchiseptica*) to an experimentally introduced human pathogen (*B. pertussis*). We therefore compared the bactericidal activity of pBD-1 and PG-1 against both *B. pertussis* and *B. bronchiseptica* in vitro using 5 and 10 μg/ml of pBD-1 and 1, 5, and 10 μg/ml of PG-1. Interestingly, while ≥5 μg/ml pBD-1 inhibited the growth of *B. pertussis* by 100% after 1 h, the growth of *B. bronchiseptica* was unaffected at this time point ($P < 0.001$) (Fig. 11). In addition, *B. bronchiseptica* was resistant to pBD-1 even at very high concentrations (50 μg/ml) and for up to 6 h of incubation (data not shown). PG-1 exhibited very strong antimicrobial activity against *B. pertussis*, with complete elimination of 5×10^6 to 7×10^6 CFU in 1 h. In contrast, *B. bronchiseptica* was resistant to this peptide (data not shown). Thus, these results indicated that *B. bronchiseptica*, a natural pathogen of pigs, has evolved means of evading the porcine innate immune system. We therefore measured the bactericidal activity of hBD-2, the human defensin that is most homologous to pBD-1, against both *B. pertussis* and *B. bronchiseptica*. While hBD-2 had no bactericidal activity against *B. bronchiseptica* in vitro at concentra-

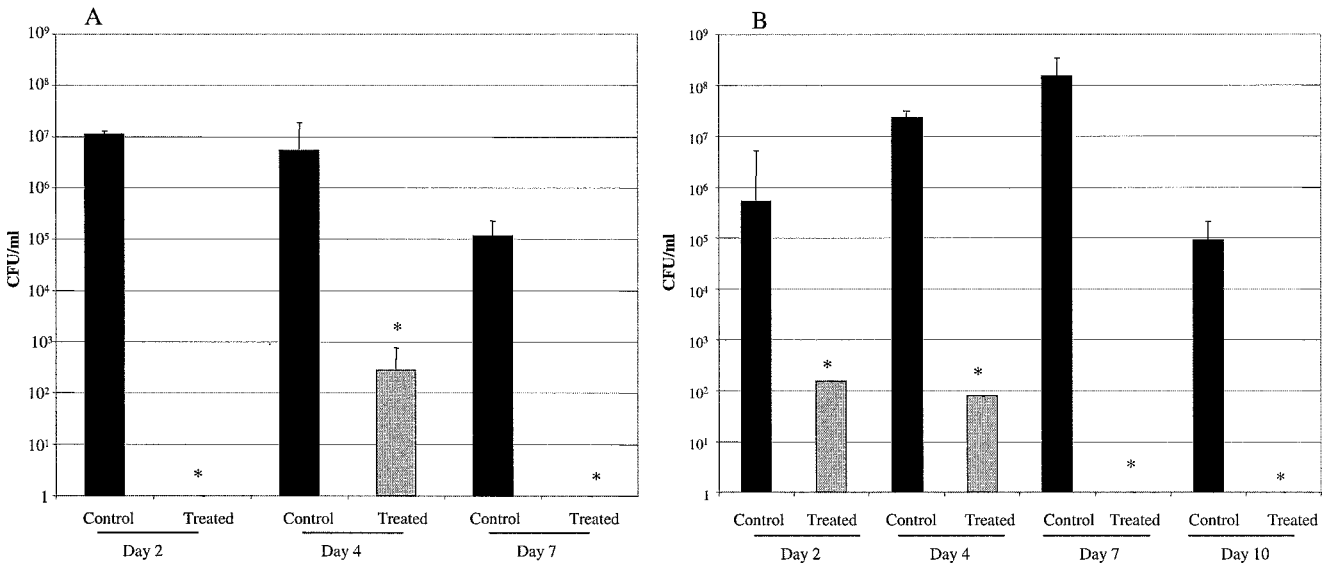

FIG. 10. Effect of in vivo treatment with pBD-1 on the bacterial load in the lung. (A) At each time point, BAL specimens from two to three piglets per saline-treated group (Control) or pBD-1-treated (Treated) group were plated onto BG agar plates to determine the number of viable bacteria within the BAL fluid. Data are expressed as the means ± SEM. $*P < 0.001$. (B) Macroscopically altered tissues were collected, weighed, homogenized, and plated onto BG plates to determine bacterial counts. Data are expressed as the means ± SEM. $*P < 0.005$.

tions of 5 and 10 μg/ml, it exhibited some inhibitory effect against *B. pertussis* in the same period of time ($P < 0.001$) (Fig. 11). However, hBD-2 (20 μg/ml) was not able to eliminate 100% of the bacteria even after 4 h of incubation (data not shown). These results suggest that both *B. pertussis* and *B. bronchiseptica* have evolved in their respective host environments and that both have developed strategies to evade the innate immune defense. Thus, the development of potential resistance against HDPs represents a challenge for the development of future innate immune treatments. Clearly, more studies are required to determine the mechanisms of resistance.

In summary, our results demonstrate that the presence of pBD-1 was associated with complete protection against respiratory infection with *B. pertussis*. Furthermore, these results demonstrate that pathogens may have developed novel strategies for overcoming recognition by the innate immune system in their natural host environments.

DISCUSSION

Pertussis represents an important respiratory disease of infants and young children and still accounts for significant numbers of morbidity and mortality worldwide. Here, we show that the presence of pBD-1, a porcine defensin homologue of hBD-2, was associated with protection against infection with *B. pertussis*. Our results demonstrate the great potential of HDPs as an alternative to antibiotics for the treatment of life-threatening infections in young children and infants. Moreover, our results demonstrate the importance of innate immunity in the pathogenesis of pertussis.

In contrast to newborn piglets, older piglets were fully protected against infection with *B. pertussis* and, in fact, very rapidly cleared infection with 5×10^9 CFU of *B. pertussis* within 24 h. These results clearly demonstrated the importance of innate immunity for protecting the pig against infection with *B. pertussis*. Similarly, adult humans or mice typically display much milder clinical symptoms of pertussis than young infants or neonatal mice, which often succumb to an infection. Thus, a mature and fully developed innate immune system seems to be essential for surviving infection with *B. pertussis*. Indeed, additional evidence to support the importance of innate immunity came from recent studies using mice, which demonstrated that the secretion of gamma interferon at the upper respiratory surfaces following infection with *B. pertussis* was responsible for confining the bacteria to the lungs and the subsequent activation of infected macrophages (51). Furthermore, complement-mediated killing and neutralization by antimicrobial peptides and surfactant proteins A and D (pulmonary collectins) are thought to represent important mechanisms for preventing infections with bordetellae (17, 38, 43). Although most of these studies were performed in vitro, together, they indicate the importance of innate immunity in confining and preventing infections with *B. pertussis*. However, the effectiveness of innate immunity is often restricted by the development of bacterial resistance. For example, the *B. pertussis* lipopolysaccharide molecule is thought to shield bacteria from neutralization by the pulmonary collectins Sp-A and Sp-D or complement-mediated killing (38, 43). Thus, more studies are required to determine the relative importance of innate immunity against pertussis.

In the present study, we demonstrated that protection was associated with the presence of pBD-1 at the upper respiratory surfaces. pBD-1 was previously described to be constitutively expressed throughout various porcine tissues (61). However, infection with *B. pertussis* induced a significant up-regulation of pBD-1 in the lungs of newborn piglets, which is consistent with observations made previously by Sang et al. (41) that indicated that pBD-1 may in fact represent an inducible defensin. In the present study, pBD-1 transcripts were detected in tongue, in-

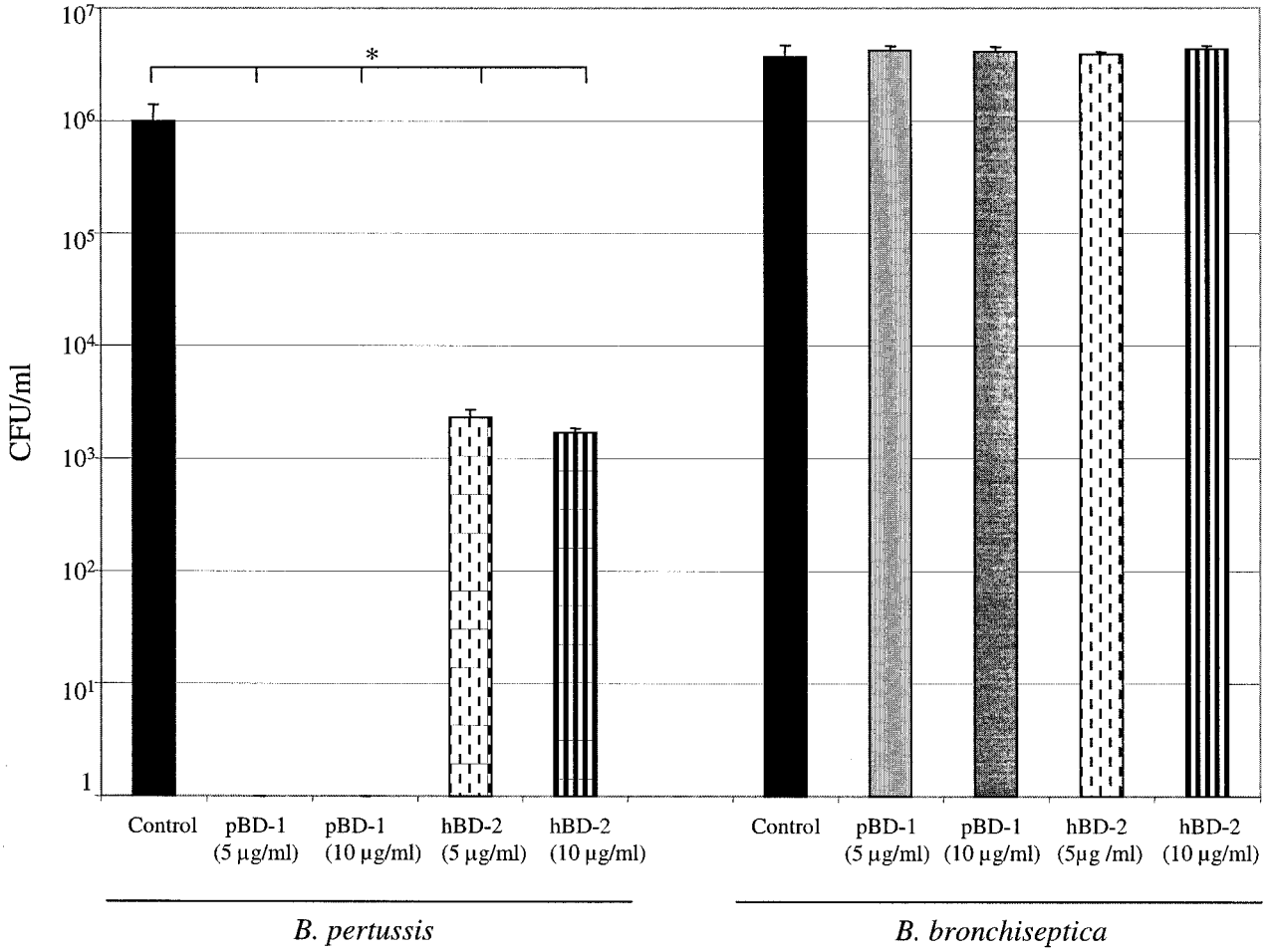

FIG. 11. Susceptibility of *B. pertussis* and *B. bronchiseptica* to pBD-1 and hBD-2. A total of 5×10^6 to 7×10^6 CFU/ml of *B. pertussis* and *B. bronchiseptica* were exposed to 5 and 10 μg/ml of pBD-1 (gray columns) and 5 or 10 μg/ml of hBD-2 (hatched column) or grown in SS medium (black column) for 1 h. The number of bacteria were determined by plate counts. *, $P \leq 0.0001$. Data are presented as the means ± SEM.

testine, nasal mucosa, trachea, and lung tissues of older piglets. In contrast, in newborn animals, pBD-1 gene expression was restricted to the tongue epithelium, with no transcripts detected in the upper respiratory tract. This observation was confirmed by RP-HPLC analysis, which revealed the presence of pBD-1 only in BAL specimens collected from older animals. This suggested that lower levels of pBD-1 contributed to the increased disease susceptibility of newborn piglets. Indeed, differential regulation of HDPs has been correlated with various disease outcomes in several disease models. Whereas upregulation of HDPs was thought to represent a marker for protection, down-regulation or even the absence of HDPs often leads to full development of disease (23, 34). For example, murine beta-defensin 1-deficient mice demonstrate a delayed clearance of *Haemophilus influenzae* from the lungs following respiratory infection (23, 32, 34). Moreover, human patients with alpha-defensin deficiency or lower levels of histatin peptides suffer from recurrent and severe bacterial and fungal infections (1, 23). Thus, our results are consistent with the concept that secreted HDPs constitute the first line of defense against respiratory pathogens (45).

HDPs have a variety of immunological functions, with direct antimicrobial activity being only one of them. For example, HDPs act as signaling molecules that can activate host cellular processes that are involved in immune defense as well as wound healing (9, 20, 24). It is becoming more evident that mammalian HDPs have strong immunostimulatory and immunomodulatory activities, including the ability to stimulate chemotaxis of immature DCs and T cells, activation of antigen-presenting cells, and induction of glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells (7, 17, 36, 56, 57). Thus, HDPs appear to represent an important link between innate and acquired immunity and are potent immune modulators and adjuvants for vaccines. In addition to the large number of in vitro studies, many studies have demonstrated the therapeutic potential of HDPs for treating infectious diseases in vivo. Typically, in these studies, HDP expression levels were increased either by injection of recombinant or synthesized HDPs or by transgenic overexpression of the HDP. For example, intratracheal instillation or systemic injection of LL-37, a peptide derived from the human cathelicidin hCAP-18, resulted in a significant reduction of the bacterial load and inflammatory response and improved survival rates following

pulmonary challenge with *Pseudomonas aeruginosa* (5). Similar results were obtained following intravenous injection with *Escherichia coli* (5). In sheep, pulmonary administration of the sheep macrophage antimicrobial peptide 29 (SMAP-29) resulted in protection against acute pneumonia caused by *Mannheimia hemolytica* (10). Furthermore, transgenic expression of hBD-5 in mouse intestinal Paneth cells led to protection against a lethal challenge with *Salmonella enterica* serovar Typhimurium (40). The possible mechanism by which pBD-1 confers protection against *B. pertussis* in the present study is not yet clear. First, in view of the very small amounts of pBD-1 (only 500 μg), it is extremely unlikely that the observed effects can be explained solely by a direct antibacterial effect of deposited pBD-1. Second, by changing the pulmonary tube between injections, we ensured that pBD-1 and bacteria did not come into direct contact before delivery into the lung. Third, preliminary studies of mice following intranasal deposition of pBD-1 indicated the recruitment of neutrophils and mononuclear cells around bronchioles and into the airways as early as 6 h after deposition (data not shown). These observations suggest that leukocytes that have been recruited as a result of pBD-1 treatment may clear the local infection. In agreement with this view, Welling et al. previously demonstrated in an experimental peritoneal and thigh muscle bacterial infection model that human neutrophil peptide 1 displayed marked in vivo antibacterial activity in mice and that this activity appeared to be mediated by local leukocyte accumulation (55). Also, preliminary studies in our laboratory using porcine PBMCs and lymph node cells demonstrated up-regulation of typical Th1-type cytokines such as IL-12 and gamma interferon after in vitro stimulation with pBD-1 (data not shown). Moreover, pretreatment with pBD-1 4 h prior to challenge infection resulted in smaller lesions and significantly lower bacterial numbers. pBD-1 significantly diminished the number of *B. pertussis* CFU recovered from treated animals compared with those recovered from saline-treated piglets. These findings indicate that protection was mediated, at least in part, by stimulation of the innate immune response. In this particular study, however, lower bacterial numbers were recovered from saline-treated animals than in previous studies, which might be due to the repeat anesthesia and intubation procedures. Further studies are required to explain whether stress related to these complex procedures can reduce the number of bacteria in the lung. Our findings suggest that protection of pigs against *B. pertussis* may be mediated by a combination of mechanisms including direct antimicrobial activity as well as immunostimulatory activity. Further studies are required to determine the optimal peptide concentration and time point to enhance the innate immune response against infection.

B. pertussis and *B. bronchiseptica* are respiratory tract pathogens that are derived from the same ancestor. However, while *B. pertussis* is an obligate human pathogen that also infects mice and pigs under experimental conditions, *B. bronchiseptica* has a broader host spectrum that includes birds, mammals, and immunocompromised people (21). Interestingly, although closely related, both pathogens exhibited a very different pattern of resistance against pBD-1 and hBD-2. While *B. bronchiseptica*, a pathogen of pigs and humans, was fully resistant to both pBD-1 and hBD-2, *B. pertussis* was highly susceptible to neutralization by pBD-1 but not fully resistant to hBD-2. Thus, these results indicate that these two bacteria have evolved in their respective microenvironments and have developed mechanisms to overcome recognition by the respective innate immune systems. This observation clearly challenges the understanding of HDPs, which suggests that neutralization of bacteria occurs in an unspecific fashion. Furthermore, this observation is of great importance for future development of HDP-based immune therapeutics, since it demonstrates that AMP activity may be relatively restricted to individual bacteria. To address this hypothesis, we are currently testing a number of bacterial mutants that display altered lipopolysaccharide molecules in order to identify the interactions between pBD-1 and the bacterial membrane.

In summary, we demonstrated that in vivo treatment with pBD-1 conferred protection against respiratory infection with *B. pertussis* in newborn piglets. Our results therefore demonstrate the importance of innate immunity against pertussis and indicate that HDPs represent an alternative to antibiotics. Moreover, our results also indicate that the mechanisms of protection may be a combination of both direct antimicrobial activity and immunostimulatory activity and that bacteria evolve to the specific microenvironments in their respective hosts.

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