Refereed Article

Determination of Alpha-toxin Antibodies Against *Staphylococcus aureus* and Phagocytic Ability Postimmunization in Rabbits

Hong Ryul Han, Hee Myung Park, Doo Kim[1]
College of Veterinary Medicine, Seoul National University
College of Veterinary Medicine, Kangwon National University[1]
Republic of Korea

Summary

Thirty rabbits were inoculated intramuscularly three times every 2 weeks with a staphylococcal subunit bacterin preparation (10 HU of alpha-toxin, 600 μg of capsular polysaccharide, 200 μg of fibronectin binding protein). The levels of specific antibody against alpha toxin in immunized rabbits were more significantly increased over 4 weeks than in control animals that showed no responses. However in the results of study for bacterial clearance rate, there were no significant differences although the bacterial numbers of immunized group were lower than those of control group. And this study showed that bacterial numbers in excised organ also were lower in all immunized rabbits than in PBS-control group though there were no significant differences statistically. The present study was to show the protective and productive ability of alpha-toxin included in subunit vaccine for bovine mastitis vaccine candidate.

Introduction

Alpha-toxin is an exotoxin produced by many strains of *Staphylococcus aureus* of both human and animal origins. As described by Woodin,[10] α-toxin has specific cytotoxic activity only on phagocytic cells. Removal of *S. aureus* from the mammary gland is accomplished mainly via phagocytosis. thus, the capability of α-toxin to kill phagocytes may prolong the infection by reducing the bacterial clearance rate. *In vitro* work has shown that specific anti-α-toxin antibodies are able to neutralize the toxin and prevent cytolytic effects on phagocytes.[3,4,7] Therefore, the presence of specific antibodies in serum may be useful in preventing the killing of phagocytes by α-toxin. **The purpose of this report was to develop mastitis subunit vaccine and to quantify specific antibodies to α-toxin in rabbit serum samples. Also to determine a optimal adjuvant for increasing anti-α-toxin antibody to confirm the protective ability in challenge inoculation of *S. aureus* via jugular vein.**

Materials and Methods

Staphylococcus aureus Wood 46 strain was incubated at 37°C in the optimal growth medium for 24 hours.[6] Briefly, supernatants were collected and stored at 4°C immediately. Ammonium sulfate was used to precipitate alpha-toxin. Precipitates were centrifuged with 2500 xg for 15 minutes, pooled and dialysed with dialysis bag (MW: 15,000) for 72 hours in PBS (pH 7.0).

The hemolytic assay is a serial two-fold dilution of 50% hemolysis end point assay described by Bernheimer and Schwartz.[9]

The crude α-toxin was further evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide gel).

A staphylococcal vaccine was produced by combining capsular polysaccharide and fibronection binding protein preparation with inactivated staphylococcal α hemolysin and incomplete Freund adjuvant.

Blood samples from individual rabbits were collected aseptically 1 week before vaccination, and every week. Serum was frozen at -20°C until analyzed.

S. aureus Wood 46 strain was grown overnight (18 h to 24 h) in tryptic soy broth (Difco Laboratories, Mich). Bacterial cells were suspended, corresponding to 10^8 cells/ml with phosphate-buffered saline (PBS). The bacteria was inoculated intravenously via jugular vein and blood samples were collected at 1, 3, 6, 12, and 24 hrs, respectively. And then 100μl of blood samples obtained each time were inoculated on the tryptic soy agar plates and the bacterial colonies were counted

Rabbits of each group were exsanguinated, and a

sample of their blood and urine was cultured for bacterial counts. Rabbits exsanguinated at 24 hrs for blood counts were also evaluated for liver, kidney and spleen. Organs were excised, weighed, washed with ethyl alcohol to eliminate surface-attached organisms and then with sterile PBS, and cultured on columbia agar plates. The plates were counted 24 h later and expressed as CFU per gram of tissue.

The Multiple ANOVA test was used to evaluate the differences in optic density and bacterial clearance rate between each group of cows.

Results

Staphylococcus aureus strain Wood 46 exhibited exponential growth in the first 11 hrs when grown aerobically on casein-yeast extract medium. Production of toxin was peaked at 18 hrs following inoculation, and when bacteria reached the stationary phase toxin released into the growth medium. Likewise, the production of protein was the same as above result.

Table 1. Comparison of bacterial growth rate, protein concentrations with alpha-toxin hemolytic activity following 0, 8, 11, 16, 18, 33, 46 hrs on casein-yeast extract medium.

Postinoculation	A(absorbance)	B(mg/dl)	C(HU)
0 hr	0.039	9.5	0
8 hrs	1.325	13	2400
11 hrs	1.346	12	1800
16 hrs	1.040	11.5	4800
18 hrs	1.241	9	9600
33 hrs	1.228	7	3600
46 hrs	0.91	8	1200

A: Absorbance of bacterial growth rate (578 nm)
B: Total protein concentration of 1ml culture medium, 562nm
C: Hemolytic activity using 10% rabbit erythrocyte suspension, 412nm with time (end-point method)

The crude toxin harvested after 18 h and precipitated with ammonium sulfate.

However, at this stage contaminated proteins of molecular weight higher than that of alpha-toxin monomer (33 kDa) were present.

Results of the enzyme immunosobent assay are shown in Table 2. Each of groups shows increased alpha toxin specific antibody in serum when compared with PBS control group. Control rabbits showed no significant increase of IgG (whole molecule) specific for staphylococcal alpha toxin. Additionally, there was no significant variation of increased serum antibody titer between groups. Peak levels were reached 32 days following immunization and, particularly the levels of antibody was significantly increased following last booster ($P<0.001$).

10^8 CFU/ml of *S. aureus* Wood 46 was inoculated via jugular vein and then blood samples of each groups were collected at 1, 3, 6, 12 and 24 hrs respectively.

Table 2. ELISA Results for IgG specific for staphylococcal alpha toxin. Rabbits wre subjected to staphylococcal bacterin-toxoid stimulation three times every 2 weeks.

Time	Groups				
	G2(n=6)	G4(n=6)	G5(n=6)	G7(n=6)	G9(n=6)
1 wk	0.41±0.076	0.42±0.030	0.42±0.021	0.42±0.059	0.41±0.025
2 wks	0.41±0.023	0.36±0.026	0.41±0.019	0.45±0.057	0.41±0.051
3 wks	0.61±0.107	0.61±0.017	0.66±0.028	0.68±0.095	0.41±0.078
4 wks	0.64±0.034	0.73±0.071	0.69±0.047	0.66±0.021	0.52±0.032
5 wks	0.64±0.029	0.64±0.024	0.61±0.034	0.64±0.032	0.47±0.045
6 wks	1.29±0.070	1.37±0.110	1.29±0.071	1.37±0.086	0.53±0.061
7 wks	1.26±0.049	1.35±0.073	1.38±0.194	1.17±0.129	0.54±0.091
8 wks	1.33±0.077	1.41±0.099	1.30±0.045	1.32±0.067	0.40±0.076

G2: alpha toxin group
G4: alpha toxin combined Fibronectin binding protein
G5: alpha toxin combined with capsular polysaccharide (Staphylococcus aureus smith strain)
G7: alpha toxin combined with fibronectin binding protein and capsular polysaccharide
G9: PBS control

The trends of bacterial number was shown in figure 1 and 2. Generally the bacterial number was decreased until postchallenge 6 hrs and then slightly increased trends was observed 12 hrs and 24 hrs postchallenge. In both groups, however, the bacterial number of PBS control group was a little higher than those of immunized groups. No statistical significance was not observed.

Values are per milliliter for urine and per gram for liver, kidney, and spleen. Results are expressed as common logarithmic scale mean bacterial counts for all animals. Kidneys and liver were excised, weighed with 70% ethanol, homogenized in 1 ml of PBS, and cultured for bacterial counts.

Also the bacterial counts of each groups were shown in figure 2. The results for the bacterial shedding through urine of immunized and non-immunized rabbits were shown in figure 2. This results described above were consistent with the significant lower baterial cell numbers in urine than those of control group.

Discussion

When used in vaccination, the optimal preparation time of alpha-toxin antigen was 18 h following inoculation due to destruction of alpha-toxin by a variety of proteinase. The result agrees with reports by Lind *et al*[8] who showed the simple method of alpha-toxin purification.

A partially purification method of alpha-toxin of this present study was simpler than any other methods. This purification method has no time consuming

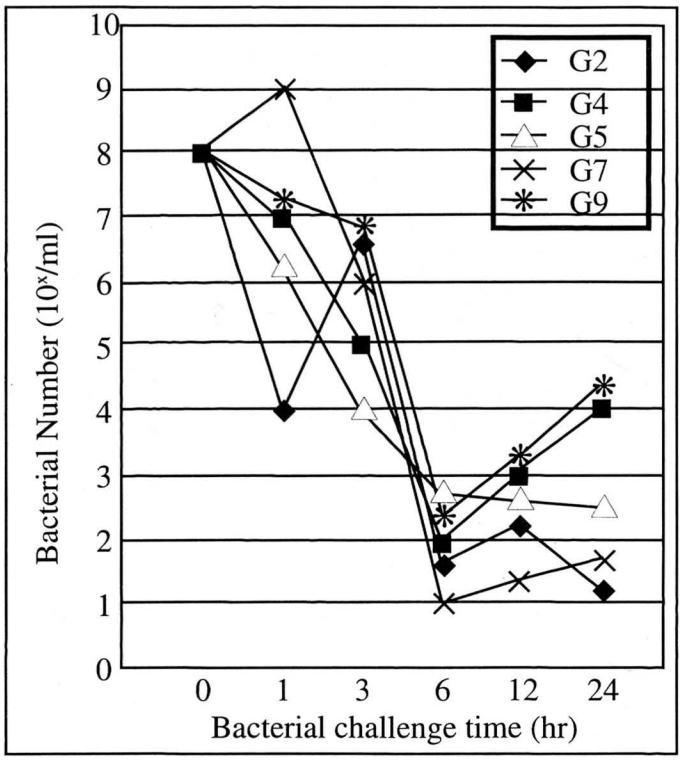

Figure 1. Effect of IM active immunization on the blood counts of rabbits challenged with 10^8 cfu/ml of *S. aureus* Wood 46.

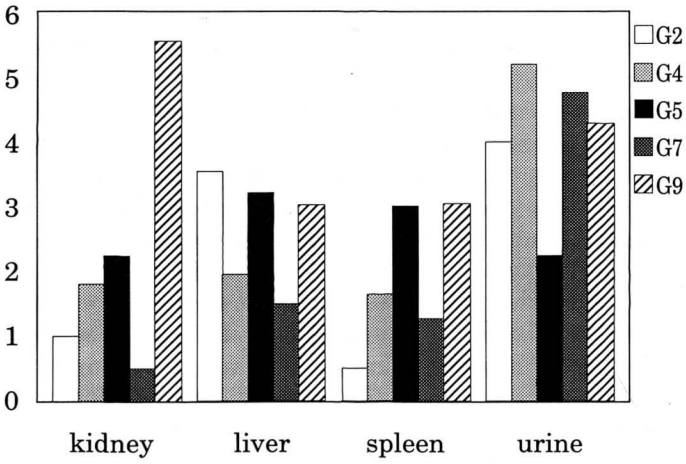

Figure 2. Effect of IM immunization on bacterial counts from liver, kidney, spleen and urine shedding of rabbits challenged with 10^8cfu/ml of *S. aureus* Wood 46.

and laborious efforts. However, the confirmation step was necessarily needed when purified by ammonium precipitation method.

The use of supernatants following centrifugation of medium for determination of hemolytic activity has no good results due to a small amount of alpha-toxin. Therefore when determined hemolytic units and activities, partially purified alpha-toxin should be used following ammonium precipitate. i.e. the concentration step was needed.

The increased serum anti-alpha toxin levels in *S. aureus*-immunized rabbits reflected increased systemic protective ability of anti-alpha toxin antibodies.[2] This agree with reports by Towers who showed that serum antibody levels to alpha toxin were increased in persons 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 increases in serum anti-alpha toxin in *S. aureus*-immunized rabbits were the result of a systemic antigenic stimulus.

The alpha toxin preparation used as vaccine subunit, although only partially purified, had good hemolytic activity. 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 and to neutralize minimal amounts of toxin.

The bacterial counts in excised organ of G2, G4, G5, G7 immunized groups were significantly lower than those of control group. This result showed consistency with the study results of Fattom *et al*[5] other than the use of different antigen for immunization.

These results showed that although there was no statistically significant difference, active immunization elicited by *S. aureus* alpha toxin are protective in rabbits models. Also these results confirm the role of alpha toxin as virulence factors and protective antigens and provide an impetus for further development of the vaccine and vaccine induced antibodies for the prevention of *S. aureus* infection in cows.

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

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