한국임상수의학회지 15(2) : 370-377 (1998)
Korean J. Vet Clin Med. 15(2) : 370-377 (1998)

Immune Response of Alpha-toxin, Capsular Polysaccharide (CPS) and Recombinant Fibronectin-Binding Protein (r-FnBP) of *Staphylococcus aureus* in Rabbit

Hee-Myung Park[1], Han-Sang Yoo, Doo Kim* and Hong-Ryul Han
Department of Internal Medicine, College of Veterinary Medicine, Seoul National Univeristy
**Kangwon National University*

황색포도상구균의 Alpha-toxin, Capsular Polysaccharide (CPS)와 재조합 Fibronectin-Binding Protein (r-FnBP) 항원을 이용한 토끼에서의 면역반응

박희명[1] · 유한상 · 김 두* · 한홍율
서울대학교 수의과대학 내과학교실, *강원대학교 내과학교실

요 약 : 본 연구는 황색포도상구균의 병원성 인자인 alpha-toxin, casular polysaccharides (CPS)와 fibronectin-binding protein (FnBP)을 이용해 실험동물인 토끼에서의 항체가 형성능과 공격접종 후 방어능에 관한 연구를 위하여 수행되었으며 향후 젖소 유방염 아단위 항원을 이용한 백신개발 가능성을 탐색하고자 수행되어졌다. 황색포도상구균의 alpha-toxin, capsular polysaccharides (CPS)와 fibronectin-binding protein (FnBP) 항원을 이용해 효소면역측정법을 통한 혈중 IgG 항체가수준을 측정하였으며 alpha-toxin, capsular polysaccharides (CPS)와 fibronectin-binding protein (FnBP)으로 면역시킨 토끼에서 대조군의 토끼보다 혈중 항체가 수준이 1차 면역 이후 유의성 있게 높았다 ($p<0.05$). 백신에 사용된 alpha-toxin, capsular polysaccharides (CPS)와 fibronectin-binding protein (FnBP) 항원중 capsular polysaccharides (CPS)가 다른 alpha toxin과 fibronectin-binding protein (FnBP)의 혈중항체가 수준과 비교하여 볼 때 비교적 낮은 수준이었다. 세균을 혈중으로 공격접종한 후 대조군과 백신접종군의 혈중내 세균제거율에 있어 대조군에 비해 백신접종군에서 유의성 있게 낮은 균수를 보였다 ($p<0.05$). 또한 장기내 균수측정실험결과 대조군의 장기보다 백신접종군에서 유의성 있게 세균수가 낮게 출현하였다 ($p<0.05$). 결론적으로 본 연구결과 황색포도상구균의 3가지 항원 alpha-toxin, capsular polysaccharide와 재조합 fibronectin binding protein을 이용한 실험동물에서 아단위 유방염 백신은 방어능이 있다고 생각된다.

Key words : alpha-toxin, capsular polysaccharide (CPS), recombinant fibronectin-binding protein (r-FnBP), *Staphylococcus aureus*

Introduction

MASTITIS is one of the major economic problems in dairy industry (Norcross and Opdebeeck, 1983; Opdebeeck and Norcross, 1983). Although the disease is multifactorial, *Staphylococcus aureus* has been considered as the primary agent and it produces several virulence factors such as capsular polysaccharides (Guidry, 1991; Fattom *et al.*, 1993), alpha-toxin (Herbelin *et al.*, 1997) and fibronectin binding protein (Nelson, 1991). Of the virulence factors, attention has been made on capsular polysaccharide

*본 연구는 한국과학기술부의 1996년도 특정연구개발사업 연구비지원에 의하여 수행되었음.

[1]Corresponding author.

since it can inhibit phagocytosis by neutrophils (Karakawa and Sutton, 1988; Poutrel and Boutonnier, 1988).

An earlier study (Paape and Wergin, 1979) reported that encapsulated *S aureus* isolated from milk of cows with mastitis inhibited phagocytosis by neutrophils and capsule production was enhanced by serial passage through bovine mammary glands. The capsular polysaccharide has been considered as a potential candidate for a vaccine to minimize mastitis in cows (Rather and Davis, 1986; Opdebeeck *et al.*, 1988a; Opdebeeck *et al.*, 1988b).

Alpha-toxin is an exotoxin produced by many strains of *S. aureus* of both human and animal origins. As described by Wood (1961), alpha-toxin has specific cytotoxic activity only on phagocytic cells, ie, neutrophils and macrophages. Although the majority of *S. aureus* strains isolated from bovine mastitic milk samples produce hemolysin, the role of this toxin in the pathogenesis of bovine mastitis for *S. aureus* is not clear. Removal of *S. aureus* from the mammary gland is accomplished mainly via phagocytosis; thus, the capability of alpha-toxin to kill phagocytes may prolong the infection by reducing the bacterial clearance rate. *In vitro* studies have shown that specific anti-alpha-toxin antibodies were able to neutralize the toxin and prevented cytolytic effects on phagocytes (Opdebeeck and Norcross, 1981; David and Neil, 1985; Cifrian *et al.*, 1996). Therefore, the presence of specific antibodies in milk may be useful in preventing the killing of phagocytes by alpha-toxin.

Fibronectin-binding proteins are thought to mediate attachment of *S. aureus* to host cells which may initiate disease (Beachey, 1981; Christensen *et al.*, 1985). Most isolated *S. aureus* specifically bind to extracellular matrix fibronectin (Kuypers and Proctor, 1989). FnBP has been shown to mediate binding of *S. aureus* to soluble fibronectin (Vercellotti *et al.*, 1984; Froman *et al.*, 1987; Mamo *et al.*, 1988). It has been reported that FnBP promotes adhesion of *S. aureus* to host tissue and may act as virulence factors. Thus, FnBP on *S. aureus* involved in the pathogenesis could be important in the development of subunit vaccine against bovine mastitis (Lopes *et al.*, 1985).

The purpose of this report was to evaluate the antibody levels of alpha-toxin, capsular polysaccharide and FnBP in immunized rabbit serum with an experimental vaccine against *S. aureus* which contributes to developing the bovine mastitis subunit vaccine in the future.

Materials and Methods

Bacterial strains

Staphylococcus aureus Wood 46 and Smith strain which produced large amount of alpha toxin and type 2 capsular polysaccharide were used, respectively. The strains were kindly gifted from Dr. Per Jonsson, the Uppsala University of Sweden.

Experimental animals

New Zealand white rabbits weighing 1.5 to 2.0 kg, were housed 2 animals per cage on the basis of cage size. These rabbits were fed ad libitum (rabbit pellet diet, Suwon, Korea), and provided with tap water. Rabbits were housed in an environment of 20±3.4°C. All rabbits were quarantined for 20 days prior to initiation of the study.

Partial purification and hemolytic activity of alpha-toxin

Staphylococcus aureus Wood 46 strain was incubated at 37°C in the optimal growth medium (casamino acid 64 g, DIFCO; D-glucose 8 g, Junsei Chemicals; thiamine 0.4 mg, Sigma; nicotinic acid 3.7 mg, Sigma; 2600 ml of 10% (w/v) yeast extract medium, DIFCO; adding DW to final volume to 3.2 L) for 24 hours and pelleted to remove bacteria by centrifugation with 10,000×g for 30 minutes (Glasstone *et al.*, 1961). The supernatant was passed through a 0.45-μm filter unit (Nalgene Co, Rochester, NY). The total protein concentration of the crude toxin was determined by BCA method (Pierce Inc). Supernatants were collected and stored at 4°C immediately. Ammonium sulfate (Wako Chemicals, Japan) was used to precipitate alpha-toxin and other proteinous materials and then incubated at 4°C for 4 days. Precipitates were centrifuged at 2500×g for 15 minutes, pooled and dialysed with dialysis bag (MW: 15,000, Spectrum, USA) for 72 hours against PBS

(pH 7.0). The crude alpha-toxin was analysed by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970).

The hemolytic assay is a serial two-fold dilution of 50% hemolysis end point assay described by Bernheimer and Schwartz (1963) and Harshman *et al.* (1988).

Production and partial purification of capsular polysaccharide

Staphylococcus aureus Smith strain was grown in nutrient broth supplemented with 10% (v/v) bovine milk whey for 24 h at 37°C with agitation at 300 g. The production of capsular polysaccharide was confirmed by direct examination of the bacteria with TEM (Hitachi Ltd., Tokyo, Jpn).

Capsular materials were isolated from bacterial extracts and culture supernatant. At first, the bacteria grown in the media was filtered with 0.45 μm membrane (Millipore, Badfore, MA, USA) filter after autoclaving twice. In order to remove nucleic acid and cellular proteins, the filtered solution was extracted with phenol : chloroform : iso-amylalcohol (75 : 25 : 1, v/v) (Sigma) overnight at 4°C after treatment with Dnase (80 g/ml, Sigma), Rnase (80 g/ml, Sigma) and Proteinase (0.1 mg/ml, Sigma). The solution was applied on ion-exchange chromatography. Crude capsular polysaccharide was loaded on a column (2.6×30 cm, Pharmacia Biotech) of DEAE-Sephacel (Sigma) equilibrated with 0.05 M sodium acetate buffer (pH 6.0) and eluted with sodium acetate buffer with a linear gradient from 0 to 0.5 M NaCl. The eluted materials were analyzed by a spectrophotometer at 260 and 280 nm (Skan Soft, USA) and electrophoresis. Elutes containing capsular polysaccharide were pooled and concentrated with PEG 8000 (Sigma).

Preparation of Fibronectin-binding protein (FnBP)

FnBP was prepared as the same method described by Kim *et al.* (1997). Brifely, the *fnbp* gene was amplified from the chromosomal DNA of *S. aureus* Wood 46 strain using the polymerase chain reaction, and cloned into pGEX-4T-2. Then the recombinant FnBP fused with glutathione-S-transferase was produced from E coli by affinity chromatography, and examined its antigenicity by Western immunoblotting method.

Vaccination protocol

A staphylococcal vaccine was produced by combining capsular polysaccharide and fibronection binding protein, inactivated alpha hemolysin and incomplete Freund's adjuvant. For vaccination, the toxin and FnBP were inactivated by exposure to 0.5% (v/v) formaldehyde for 16 hours at 20°C and then was lyophilized and suspended in sterile saline solution to the desired concentration. Thirty rabbits were allocated to 8 groups. The vaccine was administered two times by intramuscularly injection, with a 2-week interval between vaccinations. Rabbits in immunized groups were given doses of 10 hemolytic unit (HU) of alpha toxin and 600 μg of capsular polysaccharide, 200 μg of fibronectin binding protein in single or combination and rabbits in the control group were given PBS.

Quantitation of anti alpha-toxin antibody

Blood from individual rabbits were sampled aseptically 1 week before vaccination, and weekly Thereafter. Sera were stored at -20°C until use.

Anti alpha-toxin antibody was determined by an indirect ELISA. Briefly, alpha-toxin partially purified by ammonium precipitation method was diluted to a concentration of 0.05 μg/ml in sodium citrate buffer (0.6 M, pH 9.6), and then was coated in wells of a polystyrene cuvette overnight at 4°C. Uncoated antigen was removed by rinsing with phosphate-buffered saline solution (0.01 M, pH 7.2) with 0.5% Tween-20. After 1-hour incubation with serially diluted rabbit sera, peroxidase-conjugated goat anti-rabbit IgG (diluted 1 : 1000, Sigma) was added, followed by a 30-minute incubation. Finally, 100 μl of substrate (18.75 ml of 0.1 M citric acid, pH 4; 9.275 ml of 0.2 M Na_2HPO_4, pH 5.0; 30 μl of 30% H_2O_2, 30 mg of O-phenylenediamine; Sigma) was added to each well, and the plate was read by using an ELISA automatic analyzer (Skan soft I, USA) at 5 minutes after addition of substrate. Mean values of readings from duplicate samples placed in wells on opposite sides

of the cuvette were calculated to obtain an optical density value for each serum sample.

Quantitation of anti CPS antibody concentration

Anti-CPS antibody concentration was evaluated by an indirect ELISA. Briefly, CPS partially purified by the method of ammonium precipitation was diluted to a concentration of 0.05 μg/ml in sodium citrate buffer (0.6 M, pH 9.6), and then CPS was coated by incubation in wells of a polystyrene cuvette overnight at 4°C. Wells were rinsed with phosphate-buffered saline solution (0.01 M, pH 7.2) with 0.5% Tween-20 (P7949, Sigma) and serially diluted rabbit sera were added. After a 1-hour incubation, peroxidase-conjugated goat anti-rabbit IgG (diluted 1 : 1000) was added, followed by a 30-minute incubation. Finally, 100 μl of substrate (18.75 ml of 0.1 M citric acid, pH 4; 9.275 ml of 0.2 M Na_2HPO_4, pH 5.0; 30 μl of 30% H_2O_2, 30 mg of OPD) was added to each well, and the plate was read by using an ELISA automatic analyzer (Skan soft I, USA) at 5 minutes after addition of substrate. Mean values of readings from duplicate samples placed in wells on opposite sides of the cuvette were calculated to obtain an optical density value for each serum sample.

Quantitation of anti GST-FnBP antibody

Recombinant GST-FnBP was diluted to a concentration of 1.25 μg/ml in carbonate-bicarbonate buffer (pH 9.6) and then incubated in wells of polystyrene cuvette at 37°C for 2 hrs. Wells were rinsed with PBS and PBST with 1% Bovine Serum Albumin (BSA) was used as a block at 37°C for 30 minutes. Serum was diluted (1 : 200) with PBST with 0.5% BSA (electrophorsis grade, Sigma, USA) and dispensed in 50 μl aliquots to each well at 37°C for 1 hr and then rinsed four times. Fifty microliter of substrate including O-Phenylenediamine (P5412, Sigma, USA) was added to each well at room temperature for 10 min and the plate was read 5 minutes later using an ELISA reader (ANTHOS HTIII) 5 minutes later.

Challenge experiment

The bacterial challenge for the subsequent experiments was prepared as follows. *S. aureus* Wood 46 strain was grown overnight (18 to 24 h) in tryptic soy broth (Difco Laboratories, Detroit, MI, USA). Bacterial cells were adjusted to 108 cells/ml (540 nm spectrophotometrically) with phosphate-buffered saline (PBS, pH 7.2). The bacteria was inoculated intravenously via jugular vein and blood samples were collected at 1, 3, 6, 12, and 24 h, respectively. 100 μl of blood samples obtained each time were inoculated on the tryptic soy agar (Difco Laboratories, Detroit, MI, USA) plates and the number of bacterial colonies counted.

Rabbits in each group were exsanguinated, and a sample of their blood and urine was cultured for bacterial counts. Rabbits exsanguinated at 24 h for bacterial counts in blood were also evaluated for the presence of bacteria in liver and spleen. Organs were excised, weighed, washed with ethyl alcohol (99.9%, Hayman Limited, ESSEX, ENGLAND) to eliminate surface-attached organisms, homogenized with sterile PBS (pH 7.2), and cultured on columbia agar plates. The plates were counted 24 h later and expressed as CFU per gram of tissue.

Statistical analyses

The Multiple ANOVA test was used for statistical evaluation of differences in optical density readings between each group of rabbits for pre- and post-vaccination serum samples. Also the same test was used to determine the difference of bacterial clearance rate between each group of rabbits for pre- and postvaccination. A value of $p<0.05$ was considered to be significant.

Results

The toxin and other minor protein bands of each partial purification step was observed by SDS-PAGE. However, at this stage contaminated proteins of molecular weight higher than that of alpha-toxin monomer (33 kDa) were present. Results of the enzyme immunosorbent assay against alpha-toxin are shown in Fig 1. Each of groups showed increased antibody levels specific for alpha toxin without significant variation when compared with PBS control group which showed no significant increase of IgG

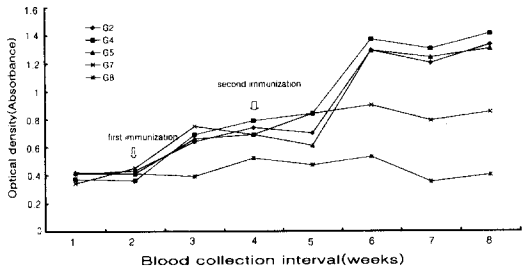

Fig 1. ELISA results for IgG specific for staphylococcal alpha toxin in rabbits immunized with subunit vaccine 2 times every 2 weeks (G2: alpha toxin, G4: alpha toxin+ FnBP, G5: alpha toxin+CPS, G7: alpha toxin+FnBP+ CPS, G8: PBS).

specific for alpha toxin. Peak levels were reached 2 weeks following immunization and, particularly the levels of antibody was significantly increased following second immunization ($p<0.001$).

A major band of capsular polysaccharide (97 kDa) was observed by SDS-PAGE following by silver staining after removing teichoic acid from the solution by oxidation with sodium metaperiodate and purifying CPS by gel filtration.

Immune responses against CPS was analysed by ELISA (enzyme immunosorbent assay) (Fig 2). Each of groups shows increased CPS specific antibody levels in serum when compared with PBS control group which showed no significant increase of antibody specific for staphylococcal CPS. However, antibody level of group 4 (alpha toxin+CPS) was the highest. Peak levels were reached 4 weeks following primary

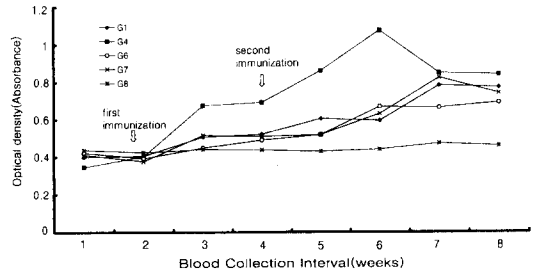

Fig 2. ELISA results for IgG specific for staphylococcal Capsular polysaccharide subjected to bacterin-toxoid stimulation two times every 2 weeks (G1: CPS, G4: alpha toxin+ CPS, G6: CPS+FnBP, G7: alpha toxin+CPS+FnBP, G8: PBS).

immunization and, particularly the level of antibody was significantly increased ($p<0.001$).

The *fnbp* gene were amplified from signal sequence to membrane-spanning domain (3079 bp), from D1 to membrane-spanning domain (961 bp), and from D1 to D3 (475 bp) by PCR. After cloning amplified *fnbp* gene into pGEX-4T-2 expression vector, the recombinant GST-FnBP was produced from signal sequence to membrane-spanning domain (236 kDa), from D1 to membrane-spanning domain (129 kDa) and from D1 to D3 (49 kDa).

Anti-FnBP levels of formalinized FnBP groups were significantly higher than those of PBS-control group from 1 weeks after first immunization ($p<0.05$) (Fig 3). There were no significantly differences in antibody levels between formalinized-FnBP groups. The levels were peacked at 5 weeks through the study and, particularly the levels of antibody were significantly increased following second boosting ($p<0.001$).

In the challenge study, bacterial counts from blood samples was performed at the different time points after challenge with 10^8 cfu/ml of *S. aureus*. The trends of bacterial counting are shown in Fig 4. The bacterial number of immunized groups in blood was lower than that of control group after bacterial challenge. Of all groups, group 7 (alpha toxin+CPS+ FnBP) showed the lowest bacterial number in blood 24 hours after challenge ($p<0.001$). However, the bacterial number between the vaccinated groups was not significantly different.

Spleen and liver were excised, washed with 70% ethanol, homogenized in 1 ml of PBS, and cultured

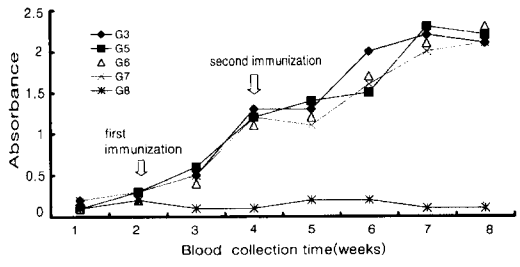

Fig 3. ELISA results for IgG specific for formalin treated GST-FnBP in rabbits immunized with subunit vaccine (G3: FnBP, G5: alpha-toxin+FNBP, G6: FnBP+CPS, G7: alpha-toxin+FnBP+CPS, G8: PBS).

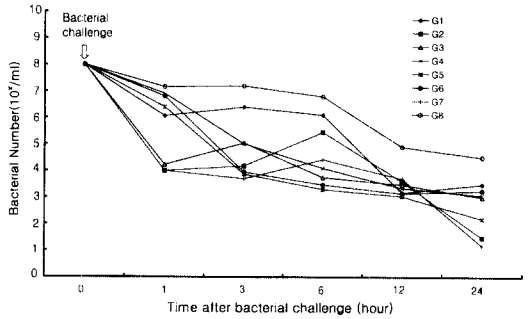

Fig 4. Effect of IM immunization on the blood counts of rabbits challenged with 10^9 cfu/ml of *S. aureus* Wood 46 (G1: CPS, G2: alpha toxin, G3: FnBP, G4: alpha toxin+CPS, G5: alpha toxin+FNBP, G6: CPS+FnBP, G7: alpha toxin+FnBP+CPS, G8: PBS).

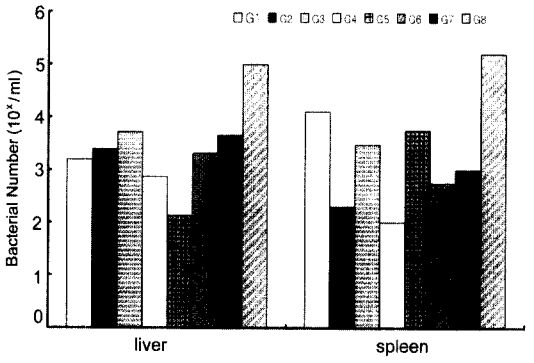

Fig 5. Effect of IM immunization on bacterial counts from liver and spleen of rabbits challenged with 10^8 cfu/ml of *S. aureus*. Wood 46 (G1: CPS, G2: alpha toxin, G 3: FnBP, G4: alpha toxin-CPS, G5: alpha toxin+FnBP, G6: CPS+FnBP, G7: alpha toxin-CPS+FnBP, G8: PBS).

for bacterial counts. Results are expressed as common logarithmic scale mean bacterial counts for all animals (Fig 5). Generally, the bacterial number recovered from excised organs showed no significant difference among the vaccinated groups. However vaccinated groups had significantly lower bacterial numbers than that of PBS-control group in both spleen and liver ($p < 0.05$).

Discussion

Hemolytic activity of alpha-toxin and protein concentration was at their maximum in the growth medium at 18 h postinoculation. The result is consistent with a previous report by Lind *et al.* (1987) who reported the simple method of alpha-toxin purification. Therefore, we harvested the bacteria and alpha-toxin antigen at 24 h postinoculation for the preparation of vaccine. Advantages of the procedure described here were rapidity in purification of alpha-toxin and stabilizing of the toxin by the addition of ammonium sulfate (65% saturation).

The increased level of serum antibody against alpha-toxin (Fig 1) reflected the increased systemic protective ability against alpha-toxin in rabbits immunized with the vaccine. This agreed with reports by Balaban *et al.* (1998) who showed that serum antibody levels to RAP (RNAIII activating protein) were increased in mouse with *S. aureus* infections. It thus seemed that alpha toxin produced in blood reached systemic lymphoid tissue in sufficient concentrations to generate a systemic immune response.

The alpha toxin preparation can be used as vaccine subunit, although only partially purified. These results indicated, however, that if alpha toxin were included in a vaccine with the intent to reduce the frequency of *S. aureus*-induced mastitis, then sufficient toxin should be included in vaccine to produce anti-alpha toxin level sufficient to neutralize minimal amounts of toxin.

As a report of Poutrel *et al.* (1988), and International Dairy Federation (1986), most CPS from bacteria were released by autoclaving. Clear supernatants were obtained by centrifugating autoclaved bacterial culture.

The level of serum antibody against CPS, as determined by ELISA was closely related with systemic protective ability in the rabbit immunized with the vaccine. However, serum antibody levels was relatively lower than those of alpha-toxin and FnBP. Therefore further study of this vaccine was needed to increase levels of serum antibody against capsular polysaccharide through conjugation with alpha-toxin or FnBP.

The CPS preparate could be used as a subunit vaccine, although only partially purified. However, these results indicated that if CPS were included in a vaccine with the intent to reduce the new infection rate of *S aureus*-induced mastitis, then sufficient CPS should be included *in vaccine* to produce sufficient anti-CPS level.

The lower number of bacteria recovered from blood suggests that opsono-phagocytosis may play an important role. In antibodies against FnBP may directly block the adherence of the bacteria to host tissue through a fibronectin mediated mechanism.

The number of bacteria in excised organs were significantly lower in immunized group than in control group. This result showed consistency with the study results of Fattom et al (1996) other than the use of different antigen for immunization.

These results showed that active immunization elicited by *S. aureus* alpha toxin, capsular polysaccharide and fibroncetin binding protein are protective in rabbit models. Also, these results confirmed the roles of alpha toxin, capsular polysaccharide and fibroncetin binding protein as a virulence factor and provided an promising prospective of the vaccine for the prevention of *S. aureus* infection in cows.

The efficacy of this vaccine and of antibodies derived from vaccinated rabbits will determine their usefulness and the validity and application of protective immunity in *S. aureus* pathogens and infections. And it is expected that it will be possible to demonstrate protective immunity in mastitic cows with *S. aureus* infections if further studies were made to determine the route of administration, the optimal immunized dose and the effective adjuvant.

Considering that most *S. aureus* isolated from the mastitic cows produce the antiphagocytic properties of CPS, and that the essential role of FnBP in the adhesion to host cells and anti-toxin effects of alpha-toxin, these antigens should be considered for development of efficient vaccines against bovine mastitis. Also further studies on bovine *Staphylococcus aureus* subunit vaccine are needed. Until recently, although most studies were focused on alpha-toxin and capsular polysaccharide subunit vaccine, we will study the subunit vaccine including alpha-toxin, capsular polysaccharide and fibronectin binding protein to overcome the disadvantages.

Acknowledgements

We are grateful to staffes in Kangwon National University. And we thank to the Ministry of Agriculture and Forestry for financial support.

References

1. Balaban N, Goldkorn T, Rachael TN, Dang HB, Steven S, Ridgley RM, Rasooly A, Wright SC, Larrick TW, Rasooly R, Carson JR. Autoinducer of virulence as a Target for vaccine and therapy against *Staphylococcus aureus*. Science 1998; 280(17): 438-440.
2. Beachey EH. Bacterial adherence. Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 1998; 143: 325-45.
3. Bernheimer AW, Schwartz LL. Isolation and Composition of Staphylococcal Alpha Toxin. J Gen Microbiol 1963; 30: 455-457.
4. Christensen GD, Simpson WA, Beachy H. Ahhesion of bacteria to animal tissues. In: DC Savage and M Fletcher, eds., Bacterial adhesion. New York: Plenum Press. 1985; 279-305.
5. Cifrian E, Guidry AJ, O'Brian NO, Marquardt WW. Effect of antibodies to staphylococcal alpha and beta toxins and *Staphylococcus aureus* on the cytotoxicity for and adherence of the organism to bovine mammary epithelial cells. Res Vet Sci 1996; 57: 1308-1311.
6. David AL, Neil LN. Enzyme-linked immunosorbent assay for detection of milk immunoglobulins to leukocidin toxin of *Staphylococcus aureus*. Am J Vet Res 1985; 46: 1728-1732.
7. Fattom A, Schneerson R, Karakawa, WW, Fitzgerald D, Pastum I, Li X, Shiloach J, Bryla DA, Robbins JB. Laboratory and clinical evaluation of conjugate vaccines composed of *S. aureus* type 5 and 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exoprotein A. Infect Immun 1993; 61: 1023-1032.
8. Fattom AI, Sarwar JA, Ortiz, Naso R. A *Staphylococcus aureus* capsular polysaccharide (CP) Vaccine and CP-specific antibodies protect mice against bacterial challenge. Infect Immun 1996; 64(5): 1659-1665.
9. Froman G, Switalski L, Speziale P, Hook M. Isolation and characterization of a fibronectin receptor from *Staphylococcus aureus*. J Biol Chem 1987; 262: 6564-6571.
10. Glasstone GP, Mudd S, Hochtein HD. The assay of antistaphylococcal leucocidal components (F and S) in human serum. Br J Exp Pathol 1961; 42: 295-312.
11. Guidry AJ, Oliver SP, Squiggins KE, Erbe EF, Dowlen HH, Hambleton CN, Berning LM. Effect

of anticapsular antibodies on neutrophil phagocytosis of *Staphylococcus aureus*. J Dairy Sci 1991; 74: 3360-3369.
12. Harshman S, Sugg N, Cassidy P. Preparation and Purification of Staphylococcal Alpha Toxin. J Enzymol 1988; 165: 3-7.
13. Herbelin C, Poutrel B, Gilbert FB, Rainard P. Immune recruitment and bactericidal activity of neutrophils in milk of cows vaccinated with staphylococcal alpha toxin. J Dairy Sci 1997; 80: 2025-2034.
14. International Dairy Federation. Bovine mastitis. Definition and guidelines for diagnosis. No 211, Int. Dairy Fed. Bull, Brussels, Belgium. 1993: p. 3.
15. Karakawa WW, Sutton A. Capsular antibodies induce type-specific phygocytosis of encapsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. Infect Immun 1988; 56: 1090-1095.
16. Kim D, Chenong CR, Park HM. Production of the recombinant fibronectin-binding protein of *Staphylococcus aureus*. Korean J Vet Res 1997; 37(4): 875-882.
17. Kuypers JM, Proctor RA. Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. Infect Immun 1989; 57: 2306-12.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.
19. Lind I, Ahnert HG, Fuchs G, Gratzl M. Purification of Alpha-Toxin from *Staphylococcus aureus* and Application to Cell Permeabiliztion. J Enzymol 1987; 164, 84-89.
20. Lopes JD, Dos RM, Brentans RR. Presence of laminin receptors in *Staphylococcus aureus*. Science 1989; 229: 275-7.
21. Mamo W, Fromman G, Wadstrom T. Interaction of subepithelial connective tissue components with *Staphylococcus aureus* and coagulase-negative staphylococci from bovine mastitis. Vet Microbiol 1988; 18: 163-176.
22. Nelson L, Flock JI, Lindberg M, Ler M, Wadstrom T. Adhesins in staphylococcal mastitis as vaccine components. Flem Vet J 1991; 62: suppl (1) 111-125.
23. Norcross NL, Opdebeeck JP. Encapsulation of *Staphylococcus aureus* isolated from bovine milk. Vet Microbiol 1983; 8: 97-404.
24. Opdebeeck J, Norcross NL. Enzyme immunoassay for detection of antibodies specific for staphylococcal alpha-haemolysin in bovine milk. Res Vet Sci 1981; 30: 83-86.
25. Opdebeeck JP, Norcross NL. Enzyme-linked immunosorbent assay for detection of antibodies specific for staphylococcal alpha-haemolysin in bovine milk. Am J Vet Res 1983; 44: 986-988.
26. Opdebeeck JP, O'Boyle DA, Frost AJ. Immunogenic properties of *Staphylococcus aureus* and *Streptococcus agalactiae* administered separately and in combition to lactating cows. Aust Vet J 1988a; 5: 194-195.
27. Opdebeeck JP, Watson DL, Frost AJ. Comparisons of *Staphylococcus aureus* grown *in vitro* and *in vivo*. Vet Microbiol 1988b; 16: 87-91.
28. Paape M, Wergin WP. Leukocytic defense mechanisms in the udder. J Dairy Sci 197; 62: 135-153.
29. Poutrel J, Bouttonier JA, Sutra L, Fournier JM. Prevalence of capsular polysaccharide types 5 and 8 among *S. aureus* isolates from cow, goat, and ewe milk. J. Clin. Microbiol 1988; 26: 8-14.
30. Rather PN, Davis AP. Slime prodution by bovine milk *Staphylococcus aureus* and identification of coagulase-negative staphylococcal isolates. J Clin Microbiol 1986; 23: 858-862.
31. Vercellotti GMN, Lussenhop D, Peterson PK. Bacterial adherence to fibronectin and endothelial cells: a possible mechanism for bacterial tissue tropism. J Lab Clin Med 1984; 103: 34-43.
32. Wood AM. Assay of the two components of Staphylococcal leucocidin and their antibodies. J Pathol Bacteriol 1961; 81: 63-68.