Therapeutic Chlamydophila abortus and C. pecorum Vaccination Transiently Reduces Bovine Mastitis Associated with Chlamydophila Infection*

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Infections with Chlamydophila abortus and C. pecorum are highly prevalent in cattle and have been associated with bovine mastitis. A prospective cohort study was conducted with a herd of 140 Holstein dairy cows to investigate the influence of Chlamydophila infection on subclinical inflammation of the bovine mammary gland as characterized by somatic cell numbers in milk. PCR detection of C. abortus and low serum antibody levels against Chlamydophila spp. were significantly associated with subclinical mastitis. To examine the effect of the infection by response modification, immune perturbation was done by two subcutaneous administrations of an experimental vaccine preparation of inactivated C. abortus and C. pecorum elementary bodies. Vaccination against Chlamydophila highly significantly decreased milk somatic cell numbers, thus reducing bovine mastitis, and increased antibody levels against Chlamydophila but did not eliminate shedding of C. abortus in milk as detected by PCR. The protective effect peaked at 11 weeks after vaccination and lasted for a total of 14 weeks. Vaccination with the Chlamydophila vaccine, a mock vaccine, or a combination vaccine against bovine viral diseases highly significantly increased C. abortus shedding in milk for 1 week, presumably mediated by the vaccine adjuvant. In summary, this study shows an etiological involvement of the widespread Chlamydophila infections in bovine mastitis, a herd disease of critical importance for the dairy industry. Furthermore, this investigation shows the potential for temporary improvement of chlamydial disease by therapeutic vaccination. Chlamydophila vaccination of cattle might serve as a testing ground for vaccines against human chlamydial infections.

Mastitis, the inflammation of the mammary gland, is the most prevalent production disease in dairy cows and is among the livestock diseases that cause the greatest economic losses in animal agriculture (48). In the United States, mastitis is estimated to cause an annual loss approaching 2 billion dollars (46). Losses are due mainly to reductions in milk quality and to a lesser extent in milk quantity. Classically, infections with bacteria such as Streptococcus agalactiae, Staphylococcus aureus, and Escherichia coli have been the main cause of bovine mastitis (47). Intensive husbandry practices have been associated with an increased incidence of mastitis caused by atypical bacterial agents such as Streptococcus dysgalactiae and Mycoplasma bovis (35, 47). Despite decades of intensive research on bovine mastitis and extensive prophylactic and therapeutic measures, bovine mastitis remains a major problem in the dairy industry, and causal agents remain undiagnosed in a large proportion of cases (“sterile mastitis”).

Exposure to infection with obligate intracellular Chlamydophila bacteria is probably ubiquitous in cattle worldwide, with high seroprevalence rates (approaching 100% in some investigations) (4, 25, 55). Two Chlamydophila species, C. abortus and C. pecorum, are routinely detected in cattle (17, 43). Acute infections with these bacteria have been associated with numerous distinct clinical disease entities in cattle, most prominently abortion and fertility disorders, sporadic encephalomyelitis, keratoconjunctivitis, pneumonia, enteritis, and polyarthritis, (1, 19, 31, 32, 34, 53, 54, 59, 60, 61). However, the vast majority of Chlamydophila infections in cattle, particularly low-level infections frequently detected after introduction of sensitive PCR methods, are not associated with obvious clinical disease (9, 24). A well-balanced host-parasite relationship appears to represent the common nature of chlamydial infection (50). Thus, while it is clear that high-dose experimental inoculations and natural infections with Chlamydophila spp. result in defined disease manifestations, the health impact of the ubiquitous subclinical infections remains unknown.

Experimental inoculation of C. abortus via the teat canal produces a severe acute mastitis of the inoculated mammary glands accompanied by fever and anorexia (6, 33, 39). After initial fibrinous and serous secretion and pronounced swelling of the udder in the first week, the disease appears to be self-limiting, leading to a state of reduced milk production and mammary gland atrophy. C. abortus has also sporadically been associated with naturally occurring bovine mastitis (26, 27, 57), but systematic investigations of the involvement of C. abortus in bovine mastitis have not been reported. In a recent study on
the epidemiology of Chlamydia infection in calves, Jee et al. (24) detected C. abortus in the milk of 15% of dams without any signs of disease. One-hundred-microliter milk samples from a single udder quarter were tested per week for 12 weeks postpartum. Thus, the sampling intensity was low, and a higher prevalence of Chlamydia spp. in milk might be detected with a higher sampling intensity. Nevertheless, these results indicate that low-level natural infection of the bovine mammary gland with Chlamydia spp. most likely is common.

For obvious economic reasons, bovine mastitis has been intensely studied since the advent of culture of bacteria on artificial media, and numerous parameters have been established for routine monitoring of udder health (16, 18, 36). However, continuous simultaneous detection of several determinants of raw milk quality and cost. This well-established parameter for continuous, noninvasive monitoring of inflammation of the mammary gland offers an intriguing potential for the study of the effects of clinically unapparent chlamydial infections. Continuous simultaneous detection of chlamydial infection and inflammatory status of the mammary gland by PCR and SCC, respectively, would allow for long-term assessment of the impact of chlamydial infection on the health of an isolated organ. This is important not only for cattle but also for the understanding of human chronic inflammatory diseases such as pelvic inflammatory disease and reactive arthritis or for coronary heart disease, for which a strong association with Chlamydia trachomatis and Chlamydia pneumoniae infection, respectively, has been found (8, 41, 42).

The investigation described here was conducted as a prospective study with a herd of 147 dairy cows about the interrelation between chlamydial infection and subclinical inflammation of the bovine mammary gland. To maximize the potential for significant outcomes, the study was designed with an intervention approach by perturbation of the Chlamydophila-specific immune response. For this purpose, an inactivated, whole-organism adjuvanted vaccine composed of C. abortus and C. pecorum elementary bodies was used (7). We report here frequent C. abortus infection of the bovine mammary gland, a significant inflammatory response to the unapparent infections indicated by increased milk SCC, and a highly significant, 3-month-long reduction of milk SCC in dairy cows with Chlamydia infection that were vaccinated against Chlamydia spp.

MATERIALS AND METHODS

Experimental animals. A herd of 147 Holstein (91%) and Red Holstein (9%) cows in Germany was used in this study. The cows had a mean age of 4.8 years (range, 2.3 to 10.4 years) and a mean of 2.4 lactations (range, 1 to 8 lactations). Cows were maintained in box stalls and fed hay and corn silage ad libitum, supplemented with dried beet shavings, molasses, and minerals. Consumption of a grain-based concentrate was controlled. Replacement heifers were acquired from other producers. Milking was performed twice daily in a 15-cow herringbone milking parlor using standard hygiene and teat-dipping procedures. Forty-two percent of cows after first delivery had milk SCCs higher than 1 x 10^5/ml, and 31% of all cows had milk SCCs above 4 x 10^5/ml. Staphylococcus aureus mastitis, a common cause of bovine mastitis herd problems, was not observed. The average interval to first insemination was 124 days, the average interval between calves was 448 days, and the insemination index was 1.9. Lameness caused by arthritis, tendonitis, or digital dermatitis required frequent intervention.

Experimental design. The investigation was designed as a prospective intervention study (14). A total of 140 cows were enrolled in the study, with 70 cows each randomly assigned to the Chlamydia vaccine or the mock control vaccine group. Cows were immunized on days 0 and 35 of the study by subcutaneous administration of a 2-ml vaccine dose. In addition, all animals received an intramammary dose of an infectious bovine rhinotracheitis-bovine respiratory syncytial virus-parainfluenza 3 virus (IBR-BRSV-PI3V) combination live attenuated vaccine (Bayer AG, Leverkusen, Germany) on days 104 and 133, inactivated bovine virus diarrhea virus (BVDV) vaccine on day 104, and live attenuated BVDV vaccine (Merial, GmbH, Hallbergmoos, Germany) on day 140. The clinical status of all cows was determined in the week prior to the first vaccination day (day 0), and the body condition relative to the body condition expected for the time of lactation (relative body score [RBS]) was scored by a combination of measures of body fat. The RBS determination was repeated in week-long examination periods ending on days 28, 70, and 174. Conjunctival and vaginal swab specimens were collected for Chlamydophila PCR assays in the week prior to day 0. Serum samples for determination of anti-Chlamydia antibody titers were collected on days 0, 41, 68, and 194. Combined quarter milk samples for SCC determination were obtained from all cows during determination of milk yield on days 0, 12, and 44 and subsequently at monthly intervals. Additional quarter milk samples for Chlamydophila PCR assays were collected from random subsets of Chlamydia- and mock-vaccinated cows on days 0, 1, 4, 7, 10, 94, and 109. All animal experimental procedures were performed by veterinarians, followed federal and state laws, and were supervised by state veterinarians.

Chlamydia vaccine. The C. abortus BovEnd 19/88 (Bayer AG, Leverkusen, Germany) and C. pecorum LW613 (51) strains were cultivated in monolayer cell cultures maintained in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum and partially purified (29). Chlamydial elementary bodies were inactivated (3), and 10^6 50% tissue culture infective doses of mixed chlamydiae per dose were used to prepare an aqueous adjuvanted vaccine (52). A mock vaccine was prepared from identically treated cell medium of uninfected cells.

Clinical and laboratory analyses. Milk SCCs were determined by fluoro-optoelectronic cell counting by use of a Fossomatic FC (Foss A/S, Hillerød, Denmark) somatic cell counter (45, 47). Standard bacterial cultures of milk were performed for cows that showed consistently high SCCs or clinical mastitis (12). Body condition score (BCS) was determined at the expected lactation-dependent body condition (RBS) was determined by the scoring method of Edmondson et al. (15). Data are shown as actual minus expected body score; therefore, a score of 0 indicates no difference between the actual and expected body conditions, a negative score indicates underconditioning, and a positive score indicates overconditioning. Anti-Chlamydia pneumoniae immunoglobulin G1 (IgG1) serum antibody levels were determined by binding to inactivated Chlamydia psittaci antigen in an enzyme immunosay by use of the CHEKIT-Chlamydia kit (Bommi Diagnostics AG, Liebefeld-Bern, Switzerland). Antibody levels were expressed as percentages of values for a positive control serum.

Chlamydia PCR. Chlamydia infection status was assessed by nested Chlamyphia ompA PCR of vaginal and conjunctival swab specimens and of combined quarter milk specimens (26, 40). Swab tips were transferred to microcentrifuge tubes containing 500 l of lysis buffer (0.05% Tween 20, 0.1 M Tris-HCl, pH 8.5), vortexed, and inserted into 1-ml pipette tips for recovery of residual lysis buffer by centrifugation at 12,000 x g for 1 min. The combined liquid was sedimented at 12,000 x g for 15 min, and the sediments were resuspended in 50 l lysis buffer and digested with proteinase K (10 mg/ml) at 60°C for 2 h. After inactivation of proteinase K (97°C, 15 min), samples were centrifuged at 12,000 x g for 5 min to remove debris, and 5 l of the supernatant was used for PCR. Milk specimens were processed using the QiAamp DNA stool kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions and subjected to PCR as described above.

Variable domains III and IV of the Chlamydia ompA gene were targeted using a nested PCR (26) modified by Sachse and Hotzel (40). In the first round, 5 l of DNA extract was amplified using primer pair 191CHOMP/CHOMP5971.
The initiation of the study, *Chlamydophila* PCRs from milk specimens amplified *C. abortus* 49% of all cows were positive in at least one of the day 0 serum IgG1 antibody enzyme immunoassay. *Chlamydophila* anti-seroconversion and conjunctival swab specimens obtained on day 0 and by day 12 of each cow was determined by serum antibodies, and mock-vaccinated animals. All cows had anti-*Chlamydophila* immunity in the time course of milk SCC, milk SCCs of these groups were analyzed by factorial ANOVA, and cows with bacterial culture-positive (i.e., nonchlamydial) clinical mastitis were excluded from the analysis.

Table 1 shows that cows infected with *Chlamydophila* on day 0 had consistently, and largely significantly (*P* ≤ 0.027), higher SCCs than noninfected cows on day 0 or 12. Also, cows with low anti-*Chlamydophila* antibody levels had significantly higher SCCs than cows with high antibody levels (*P* ≤ 0.036) (Table 1). Animals that had low anti-*Chlamydophila* antibody levels had higher SCCs throughout the observation period (*P* = 0.013 for combined repeated-measures data) than animals with high antibody levels (data not shown). The effect of the interaction between day 0 *Chlamydophila* PCR reactivity and anti-*Chlamydophila* antibody levels on the combined day 0 and day 12 repeated-measures SCC data is presented in Fig. 1. Cows that had low *Chlamydophila* antibody levels and were *Chlamydo-philic* PCR positive before vaccination had highly significantly higher somatic cell counts than the cows that had high *Chlamydophila* antibody levels and were PCR positive before vaccination had highly significantly higher somatic cell counts than the cows that had high *Chlamydophila* antibody levels and were PCR positive (*P* = 0.001). Stratification of the animals for age, lactation stage and number, relative body score, and *Chlamy- dophila* or mock vaccination did not change the trends of the results. Thus, these parameters were not confounding the influence of *Chlamydo-philic* infection on milk SCC. Overall, SCC data as an indicator of udder health indicate that this infection has a significant negative effect on the health of the bovine mammary gland.

**Vaccination against *Chlamydo-philic* reduces milk SCC.** To further examine the influence of *Chlamydo-philic* infection on the inflammatory status of the bovine mammary gland, the anti-*Chlamydo-philic* immune response of the herd was modified by vaccination with an inactivated whole-organism *C. abortus*- *C. pecorum* vaccine or a control vaccine without chlamydial antigen. Experimental cows were vaccinated on days 0 and 35 with either *Chlamydo-philic* vaccine or mock vaccine, and differences between animals with perturbed and unmodified anti-*Chlamydo-philic* immunity in the time course of milk SCC, milk data were analyzed by factorial ANOVA, and cows with bacterial culture-positive (i.e., nonchlamydial) clinical mastitis were excluded from the analysis.
yield, anti-Chlamydophila serum antibodies, and relative body condition (RBS) were monitored.

Chlamydophila vaccination elicited a strong, specific immune response resulting in significantly \( (P < 0.018) \) increased anti-Chlamydophila IgG1 antibody levels compared to those in mock-vaccinated cows (Fig. 2A). The effect of Chlamydophila vaccination on milk SCC is shown in Fig. 2B. Chlamydophila-vaccinated cows had highly significantly \( (P < 0.007) \) decreased SCCs, with an average of 123,000 cells/ml milk at all time points after vaccination, compared to mock-vaccinated cows with an average of 230,000 cells/ml milk. Peak reduction was observed on day 76, from 230,000 cells/ml in mock-vaccinated to 83,000 cells/ml in Chlamydophila-vaccinated cows.

The effects of Chlamydophila vaccination on milk yields show a trend of increased yields beginning 44 days after vaccination; however, the results are not statistically significant \( (P = 0.471) \) (Fig. 3A). Similarly, the relative body condition of Chlamydophila-vaccinated cows late after vaccination tended to be better that that of mock-vaccinated cows (Fig. 3B). Again, these results fail to reach significance \( (P = 0.069) \).

Vaccination against Chlamydophila spp. briefly increases, and fails to eliminate, Chlamydophila shedding. The influence of day 0 vaccination on the PCR detection of Chlamydophila spp. was analyzed in milk samples of a random subset of Chlamydophila- and mock-vaccinated cows. Both vaccines were associated with significant \( (P < 0.01) \), 1-week-long increases in the percentage of cows in which \( C. abortus \) DNA was detected in milk (Fig. 4), and no difference between the vaccines was observed. Vaccination with live anti-IBRV-BRSV-PI3V vaccine combined with inactivated BVDV vaccine on day 94 was associated with a similar increase in chlamydial shedding in milk. While milk excretion of \( C. abortus \) organisms reverted to baseline shedding on day 10 after vaccination, shedding of chlamydiae never completely stopped, and no difference in shedding between Chlamydophila- and mock-vaccinated cows was evident. Thus, any vaccination induced Chlamydophila shedding in milk for approximately 1 week, and the Chlamydophila vaccine did not eliminate Chlamydophila spp. more effectively than the mock vaccine.

A subset of cows respond to Chlamydophila vaccination with increased SCCs. The risks of enhancing immune-mediated chlamydial disease by antichlamydial vaccination have been well described (56). In a final analysis, we screened only Chlamydophila-vaccinated cows for animals that responded with increases rather than decreases in milk SCC. Hyperresponder cows were identified by a twofold or higher increase in day 76 SCC over day 0 milk SCC. Four hyper responders (7%)}
were identified among the 67 cows remaining by day 76 in the study (Fig. 5). These cows showed a trend in milk SCC over time that significantly differs from that of the rest of the herd \((P / H_{11005} 0.002)\). The milk SCC of the standard responders declined until day 76, while an increase in milk SCC was observed in the hyperresponders. Differences in antibody levels, milk production, and RBS between hyperresponding and standard-responding cows were not significant throughout the observation period.

**DISCUSSION**

In this experimental herd, the initial epidemiological survey found 100% seroprevalence and, using conjunctival, vaginal, and milk samples obtained at a single time point, 49% PCR prevalence of *Chlamydia* infection. These data indicate that every cow is continuously exposed to *Chlamydia* spp. Cows likely cycle through periods of relative resistance after an infection episode, indicated by increased anti-*Chlamydia* antibody levels and PCR negativity. This is followed by relative susceptibility to *Chlamydia* spp., associated with lower antibody levels and increased PCR positivity (11, 24).

The increased milk SCCs on days 0 and 12 in *Chlamydia* PCR-positive animals demonstrate that the unapparent *Chlamydia* infection and the inability of the immune response to efficiently eliminate it are not innocuous to the host. The high SCCs clearly indicate that the *Chlamydia* infection stimulates a subtle but quantifiable inflammatory response. This is particularly true for animals with the highest susceptibility, which are *Chlamydia* PCR positive and have low anti-*Chlamydia* antibody levels (Fig. 1).

Perturbation of the herd anti-*Chlamydia* immunity corroborated the inflammatory effect of clinically unapparent *Chlamydia* infection (Fig. 2). Vaccine-mediated immune stimulation, evident in increased serum anti-*Chlamydia* antibodies, was highly significantly associated with decreased numbers of milk somatic cells in *Chlamydia*-vaccinated cows (SCC of 123,000/ml) compared to mock-vaccinated animals (SCC of 230,000/ml). Even subtle inflammation, in the context of the bovine mammary gland, has major consequences by reducing the quality and quantity of milk and results in economic losses for animal agriculture. While the trend of a vaccine-mediated increase in milk yield is not significant (Fig. 3A), it is consistent with a large body of evidence that links SCC reduction with higher milk production. Data on estimated milk gains in relation to milk SCC suggest a milk gain of...
An intriguing observation is the antigen-independent, week-
long increased *C. abortus* shedding in milk after *Chlamydia phila*
vacination, mock vacination, or multivalent vacination
against unrelated bovine viruses (Fig. 4). While the mechanism
triggering this burst of chlamydial discharge is unknown, a
likely candidate for the trigger is the adjuvant content of the
vaccines. It is well established that adjuvants mimic pathogen-
associated molecular patterns, bind receptors such as Toll-like
receptors, and initiate a signaling cascade resulting in activa-
tion of innate immune effector mechanisms that ultimately
direct and augment antigen-specific immunity (44). Changes in
host cell metabolism associated with adjuvant action may ini-
tially enhance chlamydial replication or release from infected
cells. However, this chlamydial release does not provide a
specific antigenic stimulus that modulates adaptive immunity
such that *C. abortus*-mediated inflammation of the mammary
gland is eventually mitigated. Only the *Chlamydia phila*
vaccine acted as a “therapeutic vaccine” and modulated the existing
*Chlamydia phila*-specific host response such that inflammation
of the mammary gland was reduced for approximately 100 days
(Fig. 2B).

It is tempting to speculate about the mechanisms involved in
the anti-inflammatory, therapeutic effect of *Chlamydia phila*
immunization of animals with significant immunity to, and con-
current infection by, *C. abortus* (20, 49). The adjuvant com-
ponent of the *Chlamydia phila* vaccine is thought to stimulate both
Th1 and Th2 immune responses (7, 23, 30). Th1 immunity is an
absolute requirement for clearance of chlamydial infections,
while Th2 immunity mitigates Th1-associated inflammation
but prevents chlamydial clearance. Thus, the precise mecha-
nism(s) of disease protection is unclear, be it either (i) Th1-
mediated elimination of *C. abortus*, (ii) Th2-mediated mitiga-
cion of *C. abortus*-induced inflammation, (iii) a balanced
combination of both mechanisms, or (iv) an enhanced cell-
mediated immune response associated with one of these mech-

isms.

Early vaccination attempts against the human ocular disease
trachoma, caused by *Chlamydia trachomatis*, unexpectedly re-
sulted in an increase in disease severity in a subset of the study
population, which was caused by a delayed-type hypersensitiv-
ity response (56). This has, to this day, prevented further hu-
man vaccine trials and confined vaccine studies to animal mod-
els. We examined *Chlamydia phila*-vaccinated cows for evidence
of a similar exacerbation of the inflammatory response and
found four cows that reacted with significantly increased SCCs
without any signs of bacterial mastitis (Fig. 5). SCCs in these
hyperresponding cows continuously increased until day 106
and subsequently decreased again. Other parameters, such as
anti-*Chlamydia phila* antibodies, milk yield, and relative body
condition, were not significantly different from those of the
standard responders. While a hypersensitivity mechanism po-
tentially is involved, the results also may indicate a disease
mechanism that is independent of the *Chlamydia phila*
vaccination. Clearly, further and larger studies are required to address
this question.

The clinical utility of a vaccine for medical use is contingent
on the absence of serious side effects such as disease exacerb-
bation. This has prompted a decades-long, still-unsuccessful
search for an effective but also safe vaccine against human
*Chlamydia trachomatis* infection (5, 22). In contrast, the utility

![Graph showing hyperresponders and standard-responders](http://iai.asm.org/)
of a livestock vaccine is contingent upon improvement of herd disease rather than the absence of side effects. The protective effect of the *Chlamydiophila* vaccine makes therapeutic vaccination (“antigen-specific immune modulation”) for reduction of bovine somatic milk cells an attractive choice for the livestock industries compared to the use of antibiotics or other drugs for this purpose. The temporal restriction of the vaccine effect will require frequent revaccination and targeted use of this vaccine during periods of high risk, but it will also limit negative side effects. In addition, routine continuous monitoring of SCC in dairy herds will rapidly identify potentially hyperresponding cows and thus prevent their repeated vaccination. Use of a *Chlamydiophila* vaccine in cattle may also aid to evaluate, and likely mitigate, the impact of subclinical chlamydial infection on other bovine herd health problems (52, 58).

In addition to the intrinsic value for control of economic losses in animal agriculture, the *Chlamydiophila* vaccine and its use in the natural host population against subclinical mastitis in dairy cows offer intriguing advantages. Long-term noninvasive sampling and enhanced expression phenotyping afforded by the emerging bovine (*Bos taurus*) genome sequence (http://www.ncbi.nlm.nih.gov/GenBank) will allow sophisticated calibration of therapeutic vaccine parameters such as adjuvants, antigen composition of subunit vaccines, application dosages and intervals, and coadministration of antimicrobial, anti-inflamatory, or immunomodulatory drugs. Strategies defined for this natural disease that control chronic inflammation caused by bovine *Chlamydiophila* infection might well inform rational approaches to manage human chlamydial infections and the consequences of their association with chronic inflammatory diseases such as pelvic inflammatory disease, reactive arthritis, or atherosclerosis (2, 8, 37, 42).

REFERENCES

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