Immune Responses to *Mycoplasma bovis* Vaccination and Experimental Infection in the Bovine Mammary Gland

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ABSTRACT

This study characterized the immune responses in four vaccinated and four control cows in response to vaccination and experimental intramammary inoculation with *Mycoplasma bovis*. Specific antibody responses occurred in serum and milk in response to vaccination and experimental infection. Lymphocytes from peripheral blood, but not from the mammary gland of vaccinated cows had increased responsiveness to mitogens. No lymphocytes tested were responsive to *M. bovis* antigen. Both vaccination and experimental infection resulted in skin test reactivity. These results imply that vaccination results in immune responses which may alter the course of experimental *M. bovis* mastitis, but may contribute to cellular inflammation.

RÉSUMÉ

Cette expérience portait sur huit vaches, dont seulement quatre étaient vaccinées contre *Mycoplasma bovis*; elle visait à déterminer leur réponse immunitaire à la vaccination et à une infection intramammaire expérimentale avec le mycoplasme précité. Ces deux interventions déclenchèrent l'apparition d'anticorps, dans le sérum et dans le lait. Les lymphocytes circulants des vaches vaccinées, mais non ceux de leurs glandes mammaires, affichèrent une réponse plus marquée aux mitogènes. Aucun des lymphocytes testés à cette fin ne répondit à l'antigène *M. bovis*. La vaccination et l'infection expérimentale provoquèrent une réaction au test cutané. Les résultats de cette expérience rélèvent que la vaccination se traduit par une réaction immunitaire, susceptible d'altérer le cours de la mammite expérimentale à *M. bovis* et de contribuer à l'inflammation cellulaire.

INTRODUCTION

Mycoplasmal infection causing bovine mastitis is increasing in prevalence and geographic distribution, and is of increasing concern to the dairy industry (1,2). *Mycoplasma bovis*, which causes the most common and most severe form of mycoplasmal mastitis, has been isolated from mastitic udders and bulk tanks during herd outbreaks in many dairy producing areas throughout the world (3). Since there is no effective antimicrobial therapy nor mass screening method for detecting infected animals and/or herds, prophylactic vaccination could prove to be useful in controlling *M. bovis* mastitis (3,4).

There are some indications that prior exposure to *M. bovis* can affect the severity and duration of experimental and natural infection (5-9). This study systematically characterized the humoral and cellular immune responses in vaccinated and control cows in response to vaccination and experimental intramammary inoculation with live *M. bovis* organisms.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Eight Holstein cows in late lactation with confirmed conception dates within three weeks of one another were obtained from a local commercial dairy farm with no prior history of mycoplasmal disease. The cows were managed essentially as on the farm of origin. The cows were monitored for one week before milking was stopped eight weeks prior to the expected parturition date. Experimental treatments and observations were made on all the cows at the same time each day or week. Weeks were numbered from 0-20, beginning with the first experimental treatment at week 0. Experimental intramammary challenge exposure was performed one week after all the cows had calved (Week 12).

EXPERIMENTAL DESIGN

Four cows were selected and numbered (nos. 1-4) for vaccination and four cows were selected and numbered (nos. 1-4) for controls using a random number table. The only randomization was with regard to cow group (vaccinate or control) and number (nos. 1-4, treatment designation). A latin squares design was used for designating quarter vaccination (either real or sham) and experimental challenge exposure (10).

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No systematic difference was noted between unchallenged quarters which had been vaccinated and those which had not been vaccinated for most of the variables under consideration so the data from the four vaccinated cows were analyzed by categorizing the data from all the challenged quarters (infused with killed *M. bovis* antigen or not, n = 8) into one group, and all unchallenged quarters (infused with killed *M. bovis* antigen or not, n = 8) into a second group. Similarly the data from the four control cows were analyzed by categorizing the data from all challenged quarters into one group and all unchallenged quarters into a second group regardless of quarter placebo infusion.

VACCINATION AND SKIN TEST

Mycoplasma bovis strain California 201 was used as vaccine and skin test antigen and for experimental challenge exposure. The serological and biochemical relationships of this isolate with other strains, and the method of preparation have been reported (11,12).

The four vaccinated cows were inoculated with 2 mL of antigen (1 mg formalin killed *M. bovis* protein) in Freund's complete adjuvant at three locations subcutaneously at weeks 0, 2 and 4, and with 3 mL (1 mg formalin inactivated *M. bovis* protein) in designated quarters by intramammary infusion at weeks 6 and 8. These cows were also skin tested at weeks 0, 12 and 19.5 with 0.1 mg *M. bovis* protein in 0.2 mL phosphate buffered saline (PBS) at three locations as previously described (13,14). The four control cows were given a similar placebo inoculation (without *M. bovis* protein) at the same times, but were skin tested with *M. bovis* antigen as above at week 19.5. (Skin test values are expressed as the mean increase in skinfold thickness at the injection sites compared to preinjection measurements). All cows were challenge exposed in designated quarters with 1.5 x 10^6 colony-forming units (cfu) of live *M. bovis* in PBS containing 20% fetal bovine serum at week 12.

SEROTEST

Serum and whey samples were analyzed for *M. bovis*-specific antibodies using the indirect hemagglutination procedure as previously described (15).

PREPARATION OF LYMPHOCYTES FROM MAMMARY GLAND AND PERIPHERAL BLOOD

Mammary gland lymphocytes (MGL) were obtained at weeks 0, 6, 8, 10 and 19 by centrifuging (300 x *g*, 10 min) whole milk collected at the morning milking. The cell pellet was washed twice in PBS, and resuspended to the desired concentration of MGL.

Peripheral blood lymphocytes (PBL) were obtained at weeks 0, 2, 4, 6, 8, 10, 12.5, 15, 17.5 and 19. Twenty milliliters of whole peripheral blood collected with ethylene diamine tetraacetate (EDTA) were diluted with an equal volume of Spinners minimal essential medium (SMEM, Microbiological Associates, Bethesda, Maryland). Ten milliliters of diluted blood were layered over 5 mL Histopaque (Sigma, St. Louis, Missouri) for centrifugation at 400 x *g* for 30 min. The band of cells located between the plasma fraction and the packed erythrocytes was washed twice with SMEM and resuspended to the desired concentration of PBL.

LYMPHOCYTE STIMULATION TEST

The lymphocyte stimulation test (LST) was performed on PBL at weeks 0, 4, 6, 8, 10, 12.5, 15, 17.5 and 19, and on MGL at weeks 0, 6, 8, 10 and 19 as described elsewhere (14,16,17). Briefly, in a 96-well plate, 2 x 10^5 lymphocytes were cultured per well in 250 μL of RPMI-1640 containing 10% fetal bovine serum (Grand Island Biological Co., Santa Clara, California), and 1% of a 10,000 U/mL penicillin + 10,000 μg/mL streptomycin solution (Grand Island Biological Co., Santa Clara, California). The mitogens used were phytohemagglutinin (PHA-Difco, Detroit, Michigan) at 80 μg/mL, conconavalin A (CON A-Pharmacia, Piscataway, New Jersey) at 40 μg/mL, and pokeweed mitogen (PWM-Grand Island Biological Co., Santa Clara, California) at 40 μg/mL. The antigen was *M. bovis* strain 201 (heat killed) at 1 x 10^8 cfu/

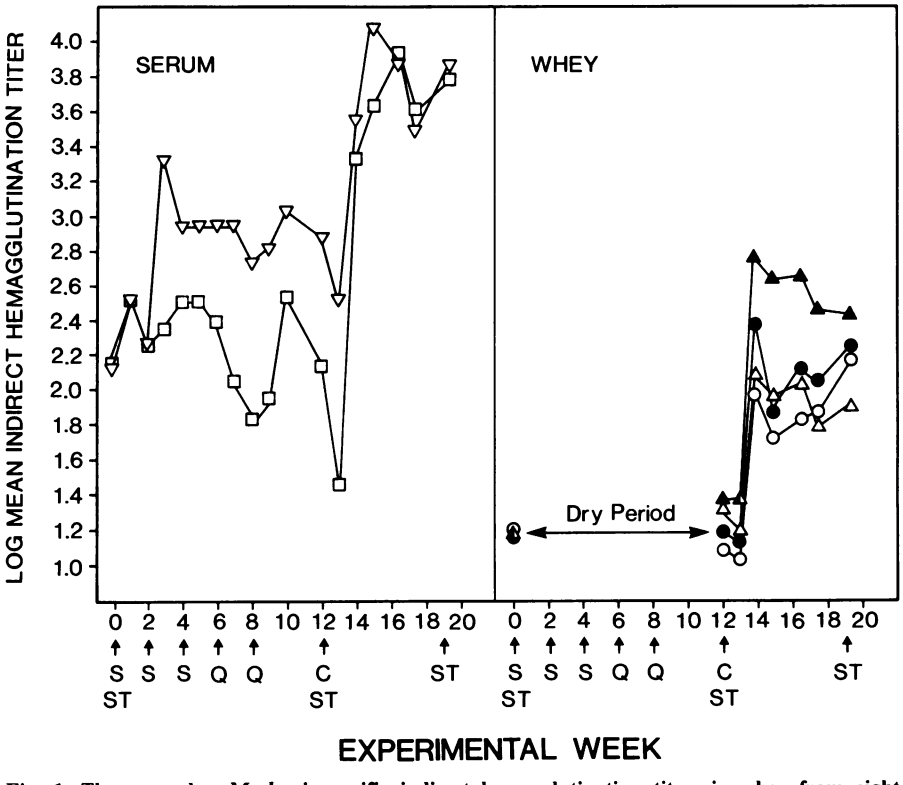

Fig. 1. The mean log *M. bovis*-specific indirect hemagglutination titers in whey from eight unchallenged (△) and eight challenged (▲) quarters, and serum (▽) in four vaccinated cows; and from eight unchallenged (○) and eight challenged (●) quarters, and serum (□) in four control cows experimentally challenged with *M. bovis*. (S = systemic vaccination, Q = quarter vaccination, ST = skin test, C = experimental challenge).

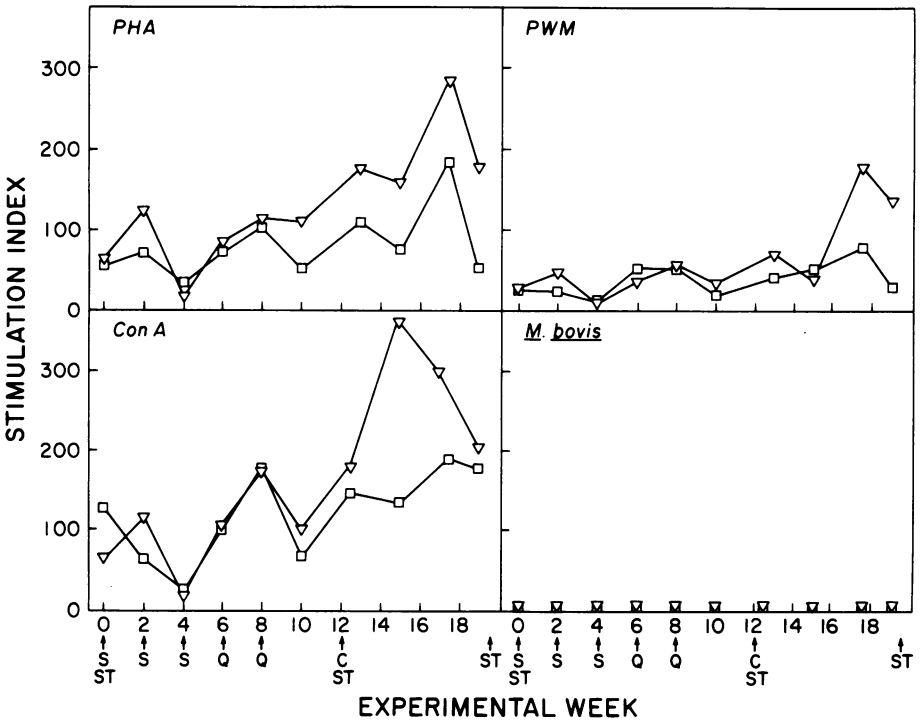

Fig. 2. The mean *in vitro* stimulation by mitogens and *M. bovis* antigen of peripheral blood lymphocytes from four vaccinated (▽) and four control (□) cows experimentally challenged with *M. bovis* (S = systemic vaccination, Q = quarter vaccination, C = experimental challenge, ST = skin test).

mL. Mitogens and antigen were tested in triplicate. Following incubation of lymphocyte cultures at 37°C in a 5% CO_2 atmosphere with mitogen or antigen (72 and 96 h respectively), 25 μL containing 0.3 mCi of ^{3}H-thymidine (specific activity 6.7 Ci/mmol-New England Nuclear, Boston, Massachusetts) were added to each well for an additional 14 h. The cell cultures were harvested and counted in a liquid scintillation spectrometer. The data are expressed in terms of the stimulation index (SI) which represents the ratio of cpm incorporated by mitogen or antigen containing cultures to the cpm incorporated by control cultures.

RESULTS

SPECIFIC ANTIBODY RESPONSE

Vaccinated cows developed measurable, specific, serum antibodies in response to vaccination when compared to control cows (Fig. 1). Both vaccinated and control cows developed measurable, specific serum antibodies in response to experimental challenge.

Vaccinated cows developed slightly increased specific whey antibodies in response to vaccination when compared to control cows (Fig. 1, Week 12). Quarters in both vaccinated and control cows developed whey antibodies in response to experimental challenge.

LYMPHOCYTE RESPONSE

The PBL from the vaccinated cows developed mitogen responses which were higher than those of PBL from control cows during the course of the study period (Fig. 2). Results from a simple regression analysis of the mean LST for each mitogen over time (data not shown) indicated that, in general, PBL from vaccinated cows became more responsive to mitogen stimulation over time than did PBL from control cows. The PBL from both vaccinated and control cows did not respond to stimulation with *M. bovis* antigen at any time. The MGL from vaccinated and control cows were hyporesponsive to mitogen and antigen stimulation (Fig. 3), with SI lower than those of the corresponding PBL cultures at all times tested.

SKIN TEST

Measurements of the increase in skinfold thickness indicated that cows developed specific skin test reactivity in response to vaccination (vaccinated cows week 12), and to infection (control cows week 19.5) (Fig. 4). The control cows were tested with a placebo at weeks 0 and 12, and thus their initial (Week 0) and postvaccination (Week 12) reactivities do not reflect their skin test response to *M. bovis*.

DISCUSSION

The role of the immune responses in resistance to infection and expression of *M. bovis* mastitis is not well understood. Although studies of *M. bovis*-specific immune responses have failed to differentiate between resistant and susceptible animals, evidence for immune prophylaxis has been derived from clinical observations, reinfection studies, and field outbreak intervention vaccine trials (5-9). This study demonstrated that immune responses to *M. bovis* could be induced using killed antigen which enhanced the cellular inflammation.

Vaccination with killed *M. bovis* systemically and in the mammary gland elicited local and systemic antibody, and those quarters in vaccinated cows which had been vaccinated during the dry period with killed *M. bovis* had higher specific antibody levels than quarters in the same cows which had not been given quarter vaccination (data not shown).

Vaccination does not protect against experimental challenge exposure, nor against spread of infection from infected to uninfected quarters (10). There is little evidence that immunization with killed mycoplasma will prevent mammary infection, although recent prior infection with live organisms has done so temporarily in some cases (3-6). Although antibody kills *M. bovis* organisms *in vitro* (18,19), preexisting antibody levels in quarters which had been vaccinated did not abrogate the infection compared to unvaccinated quarters in vaccinated cows or similar quarters in control cows. Some of the antibody-mediated mycoplasmacidal activity may have been restored in

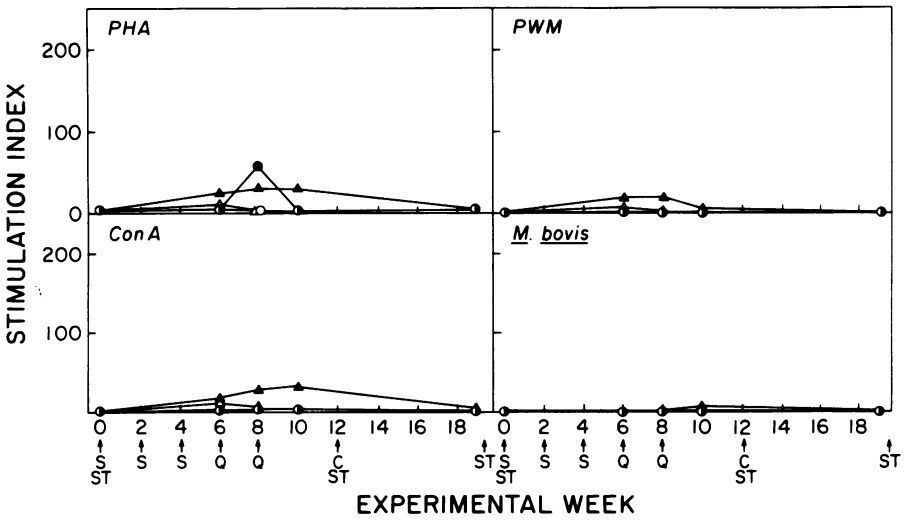

Fig. 3. The mean stimulation by mitogens and *M. bovis* antigen of mammary gland lymphocytes from eight unchallenged (△) and eight challenged (▲) quarters in four vaccinated cows, and from eight unchallenged (○) and eight challenged (●) quarters in four control cows experimentally challenged with *M. bovis*. (S = systemic vaccination, Q = quarter vaccination, C = experimental challenge, ST = skin test).

mastitic glands presumably due to the influx of blood components resulting in resolution of *M. bovis* infection in vaccinated cows (10).

Cell-mediated immunity (CMI) contributes to resistance to *M. bovis* and other mycoplasmal infections, and is probably active in the mammary gland (5,20,21). Incubation of PBL or phagocytes in the presence of milk decreases their activity (22,23). Phagocytic cells, normally mycoplasmacidal, may lose this capacity in the lumen of the mammary gland, and MGL have reduced mitogenic responsiveness as shown in this study and elsewhere (17,24,25). Mammary gland lymphocyte hyporesponsiveness appears to be a general phenomenon, and may limit CMI against *M. bovis* infection. Although probably hyperactive in the interstitium of the mammary gland, lymphocytes in the lumen appear to have lost some of their functional capabilities, which may explain the contrast between the massive cellular responses seen histologically in the interstitium of the mammary gland and in skin test reactions evident in this study and elsewhere, and the hyporeactivity of MGL recovered from secretions (5,17).

Experimental infections indicate that no detectable inflammation occurs until three to seven days after experimental challenge despite high levels of antibody in the glands of vaccinated cows and considerable amounts of antigen introduced at the time of challenge (10,26). This suppressed response in the mammary gland is in sharp contrast to the skin test response which was quite pronounced by 12 h postinjection in the same cows. Evidently inflammation is delayed until its suppression is overcome, probably by the influx of humoral and cellular blood components. Experimental infection levels are chronologically and proportionally related to the levels of cellular inflammation and decrease in milk production (10,27). This inflammatory reaction may damage host tissue as a result of the close association of *M. bovis* with host cells and/or antigenic exchange between host cells and *M. bovis*, but also may participate in resolving *M. bovis* infection.

When compared to control cows, vaccinated cows usually had distinctive immune responses. Although serum and whey antibody responses were induced during vaccination and infection, responsiveness of cellular components isolated from the mammary gland remained low. Resolution of infection in vaccinated cows may be accompanied by adverse inflammatory reactions. Effective vaccine strategies should evoke protective immunity with minimal hypersensitive, inflammatory reactions. A better understanding of the specificity and components of protective immunity should lead to better strategies for prophylactic vaccination against *M. bovis* mastitis.

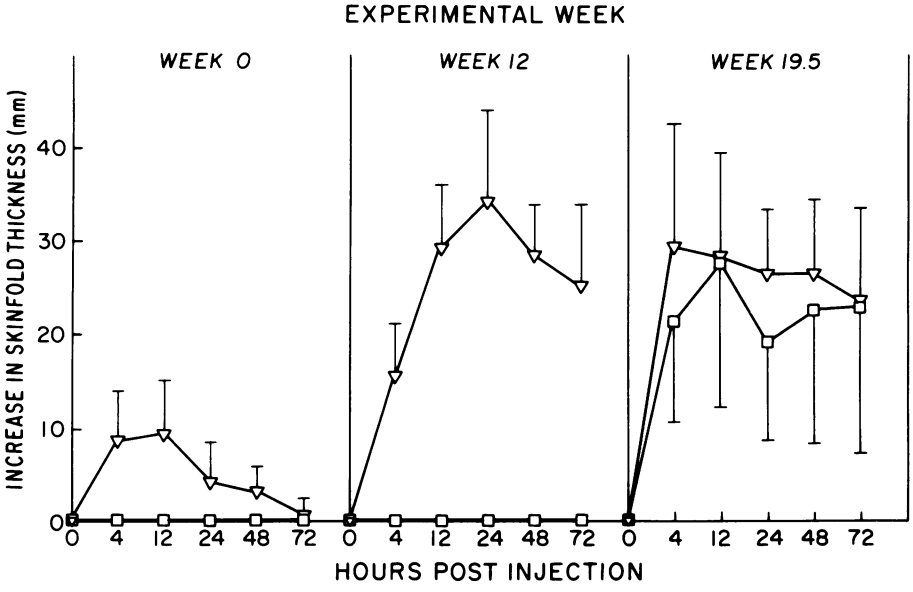

Fig. 4. The mean increase in skinfold thickness at three different times in response to *M. bovis* antigen in four vaccinated (▽) and four control (□) cows experimentally challenged with *M. bovis*. Vertical bars indicate SD.

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REFERENCES

1. **JASPER DE.** Mycoplasma and mycoplasmal mastitis. J Am Vet Med Assoc 1977; 170: 1167-1172.
2. **JASPER DE.** The role of *Mycoplasma* in bovine mastitis. J Am Vet Med Assoc 1982; 181: 158-162.
3. **JASPER DE.** Bovine mycoplasmal mastitis. Adv Vet Sci Comp Med 1981; 25: 121-152.
4. **BOUGHTON E.** *Mycoplasma bovis* mastitis. Vet Bull 1979; 49: 377-387.
5. **BENNETT RH, JASPER DE.** Factors associated with differentiation between cattle resistant and susceptible to intramammary challenge exposure with *Mycoplasma bovis*. Am J Vet Res 1978; 39: 407-416.
6. **BENNETT RH, JASPER DE.** Systemic and local immune responses associated with bovine mammary infections due to *Mycoplasma bovis* resistance and susceptibility in previously infected cows. Am J Vet Res 1978; 39: 417-423.
7. **HOWARD CJ, GOURLAY RN, TAYLOR G.** Immunity to mycoplasma infections of the calf respiratory tract. In: Butler JE, ed. The Ruminant Immune System. New York: Plenum Press, 1981: 711-726.
8. **CHIMA JC, WILKIE BN, RUHNKE HL, TRUSCOTT RB, CURTIS RA.** Immunoprophylaxis of experimental *Mycoplasma bovis* arthritis in calves. Protective efficacy of live organisms and forminalized vaccines. Vet Microbiol 1980; 5: 113-122.
9. **BIANCARDI G.** Mastite micoplasma bovina: contributo alla profitassi mediante vaccionne. VII Int Med Dis Cattle, Milan, 1974: 101-106.
10. **BOOTHBY JT, JASPER DE, THOMAS CB.** Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on the mycoplasmal infection and cellular inflammatory response. Cornell Vet 1986; 76: 188-197.
11. **BOOTHBY JT, JASPER DE, ROLLINS MH.** Characterization of antigens from mycoplasmas of animal origin. Am J Vet Res 1983; 44: 433-439.
12. **CARROLL EJ, ROLLINS MH, JASPER DE.** The immune response of rabbits to 3 strains of *Mycoplasma agalactiae* var *bovis* isolated from mastitic bovine udders. Cornell Vet 1976; 66: 143-151.
13. **BENNETT RH, CARROLL EJ, JASPER DE.** Skin-sensitivity reactions in calves inoculated with *Mycoplasma bovis* antigens: humoral and cell-mediated responses. Am J Vet Res 1977; 38: 1721-1730.
14. **BOOTHBY JT, JASPER DE, ZINKLE JG, THOMAS CB, DELLINGER JD.** Prevalence of mycoplasmas and immune responses to *Mycoplasma bovis* in feedlot calves. Am J Vet Res 1983; 44: 831-833.
15. **CHO HJ, RUHNKE HL, LANGFORD EV.** The indirect hemagglutination test for the detection of antibodies in cattle naturally infected with mycoplasmas. Can J Comp Med 1976; 40: 20-29.
16. **MUSCOPLAT CC, CHEN AW, JOHNSON DW. ALHAJI I.** *In vitro* stimulation of bovine peripheral blood lymphocytes: standardization and kinetics of the response. Am J Vet Res 1974: 38: 1557-1561.
17. **SCHORE CE, OSBURN BI, JASPER DE, TYLER DE.** B and T lymphocytes in the bovine mammary gland: rosette formation and mitogen response. Vet Immunol Immunopathol 1981; 2: 561-569.
18. **HOWARD CJ, TAYLOR G, COLLINS J, GOURLAY RN.** Interaction of *Mycoplasma dispar* and *Mycoplasma agalactiae* var *bovis* with bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes. Infect Immun 1976; 14: 11-17.
19. **HOWARD CJ.** Mycoplasmacidal action of bovine complement mediated by bovine IgG_1, IgG_2, or IgM. Vet Microbiol 1981; 31: 317-330.
20. **TAYLOR G, TAYLOR-ROBINSON D.** The part played by cell-mediated immunity in mycoplasma respiratory infections. Dev Biol Stand 1975; 28: 195-210.
21. **BENNETT RH, JASPER DE.** Immunosuppression of humoral and cell-mediated responses in calves associated with inoculation of *Mycoplasma bovis*. Am J Vet Res 1977; 38: 1731-1738.
22. **ESHELMAN JE, EBERHART RJ, SCHOLZ RW.** Effects of cream on bactericidal and metabolic functions of bovine polymorphonuclear neutrophils. Am J Vet Res 1981; 42: 738-742.
23. **SCHORE CE, OSBURN BI, JASPER DE, TYLER DE.** Comparative studies on peripheral blood and milk lymphocytes. In: Butler JE, ed. The Ruminant Immune System. Adv Exp Med Biol 1981; 137: 761.
24. **PAAPE MJ, WERGIN WP, GUIDRY AJ, SCHULTZE WD.** Phagocytic defense of the ruminant mammary gland. Adv Exp Med Biol 1981; 137: 555-578.
25. **PAAPE MJ, WERGIN WP.** The leukocyte as a defense mechanism. Am J Vet Med Assoc 1977; 170: 1214-1223.
26. **BENNETT RH, JASPER DE.** Bovine mycoplasma mastitis from intrammammary inoculation of small numbers of *Mycoplasma bovis*. I. Microbiology and pathology. Vet Microbiol 1978; 2: 341-355.
27. **BOOTHBY JT, JASPER DE, THOMAS CB.** Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on milk production and milk quality. Can J Vet Res 1986; 50: 200-204.