

## Therapeutic *Chlamydophila abortus* and *C. pecorum* Vaccination Transiently Reduces Bovine Mastitis Associated with *Chlamydophila* Infection<sup>∇</sup>

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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 quantity and to a lesser extent in milk quality. 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, kerato-conjunctivitis, pneumonia, enteritis, and polyarthrititis, (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

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the epidemiology of *Chlamydomphila* 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 *Chlamydomphila* 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 *Chlamydomphila* 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). Uniformly accepted among these parameters is the number of somatic cells in milk (somatic cell count [SCC]). Milk from a healthy bovine mammary gland contains fewer than 100,000 somatic cells per milliliter, and there is consensus that the presence of more than  $10^5$  somatic cells per ml bovine milk indicates inflammation of the mammary gland. Milk with 200,000 or more cells per ml is of reduced value because manufacturing properties are compromised, particularly for cheese production (13, 28, 48). Clinically manifest mastitis is typically associated with SCCs of above  $10^6$  per ml (18). Milk SCCs of individual dairy cows are routinely monitored as one 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 *Chlamydomphila*-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 *Chlamydomphila* infection that were vaccinated against *Chlamydomphila* 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 transponder 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  $1 \times 10^5$ /ml, and 31% of all cows had milk SCCs above  $4 \times 10^5$ /ml. *Staphylococcus aureus* mastitis, a common cause of bovine mastitis herd problems, was not observed in the herd. 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 *Chlamydomphila* 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 intramuscular dose of an infectious bovine rhinotracheitis-bovine respiratory syncytial virus-parainfluenza 3 virus (IBRV-BRSV-PI3V) combination live attenuated vaccine (Bayer AG, Leverkusen, Germany) on days 104 and 133, inactivated bovine virus diarrhoea virus (BVDV) vaccine on day 104, and live attenuated BVDV vaccine (Meril, GmbH, Hallbergmoos, Germany) on day 140. The clinical status of all cows was determined in the week prior to the first vaccination (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 *Chlamydomphila* PCR assays in the week prior to day 0. Serum samples for determination of anti-*Chlamydomphila* antibodies 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 *Chlamydomphila* PCR assays were collected from random subsets of *Chlamydomphila*- 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.

***Chlamydomphila* 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 relative to 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-*Chlamydomphila* immunoglobulin G1 (IgG1) serum antibody levels were determined by binding to inactivated *Chlamydia psittaci* antigen in an enzyme immunoassay by use of the CHEKIT-*Chlamydia* kit (Bommeli Diagnostics AG, Liebefeld-Bern, Switzerland). Antibody levels were expressed as percentages of values for a positive control serum.

***Chlamydomphila* PCR.** *Chlamydomphila* infection status was assessed by nested *Chlamydomphila 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  $\mu$ 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  $12,000 \times g$  for 1 min. The combined liquid was sedimented at  $12,000 \times g$  for 15 min, and the sediments were resuspended in 50  $\mu$ 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  $12,000 \times g$  for 5 min to remove debris, and 5  $\mu$ 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 *Chlamydomphila ompA* gene were targeted using a nested PCR (26) modified by Sachse and Hotzel (40). In the first round, 5  $\mu$ l of DNA extract was amplified using primer pair 191CHOMP/CHOMP371.

TABLE 1. Association of milk somatic cell counts with PCR detection of and antibodies against *Chlamydomphila* spp.

Test	Result	Milk SCC on day:					
		0			12		
		<i>n</i>	Mean (10 <sup>3</sup> /ml) <sup>a</sup>	<i>P</i> <sup>b</sup>	<i>n</i>	Mean (10 <sup>3</sup> /ml)	<i>P</i>
Conjunctival PCR, day 0 <sup>c</sup>	Negative	82	140.0	0.288	75	119.2	<b>0.027</b>
	Positive	33	199.3		31	221.2	
Vaginal PCR, day 0	Negative	78	126.0	<b>0.012</b>	73	116.3	<b>0.017</b>
	Positive	37	239.4		33	224.8	
Conjunctival + vaginal PCR, day 0	Negative	61	127.3	0.083	57	106.0	<b>0.011</b>
	Positive	54	193.4		49	202.1	
Anti- <i>Chlamydomphila</i> serum IgG1	High	66	147.3	<b>0.036</b>	56	121.8	<b>0.014</b>
	Low	65	245.8		61	222.1	

<sup>a</sup> Data represent antilogs of the means of the log-transformed SCCs.

<sup>b</sup> Boldface indicates a significant difference between the two means (Tukey HSD test).

<sup>c</sup> Cows with bacteriologically positive mastitis were excluded.

Subsequently, 1 µl of the PCR product served as template in the second round, which used primers 201CHOMP and CHOMP336 (40). For species differentiation, first-round PCR products of all positive samples were subjected to *C. psittaci/C. abortus/C. caviae/C. felis*- and *C. pecorum*-specific nested amplification using primer pairs 218PSITT/CHOMP336s and 204PECOR/CHOMP336s, respectively.

**Statistical analysis.** All statistical analyses were performed with the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK). SCCs, milk yields, RBSs, and anti-*Chlamydomphila* antibody levels for the *Chlamydomphila*- and mock-vaccinated groups were normalized to the population mean such that the means of the day 0 results were identical for both *Chlamydomphila*- and mock-vaccinated animals. Data for all subsequent time points were multiplied by the day 0 factor used for *Chlamydomphila*- and mock-vaccinated groups, respectively, to adjust the group mean to the population mean. Normalization changed all data by less than 5%. To identify confounding factors, the data were also stratified for age of the cows, lactation number and stage, and *Chlamydomphila* PCR detection. SCC data were logarithmically transformed. During the study period, 10 *Chlamydomphila*-vaccinated cows and 8 mock-vaccinated cows progressed from late lactation through a 6-week dry period and then delivered a calf and entered a new lactation. Because of the fundamentally different lactation characteristics, these cows were treated as separate cases for the late lactation period and the new, early lactation period. The set of data just prior to parturition was considered day 0 data for the new lactation. Normal distribution of data was confirmed by Shapiro-Wilk's *W* test, and homogeneity of variances by Levene's test. Data were analyzed by repeated-measures analysis of variance (ANOVA). Comparisons of means under the assumption of no a priori hypothesis were performed by the Tukey honest significant difference (HSD) test. *Chlamydomphila* PCR data were also analyzed by the Fisher exact two-tailed test. Differences at a *P* value of ≤0.05 in all tests were considered significant.

## RESULTS

**Clinically unapparent *Chlamydomphila* infection is associated with increased inflammation of the bovine mammary gland.** At the initiation of the study, the *Chlamydomphila* infection status of each cow was determined by *Chlamydomphila* PCR of vaginal and conjunctival swab specimens obtained on day 0 and by anti-*Chlamydomphila* serum IgG1 antibody enzyme immunoassay. All cows had anti-*Chlamydomphila* serum antibodies, and 49% of all cows were positive in at least one of the day 0 *Chlamydomphila* PCRs. PCR typing revealed that all positive PCRs from milk specimens amplified *C. abortus* DNA fragments. Cows were stratified into *Chlamydomphila* PCR-positive and -negative groups on day 0, and into groups with high (above median [more than 75% of the optical density of pos-

itive control serum]) and low (equal to or below median) anti-*Chlamydomphila* antibody levels. 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 *Chlamydomphila* on day 0 had consistently, and largely significantly ( $P \leq 0.027$ ), higher SCCs than noninfected cows on day 0 or 12. Also, cows with low anti-*Chlamydomphila* antibody levels had significantly higher SCCs than cows with high antibody levels ( $P \leq 0.036$ ) (Table 1). Animals that had low anti-*Chlamydomphila* 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 *Chlamydomphila* PCR reactivity and anti-*Chlamydomphila* antibody levels on the combined day 0 and day 12 repeated-measures SCC data is presented in Fig. 1. Cows that had low *Chlamydomphila* antibody levels and were *Chlamydomphila* PCR positive before vaccination had highly significantly higher somatic cell counts than the cows that had high *Chlamydomphila* antibody levels and were *Chlamydomphila* PCR positive ( $P = 0.001$ ). Stratification of the animals for age, lactation stage and number, relative body score, and *Chlamydomphila* or mock vaccination did not change the trends of the results. Thus, these parameters were not confounding the influence of *Chlamydomphila* 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 *Chlamydomphila* reduces milk SCC.** To further examine the influence of *Chlamydomphila* infection on the inflammatory status of the bovine mammary gland, the anti-*Chlamydomphila* 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 *Chlamydomphila* vaccine or mock vaccine, and differences between animals with perturbed and unmodified anti-*Chlamydomphila* immunity in the time course of milk SCC, milk

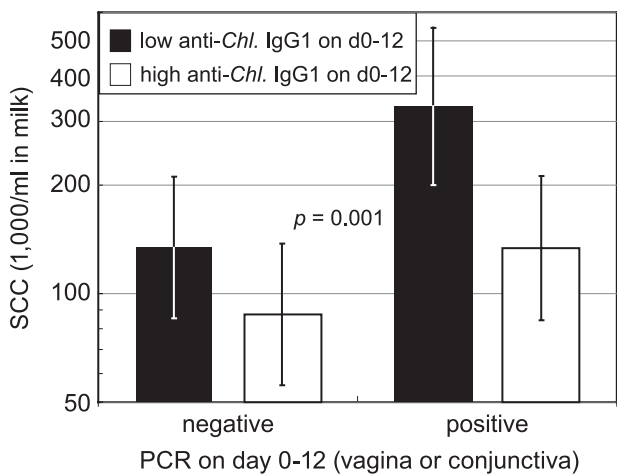


FIG. 1. Effect of the interaction between day 0 *Chlamydomphila* PCR and anti-*Chlamydomphila* serum IgG1 on milk SCC on days 0 and 12. *Chlamydomphila* PCR-positive cows with low *Chlamydomphila* antibody levels before vaccination have significantly higher somatic cell counts on days 0 and 12 than cows that are *Chlamydomphila* PCR negative and have high anti-*Chlamydomphila* antibody levels ( $P = 0.001$ ; combined day 0 and 12 data in repeated-measures ANOVA and Tukey HSD test). Data are shown as the antilog of mean log SCC  $\pm$  95% confidence interval.

yield, anti-*Chlamydomphila* serum antibodies, and relative body condition (RBS) were monitored.

*Chlamydomphila* vaccination elicited a strong, specific immune response resulting in significantly ( $P = 0.018$ ) increased anti *Chlamydomphila* IgG1 antibody levels compared to those in mock-vaccinated cows (Fig. 2A). The effect of *Chlamydomphila* vaccination on milk SCC is shown in Fig. 2B. *Chlamydomphila*-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 *Chlamydomphila*-vaccinated cows.

The effects of *Chlamydomphila* 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 *Chlamydomphila*-vaccinated cows late after vaccination tended to be better than that of mock-vaccinated cows (Fig. 3B). Again, these results fail to reach significance ( $P = 0.069$ ).

**Vaccination against *Chlamydomphila* spp. briefly increases, and fails to eliminate, *Chlamydomphila* shedding.** The influence of day 0 vaccination on the PCR detection of *Chlamydomphila* spp. was analyzed in milk samples of a random subset of *Chlamydomphila*- and mock-vaccinated cows. Both vaccines were associated with significant ( $P \leq 0.01$ ), 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

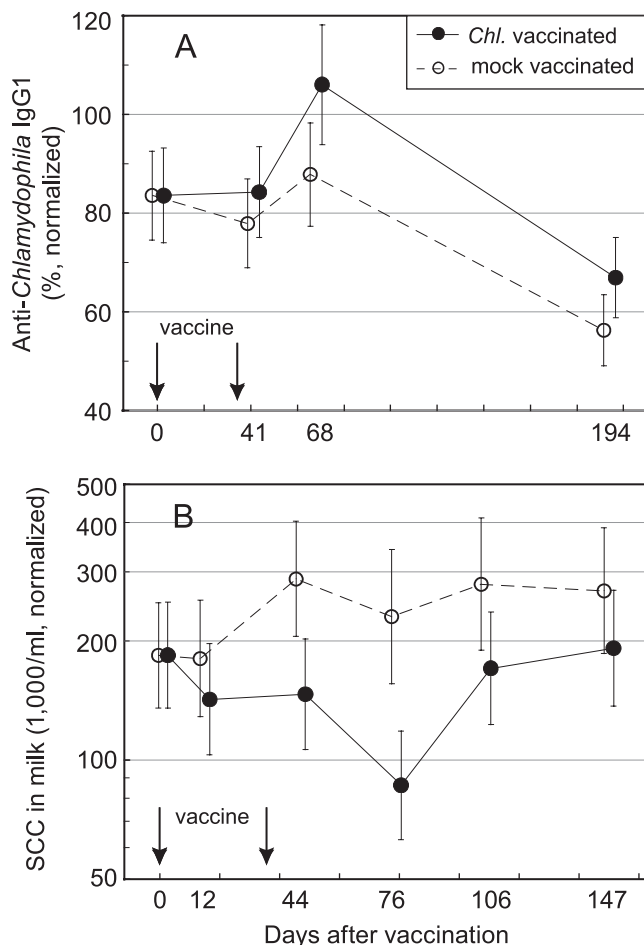


FIG. 2. Effect of *Chlamydomphila* vaccination on anti-*Chlamydomphila* serum antibodies and milk somatic cell counts. Data are shown as the antilog of mean log SCC  $\pm$  95% confidence interval and were normalized for identical day 0 means of *Chlamydomphila*- and mock-vaccinated animals (vaccine on days 0 and 35). A. *Chlamydomphila*-vaccinated cows have significantly higher anti-*Chlamydomphila* serum IgG1 levels than mock-vaccinated cows ( $P = 0.018$ ; combined time points after day 0 in repeated-measures ANOVA and Tukey HSD test). Levels of anti-*Chlamydomphila* serum IgG1 antibodies are shown as percent optical density in comparison to a low-positive control serum. All cows had positive prevaccination antibody levels. B. *Chlamydomphila*-vaccinated cows have significantly lower milk SCC than mock-vaccinated cows ( $P = 0.007$  for all combined time points after day 0 in repeated-measures ANOVA and Tukey HSD test). Error bars indicate 95% confidence intervals.

difference in shedding between *Chlamydomphila*- and mock-vaccinated cows was evident. Thus, any vaccination induced *Chlamydomphila* shedding in milk for approximately 1 week, and the *Chlamydomphila* vaccine did not eliminate *Chlamydomphila* spp. more effectively than the mock vaccine.

**A subset of cows respond to *Chlamydomphila* 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 *Chlamydomphila*-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 hyperresponders (7%)

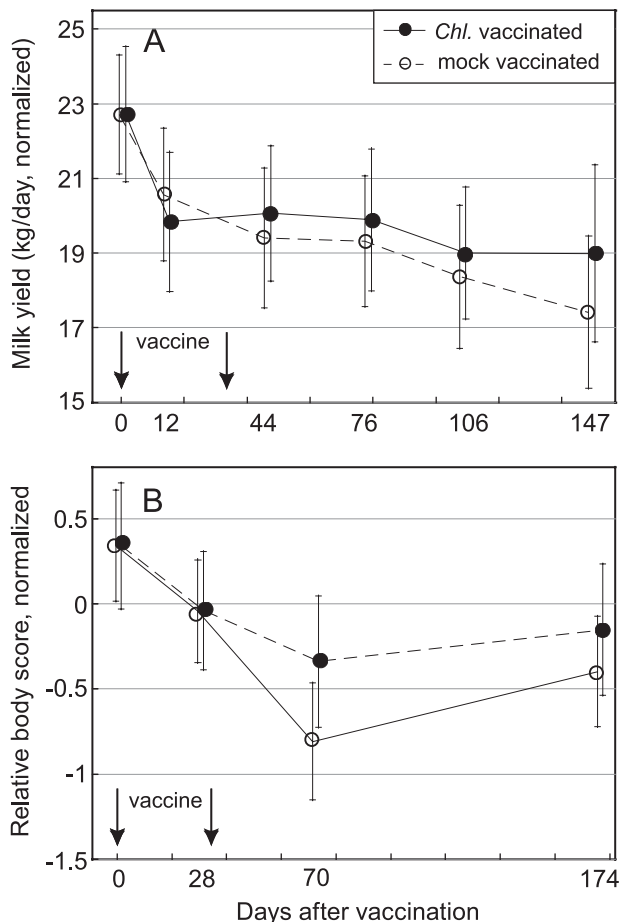


FIG. 3. Effect of *Chlamydomphila* vaccination on milk production and body condition. A. *Chlamydomphila*-vaccinated cows do not produce significantly more milk than mock-vaccinated cows ( $P = 0.471$  for days 44 to 147 in repeated-measures ANOVA). B. *Chlamydomphila*-vaccinated cows tend to have a better body condition on days 70 through 174 than cows that were mock vaccinated, but the difference does not reach statistical significance ( $P = 0.069$  for days 70 to 174 in repeated-measures ANOVA and Tukey HSD test). Error bars indicate 95% confidence intervals.

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 ( $P = 0.002$ ). 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 *Chlamydomphila* infection. These data indicate that every cow is continuously exposed to *Chlamydomphila* spp. Cows likely cycle through periods of relative resistance after an infection episode, indicated by increased anti-*Chlamydomphila*

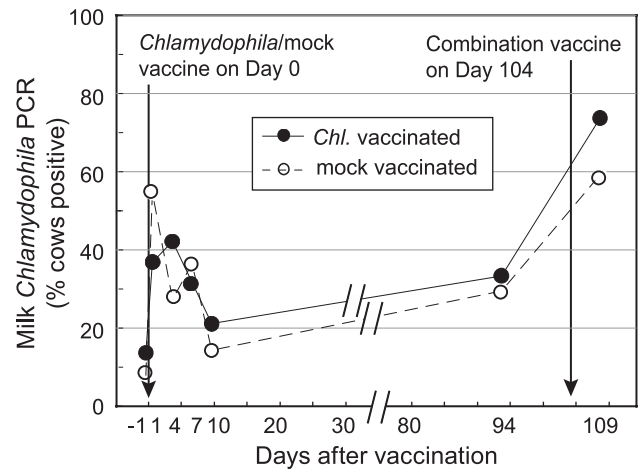


FIG. 4. Effect of vaccinations on detection of *Chlamydomphila* in milk. Cows were vaccinated on day 0 with *Chlamydomphila* vaccine or mock vaccine, and all cows on day 104 were vaccinated with a combination of live attenuated IBRV-BRSV-PI3V vaccine and inactivated BVDV vaccine. After both vaccinations, the percentage of cows with positive *Chlamydomphila* milk PCR among the combined PCR-tested *Chlamydomphila*-vaccinated ( $n = 22$ ) and mock-vaccinated ( $n = 19$ ) cows increased significantly. The difference between the percentage of *Chlamydomphila* milk PCR-positive animals on day 0 versus day 1, 4, or 7 ( $P < 0.01$  by Fisher exact two-tailed test) or on day 94 versus day 109 ( $P = 0.01$ ) is highly significant. No significant difference in the *Chlamydomphila* milk PCR results on any test day was observed between *Chlamydomphila*- and mock-vaccinated cows. Both *Chlamydomphila* and irrelevant vaccinations therefore increase *Chlamydomphila* detection in milk for approximately 1 week, but the *Chlamydomphila* vaccine does not eliminate or reduce *Chlamydomphila* shedding significantly compared to an irrelevant mock vaccine.

antibody levels and PCR negativity. This is followed by relative susceptibility to *Chlamydomphila* spp., associated with lower antibody levels and increased PCR positivity (11, 24).

The increased milk SCCs on days 0 and 12 in *Chlamydomphila* PCR-positive animals demonstrate that the unapparent *Chlamydomphila* 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 *Chlamydomphila* infection stimulates a subtle but quantifiable inflammatory response. This is particularly true for animals with the highest susceptibility, which are *Chlamydomphila* PCR positive and have low anti-*Chlamydomphila* antibody levels (Fig. 1).

Perturbation of the herd anti-*Chlamydomphila* immunity corroborated the inflammatory effect of clinically unapparent *Chlamydomphila* infection (Fig. 2). Vaccine-mediated immune stimulation, evident in increased serum anti-*Chlamydomphila* antibodies, was highly significantly associated with decreased numbers of milk somatic cells in *Chlamydomphila*-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

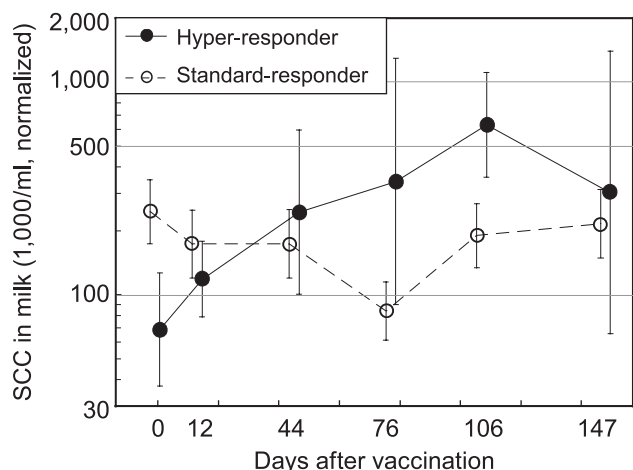


FIG. 5. Hyperresponders identified among *Chlamydomphila*-vaccinated cows. Hyperresponders among *Chlamydomphila*-vaccinated cows were identified by a twofold or greater increase of day 76 milk SCC over prevaccination SCC. Data are shown as the antilog of mean log SCC  $\pm$  95% confidence interval. The difference in the trend of milk SCC over time between the hyperresponders ( $n = 4$ ) and standard responders ( $n = 63$ ) is highly significant ( $P = 0.002$  by repeated-measures ANOVA and Tukey HSD test).

approximately 200 kg per year for a cow with an SCC of 120,000 cells/ml milk versus a cow with 230,000 cells/ml (46, 48). *Chlamydomphila* vaccination also potentially improves overall health, as suggested by the trend for higher relative body scores in *Chlamydomphila*-vaccinated cows, although it fails to reach significance. Clearly, larger studies are required to conclusively demonstrate improvement in milk yields and body condition.

The milk SCC reduction effect of the *Chlamydomphila* vaccine disappears between days 76 and 106, as is evident in Fig. 2B and even more clearly in the serial correlation between SCCs. Between days 76 and 106, anti-*Chlamydomphila* antibody levels in *Chlamydomphila*-vaccinated cows were still significantly higher than those in mock-vaccinated cows (Fig. 2A). These data support the notion that antibody effects are not the protective mechanism of the *Chlamydomphila* vaccine. Rather, a body of experimental and epidemiological data suggests that antibodies are only surrogate markers for an immune mechanism that protects the vaccinated animals against *Chlamydomphila*-induced disease. This mechanism presumably is Th1 cellular immunity, which is required to clear chlamydial infection (21, 38), and the limited time frame of the protective effect presumably is the corollary of the limited life span of immune effector cells.

The modified anti-*Chlamydomphila* immune response elicited by therapeutic vaccination of infected animals does not eliminate *C. abortus*, as indicated by consistently positive results of milk *Chlamydomphila* PCRs for *Chlamydomphila*- and mock-vaccinated cows (Fig. 4). Nevertheless, it may well be that the *Chlamydomphila* vaccine-induced immune response reduces chlamydial loads but does not completely eliminate the organisms. The nested PCR method used in this study does not allow discrimination between different chlamydial burdens. It will be interesting to quantify chlamydial milk loads with quantitative PCR methodology in future studies (10, 24).

An intriguing observation is the antigen-independent, week-long increased *C. abortus* shedding in milk after *Chlamydomphila* vaccination, mock vaccination, or multivalent vaccination 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 activation of innate immune effector mechanisms that ultimately direct and augment antigen-specific immunity (44). Changes in host cell metabolism associated with adjuvant action may initially 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 *Chlamydomphila* vaccine acted as a "therapeutic vaccine" and modulated the existing *Chlamydomphila*-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 *Chlamydomphila* immunization of animals with significant immunity to, and concurrent infection by, *C. abortus* (20, 49). The adjuvant component of the *Chlamydomphila* 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 mechanism(s) of disease protection is unclear, be it either (i) Th1-mediated elimination of *C. abortus*, (ii) Th2-mediated mitigation 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 mechanisms.

Early vaccination attempts against the human ocular disease trachoma, caused by *Chlamydia trachomatis*, unexpectedly resulted in an increase in disease severity in a subset of the study population, which was caused by a delayed-type hypersensitivity response (56). This has, to this day, prevented further human vaccine trials and confined vaccine studies to animal models. We examined *Chlamydomphila*-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-*Chlamydomphila* antibodies, milk yield, and relative body condition, were not significantly different from those of the standard responders. While a hypersensitivity mechanism potentially is involved, the results also may indicate a disease mechanism that is independent of the *Chlamydomphila* 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 exacerbation. 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

of a livestock vaccine is contingent upon improvement of herd disease rather than the absence of side effects. The protective effect of the *Chlamydomphila* 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 *Chlamydomphila* 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 *Chlamydomphila* 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.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-inflammatory, or immunomodulatory drugs. Strategies defined for this natural disease that control chronic inflammation caused by bovine *Chlamydomphila* 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).

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