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Effects of Autogenous Toxoid-bacterin in Lactating Cows with *Staphylococcus aureus* Subclinical Mastitis

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ABSTRACT. To evaluate clinical effects of autogenous toxoid-bacterin treatment for *Staphylococcus aureus* subclinical mastitis in lactating cows, 22 cows which had at least one *S. aureus* infected quarter were selected from among cows at a *S. aureus* prevalent dairy farm. Eleven cows were injected with their own autogenous toxoid-bacterin and the others were maintained as non-injected control. In the toxoid-bacterin injected group, 27% of infected quarters were cured during the 12-week trial, compared to 5% in the control group. New intramammary infections with *S. aureus* were only detected in 3 quarters of the control group. Mean IgG antibody titer against *S. aureus* somatic antigens and α-toxin in serum and milk were significantly increased in the toxoid-bacterin injected group ($p<0.05$) and remained higher than those of the control group which showed no significant changes ($p<0.05$). In contrast to the control group, from 3 weeks after the second injection of the toxoid-bacterin injected group, mean *S. aureus* cfu/m*l* in milk samples from injected quarters with *S. aureus* was significantly decreased until the end of the study ($p<0.05$). In the toxoid-bacterin injected group, significant decreases of mean SCC were detected from milk samples from infected quarters with *S. aureus* from week 7 to week 10 ($p<0.05$). These data show that autogenous toxoid-bacterin treatment against *S. aureus* subclinical mastitis in lactating cows may increase the cure rate of the infections, reduce the severity of the infections and also prevent occurrence of the new infections.

KEY WORDS: autogenous toxoid-bacterin, IgG antibody, mastitis, *Staphylococcus aureus*.

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Bovine mastitis is one of the most problematic disease and continues to have major economic impact on the dairy industry throughout the world [10, 11]. Numerous agents can cause mastitis problems in dairy cows but *Staphylococcus aureus* is the most important etiological agent of bovine mastitis [3, 8, 25]. Therefore, although various management methods for decreasing the prevalence of *S. aureus* mastitis have been improved, many dairies still have some level of infection with *S. aureus* [14, 17, 25]. To cope with this problem, vaccination to prevent *S. aureus* mastitis has been the subject of concern of many researchers [6, 13, 18]. These vaccines have been employed as live [28] or killed *S. aureus* [4], isolated capsular materials [16], toxoids [1], or combined preparations of killed cells and toxoids [20–22, 29]. These vaccines have been reported to increase specific antibody to *S. aureus* antigens both in serum and milk, but only partially prevent new intramammary infections.

Autogenous bacterins are a type of vaccine made from bacteria isolated from infected animals and are inoculated back into those animals. Staphylococcal autogenous bacterins have been used for the treatment of staphylococcal infection in humans [9, 19], and animals [5, 24], for reducing the severity of the infection. In these trials, the authors concluded that autogenous bacterin treatment could increase recovery rate in clinical cases.

Therefore, the purpose of the study reported here was to determine the effects of autogenous toxoid-bacterins on cure rates, changes of antibody level, changes of bacterial counts and somatic cell count (SCC) in milk, when applied to *S. aureus* subclinical mastitis in lactating cows.

MATERIALS AND METHODS

Experimental animals: Two groups of eleven 2- to 3-year-old lactating Holstein cows were used in the study: the toxoid-bacterin injected group (group I) and the non-injected group (group II). All cows were infected subclinically with *S. aureus* in at least one quarter of the udder, and had not received any drugs for 3 months before the study. The status of udder health for each cow was known from the results of bacteriological tests conducted periodically on aseptically collected milk samples in the laboratory. Cows were housed at a commercial farm, with a 12-month mean bulk tank milk SCC of $\leq$ 450,000 cells/m*l*, and with rolling herd average milk production of 14 kg/cow on the basis of twice daily milking. Mean ± SEM body weight at the time of entry into the study was 545 ± 22 kg in group I, and 552 ± 27 kg in group II, respectively. Cows in group I were injected with their own toxoid-bacterin and boosted after 4 weeks, whereas cows in group II were maintained without any treatment throughout the study period of 12 weeks. All cows had no history of concurrent disease other than quarters, and had unremarkable physical examination findings. In an attempt to maintain similar experimental characteristics between groups, the cows were randomly allocated to one of the two groups based on their number of lactation and days in milk. All individual quarter milk samples were cultured bacteriologically and determined for SCC. Cows were fed and milked under the usual farm management policy.

Autogenous toxoid-bacterin: To prepare autogenous toxoid-bacterin for each infected cows, *S. aureus* showing α and β hemolytic pattern on blood agar was isolated from one of

the infected quarters of each cow and confirmed by a coagulase test, DNase test and mannitol agar test. Bacteria were cultured in a brain-heart infusion broth with 10% (vol/vol) sterile bovine milk whey to enhance pseudocapsule production. After incubation for 24 hr at 37°C, the cultures were sterilized by addition of formaldehyde to 1% (vol/vol). Sterility was ensured by incubation of cultures on brain-heart infusion agar plates for 24 hr. If there were no growing bacteria on the plate, the cultures were regarded as sterilized. After sterility was ensured, the cells were harvested and washed 3 times with sterile normal saline by centrifugation (4,000 rpm, 4°C, 30 min). Finally, the cells were suspended in sterile normal saline, and bacterial concentrations were adjusted to 10^{10} colony forming unit (cfu)/m*l* at 450 nm with a spectrophotometer.

To prepare crude toxoid components, the same bacteria used for bacterins were grown for 48 hr at 37°C in a brain-heart infusion broth. Bacteria were removed by centrifugation (4,000 rpm, 4°C, 30 min) and the supernatant was concentrated by lyophilization to 12% of the original volume. Formaldehyde was added to 1% (vol/vol) and the mix was maintained for 48 hr at 4°C.

Each dose of the toxoid-bacterin consisted of 1 m*l* of bacterin, 1 m*l* of toxoid, and 50 mg dextran sulfate (m.w. 500,000), which was emulsified with 2 m*l* of Freund's incomplete adjuvant (Sigma, U.S.A.) giving a total volume of 4 m*l*.

Safety test of toxoid-bacterin: The safety of each toxoid-bacterin was tested by injecting 1 m*l* of toxoid-bacterin to an 8-week-old ICR mouse, subcutaneously. If the mouse remained healthy for 1 week, the toxoid-bacterin was regarded as safe.

Injection of autogenous toxoid-bacterin: Eleven selected lactating Holstein cows were injected subcutaneously with their own toxoid-bacterin in the area of the supramammary lymph node and received a booster after 4 weeks. Another 11 cows were maintained as control.

Sampling and laboratory analysis: Individual quarter milk samples from toxoid-bacterin injected cows and control cows were obtained weekly for bacteriological culture and the determination of SCC. SCC of individual quarter milk was also examined by a Fossomatic 90 (Foss Electric, Denmark). About 0.1 m*l* of individual quarter milk samples were streaked on bovine blood agar plates for bacteriological cultures. The plates were incubated aerobically at 37°C and examined after 24 hr incubation. Staphylococcal colonies were identified on the basis of colony morphology, hemolytic patterns and microscopic appearance and the colonies were counted. The remainder of individual milk samples were centrifuged (3,000 rpm, 4°C, 15 min) and skim milk were separated. The skim milk was stored at –20°C for detecting antibody titer. Blood was also collected at two weeks intervals and sera were stored at –20°C until used for antibody detection.

Antibody detection by indirect ELISA: The sera and milk samples were analyzed for specific IgG antibodies to *S. aureus* somatic antigens and to α-toxin in an indirect ELISA.

To prepare staphylococcal somatic antigens for each toxoid-bacterin injected cow, the same bacteria used for each bacterin preparation were grown and harvested as described in the preparation of the toxoid-bacterin. To prepare staphylococcal somatic antigens for each control cow, one of the *S. aureus* infected quarters was selected in each cow and *S. aureus* was isolated by the method described in the preparation of the toxoid-bacterin. Somatic antigen preparation for control cows was same as that of toxoid-bacterin injected cows. All staphylococcal somatic antigens were suspended (10^{10} cfu/m*l*) in coating buffer (0.05 M carbonate buffer, pH 9.6) immediately before the plates were coated. The α-toxin antigens (Sigma, U.S.A.) were also suspended (20 μg/m*l*) in coating buffer immediately before the plates were coated on.

The wells of the microtiter plates were coated by incubation with 100 μl antigen overnight at 4°C. The coated wells were washed 4 times with phosphate buffered saline with 0.05% Tween 20 (PBS-T). The serum samples were diluted 1:500 in phosphate buffered saline (PBS) for somatic cell ELISA and 1:200 for α-toxin ELISA. The skim milk samples were diluted 1:3 in PBS for both ELISAs.

The plates were incubated with 100 μl well of diluted serum and skim milk at 37°C for 2 hr. After incubation of the plates were washed 4 times with PBS-T. Subsequently, peroxidase-conjugated rabbit anti-bovine IgG (Sigma, U.S.A.) diluted 1:1,000 in PBS was added and incubated at 37°C for 1 hr. The plates were washed again 4 times with PBS-T, and 100 μl of 0.4 mg/m*l* o-phenylenediamine in H_2O_2 inserted (0.04%) citrate buffer (pH 5.0) was added. The enzyme reaction was allowed to continue for 30 min and stopped by addition of 50 μl H_2SO_4. The absorbance was read at 405 nm with automatic ELISA reader (Titertek Multiscan Mcc/340).

Statistical analysis: The mean absorbance between the toxoid-bacterin injected group and control group was compared by Student's *t*-test. The differences of weekly mean SCC, cfu/m*l* in individual quarter milk samples and mean absorbance for each group were also analyzed by Student's *t*-tests. A value of $p<0.05$ was considered statistically significant.

RESULTS

Changes of intramammary infection state: The changes in the intramammary infection states of the toxoid-bacterin injected group and control group are summarized in Table 1. In the toxoid-bacterin injected group, 9 (27%) of 33 infected quarters were cured during the experimental period compared to 5% (1/18) in the control group.

New intramammary infections with *S. aureus* were detected in 3 quarters in the control group but no new intramammary infection was detected in the toxoid-bacterin injected group.

IgG levels against S. aureus somatic antigens in serum and milk: Mean IgG antibody titer against *S. aureus* somatic antigens in serum are shown in Fig. 1. Mean IgG antibody titer was significantly increased in the toxoid-bacterin injected group and remained higher than that of the control group after toxoid-bacterin injection ($p<0.05$), but the control group showed no significant changes throughout the trial ($p<0.05$). The mean IgG antibody titer in milk was also significantly

Table 1. Changes of intramammary infection state

Treatment (n)	No. of infected quarters	No. of persisted quarters	No. of cured quarters (%)	No. of new infected quarters
Toxoid-bacterin (11)	33	24	9 (27)	0
Control (11)	18	17	1 (5)	3

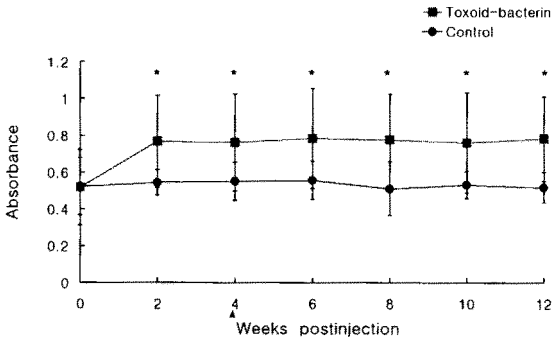

Fig. 1. Changes of mean IgG antibody titer against *S. aureus* somatic antigens in serum. *: significant difference between the toxoid-bacterin group and the control group ($p<0.01$). ▲: boost. Bar represents ± 2 standard deviation.

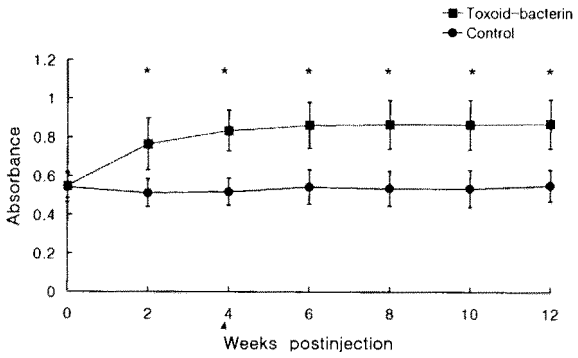

Fig. 2. Changes of mean IgG antibody titer against *S. aureus* somatic antigens in milk. *: significant difference between the toxoid-bacterin group and the control group ($p<0.01$). ▲ : boost. Bar represents ± 2 standard deviation.

increased in the toxoid-bacterin injected group after toxoid-bacterin injection and maintained a higher titer than that of the control group ($p<0.05$), which had no significant changes (Fig. 2; $p<0.05$).

IgG levels against α-toxin in serum and milk: In serum samples, the toxoid-bacterin injected group showed significant responses in IgG antibodies against α-toxin after the first toxoid-bacterin injection, and mean IgG antibody titer remained higher than that of the control group ($p<0.05$). In the control group, there were no significant changes of mean IgG antibody titer against α-toxin throughout the trial (Fig. 3; $p<0.05$).

In milk samples, mean IgG antibody titer in the toxoid-bacterin injected group was increased after the first injection, and statistically significant changes were detected from 2 weeks after the second injection (week 6) (Fig. 4; $p<0.05$). In the control group, mean IgG antibody titer against α-toxin in milk was not significantly changed throughout the trial ($p<0.05$). Significant differences of mean IgG antibody titer against α-toxin in milk between the toxoid-bacterin injected group and control group were detected from 6 weeks (week 10) after the second injection ($p<0.05$).

Changes of mean S. aureus colony forming unit (cfu) in individual quarter milk: In contrast to the control group, 3 weeks after the second injection of the toxoid-bacterin injected group, mean *S. aureus* cfu/m*l* in milk samples from infected quarters with *S. aureus* was significantly decreased until the end of the study (Fig. 5; $p<0.05$).

Changes of mean SCC in individual quarter milk: In the

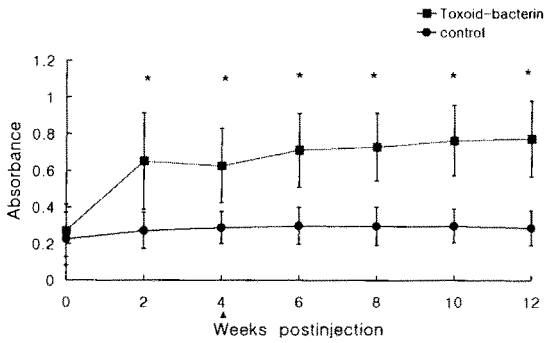

Fig. 3. Changes of mean IgG antibody titer against *S. aureus* α-toxin in serum. *: significant difference between the toxoid-bacterin group and the control group ($p<0.05$). ▲ : boost. Bar represents ± 2 standard deviation.

toxoid-bacterin injected group, mean SCC in milk samples from infected quarters with *S. aureus* was slightly changed every week (Fig. 6), and significantly decreased changes were detected from week 7 to week 10 ($p<0.05$). There were no significant changes of mean SCC in individual quarter milk samples from non-infected quarters ($p<0.05$).

In the control group, mean SCC in milk samples from infected and non-infected quarters with *S. aureus* was also variable during the experiment (Fig. 6), but significant changes were not detected ($p<0.05$).

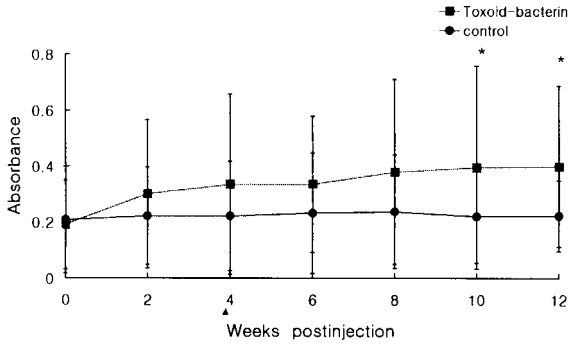

Fig. 4. Changes of mean IgG antibody titer against *S. aureus* α-toxin in milk. *: significant difference between the toxoid-bacterin group and the control group ($p<0.05$). ▲ : boost. Bar represents ± 2 standard deviation.

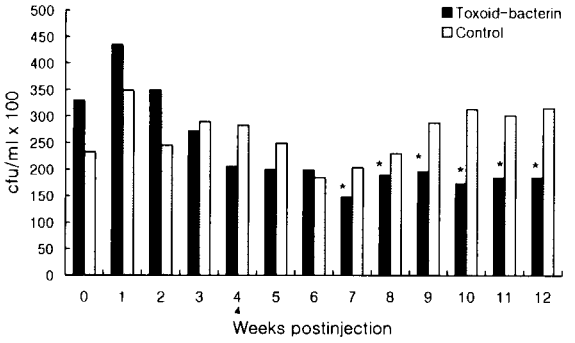

Fig. 5. Changes of mean *S. aureus* cfu/m*l* in individual quarter milk. *: significant difference compared with preinjection state ($p<0.05$). ▲ : boost.

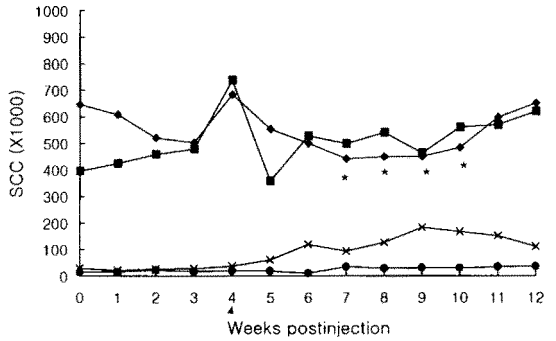

Fig. 6. Changes of mean SCC in individual quarter milk. *: significant difference compared with preinjection state ($p<0.05$). ▲ : boost. ◆ : infected quarters of the toxoid-bacterin injected group. ■ : infected quarters of the control. ● : non-infected quarters of the toxoid-bacterin injected group. ×: non-infected quarters of the control.

DISCUSSION

Traditionally, treatment of *S. aureus* mastitis has been limited to intramammary infusion of antibiotics on dry cows and lactating cows with clinical or subclinical mastitis. The use of intramammary antibiotic therapy for *S. aureus* during lactation however has given very poor results.

Since *S. aureus* respond poorly to antibiotics, vaccines against this organism have been studied extensively and are now focused on enhancing specific antibodies against pseudocapsular antigens [20–22, 29–31].

Pseudocapsule allows complement and antibodies to reach and penetrate the cell wall, but it interferes with recognition of the complexed antibody by polymorphonuclear neutrophil (PMN), thereby preventing activation of complement (C_3) [23]. In addition, pseudocapsules are poor immunizing agents because they are week immunogens and T-cell independent [26]. However, when antibodies are produced against capsule, they are effective opsonins for PMN in cattle [15], and can increase the phagocytic efficacy of PMN [15, 16]. Therefore to enhance pseudocapsule production and to prepare autogenous toxoid-bacterin, isolated *S. aureus* was cultured in a brain-heart infusion broth which was added to 10% (vol/vol) sterile bovine milk whey in the present experiment. The production of pseudocapsule during preparation of toxoid-bacterin was not determined in this study. However, pseudocapsule might have been contained in the preparation of autogenous toxoid-bacterin used in this study, and this possibility was supported by the results which showed prevention of the occurrence of new infection and a reduction in the severity of infection through the increase of antibody titer in the toxoid-bacterin injected group.

In the present experiment, the cure rate of *S. aureus* infection was 27% in the toxoid-bacterin injected group. This cure rate was slightly lower than that for antibiotic treatment [7], but in the non-treated control group the cure rate was only 5%. However, antibiotic therapy for *S. aureus* mastitis during lactation can cause milk residue problems. For this reason, autogenous toxoid-bacterin treatment for *S. aureus* subclinical mastitis during lactation, as tested in the present experiment could be a substitute for antibiotic therapy which does not create the problem of antibiotic residues in milk.

In contrast to the control group, 3 weeks after the second injection of the toxoid-bacterin injected group, mean *S. aureus* cfu/m*l* in milk samples from infected quarters with *S. aureus* was significantly decreased until the end of the study ($p<0.05$). These results reflect that some *S. aureus* infected quarters were cured and the autogenous toxoid-bacterin injection was effective in elimination of existing *S. aureus* infection to some extent. New intramammary infections with *S. aureus* were not detected in the toxoid-bacterin injected group but were detected in 3 quarters in the control group. These results may be attributable to increasing opsonization and phagocytosis by PMN as a result of increased specific IgG antibodies against *S. aureus* in serum and milk [6, 27].

In the toxoid-bacterin injected group, significant decreases in mean SCC of milk samples from infected quarters with *S.*

aureus were detected from week 7 to week 10 ($p<0.05$). These results suggest that the toxoid-bacterin treatment could reduce the severity of infection. Anderson [2] reported that the risk of higher milk SCC might occur due to enhanced immunity after *S. aureus* vaccination. But, in the present study, higher milk SCC was not observed in the toxoid-bacterin injected group.

α-Toxin is a product of *S. aureus* and is believed to be responsible for the gangrenous from of *S. aureus* mastitis [2]. Specific antibodies against α-toxin are effective at reducing *S. aureus* adherence to mammary epithelial cells and epithelial cell damage [12]. In the present experiment, specific IgG antibody titer against α-toxin was increased in serum and milk of the toxoid-bacterin injected group. These increased antibodies against α-toxin can minimize tissue damage by neutralizing α-toxin produced by certain *S. aureus* infections and can reduce *S. aureus* adherence to mammary epithelial cells.

In a previous study, subcutaneous vaccination in the area of the supramammary lymph node enhanced local production of antibody in the udder [20]. Some swellings at the injection site however were observed in most of the injected cows after the first and second injections. This result was similar to that of another study [21]. However, none of the toxoid-bacterin injected cows showed signs of pain when the swellings were palpated. Systemic adverse reactions like reduced appetite, short time lethargy after *S. aureus* vaccination were also reported previously [21], however these adverse reactions did not occur in the present experiment.

The results of the present study showed that autogenous toxoid-bacterin treatment for subclinical *S. aureus* mastitis in lactating cows could be an alternative management method for increasing the cure rate of the infections, reducing the severity of the infections and preventing the occurrence of new infections in highly *S. aureus* prevalent dairy farms without remarkable adverse effects.

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