CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2008, p. 540–543 Vol. 15, No. 3
1556-6811/08/$08.00+0 doi:10.1128/CVI.00466-07

Passive Transfer of Maternal *Mycoplasma hyopneumoniae*-Specific Cellular Immunity to Piglets[▽]

Meggan Bandrick, Maria Pieters, Carlos Pijoan, and Thomas W. Molitor*

Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108

Received 24 November 2007/Returned for modification 15 December 2007/Accepted 2 January 2008

Immunity in the neonatal animal is primarily maternally derived, either by lymphocytes that pass into the newborn across the placenta or following colostrum ingestion. However, the effect of this passively transferred cellular maternal immunity on the newborn's immune repertoire is not clearly understood. Various studies have shown that colostral lymphocytes are activated and possess functional abilities; however, no studies have shown the transfer of colostral antigen-specific T-cell-specific responses in a newborn. In this study we examined the transfer of vaccine-induced *Mycoplasma hyopneumoniae* cellular immunity from immune dams to newborn piglets. Newborn piglets from vaccinated and nonvaccinated dams were assessed in two ways for cellular immune responses specific to *M. hyopneumoniae*: (i) delayed-type hypersensitivity (DTH) testing and (ii) in vitro lymphocyte proliferation, assayed on piglet blood lymphocytes and sow colostral lymphocytes. DTH responses to *M. hyopneumoniae* were detected only for offspring of vaccinated sows, whereas DTH responses to the nonspecific mitogen phytohemagglutinin were seen for all piglets. *M. hyopneumoniae*-specific proliferation was seen for colostral lymphocytes from vaccinated sows and for blood lymphocytes from neonatal piglets of vaccinated dams but not for blood lymphocytes from piglets of nonvaccinated sows. Functional antigen-specific T cells were transferred to offspring from vaccinated sows and participated in the neonatal immune response upon stimulation. These data have implications for defining disease intervention strategies.

The immediate postnatal period is a critical time in the development of young animals' immune systems because it involves a major shift from reliance on innate immunity to adaptive immunity. During this transition period, neonates are protected by passively acquired maternal immunity. In most species, the fetus acquires passive immunity in utero when immune factors cross the placenta. However, some animals, specifically those with epitheliochorial (swine and equine) or hematochorial (bovine) placentation, first receive maternal immunity at birth through colostrum ingestion. Immunomodulatory factors are integral parts of colostrum and include hormones and cytokines, as well as antibodies and a variety of cells (reviewed in reference 22). While an extensive literature exists regarding the immunoglobulin composition of porcine mammary secretions, little attention has been given to colostral cells. There are more than 2×10^6 cells per ml in colostrum, approximately 20% of which are lymphocytes, and an estimated 500 million maternal cells transverse the intestinal epithelium daily (3, 10). Interestingly, the transfer of lymphocytes from colostrum into the circulation of the neonate is ordered, not random, indicating an evolutionary importance of maternal lymphocytes (21, 23). The present study investigated whether the colostrum of vaccinated sows (VS) transfers functional antigen-specific lymphocytes to newborn piglets.

Contributions by studies of different species have made it clear that colostral lymphocytes are phenotypically memory and activated cells and can proliferate following mitogen stimulation in vitro. Transferred colostral cells also have functional ability. Transfer of sensitivity to tuberculin has been demonstrated for human infants from vaccinated mothers by testing delayed-type hypersensitivity (DTH), a T-lymphocyte-mediated response (16). In addition, the transfer of sensitivity to nematode and fungal antigens to neonatal mice born to immunized mothers has been demonstrated (9, 12). Still, humans and mice are exposed to maternal immunity during gestation, and the detection of antigen-specific reactivity at birth may be a result of antigen priming while in utero. Therefore, the responding cells in these reports may be neonatal cells that were primed during gestation and not maternal cells.

To date, no reports have investigated the ability of colostral cells to respond in an antigen-specific manner in the neonate. It is of great interest and practicality to harness the ability of passively transferred antigen-specific cellular immunity because of the potential role maternal cells have in protecting the neonate from disease. We evaluated the ability of passively transferred *Mycoplasma hyopneumoniae*-specific colostral cells to function in a specific manner in the newborn pig. *M. hyopneumoniae* was chosen because of its clinical importance to the swine industry (reviewed in reference 15) and the importance of cellular immunity associated with this infection (1, 13, 18, 20). Newborn piglets are naïve to *M. hyopneumoniae*, and there is no evidence of transplacental infection. Although the use of sow vaccination for *M. hyopneumoniae* is uncommon (6). sow vaccination and antibiotic treatment during lactation results in a significantly lower prevalence of *Mycoplasma*-positive piglets at weaning (14) and subsequent lower prevalence and severity of lung lesions at slaughter (4). *M. hyopneumoniae*-specific antibodies have been demonstrated for blood and milk of sows following vaccination, yet direct evidence for the passive transfer of cellular immunity specific for *M. hyopneumoniae* has not been demonstrated. A greater understanding

* Corresponding author. Mailing address: Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108. Phone: (612) 625-5295. Fax: (612) 625-6241. E-mail: molit001@umn.edu.

▽ Published ahead of print on 9 January 2008.

of the role of maternal cellular immunity in the protection of neonates and potential interference with active immunity could be used to define more-effective disease intervention strategies.

MATERIALS AND METHODS

Animals. All animals were treated in accordance with the University of Minnesota's Institutional Animal Care and Use Committee regulations. Sows were housed at a commercial farm in gestation crates until 1 week prior to farrowing, at which time they were moved to farrowing crates. Food and water were provided ad libitum. At the beginning of the study, 15 sows were randomly assigned to VS or non-VS (NVS) groups. At 5 and 3 weeks antepartum, VS were vaccinated against *M. hyopneumoniae* (Respisure; Pfizer) according to the manufacturer's instructions. Colostrum was collected from all sows at farrowing. Blood was collected from 7 piglets per sow at 0 h for a total of 105 piglets. To ensure that 0-h piglet blood was collected prior to colostrum ingestion, farrowings were monitored and piglets were placed in tubs immediately after birth. Blood was collected from piglets within 30 min of being placed in the tub and then immediately returned to their dams. Piglets were ear notched for identification purposes. Based on previous studies, only piglets that had not been cross-fostered (to ensure maternal lymphocyte transfer into circulations) were included in the 24-h blood collection. Blood was collected from 42 piglets at 24 h, corresponding to at least two piglets per sow (15 total sows).

DTH testing. DTH testing was performed on 20 non-cross-fostered piglets per group at 4 days of age. Concentrated and purified *M. hyopneumoniae* (300 μg/ml in 0.1 ml) antigen was injected intradermally into the left inguinal area (13). Phytohemagglutinin (PHA) (20 μg/ml in 0.1 ml; Sigma, St. Louis, MO) and saline (0.1 ml) were used as positive and negative controls, respectively. Injection sites were clearly marked with livestock paint. Skin fold thickness was measured 24 to 36 h later with calipers. Final DTH lesion values were determined in terms of orthogonal diameter by skin thickness.

Sample collection. Twenty milliliters of colostrum was manually collected from each functional udder in 50-ml conical tubes following alcohol swabbing of teats from all 15 sows. Blood was collected by jugular puncture into sterile heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). A total of 105 piglets were subjected to blood collection at 0 h, and 42 of those piglets were subjected to blood collection again 24 h after suckling. Piglet data were used only if blood samples from before and after suckling were taken.

Antigen preparation. *M. hyopneumoniae* was cultured in Friis' medium. At passage 15 and when the pH of the culture reached 6 or lower, the organisms were harvested by continuous-flow centrifugation at 70,000 × *g*. The harvested *M. hyopneumoniae* was resuspended in Tris-sodium chloride (TN) buffer (pH 7.2 to 7.4) in 1/100 of the original volume of the culture and washed three times by centrifugation, each with the same proportion of TN buffer. The washed *M. hyopneumoniae* was inactivated by one freeze-thaw cycle and then by sonic disruption. *M. hyopneumoniae* was solubilized with 1% NP-40 and the antigen concentration was adjusted to 4 mg/ml.

Lymphocyte stimulation. Colostrum was diluted 1:3 with sterile phosphate-buffered saline (PBS) to decrease viscosity and then washed three times prior to lymphocyte isolation. Piglet blood was diluted 1:1 in sterile PBS to improve cell recovery yield. Colostral and blood lymphocytes were isolated by Ficoll density centrifugation as described previously (10, 2). Cells were stained with the membrane stain carboxy fluorescein succinylimidyl ester (CFSE) (ICT, Bloomington, MN) to evaluate antigen-specific proliferation (11). CFSE was added to cells in PBS and allowed to incubate for 20 min at room temperature in the dark. Cells were washed two times with RPMI supplemented with 10% fetal bovine serum, 100 U penicillin G per ml, and 100 μg of streptomycin per ml and reconstituted in this medium. Viability was deemed to be greater than 97% by trypan blue exclusion. Cells were plated in "v"-bottomed 96-well plates in duplicate at a concentration of 4×10^5 cells per well. Cells were stimulated with 10 μg/ml purified *M. hyopneumoniae* antigen as described previously (20). The *M. hyopneumoniae* antigen used in vitro was the same as that used for in vivo testing but at a different concentration. Nonstimulated cultures served as negative controls; concanavalin A-stimulated cultures (5 μg/ml) served as positive controls. Cells were allowed to incubate for 4 days. Flow cytometry was used to assess lymphocyte proliferation utilizing a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The FL-4 laser was calibrated using the manufacturer's calibration beads. Nonstained, nonstimulated cells were used to establish a baseline for the proliferation assay. Event acquisition was set for 10,000 events in a region encompassing the CFSE-positive quadrants (lower quadrants only). Results were analyzed by CellquestPro software. Proliferation was determined as the mean number of cells proliferating in antigen-stimulated wells minus the mean number of cells proliferating in the nonstimulated wells. Data are expressed in percentages.

Statistics. Data were analyzed using the linear mixed-effect model in the SAS program and means were compared using the least-squares means and honestly significant difference comparison. Data were transformed to $\log_y$ values, but the original means are used in the text and figures.

RESULTS

In order to characterize the in vivo cellular immune response induced by the injection of the nonspecific mitogen PHA and the *M. hyopneumoniae* antigen, the inflammatory response was measured at 1, 24, 36, and 48 h postinjection. Hypersensitivities were not evident immediately postinjection. Hypersensitivity responses were maximal at 36 h postinjection, and the magnitude of the response as measured by orthogonal diameter and skin fold thickness declined by 48 h postinjection. The timing and characteristics of the inflammation confirmed that the responses were DTH responses (data not shown). Since responses were maximal at 36 h postinjection, 36-h responses were compared.

Newborn piglets from both the VS and NVS groups showed DTH responses to the nonspecific mitogen PHA, thus indicating cellular immune competence. No difference in the magnitude of PHA DTH response size was observed among piglets across sow vaccination groups (Fig. 1). One piglet did not respond to PHA. Inflammation was not observed at the saline injection site for any of the neonatal pigs. To investigate the in vivo cellular immune response to *M. hyopneumoniae*, concentrated *M. hyopneumoniae* antigen was injected intradermally into newborn pigs. Previous studies have demonstrated DTH responses to *M. hyopneumoniae* in infected pigs (1, 13), but this is the first to use DTH as a measure to assess passive maternal cellular immune transfer to piglets. Offspring from sows vaccinated with *M. hyopneumoniae* had significantly greater *Mycoplasma*-specific DTH responses (3.1 ± 0.1 mm^2) than offspring from NVS (0.8 ± 0.1 mm^2) ($P < 0.001$) (Fig. 1). It should be noted that one piglet from an NVS did have a detectable DTH response to *M. hyopneumoniae*.

To assess in vitro cellular immune responses, *M. hyopneumoniae*-specific proliferation was measured. Lymphocytes isolated from colostrum of VS proliferated significantly more in response to *M. hyopneumoniae* than colostral lymphocytes from NVS ($P < 0.05$) (Fig. 2). Among lymphocytes isolated from piglet blood, *M. hyopneumoniae*-specific proliferation was observed only from lymphocytes isolated from piglets of VS, and only after suckling ($P < 0.05$). There was no statistical difference between antigen-specific proliferation of colostral lymphocytes and that of lymphocytes isolated from piglets of VS after suckling. Antigen-specific proliferation was not observed from lymphocytes isolated from precolostral pigs of any group.

DISCUSSION

In this study, we have demonstrated for the first time that lymphocytes that are passively transferred from vaccinated dams to their offspring in colostrum are able to proliferate and participate in a functional response to a particular antigen, in this case *M. hyopneumoniae*. Evidence that colostral lympho-

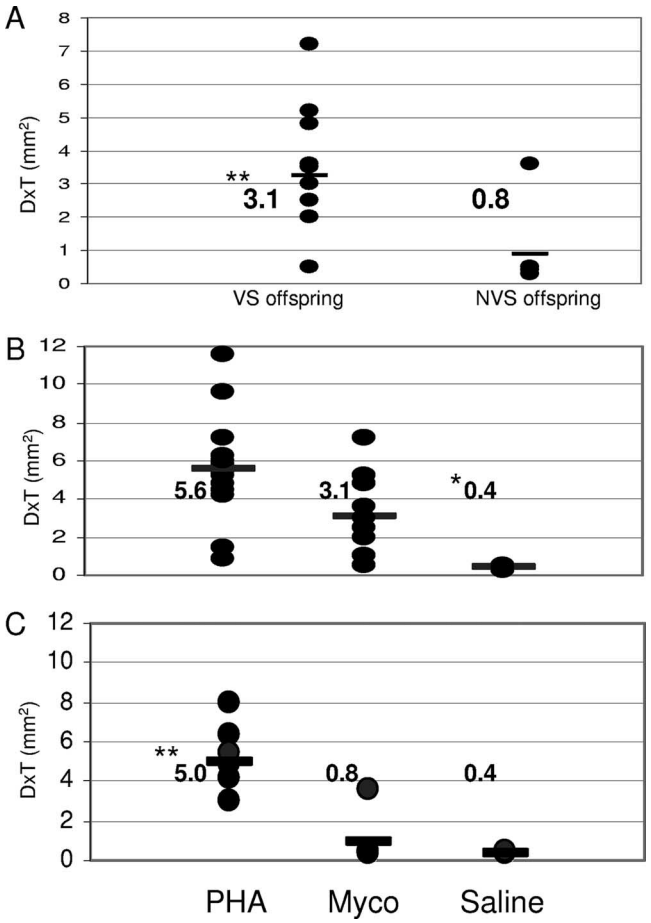

FIG. 1. DTH responses to *Mycoplasma hyopneumoniae* in young pigs. *M. hyopneumoniae* antigen, PHA, or saline was injected intradermally into the left inguinal area of 3- to 5-day-old pigs. Skin fold thickness and lesion size were measured 36 h later with calipers. (A) *Mycoplasma*-specific DTH responses in offspring of VS and NVS. (B) DTH responses in offspring of VS. (C) DTH responses in offspring of NVS. *M. hyopneumoniae*-specific DTH responses were evident only for offspring of VS. All animals responded to the nonspecific mitogen PHA and none responded to saline. * indicates significance at a P value of <0.05; ** indicates significance at a P value of <0.001. DxT, measurement value for orthogonal diameter by skin thickness.

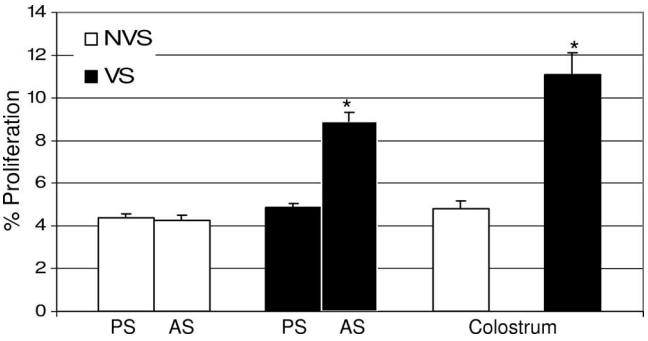

FIG. 2. *Mycoplasma*-specific lymphocyte proliferation. Lymphocytes were isolated from sow colostrum and from piglet blood before (PS) and 24 h after (AS) colostrum ingestion and stimulated with *M. hyopneumoniae* antigen. Average antigen-specific proliferation by lymphocytes isolated from PS and AS piglets and from sow colostrum. Variation is expressed as standard error; * indicates significance at a P value of <0.05.

cytes were transferred into the newborn's circulation and that colostral lymphocytes were functional came from antigen-specific in vivo DTH responses and in vitro lymphocyte proliferation. *M. hyopneumoniae*-specific DTH lesions were found only for offspring of VS. Colostral lymphocytes from VS proliferated in response to stimulation with *M. hyopneumoniae* antigen, and colostral lymphocytes from NVS did not. Lymphocytes isolated from piglets before suckling did not respond to *M. hyopneumoniae* stimulation, indicating that piglets are naïve to *M. hyopneumoniae* at birth. Moreover, lymphocytes isolated from the offspring of VS after suckling proliferated to the same degree as the colostral lymphocytes they received. Antigen-specific DTH responses and lymphoproliferation are by definition secondary immune responses. Since newborn piglets are otherwise naïve to *M. hyopneumoniae*, the DTH responses and lymphoproliferative responses we observed for neonates of VS were due to the action of antigen-specific maternal colostral cells. Therefore, maternal colostral cells transferred into the newborn's circulation and participated in the immune response in an antigen-specific manner.

Infection control in newborns often relies on maternal vaccination with the assumption that maternal immunity is transferred to the newborn and that maternal immunity is protective. Maternal vaccination is more widely practiced than newborn vaccination due to complications of the newborn's maturing immune system and of passive interference. Specifically regarding *M. hyopneumoniae*, several control studies have documented that when sow vaccination is followed by suckling pig vaccination, many piglets do not seroconvert to the vaccine, suggesting interference (5). Further, in most vaccination regimens timing is most often correlated with antibody titer without regard to cellular immunity. In fact many studies have indicated that cellular immunity is important in the response to *M. hyopneumoniae* (1, 13, 18, 20), and there appears to be no correlation between serum antibody level and protection from bacterial colonization or *Mycoplasma* disease (19). The studies reported here are the first to demonstrate a functional response of passively transferred colostral cells in the newborn to *M. hyopneumoniae*.

The transfer of colostral cells with the ability to participate in a functional immune response suggests that passively transferred maternal cellular immunity can affect immune development of the newborn piglet. The impact of passively transferred maternal immunity on the development of the newborn's immune repertoire is not clear; however, the development of adaptive immunity is undoubtedly affected by components of colostrum, notably maternal antibodies. Transferred antibodies are functional and can serve protective roles in the neonate, yet maternal antibodies can also interfere with active immune induction. Studies with rodents suggest that passively acquired cellular immunity is not subject to interference and may be helpful in overcoming the inhibitive effects associated with maternal antibodies in the newborn (17). In addition, colostral cells have been found to modulate the proliferative response of piglet lymphocytes by significantly increasing blastogenesis to pokeweed mitogen, compared to what is seen for piglets not given maternal colostral cells (23).

A common practice in commercial swine production is to cross-foster newborn piglets soon after birth. The timing of cross-fostering practices may limit the number of cells transferred into the neonate and may hinder the immune development of those animals, since colostral cells are transferred into the neonate only if the colostrum is from the piglet's own mother (21, 23). Using technetium-labeled cells (21) and fluorescein-labeled cells (23), two different groups demonstrated that heat-killed cells and cells from a source other than the piglet's own mother do not transverse the intestinal barrier. Thus, the transfer of T cells is dependent on their being viable and maternal in origin. No source or class restriction exists for antibody transfer (8) or for colostral cell transfer in other species, including primates (7). Due to the very probable importance of T cells in neonatal immune development, the timing of cross-fostering practices warrants further investigation.

It should be noted that *M. hyopneumoniae* infection was evident on the farm and that a single positive DTH response from a piglet of the NVS group was observed. This response could be a sensitivity issue; however, no animals in the study responded to saline and all responded to PHA. More likely, the piglet with the positive DTH response was the offspring of a sow that was actively infected with *M. hyopneumoniae*. In an infected sow, the population of *M. hyopneumoniae*-specific transferred cells may be great enough for a specific cell-mediated response to be mounted in the newborn.

ACKNOWLEDGMENTS

The work was performed on a commercial swine farm. We thank the Pipestone Veterinary Clinic and staff veterinarians, Pipestone System producers, and Pipestone System employees for their assistance in this study. We also thank Juan Antonio Aeson, Seth Baker, Jean Paul Cano, Alejandrina Da Silva, Tug Peterson, Alejandro Pieters, Albert Rovira, and Nathan Schafer for assistance with sample collection and Abdoraghman Alghamdi for assistance with statistical analysis. Special thanks to Shi-Jun Ma and Prototek for assistance with the *Mycoplasma* antigen used in this study.

This work was supported by Pfizer, Syngen, and the National Pork Board.

REFERENCES

1. **Adegboye, D. S.** 1978. Attempts to demonstrate cell-mediated immune responses during *Mycoplasma suipneumoniae* infection of pigs. Res. Vet. Sci. **25:**323–330.
2. **Bautista, A. E., M. P. Suarez, and T. W. Molitor.** 1999. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Arch. Virol. **144:**117–134.
3. **Evans, P. A., T. J. Newby, C. R. Stokes, and F. J. Bourne.** 1982. A study of cells in the mammary secretions of sows. Vet. Immunol. Immunopathol. **3:**515–527.
4. **Fano, E., C. Pijoan, and S. Dee.** 2007. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Can. J. Vet. Res. **71:**195–200.
5. **Grosse Beilage, E., and A. Schreiber.** 2005. Vaccination of sows against *Mycoplasma hyopneumoniae* with Hyoresp. Dtsch. Tierarztl. Wochenschr. **112:**256–261. (Abstract.)
6. **Haesebrouck, F., F. Pasmans, K. Chiers, D. Maes, R. Ducatelle, and A. Decostere.** 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet. Microbiol. **100:**255–268.
7. **Jain, L., D. Vidyasagar, M. Xanthou, V. Ghai, S. Shimada, and M. Blend.** 1989. In vivo distribution of human milk leucocytes after ingestion by newborn baboons. Arch. Dis. Child. **64:**930–933.
8. **Klobasa, W. E., and J. E. Butler.** 1981. Regulation of humoral immunity in the piglet by immunoglobulins of maternal origin. Res. Vet. Sci. **31:**195–206.
9. **Kumar, S. N., G. L. Stewart, W. M. Steven, and L. L. Seelig.** 1989. Maternal to neonatal transmission of T-cell mediated immunity to *Trichinella spiralis* during lactation. Immunology **68:**87–92.
10. **Le Jan, C.** 1994. A study by flow cytometry of lymphocytes in sow colostrum. Res. Vet. Sci. **57:**300–304.
11. **Olin, M. R., K. Choi, J. Lee, and T. W. Molitor.** 2007. Morphine modulates gamma delta lymphocytes cytolytic activity following BCG vaccination. Brain Behav. Immun. **21:**195–201.
12. **Rifkind, D., J. A. Frey, E. A. Petersen, and M. Dinowitz.** 1976. Delayed hypersensitivity to fungal antigens in mice. II. Molecular classes in immunogenic RNA extracts that transfer delayed hypersensitivity. J. Infect. Dis. **133:**523–532.
13. **Roberts, D. H.** 1973. Preliminary studies on the cell-mediated immune response in pigs to *Mycoplasma hyopneumoniae*. Br. Vet. J. **129:**427–438.
14. **Ruiz, A. R., V. Utrera, and C. Pijoan.** 2003. Effect of *Mycoplasma hyopneumoniae* sow vaccination on piglet colonization at weaning. J. Swine Health Prod. **11:**131–135.
15. **Ross, R. F.** 1999. Mycoplasmal diseases, p. 495–510. *In* B. Straw, S. D'Allaire, W. Mengeline, and D. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames, IA.
16. **Schlesinger, J. J., and H. D. Covelli.** 1977. Evidence for transmission of lymphocyte responses to tuberculin by breast-feeding. Lancet **ii:**529–532.
17. **Siegrist, C. A.** 2001. Neonatal and early life vaccinology. Vaccine **19:**3331–3346.
18. **Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi.** 1984. Mycoplasma hyopneumoniae infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. Am. J. Vet. Res. **45:**1928–1932.
19. **Thacker, E. L., B. J. Thacker, T. B. Boettcher, and H. Jayappa.** 1998. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. Swine Health Prod. **6:**107–112.
20. **Thacker, E. L., B. J. Thacker, M. Kuhn, P. A. Hawkins, and W. R. Waters.** 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am. J. Vet. Res. **61:**1384–1389.
21. **Tuboly, S., G. Bernath, R. Glavits, and I. Medveczky.** 1988. Intestinal absorption of colostral lymphoid cells in newborn pigs. Vet. Immunol. Immunopathol. **20:**75–85.
22. **Wagstrom, E. A., K. J. Yoon, and J. J. Zimmerman.** 2000. Immune component in porcine mammary secretions. Viral Immunol. **13:**383–397.
23. **Williams, P. P.** 1993. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. Can. J. Vet. Res. **57:**1–8.