Maternal Immunity Provides Protection against Pertussis in Newborn Piglets†

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Pertussis continues to be a significant cause of morbidity and mortality in infants and young children worldwide. Methods to control the disease are based on vaccination with either whole-cell or acellular vaccines or treatment with antibiotics. However, despite worldwide vaccination infants are still at the highest risk for the disease. Here we used our newly developed newborn-piglet model to investigate whether transfer of maternal immunity can protect newborn piglets against infection with Bordetella pertussis. Pregnant sows were vaccinated with heat-inactivated B. pertussis or treated with saline (controls). Newborn piglets were allowed to suckle colostrum and milk for 4 to 5 days before they were challenged with 5 × 10³ CFU of bacteria intrapulmonarily. Elevated levels of B. pertussis-specific secretory immunoglobulin A (S-IgA) and IgG antibodies were found in the colostrum and serum of vaccinated sows but not in those of control sows. Subsequently, significant levels of specific IgG and S-IgA were detected in the serum and bronchoalveolar lavage fluid of piglets born to vaccinated sows. Following infection with 5 × 10⁷ CFU of B. pertussis, clinical symptoms, pathological alterations, and bacterial shedding were significantly reduced in piglets that had received passively transferred immunity. Thus, our results demonstrate that maternal immunization might represent an alternative approach to provide protection against pertussis in young infants.

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spring and subsequently are transported to the mucosal surfaces via a comparable pathway. Moreover, because in pigs all mucosal compartments are accessible, we utilized our newly developed model of pertussis to investigate whether maternal immunity can be an alternative approach to reduce the vulnerability of young infants to the disease. The present study was undertaken to determine the role of maternal immunity in piglets challenged with B. pertussis.

MATERIALS AND METHODS

Bacterial cultures. Bacterial suspensions of strain Tohama I were stored at −70°C in Casamino Acids plus 10% glycerol. Organisms were initially grown on the surface of Bordet-Gengou (BG; Becton Dickinson & Co.) agar containing 15% (vol/vol) defibrinated sheep blood and 40 μg/ml cephalxin (Sigma-Aldrich) at 37°C for 48 h. After incubation, heavy inocula of bacteria were transferred to Stainer-Scholte (SS) medium and grown aerobically at 37°C for 48 h either as liquid cultures at 250 rpm in a Thermo Forma Shaker or as BG agar plate cultures. Bacteria were harvested from puddle plates by scraping off and resuspending bacteria in SS medium. Bacteria were collected by centrifugation at 4,500 × g for 10 min. The pellets were resuspended in phosphate-buffered saline without Mg2+ and Ca2+ (PBSA; pH 7.2) and adjusted to the indicated optical density (OD) at 600 nm with a spectrophotometer (Ultrospec 3000; Pharmacia Biotech). The bacterial suspension (adjusted to 50% from liquid culture and 50% from BG agar plates) was kept on ice until it was used for the challenge. The corresponding viable counts of these suspensions were determined by plating serial dilutions of the bacterial suspension onto BG agar plates and incubation at 37°C for 4 to 5 days.

Animals. Pregnant Landrace sows were purchased from the Skatooz Prairie Swine Centre, University of Saskatchewan. Sows were induced to farrow by intramuscular (i.m.) injection of prostaglandin (Placent; Schering, Quebec, Canada) at day 113 of gestation. Piglets were born at day 114 or 115 of gestation. Nursing piglets were kept within the same room but in separated pens and monitored very closely. The piglets were challenged at 3 to 5 days of age. All experiments were carried out in a double-blind manner. Studies were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

Collection of samples. Colostrum and milk samples were collected, and the solid fraction was removed by adding rennet tablets to a final concentration of 125 μg/ml. Samples were stirred and incubated for 3 to 4 h at 37°C to clot formation. In order to collect the whey, samples were centrifuged at 2,000 × g for 20 min. This resulted in the formation of three layers, a top layer (fat), a middle layer (whey), and a bottom solid layer. The middle layer was carefully removed and stored at −20°C until used. Sows were bled prior to priming, boosting, and farrowing. Newborn piglets were bled before suckling, at the time of challenge, and then challenged intrapulmonarily with 5 × 109 CFU of pertussis. Since intestinal lymphocytes populate the mammary gland at the end of pregnancy, the ability of young infants to the disease. The present study was undertaken to determine the role of maternal immunity in piglets challenged with B. pertussis. The lungs were removed, BAL fluid samples were taken (in 15 ml of SS medium), and sections of the lung were fixed in 10% buffered formalin and processed as previously described (14). Histopathological changes including hemorrhagic necrosis, bronchopneumonia, and pleuroneumonia were evaluated on a scale indicating moderate, severe, and moderate, respectively. Furthermore, to compare the pathological alterations in the lung, lesions were excised and weighed and compared to the total weight of the lung.

Quantification of bacteria from the lungs. The lungs were removed following euthanasia, and the extent of pathological changes was monitored macroscopically. BAL fluid was obtained by filling the lungs with 15 ml of SS medium and withdrawing as much fluid as possible (this procedure was performed once). To quantify the presence of B. pertussis in the BAL fluid, fluid samples were centrifuged at 310 × g for 10 min to remove debris and host cells; supernatant and dilutions thereof were plated onto BG agar plates in duplicate 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.

B. pertussis-specific ELISA. Polystyrene microtiter plates (Immulon 2 HB; Dynex Technologies, Chantilly, VA) were coated with 2 μg/ml (100 μl per well) sonicated, heat-killed B. pertussis or B. bronchiseptica and incubated with serially diluted BAL fluid, serum, colostrum, or milk whey. Alkaline phosphatase-conjugated goat anti- mouse immunoglobulin G (IgG; 1:5,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used to detect B. pertussis-specific IgG. Mouse anti-pig IgA (1:300 dilution; Serotec) was used to detect B. pertussis-specific IgA in samples. The reaction was amplified with biotinylated goat anti-mouse IgG (1:5,000 dilution; Zymed). Detection was carried out with streptavidin peroxidase (1:5,000 dilution; Jackson Laboratories), and the reaction was visualized with p-nitrophenylphosphate (Sigma-Aldrich). B. pertussis-specific antibody titers were determined by Microplate Manager 5.0 (Bio-Rad Laboratories) with the assay read at 450 nm by a microplate reader (Bio-Rad Laboratories).

Statistical analysis. All data from this study followed nonnormal distributions. To account for this outcome, data were ranked and then either analysis of variance (ANOVA) or Student’s t test was 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. There were more than two experimental groups in the analysis and the ANOVA was significant, the means of the ranks were compared with Tukey’s test. Categorical outcomes (cough, evidence of clinical symptoms) were compared between experimental groups with Fisher’s exact test. Probabilities less than or equal to 0.05 were considered significant.

RESULTS

Three independent animal experiments were performed to analyze the role of maternal antibodies in protection against infection with B. pertussis (Table 1). Four pregnant sows were vaccinated 4 weeks prior to farrowing and boosted at 2 weeks prior to farrowing. Control sows were treated with PBSA. Antibodies

| Table 1. Summary of vaccination and challenge experiments |
| Expt and treatment (no. of sows) | No. of piglets | No. with cough/total | No. with clinical symptoms/total |
| I | Vaccination (1) | 9 | 4/9b | 4/9b |
| Vaccination (1) | 9 | 2/9b | 3/9b |
| Control (1) | 9 | 9/9 | 9/9 |
| II | Vaccination (1) | 11 | 4/11b | 3/11b |
| Control (1) | 11 | 11/11 | 11/11 |
| III | Vaccination (1) | 9 | 2/9b | 3/9b |
| Control (1) | 11 | 11/11 | 11/11 |

b Clinical symptoms were monitored twice daily. Piglets from vaccinated mothers displayed only mild respiratory symptoms or slight rises in body temperature. In contrast, piglets born to control sows displayed fever and severe respiratory symptoms such as nasal discharge, breathing difficulties, and coughing.

P < 0.004.

P < 0.002.

P < 0.0005.
were measured in sow serum and colostrum. Piglets were bled prior to suckling colostrum and then bled again prior to challenge infection at 4 to 5 days of age.

**B. pertussis-specific antibodies in serum and colostrum of vaccinated sows.** To examine the induction of specific antibodies in sow serum following vaccination, serum was collected prior to priming, boosting, and farrowing. Significantly high levels of *B. pertussis*-specific antibodies, of both the IgG and IgA isotypes, were detected in serum from vaccinated sows prior to farrowing (*P* < 0.0001) but not in serum from nonvaccinated sows (Fig. 1). Colostrum was collected after farrowing and analyzed for the presence of *B. pertussis*-specific secretory IgA (S-IgA) and IgG antibodies by enzyme-linked immunosorbent assay (ELISA). All four vaccinated sows displayed significantly elevated levels of colostral *B. pertussis*-specific S-IgA and IgG (*P* < 0.03), whereas nonvaccinated control sows displayed either no or very low levels of nonspecific antibodies in their colostrum (Fig. 2).

**Maternally derived antibodies in serum and BAL fluid of newborn piglets.** The presence of IgA and IgG antibodies was assessed in serum from newborn piglets prior to suckling colostrum and at 4 to 5 days of age prior to challenge infection. As shown in Fig. 3, piglets born to vaccinated sows had significantly elevated levels of both IgA and IgG serum antibodies (*P* < 0.0001). In contrast, serum from piglets born to nonvaccinated control sows did not contain detectable levels of antibodies against *B. pertussis*. However, we noticed that a few animals born to vaccinated sows already contained specific antibodies in their serum, which most likely was a result of the animals being able to suckle and absorb colostral antibodies before they were bled. However, none of the piglets born to control sows had detectable levels of antibodies in their serum. In contrast, all piglets born to vaccinated sows had significant levels of *B. pertussis*-specific serum antibodies, even at the time of euthanizing (2, 4, or 7 days postchallenge; data not shown).

The existence of both antibody isotypes was also assessed in BAL fluids obtained from newborn piglets at 2, 4, and 7 days postchallenge. Piglets born to vaccinated sows had significantly higher levels of both S-IgA and IgG antibodies in their BAL fluids at days 2 and 4 postchallenge (*P* < 0.004) (Fig. 4). In contrast, no IgG antibody was detectable in BAL fluid from piglets born of nonvaccinated control sows at these times. However, there was some detectable nonspecific IgA in the BAL fluid from control piglets (days 2 and 4). Thus, these results demonstrate that maternally derived antibodies were detectable in serum and BAL fluid from piglets born to vaccinated sows.

**Maternal immunization provided protection against challenge infection.** Newborn piglets of both groups were challenged with 5 × 10⁷ CFU of live bacteria at 4 to 5 days of age. Whereas all piglets born to control sows displayed severe clinical symptoms including elevated body temperature, nasal discharge, nonparoxysmal cough, and breathing difficulties, significantly lower numbers of piglets born to vaccinated sows showed cough and clinical symptoms (*P* ≤ 0.004, Table 1). Moreover, clinical symptoms in these animals were much milder, with only slight increases in body temperature and mild respiratory symptoms (*P* ≤ 0.003). In contrast, all animals born to nonvaccinated sows showed severe clinical symptoms as early as 2 days postchallenge. Postmortem investigation on days 2, 4, and 7 revealed severe pathological
alterations, such as hemorrhagic and necrotizing bronchopneumonia, in these animals (Fig. 5A and C). In contrast, pathological alterations in piglets born to vaccinated sows were either absent or significantly reduced in size \( P < 0.0001 \); Fig. 5B and D and 6).

Thus, maternal immunization clearly provided newborn piglets protection against infection with \( B. \) pertussis.

Maternal immunity reduced the bacterial load in the lungs of infected piglets. Three or four piglets per group were eu-

![Graph A](image1.png)

**FIG. 2.** Titers of antibody against \( B. \) pertussis in sow colostrum. (A) Levels of \( B. \) pertussis-specific colostral IgA from four sows vaccinated with whole, heat-inactivated bacteria. (B) Levels of \( B. \) pertussis-specific colostral IgG from four vaccinated sows. Control animals were vaccinated with PBSA instead. ELISA results are shown for four different sows in three separate experiments, with each bar representing one sow. \( *, P < 0.03 \) versus control group.

![Graph B](image2.png)

**FIG. 3.** Passive transfer of specific antibodies via colostrum. Antibody titers were measured in sera of piglets prior to suckling and at the time of challenge (4 to 5 days old). (A) Levels of \( B. \) pertussis-specific serum IgA in piglets born to vaccinated sows compared to piglets born to nonvaccinated control sows. (B) Levels of \( B. \) pertussis-specific serum IgG in piglets born of vaccinated sows compared with control piglets. The results shown are the means \( \pm \) the standard errors of the antibody titers detected by ELISA. Each bar represents piglets born to one sow. \( *, P < 0.0001 \) versus control animals at the time of challenge.
thanized at days 2, 4, and 7 postchallenge (14). The lungs were removed following euthanasia, and BAL fluid was obtained by filling the lungs with 15 ml of SS medium. The numbers of bacteria in the BAL fluid (Fig. 7A) and lung lesions (Fig. 7B) were determined over a period of 7 days. The number of isolated bacteria in the BAL fluid collected from piglets born to vaccinated sows was significantly lower \((P < 0.05)\) at days 2, 4, and 7 after challenge infection compared to BAL fluid from piglets born to control sows. In addition, significantly lower numbers \((P < 0.0001)\) of bacteria were found in homogenized lung tissues from piglets born to vaccinated sows compared with control animals in the same time period.

DISCUSSION

In the present study, we demonstrated the contribution of maternal immunity in providing newborn piglets protection against \(B.\) \textit{pertussis} disease. Despite decades of intensive vaccination, pertussis continues to pose a real threat to public health worldwide. Current vaccination strategies fail to protect newborns and as a result neonates are at the highest risk for infection. Here we show that maternal immunization might represent an alternative strategy for protecting the very young. Piglets born to vaccinated sows exhibited strong protection against infection with \(B.\) \textit{pertussis}, and in fact, most of these animals did not develop clinical symptoms or lung lesions when challenged with \(5 \times 10^9\) CFU of \(B.\) \textit{pertussis}. Thus, transfer of passive immunity clearly has the potential of providing newborns complete protection against pertussis.

Neonates are highly susceptible to a variety of infectious diseases, including pertussis (6, 40). Several factors, including the immaturity of lung defenses and deficiencies in APCs, complement, and Th1-type cytokines, as well as impaired IgG
isotype switching, are thought to be responsible for the higher susceptibility to disease (6, 40). Providing passive protection to infants by immunizing mothers could overcome some of these problems and therefore bypass the problems of immunological immaturity in the neonate, the need for active immunization of the infant within the first months of life, and transmission of infectious diseases to other infants (13, 16, 19). Thus, until the neonatal immune system is fully established, maternal immunization could protect the neonate against infections or at least modify the severity of infectious diseases for various periods of time (40, 49).

Maternally derived immunity consists of many components, including antibodies that are transmitted either transplacentally or via colostrum and milk. In pigs, in contrast to humans, immunoglobulins are not transmitted in utero, which makes colostrum and milk the only source of antibodies and therefore limits the use of this model for analyzing passive transfer of immunity. Furthermore, since pigs are outbred animals studies to determine the cytotoxic immune response are more complicated to perform. However, cytokine expression in BAL fluid, colostrum, and serum can be easily measured by ELISA and real-time PCR. In the present study, specific anti-\textit{B. pertussis} antibodies were detected in the colostrum and milk of vaccinated sows after immunization with heat-inactivated bacteria. Subsequently, antibodies were detected in serum and BAL fluid from piglets born to vaccinated sows. Antibody titers in the BAL fluid dropped within the first 7 days postinfection, which suggests that the passively transferred antibodies were used up by the bacterial antigen present after the challenge infection. However, it was difficult to establish a quantitative correlation between specific antibody levels in either serum or BAL fluids and protection in terms of reduced lesion size and early clearance of bacteria from the lungs. This is consistent with other studies (10, 45), and in fact, difficulties still exist not only in defining quantitative serological correlates of protection (18, 20, 45) but also in determining the exact role of antibodies in disease protection. In the murine model, B cells appeared to be critical for the resolution of a primary infection with \textit{B. pertussis}, suggesting that antibodies play a crucial role in the clearance of bacteria (34). Furthermore, several studies have demonstrated that passive transfer of \textit{B. pertussis}-specific antibodies induced protection against infection (21, 37, 41, 43, 44). Similarly, anti-pertussis toxin (Ptx), anti-fimbrial (fim2 and fim3), and anti-pertactin (Prn) antibodies have been correlated with clinical protection in humans (10, 26, 45). In agreement, pre-convalescent-phase serum from mice infected with \textit{B. pertussis} and S-IgA were shown to play an important role in inhibiting the attachment and/or colonization of bacteria (50).
Together, these findings indicate that antibodies are required for the resolution of a primary infection with *B. pertussis*. However, Leef et al. (32) also reported protection against an aerosol *B. pertussis* challenge in the absence of *B. pertussis*-specific antibodies and transfer of serum from convalescent *B. pertussis*-infected mice to normal adult mice, followed by an aerosol challenge, had a minimal effect on the early growth of *B. pertussis* in their lungs (35). Thus, these examples demonstrate that the exact role of antibodies in host protection and their required absolute values for protection are still not fully understood.

In the present study, protection was related to the presence of antibodies in colostrum from vaccinated sows. However, other mechanisms whereby maternal immunity confers protection may exist and require further investigation. It is very likely that maternally derived antibodies in the BAL fluid of piglets from vaccinated sows interfered with the initial adherence of *B. pertussis* to pulmonary and tracheal epithelial cells, as well as to macrophages (4, 24, 48). In fact, it has been demonstrated that an effective antibody response to filamentous hemagglutinin can inhibit the attachment of the bacteria to the respiratory mucosal surface (7). Furthermore, it is likely that antibodies present in the BAL fluid also increased phagocytosis and subsequent killing of bacteria from the respiratory tract, as similar findings have been described in humans (26, 27, 39). However, more experiments are necessary to address the role of specific antibodies in disease protection and subsequently, if possible, determine the absolute antibody values required for protection. We believe that our model will help to address some of these important questions.

Despite the controversy about the absolute antibody values that are required for protection, it is widely accepted that immunity to *B. pertussis* is mediated by a combination of both humoral and cell-mediated immunity. In particular, CD4⁺ T cells and the presence of gamma interferon seem to be important for the clearance of bacteria from the lung (31). Furthermore, B cells can also represent a source of cytokines and chemokines, which in turn can exert a critical regulatory effect on APCs and CD4⁺-T-cell-mediated immunity (5, 17, 32).

Although we have not directly addressed the role of cell-mediated immunity in the present study, we believe that a combination of both humoral and cell-mediated immunity is required for optimal disease protection. Indeed colostrum contains a variety of other factors, including CD14, cytokines, chemokines, and cells such as neutrophils, monocytes, and lymphocytes (30). It is well established that milk-derived lymphocytes can migrate from the gut into the circulation and lymphoid tissues of the newborn, where they can deliver more specific immune functions (42, 51). In fact, it is possible that these primed immune cells may skew the immune response toward a Th1 response and promote the antibacterial activity of macrophages and neutrophils by stimulating opsonizing antibodies (34, 35, 38). In addition, immunization of the mother may induce cytokine and chemokine up-regulation in mammary glands, which increases the number of lymphocytes within the colostrum and milk transferred to the offspring. Further studies are currently under way to address these important aspects of maternal immunization.

In summary, we demonstrated that maternal immunization could provide an alternative approach for providing protective immunity to the newborn infant. With our porcine model, we were able to demonstrate that passively transferred immunity provided protection against intrapulmonary infection with *B. pertussis*. 

FIG. 7. Maternal immunization reduces the bacterial load in the lungs of newborn piglets. (A) At each time point, three or four piglets from each group were sampled. BAL fluids were collected, diluted, and plated onto BG agar plates to determine viable bacteria within the BAL fluid. (B) Macroscopically altered tissues were collected, weighed, homogenized, diluted, and plated onto BG agar plates to determine viable bacteria. The results are expressed as the mean values ± the standard deviations per lung, as counted from individual lungs of at least three piglets per group from three separate experiments. *, *P* < 0.05 in BAL fluids and *P* < 0.0001 in homogenized tissues versus the control group.
pertussis. However, further studies are necessary to address the issue of immunizing the mother during, before, or even after pregnancy, as well as understanding the role of cell-mediated immunity in disease protection.

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