BIKEN'S JOURNAL 2, 50-55 (1959)

Lysozyme, an Index of Pathogenic Staphylococci

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(Received for publication, July 15, 1959)

SUMMARY

The staphylococcal bound coagulase bears no direct relationship to insusceptibility to leucozyme A. A bound coagulase negative mutant was proved to be still leucozyme A insensitive.

A lysozyme-like enzyme was detected in the culture supernatants of all the leucozyme A insensitive staphylococci tested. Lysozyme production is a good biochemical index, if not determinant, of pathogenic staphylococci.

INTRODUCTION

In the course of studies, on the phagocytosis of staphylococci by guinea pig leucocytes, the observations of Rogers *et al.* (1952) were confirmed. That is ingested pathogenic staphylococci remained distinctly Gram positive, whereas phagocytized nonpathogenic staphylococci became Gram negative and only stained slightly. Attempts were made to identify the active substance playing the major rôle in this bacteriolysis of nonpathogenic staphylococci in leucocytes.

Of four bacteriolytic substances shown to be present in guinea pig leucocytes, leucozyme A was shown to be the active substance. The insusceptibility of pathogenic staphylococci to leucozyme A was further studied in relation to the significance of the free coagulase. The free coagulase did not directly inhibit the activity of leucozyme A towards nonpathogenic staphylococci *in vitro* (Amano *et al.*, 1956).

Leucozyme A was shown to be adsorbed by the cell walls of nonpathogenic staphylococci and not by the cell walls of pathogenic staphylococci. However, the cell walls of nonpathogenic staphylococci pretreated with the staphylococcal free coagulase could not adsorb leucozyme A. The staphylococcal free coagulase is suspected to be one of the active principles concerned with pathogenicity (Amano *et al.*, 1958).

The significance of bound coagulase was further studied. As described in the previous report (Amano *et al.*, 1958), a free coagulase negative and a bound coagulase positive mutant produced a heat labile substance, which could eliminate the leucozyme A adsorbing ability of the cell walls of nonpathogenic staphylococci. In studying the nature of the active substance, a lysozyme-like enzyme was found which was produced by leucozyme A insensitive staphylococci.

This report describes further aspects of the significance of the lysozyme-like enzyme.

Materials and Methods

1. *Bacteria :*

Newman : —— Free and bound coagulase-positive strain (N-1), kindly supplied by Dr. E. S. Duthie.

N-2 : —— Free coagulase-negative and bound coagulase-positive, a mutant of N-1.

AKM. : —— Free and bound coagulase-negative and leucozyme A sensitive strain, isolated from human urine.

2. *Preparation of leucozyme A and test for its action :*

As described previously (Amano *et al.*, 1956).

3. *Estimation of staphylococcal lysozyme activity :*

The crude enzyme preparation was prepared as follows: 10 ml. of nutrient broth was inoculated with a test strain and incubated at 37°C overnight in a shaker.

Two ml. of the centrifuged supernate were added to 0.5 ml. of U. V. killed *Micrococcus lysodeikticus* suspension, the optical density of which had been previously adjusted to 1.5 in a Coleman junior spectrophotometer with 10 mm. diameter cuvette at 550 mμ. The mixture was incubated at 37°C for 30 min. and the optical density was read. The activity was expressed according to the following formula.

$$\text{Lytic Index} = \frac{\text{O.D. (initial)} - \text{O.D. (30 min.)}}{\text{O.D. (initial)}} \times 100$$

4. *Staining procedure :*

Dried smears were fixed in a gas flame and Hucker's modification of the Gram technique was employed.

5. *Index of microscopical findings:*

— : All bacteria stained Gram positive and distinctly.

: All bacteria stained Gram negative and most of the bacteria formed an amorphous mass.

RESULTS

1) *The isolation of a bound coagulase negative mutant and its behaviour to leucozyme A :*

Since it has already been reported that a bound coagulase positive and free coagulase negative strain are still insusceptible to leucozyme A (Amano *et al.*, 1958), further attempts were made to study the significance of the bound coagulase. For this purpose a mutant deprived of the bound coagulase as well as of the free coagulase was required.

A streptomycin resistant mutant (N-3) was selected from N-2 (bound coagulase positive, free coagulase negative) on a streptomycin gradient agar. To eliminate contaminating staphylococci, the character of resistance to streptomycin was chosen and strain N-3 was used as the parent strain for isolation of the required bound cogulase negative mutant.

A nutrient broth medium containing 10 per cent fresh rabbit plasma was inoculated with strain N-3 and incubated at 37°C overnight. After the incubation the culture was shaken

vigorously and left for 2 hours. A loopful of the supernate was streaked onto a nutrient agar plate containing 200 μg. per ml. of streptomycin. After the overnight incubation, each colony was tested for the bound coagulase using the slide test.

After much effort, one colony was found to be negative for the bound coagulase test and designated as N-4.

Table 1. Leucozyme A sensitivity of strains N-2, N-3, N-4 and AKM.

	1	2	3	4	5	6	7	8
guinea pig serum (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Bacterial suspension (0.1 ml.)	N-2	N-2	N-3	N-3	N-4	N-4	AKM	AKM
Leucozyme A (ml.)	0.3	—	0.3	—	0.3	—	0.3	—
Physiological saline (ml.)	—	0.3	—	0.3	—	0.3	—	0.3
Incubated at 37°C for 2 hours								
Microscopical findings	—	—	—	—	±	—	##	—

The behaviour of N-2, N-3 and N-4 to leucozyme A are shown in Table 1. Of the population of bound coagulase negative strain N-4, a few individual coccal cells were rendered Gram negative by leucozyme A. However it should be

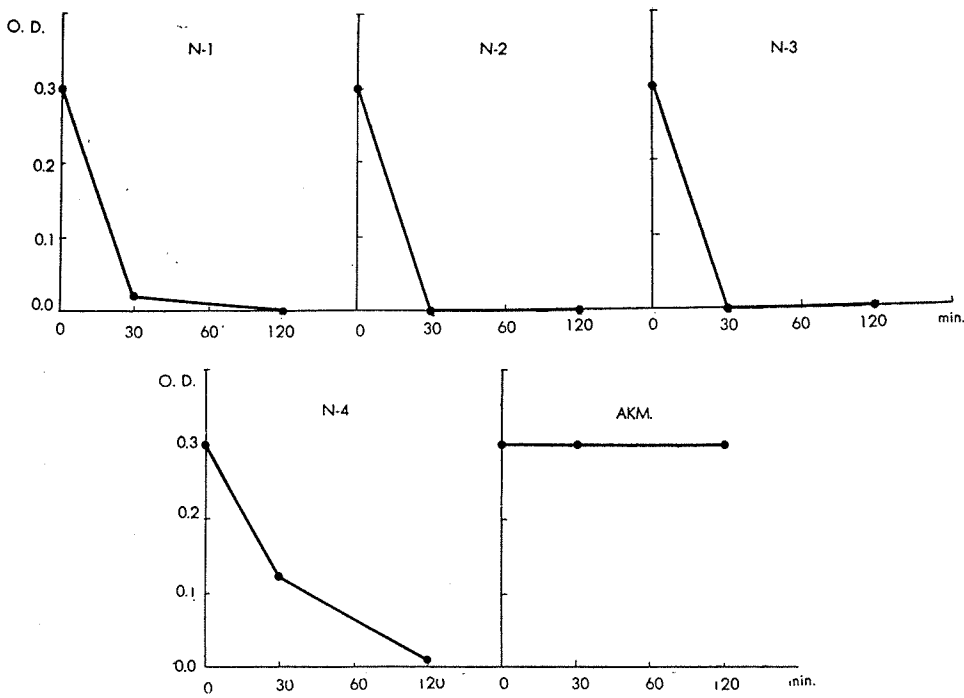

Fig. 1. The lysozyme activity of strains N-1, N-2, N-3, N-4 and AKM.

mentioned that they were still resistant to leucozyme A when compared with the nonpathogenic, leucozyme A sensitive strain (AKM).

Therefore it can be stated that the bound coagulase bears no significant relationship to leucozyme A sensitivity.

2) *Correlationship between the lysozyme-like enzyme production and the insusceptibility to leucozyme A:*

Since bound coagulase had no relationship to leucozyme A sensitivity, efforts were made to find another biochemical determinant bearing some relationship to leucozyme A sensitivity. After many trials, lysozyme-like enzyme was occasionally found to be produced by the bound coagulase negative strain N-4 but not by the nonpathogenic strain AKM. Strains N-1, N-2 and N-3, predecessors of strain N-4, also produced the enzyme. The results are presented in Fig. 1. Though the lysozyme activity of the culture supernate of strain N-4 was somewhat weaker than that of bound coagulase positive strains, it is still markedly active when compared to the nonpathogenic strain AKM.

To demonstrate a correlation between lysozyme production and insusceptibility to leucozyme A, all the stock strains were examined for lysozyme production and leucozyme A sensitivity.

Table 2. The correlation between bound coagulase, lysozyme activity and leucozyme A sensitivity

Bound coagulase \ Lysozyme activity	60 ≦ L.I.	10 < L.I. < 60	L.I. ≦ 10
+	51 (0)*	4 (0)	0
±	0	1 (0)	0
−	1 (1)	10 (0)	31 (31)
Not assayed	1 (1)	0	1 (1)

* The numbers in parentheses indicates leucozyme A sensitive strains

The results are shown in Table 2. Strains not producing lysozyme were sensitive to leucozyme A. On the other hand lysozyme producing strains were entirely insensitive.

Ten strains including strain N-4 were bound coagulase negative and lysozyme producing. The nine other strains had been isolated from human lesions. It is very interesting that these ten strains were also insusceptible to leucozyme A.

Lysozyme was denatured by heating at 100°C for 15 min. at pH 7.0 and could be completely adsorbed by a small amount of bentnite (Wako pure chemicals). After adsorption, it could be eluted by 20 per cent pyridine solution, adjusted to pH 5.0 with H_2SO_4.

3) *The isolation and behaviour to leucozyme A of a lysozyme negative mutant:*

A mutant of strain N-4 which did not produce lysozyme was required to see, whether lysozyme production bears any direct relation to insusceptibility to leucozyme A, or whether it was only an occasionally detected biochemical indicator.

An overnight broth culture of streptomycin resistant and free and bound coagulase negative strain N-4, was washed once with saline and then irradiated by U. V. to give from 50 to 100 colonies on an agar plate when 0.1 ml. of the irradiated suspension was spread. The colonies of the original plate were replicated onto a plate containing streptomycin.

The original plate was covered by 5 ml. of soft agar containing lyophilized U. V. killed cells of *M. lysodeikticus* and merthiolate at a concentration of 1 : 2,000 and incubated at 37°C for from 1 to 2 days. Around the lysozyme positive colonies clear halos could be detected. Pure cultures were taken from the colonies of the replica plate corresponding to the lysozyme negative colonies on the original plate, and again lysozyme production in the culture supernate was examined by the authentic test described above.

During the experiment, when the covered orignal plates were incubated for only a day, an apparently lysozyme negative strain was isolated and termed N-5. But strain N-5 still produced very small amount of lysozyme and was still insensitive to leucozyme A. The same procedures were repeated with strain N-5, and after much effort only one colony was shown not to produce lysozyme and was called N-6.

Table 3. Leucozyme A sensitivities of Strains N-4, N-5 and N-6

	1	2	3	4	5	6	7	8
Guinea pig serum (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Bacterial suspension ($D_{5500}=0.1$) (0.1 ml. respectively)	N-4	N-4	N-5	N-5	N-6	N-6	AKM	AKM
Leucozyme A (ml.)	0.3	—	0.3	—	0.3	—	0.3	—
Physiological saline (ml.)	—	0.3	—	0.3	—	0.3	—	0.3
Incubated at 37°C for 2 hours								
Microscopical findings	±	—	±	—	±	—	##	—

The sensitivity to leucozyme A of strains N-4, N-5 and N-6 are shown in Table 3. Strain N-6 was still as insensitive to leucozyme A as N-4, although the lysozyme activity of supernatants of strain N-6 cultures was hardly detectable.

Therefore lysozyme production is an occasionally detected biochemical indicator, but it is better than the bound coagulase as an index of insensitivity to leucozyme A.

DISCUSSION

The presence of a lysozyme-like enzyme in the supernatants of cultures of *staphylococcus aureus* was first described by Ralston *et al.* (1957). Quite recently Richmond (1959) reported that lysozyme is detectable in the supernatants of cultures of *Staphylococcus aureus* 524, and proved that it was very similar to egg white lysozme.

The lysozyme-like enzyme reported in the present paper must be similar. The present studies on the lysozyme of staphylococci developed from studies of

the pathogenicity of staphylococci. The distribution of the enzyme among strains of staphylococci was almost indentical with that of the insusceptibility to leucozyme A. Hence, the presence of lysozyme in the culture supernatants indicates the resistance of the strain to the bactericidal action by leucocytes.

The presence of lysozyme is no more than a biochemical index for the insusceptibility to leucozyme A, because a mutant (N-6) not producing lysozyme obtained from a lysozyme producing and leucozyme A resistant strain (N-4) was shown still to be insusceptible to leucozyme A. However, lysozyme production is a better index than staphylococcal bound coagulase, because nine staphylococcal strains isolated from human lesions were leucozyme A resistant and lysozyme producers, whereas they were bound and free coagulase negative.

As described in the previous report (Amano *et al.*, 1958), there was in a free coagulase negative and bound positive strain (N-2) a substance (or substances) in the cultural supernatant which was heat labile and capable of inactivating the receptors of leucozyme A from the cell walls of nonpathogenic staphylococci. As the lysozyme-like enzyme was occasionally found and was no more than a biochemical index, our further research is directed to the identification of the heat labile active substance (or substances).

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