KAWASAKI MED. J. VOL. 10 : NO. 2, 91-99 (1984)

A Comparative Study on the Resistance of Protein A-deficient Mutant and Cowan I Strains of *Staphylococcus aureus* to Hydrogen Peroxide *in Vitro*

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Accepted for Publication on March 7, 1984

ABSTRACT. A protein A-deficient strain, HL-87, was isolated from *Staphylococcus aureus* (*S. aureus*) Cowan I strain possessing protein A. Using the two strains, Cowan I and HL-87, the resistance to the bactericidal effect of hydrogen peroxide (H_2O_2) *in vitro* was investigated. It has been revealed that the viability of HL-87 strain was significantly lower than that of Cowan I strain after incubation in the presence of H_2O_2. When the catalase activity in both strains was assayed, HL-87 strain showed higher activity than Cowan I strain did, indicating that the difference in the resistance to *in vitro* H_2O_2 between the two strains did not depend on their catalase activity. These results suggested that protein A in the cell walls of Cowan I strain was directly related to the protection from the H_2O_2-bactericidal effect.

Key words : Hydrogen peroxide — Protein A — Resistance — *Staphylococcus aureus*

The protein A which is one of the major components of the cell wall of *Staphylococcus aureus* (*S. aureus*),[1] is well known for its unique properties of reacting with Fc piece of immunoglobulin G of most mammalian sera nonspecifically.[2-4] Many workers investigated the relationship between the pathogenicity of the bacteria and the presence of protein A and suggested that protein A displayed the anti-phagocytic activity against polymorphonuclear leukocytes (PMN).[5-7] Our previous studies indicated that the resistance to the bactericidal effect of hydrogen peroxide (H_2O_2) which is considered to be responsible for the bactericidal effect of PMN[8,9] was different in degree between *S. aureus* Cowan I strain possessing protein A and protein A-deficient Wood 46 strain, suggesting that the viabilities of the organisms in the presence of H_2O_2 might be due to the protein A content in the cell walls.[10] These studies were, however, carried out on genetically different strains. To determine whether protein A of *S. aureus* is really responsible for the resistance to the H_2O_2-bactericidal effect or not, we isolated a mutant strain from Cowan I strain by the method of Masuda *et al.*[11] and attempted to determine the resistance of the mutant and Cowan I strains to *in vitro* H_2O_2.

MATERIALS AND METHODS

Bacterial strains

Two strains were used throughout the experiments. One was *S. aureus*

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Cowan I strain, which was kindly supplied by Dr. Y. Arai (Saitama Medical School, Saitama, Japan). The other, HL-87 strain, was one of the protein A-deficient mutants which were isolated from Cowan I strain by a procedure modified from the method of Masuda *et al.*[11]

An overnight culture of Cowan I strain in brain heart infusion broth (BHIB) was inoculated freshly to BHIB and incubated at 37°C for 3 hr. The bacteria were washed with saline and placed in a Petri-dish, which was placed under ultraviolet light until 10^8 colony forming units (CFU)/ml of the bacterial viability were decreased down to 10^5 CFU/ml. The irradiated bacteria in 0.5 ml of the suspension were inoculated to fresh BHIB and incubated with shaking for 3 hr. Two ml of cell culture were mixed with an equal volume of heavily sensitized sheep red blood cells (H-SRBC), incubated at room temperature for 10 min and then spun down at $2{,}300 \times g$ for 1 min to remove the aggregates of the protein A positive bacteria and H-SRBC. The bacteria in the supernatant were cultivated in BHIB at 37°C overnight. This isolation procedure was repeated five times. The final supernatant was appropriately diluted and placed onto a brain heart infusion agar (BHIA) plate containing 1 per cent normal dog serum ; then, the haloless colonies were collected after overnight incubation. The hemagglutination test indicated that the bacteria collected were protein A-free cocci.

Sensitized sheep red blood cells

Two types of sensitized sheep red blood cells were prepared : One was heavily (H-SRBC) and the other normally (N-SRBC) sensitized, respectively.

Ten ml of sheep red blood cells (SRBC) in suspension (50 per cent w/v ; Nippon Bio-Test Laboratories Inc., Tokyo, Japan) were washed twice with veronal-buffered saline (VBS) and the cells were suspended in 20 ml VBS. This suspension was then mixed with an equal volume of anti-SRBC rabbit IgG solution (Japan Immunoresearch Laboratories Co., Takasaki, Japan) which was diluted with VBS to 1 : 8 before mixing. The mixture was incubated at 37°C for 30 min. The cells were washed at $170 \times g$ three times with VBS and then suspended in VBS up to 10 ml. This H-SRBC preparation was used to isolate protein A-deficient cocci from Cowan I cultures. To prepare N-SRBC, an appropriate volume of SRBC was washed twice and then suspended with VBS to adjust at 1×10^9 cells/ml. The suspension was mixed with an equal volume of anti-SRBC rabbit IgG solution which titer was adjusted at a half of the hemagglutination. The mixtures were incubated at 37°C for 30 min. After that, they were washed three times and then the treated SRBC were fed with VBS up to approximately 5×10^8 cells/ml. This N-SRBC preparation was used to assay protein A content by the hemagglutination test.

Hemagglutination test

Hemagglutination test described by Winblad and Ericson[12] was used to select the protein A-deficient mutants from the population of Cowan I strain.

The bacteria isolated by the selection with H-SRBC were washed once with phosphate-buffered saline (PBS), suspended in 200 μl PBS (approximately 2×10^8 CFU/ml), and then mixed with an equal volume of N-SRBC in an agglutination tray (Tomy Seiko, Co., Tokyo). The mixture was incubated for 2 hr at 37°C and kept at 4°C overnight. The bacteria without hemagglutination were collected as protein A-deficient mutants and designated as HL strains.

One of the isolated mutants, HL-87 strain, was used throughout the present study.

Relative amount of protein A in Cowan I and HL-87 strains, were assayed by a modified method of Spika *et al.*[13] Briefly, 1.0 ml portion (approximately 10^9 CFU/ml) of the bacterial cells cultivated in tryptosoy broth (TSB) was spun down at 2,300×g for 15 min into a pellet. The bacteria were suspended in 2.0 ml of PBS containing 5 units lysostaphin per ml and then incubated for 2 hr at 37°C. After incubation, the suspension was centrifuged to remove nonlysed bacteria and cell debris. The supernatant was made up into serial two-fold dilutions with PBS on the agglutination tray. To 200 μl of each diluted supernatant was added 200 μl of N-SRBC solution. The mixture was incubated at 37°C for 2 hr and kept at 4°C overnight. The hemagglutination titer was defined as the inverse of the highest dilution showing the visible hemagglutination.

Assay of the resistance to H_2O_2

The resistance of the mutant and Cowan I strains to the H_2O_2-bactericidal effect was determined by a modified procedure of the method reported by Babior *et al.*[14]

The bacterial suspension was prepared as follows : The bacteria cultivated in TSB for 5 hr were washed twice with PBS and then suspended in PBS. The suspension was diluted and adjusted by spectrophotometry at 0.05 optical density at 620 nm (approximately 1×10^7 CFU/ml). The suspension was further diluted with PBS to 2×10^4 CFU/ml). In some of the experiments, the suspension was serially diluted from 2×10^5 to 2×10^3 CFU/ml to determine the survival cell number in an appropriate concentration of H_2O_2. The bacterial suspension and H_2O_2 (4.41 μM/ml or 5.88 μM/ml) in PBS solution, 0.5 ml each in a droplet, were placed separately from each other on a plastic Petri-dish. The reaction was initiated by mixing the droplets by turning the Petri-dish. The mixture was then placed in a 37°C incubator for 10 min, and the reaction was terminated by the addition of 15 ml nutrient agar (NA). After incubation at 37°C for 36 to 44 hr, the colonies were counted. To examine the time course of the H_2O_2-bactericidal effect, the reaction was terminated at 10, 20, 30, 40 and 50 min after mixing and the viable cells were counted by the method mentioned above.

Assay of the catalase activity

The catalase activity was assayed by the H_2O_2-decrease in reaction mixtures with the cells. The H_2O_2-decrease was measured by a modified spectrophotometric method of Beers and Sizer.[15]

The bacterial suspensions, 5×10^7 and 1×10^8 CFU/ml, in 0.05 M phosphate buffer (PB) were prepared. One ml of each suspension was mixed with 2.5 ml of H_2O_2 solution (5 mM/ml) in a cuvette. The mixture was allowed to stand at room temperature (20±0.5°C) for 1, 5, 10, 15, 20, 25 and 30 min and then were measured for the optical density at 240 nm.

Culture media and reagents

BHIB, BHIA, TSB and NA were purchased from Nissui Pharmaceutical Co. (Tokyo). Lysostaphin was also purchased from Sigma Chemical Co. (U.S.A.). VBS,[11] PBS of Dulbecco and Vogt, but lacking Ca and Mg, and 0.05 M PB (pH 7.0) were used.

RESULTS

Properties of HL-87 strain

HL-87 strain was one of the protein A-deficient mutants isolated from Cowan I strain. When the growth of both Cowan I and HL-87 strains in TSB was followed spectrophotometrically and by the assay of viable cell count, no difference in doubling time (51.2±5.5 min) between the two strains observed.

The biological properties of both strains, such as nuclease, alpha-hemolysin, coagulase, catalase activities and mannitol utilization, assayed by the usual qualitative methods are summarized in Table 1. No difference in these properties was seen between them, while hemagglutination titer of HL-87 strain was less than 1/256 of the parental strain. From these results, it is likely that HL-87 strain is a mutant devoid of the protein A production without variation in the other biochemical properties.

TABLE 1. Biological properties of HL-87 strain [a]

strains	hemagglutination titer[b]	nuclease	alpha-hemolysin	coagulase	catalase	mannitol utilization
HL-87	N.D.[c]	+	+	+	+	+
Cowan I	256	+	+	+	+	+

a) HL-87 strain is one of protein A-deficient mutants isolated from *S. aureus* Cowan I strain.
b) Reciprocal hemagglutination titer of protein A in 10^9 bacterial cells per ml.
c) Not detectable by the hemagglutination test.

Resistance to H_2O_2

The resistance of HL-87 strain to the H_2O_2-bactericidal effect was compared with that of Cowan I strain by counting the viable cells after incubation in the presence of H_2O_2 for 10 min at 37°C. The results are illustrated in Fig. 1. There was a significant difference ($P<0.05$, paired t-test) between the two strains in the viable cell count (approximately 0.4 $\log_{10}$ CFU/ml) and HL-87 strain was less resistant than Cowan I strain in different concentrations of H_2O_2.

To examine the relationship between the starting cell number and the survival cell number, the organisms at varied concentrations (from 1×10^3 to 1×10^5 CFU/ml) were incubated with H_2O_2 (2.21 μM/ml), and then the viable cells were counted (Fig. 2). The more starting cells were incubated, the greater the viability in number. The results also showed significant differences in viability between both strains at each starting cell number, indicating that HL-87 strain was regularly less resistant than Cowan I strain.

To examine the kinetics of the H_2O_2-killing, the bacterial cells (1×10^4 CFU/ml) and H_2O_2 (2.21 μM/ml) were incubated together for 10 min to 50 min and the viability was assayed at every 10 minutes. The results are shown in Fig. 3. The viability of HL-87 strain was decreased rapidly than that of Cowan I strain. These results indicated that HL-87 strain is less resistant to the H_2O_2-bactericidal effect than Cowan I strain. It was noted that the incubation for the first 10 min was strikingly effective, and thereafter the rate of the decreases

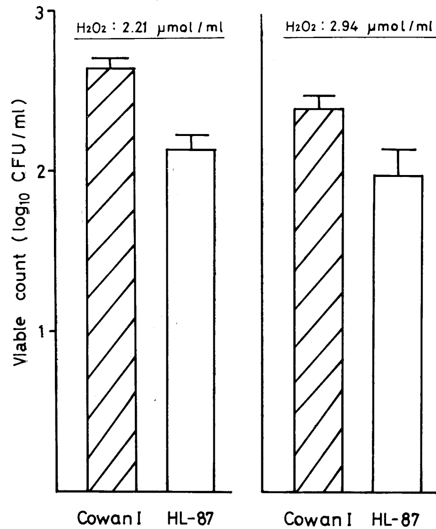

Fig. 1. Resistance of Cowan I and HL-87 strains to H_2O_2. The resistance was compared between Cowan I (///) and HL-87 (□) strains (1×10^4 CFU/ml, each) by counting the viable cells after incubation for 10 min in the presence of H_2O_2 (2.21 or 2.94 μM/ml) at 37°C. Each column represents a mean value of five experiments, and each bar indicates $\pm$ 1SD.

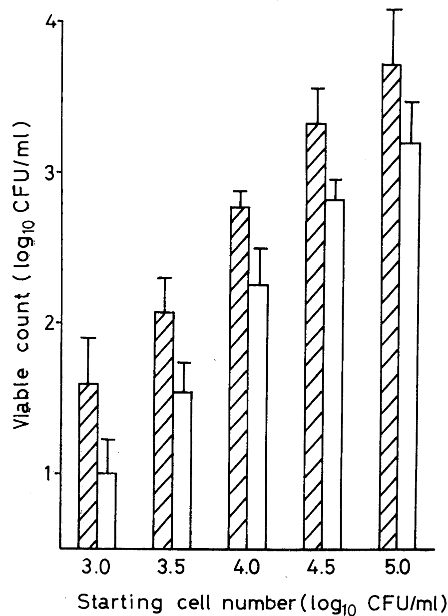

Fig. 2. Resistance of Cowan I (///) and HL-87 (□) strains at various concentrations to H_2O_2. The cells at 10^3, $10^{3.5}$, 10^4, $10^{4.5}$ and 10^5 CFU/ml were incubated at 37°C for 10 min in the presence of H_2O_2 (2.21 μM/ml), and then the viable cells were counted. Each column represents mean values of four experiments, and each bar indicates $\pm$ 1SD.

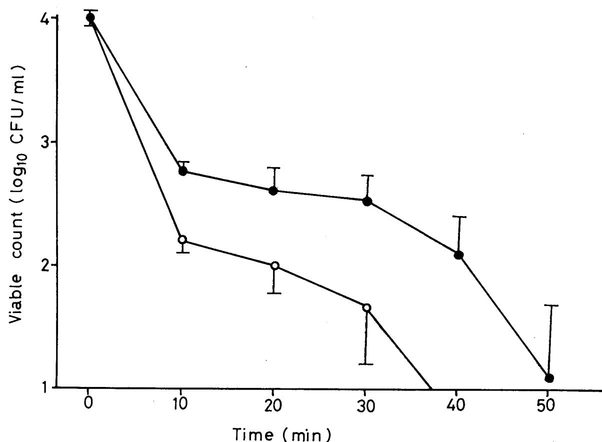

Fig. 3. Time course of the H_2O_2-bactericidal effect to Cowan I (●) and HL-87 (○) strains. The cells (1×10^4 CFU/ml) were incubated in H_2O_2 (2.21 μM/ml) at 37°C for 10, 20, 30, 40 and 50 min and the viability was assayed. The points represent mean values of four experiments, and each bar indicates $\pm$ 1SD.

slowed down in both strains. The reason for such diphasic response of the organisms against H_2O_2 is not known.

Catalase activity of the bacteria

The catalase activity of the bacteria was examined quantitatively, since Amin and Olson[16] reported that the resistance of *S. aureus* strains to the H_2O_2-bactericidal effect depended on their catalase activity.

The kinetics of the decrease of H_2O_2 in the reaction mixture during incubation is shown in Fig. 4. When the cells of Cowan I strain (1.4×10^7 CFU/ml) were incubated with H_2O_2 (3.57 mM/ml), H_2O_2 in the mixture was gradually decreased, but only 10 per cent was broken down at 30 min. On the contrary, HL-87 strain broke down H_2O_2 up to 73 per cent under the identical condition. The difference of the H_2O_2-amount between the strains was 17 per cent at 30 min. This difference was remarkably increased when the bacterial concentration in the reaction mixture was increased. As shown in the figure, H_2O_2 in the mixture containing HL-87 strain (2.8×10^7 CFU/ml) was strongly decreased down to 52 per cent at 30 min postincubation, while the decrease of H_2O_2 in the Cowan I mixture at the identical concentration was only 21 per cent. The difference in H_2O_2 concentration between the strains was 27 per cent. The viable count of each strain was not changed at 30 min postincubation. Thus, it was likely that the breakdown of H_2O_2 depended on the activity of the catalase released from the intact bacterial cells. When the activity of *Streptococcus pyogenes* (IID 689) for the H_2O_2-breakdown was assayed under the identical condition, only 3 per cent or less H_2O_2 was lost, indicating that the H_2O_2-breakdown in the presence of the staphylococci resulted from the specific catalase activity in the organisms. These results suggested that catalase activity of HL-87 strain was much higher than that of Cowan I strain, while no difference in the enzyme activity was detected in the qualitative assay as shown in Table 1 and that the resistance of both strains to H_2O_2 did not

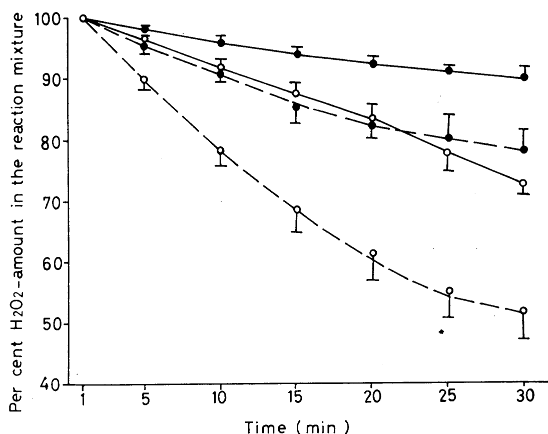

Fig. 4. The kinetics of H_2O_2-decrease resulted from the breakdown by catalase of Cowan I and HL-87 strains. The mixtures containing the cells at varied concentrations and H_2O_2 (3.57 mM/ml) in the cuvettes were allowed to stand at room temperature (20 $\pm$ 0.5°C) for 1, 5, 10, 15, 20, 25 and 30 min and then were measured for optical density at 240 nm. Mean values of four experiments are plotted as per cent of the optical density at each incubation time against that at 1 min. Bars at the points indicate $\pm$ 1SD. Symbols : ●—●, Cowan I at 1.4×10^7 CFU/ml ; ●----●, Cowan I at 2.8×10^7 CFU/ml ; ○—○, HL-87 at 1.4×10^7 CFU/ml ; ○----○, HL-87 at 2.8×10^7 CFU/ml.

depend on their catalase activities. It is, however, obscure whether all HL strains isolated by the present author possess higher catalase activity than the parental strain or not.

DISCUSSION

The present author previously reported that the resistance to the H_2O_2-bactericidal effect was different between Cowan I and Wood 46 strains.[10] However, as far as the strains genetically different each other are used, it is not easy to determine whether the resistance of the organisms to H_2O_2 is dependent on the presence of protein A in the bacteria or not, because the strains may be different not only in the protein A content, but also in the other phenotypic properties responsible for the viability in the presence of H_2O_2. This was the main reason why the protein A-deficient mutant isolated from Cowan I strain was used in the present study. The mutant, termed temporarily as HL-87 strain, possessed undetected amount of protein A. The results in the present study regularly indicated that the mutant strain was much less resistant than Cowan I strain to H_2O_2, although the mutant strain possessed much higher catalase activity which broke down the exogenous H_2O_2. Therefore, it may be concluded that protein A is, at least, one of the substances responsible for the resistance of Cowan I strain to the H_2O_2-bactericidal effect.

Recently, the present author found that the protein A production of some *S. aureus* strains was suppressed under a controlled culture condition and that

the bacteria under such condition became remarkably sensitive to H_2O_2.[17] These findings may support the conclusion mentioned above.

Many workers reported that H_2O_2 appeared to be a primary bactericidal substance in PMN,[8,9] which is well known to play an important role in the defence mechanism against the bacterial infection.[18,19] The present author demonstrated that *S. aureus* Cowan I strain had a much higher resistance to the bactericidal effect of PMN than Wood 46 strain.[10] From this fact and the results in the present study, it seems that protein A is a substance responsible for the resistance against PMN. Further evidences are required to confirm the relationship between the bactericidal effect of PMN and the presence of protein A in the bacteria.

Acknowledgments

The author wishes to thank the late Professor N. Higashi for his guidance, and also thanks Drs. A. Matsumoto and H. Mine for their informative suggestions. The author is indebted to Miss. S. Ohmori for her technical assistance.

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