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An Analysis of the Bactericidal Effect of Leucocytes Extract on *Staphylococci*

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SUMMARY

Leucozyme A, an active substance or substances from an acidic extract of guinea pig leucocytes, is adsorbed by the cell walls of non-pathogenic and coagulase-negative staphylococci. The cell walls of pathogenic and coagulase-positive staphylococci do not adsorb it. The same results were obtained when the cell walls had been heated or treated with enzymes (ribonuclease, lysozyme or trypsin).

Adsorption of leucozyme A by cell walls of coagulase-negative staphylococci was not observed after treatment of these cell walls with a cell extract or a partially purified coagulase preparation obtained from the supernatant of a culture of coagulase-positive staphylococci. Both preparations were shown to contain free coagulase. The free staphylococcal coagulase is suspected to be one of the active principles, because both the extract and the partially purified coagulase are resistant to boiling for 15 min. at neutral pH.

INTRODUCTION

Rogers and Tompsett (1952) showed by use of stained preparations and viable counting and slide culture techniques that pathogenic, coagulase-positive strains of staphylococci could survive in human polymorphonuclear leucocytes after being ingested, whereas non-pathogenic, coagulase-negative strains could not.

In the course of studies, in our laboratory, on the phagocytosis of staphylococci by leucocytes of the guinea pig, an animal which is moderately resistant to staphylococcal infection, the observations of Rogers *et al.* (1952) were confirmed to the extent that ingested pathogenic staphylococci were distinctly Gram positive, whereas phagocytized non-pathogenic staphylococci were Gram negative and only stained faintly.

As we knew that four active substances exist in guinea pig leucocytes, we tried to identify the active principle concerned with the morphological change of non-pathogenic staphylococci in the leucocytes.

As described in our previous report (Amano *et al.*, 1956), the active substance is " leucozyme A ", which can be extracted at acidic pH and is very heat stable. Although there is a strict parallelism between coagulase activity and pathogenicity of staphylococci, leucozyme A was not inhibited by staphylococcal coagulase.

This report describes further some aspects of correlation between pathogenicity and resistance to leucozyme A.

Materials and Methods

Bacteria.

KAT.:—Free and bound coagulase-positive strain, isolated from a human abscess.
KAT.-W:—A white variant of KAT., free and bound coagulase-positive.
209-P:—Free coagulase-positive and bound coagulase-weakly-positive strain, obtained from the Japan Antibiotics Research Association.
Newman-1:—Free and bound coagulase-positive strain, kindly supplied by Dr. E. S. Duthie.
Newman-2:—Free coagulase-negative and bound coagulase-positive, a variant of Newman-1.
AKM.:—Free and bound coagulase negative strain, isolated from human urine.
Air-1 and Air-2:—Free and bound coagulase-negative strains, isolated from air in our laboratory.

Bacterial suspensions, guinea-pig serum and leucozyme A preparations. Similar to those described previously (Amano *et al.*, 1956).

Concentrated and partially purified preparation of coagulase. A slight modification of the method of Duthie (1952) was employed. A broth culture of strain Newman-1 which had been shaken was centrifuged and the resulting cell free supernatant containing coagulase was cooled. All subsequent procedures were carried out at 4°C. Cadmium sulphate was added at a final concentration of 0.5% (w/v) and the mixture adjusted to pH 5.8. The flocculent precipitate was collected next day by centrifugation and dissolved by the gentle agitation in N-HCl at a final pH value of ca. 2.0. Insoluble material was removed by centrifugation. The clear solution was dialysed against a large volume of dilute-HCl at pH 2.0 for 48 hours and then against distilled water for 24 hours. The resultant precipitate was removed and the clear supernatant was lyophilized.

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

Index of microscopical findings

—: All bacteria were stained Gram positive and distinctly.
+++: All bacteria were stained Gram negative and the greater part was changed to an amorphous mass.

RESULTS

Interaction of leukozyme A with staphylococcal cells.

As shown in the previous paper, living pathogenic staphylococci had no inhibitory effect on leucozyme A (Amano *et al.*, 1956). In previous experiments, relatively small amount of bacteria was used.

Now, even larger amounts of bacteria were used and the effect of bacterial cells of coagulase-positive and -negative strains on leucozyme A was examined.

The bacteria harvested from agar slopes after 18 hours culture were thickly suspended and washed twice by saline. The optical density of the bacterial suspension at 550 mμ was corrected to $D_{5500}=0.5$ with a Coleman junior spectrophotometer. One ml of each suspension was packed by centrifugation and

mixed with 0.5 ml. of leucozyme A. These mixtures were centrifuged after incubation for 30 min. at 37°C. The supernatant of each was added to the system containing guinea-pig serum and the bacterial suspension of AKM.

Table 1. Interaction of leucozyme A with staphylococcal cells

	1	2	3	4	5	6
Packed bacterial cells	AKM.(C−)*	AKM. 100°C15′	KAT.(C+)	KAT. 100°C15′	—	—
Leucozyme A	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	—
Physiological saline	—	—	—	—	—	ml. 0.5
Following incubation at 37°C for 30 min. and centrifugaton, the supernatants were added to each system described below						
Guinea pig serum	0.25	0.25	0.25	0.25	0.25	0.25
Bacterial suspension (0.25 ml. respectively)	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.
Incubated at 37°C for 2 hours						
Microscopical findings	—	—	+	+	+++	—

* C−: Free and bound coagulase negative
C+: Free and bound coagulase positive

As shown in Table 1, leucozyme A activity was inhibited by AKM. (non-pathogenic) cells, either living or dead, rather than by KAT. (pathogenic) cells. These results suggest a possibility that leucozyme A was adsorbed by the leucozyme A sensitive strain, and not by the resistant one.

This possibility was confirmed by the following experiment using cell-walls in place of bacterial cells.

Adsorption of the leucozyme A onto the cell walls of staphylococci.

Cells of each strain of bacteria were disrupted by a Mickle disintegrator and centfriuged at 3000 r.p.m. for 20 min. to remove intact cells. This supernatant was centrifuged at 8000 r.p.m. for a further 20 min. and the sedimented cell walls were washed twice by saline and resuspended in saline. The optical density of this cell wall suspension at 550 mμ was corrected to $D_{5500}=0.2$ with a Coleman junior spectrophotometer. One ml. of each suspension was packed by centrifugation at 10000 r.p.m. for 20 min. Following procedures were the same as those of the preceding experiment.

As shown in Table 2, leucozyme A was completely lost after treatment with the cell walls of AKM., although the cell walls of KAT. had no effect on it.

It is suggested from these results that leucozyme A was adsorbed by the cell walls of the leucozyme A sensitive strains.

Relationship between the adsorption of leucozyme A by staphylococcal cell walls and its bactericidal action.

The correlation found in the preceding experiment was studied in several strains.

Table 2. Adsorption of the leucozyme A activity by the cell walls of staphylococci

	1	2	3	4
Packed bacterial cell walls	AKM.(C−)	KAT.(C+)	−	−
Leucozyme A	0.5 ml.	0.5 ml.	0.5 ml.	−
Physiological saline	−	−	−	0.5 ml.
Following incubation at 37°C for 15 min. and centrifugation, the supernatants were added to each system described below.				
Guinea pig serum	0.25	0.25	0.25	0.25
Bacterial suspension (0.25ml respectively)	AKM.	AKM.	AKM.	AKM.
Incubated at 37°C for 2 hours				
Microscopical findings	−	+++	+++	−

Teble 3. Relationship between the adsorption of leucozyme A onto staphylococcal cell walls and its bactericidal action.

Strain of bacteria	Color of colony	Coagulase		Adsorption of leucozyme A onto cell wall	Lysis of bacteria by leucozyme A
		free	bound		
KAT.	Golden yellow	++	+++	−	−
KAT.-W	White	+++	+++	−	−
Newman-1	Yellowish white	+++	+++	−	−
Newman-2	Golden yellow	−	+++	−	−
209-P	Golden yellow	+++	±	−	−
AKM.	White	−	−	+	+++
Air-1	Yellow	−	−	+	+++
Air-2	White	−	−	+	+++

As shown in Table 3, KAT., KAT.-W, Newman-1, Newman-2 and 209-P were pathogenic and coagulase-positive and not affected by leucozyme A. Leucozyme A was not adsorbed onto the cell walls of these strains.

However, leucozyme A was completely adsorbed onto the cell walls of AKM., Air-1 and Air-2, which were non-pathogenic, coagulase-negative and sensitive to leucozyme A.

It is interesting that there exists a strict parallelism between the adsorption of leucozyme A, i. e. bactericidal action of leucozyme A and the coagulase activity, especially the bound coagulase activity, of staphylococci.

The effect of heat or enzyme treatment of cell walls on the adsorption of leucozyme A.

As the preceding results were obtained with the native cell walls, the effect of heat or enzyme treatment of cell walls on the adsorption of leucozyme A was examined.

Table 4. The effects of heat or enzyme treatment of cell walls on the adsorption of leucozyme A

	1	2	3	4	5	6	7	8
Cell wall	AKM. (C−)	AKM.	AKM.	AKM.	AKM.	Newman-1 (C+)	Newman-1	Newman-1
Treatment	RNase	Lysozyme	Trypsin	100°C15′	None	Trypsin	100°C15′	None
Clumping by dilute plasma (bound coagulase)	−	−	−	−	−	−	+	+
Adsorption of leucozyme A	+	+	+	+	+	−	−	−

As shown in Table 4, adsorption of leucozyme A was not affected by boiling the cell walls for 15 min. or by treatment with certain enzymes, such as ribonuclease, lysozyme or trypsin.

Furthermore, trypsin treated cell walls of Newman-1, in which bound coagulase was completely destroyed, did not adsorb the leucozyme A, as well as did the untreated cell walls.

It is therefore suggested that the resistance of coagulase-positive staphylococci to leucozyme A is not dependent on the cell surface being coated with bound coagulase.

The effect of cell extracts of coagulase-positive strain (Newman-1) on the adsorption of leucozyme A onto the cell walls of AKM.

Another possibility as to the resistance of coagulase-positive staphylococci to leucozyme A is that certain active substances in the pathogenic staphylococci would destroy the site of adsorption of leucozyme A which has been suggested to exist in cell walls of the leucozyme A sensitive and coagulase-negative staphylococci.

To investigate this possibility the following experiment was perfomed. A 150 ml. broth culture of Newman-1 which had been shaken was centrifuged and the sedimented cells were washed twice with saline and resuspended in 5 ml. of saline. This suspension was disrupted with glass beads for 30 min. in a Mickle disintegrator. Ten ml. of saline was added, and the suspension centrifuged at 12000 r.p.m. for 20 min.. The clear supernatant was used as the cell extact of Newman-1. A cell extract AKM. was prepared by the same method.

As shown in Table 5, cell walls of AKM. treatd by a cell extract of Newman-1 were no longer able to adsorb the leucozyme A. The same effect was observed with a boiled cell extract of Newman-1. Furthermore, coagulase activity in the cell extract was still strongly positive after boiling the extract for 15 min.. On the other hand, the cell extract of AKM. had no effect on the cell walls of AKM.

It is therefore suggested that the leucozyme A adsorbing ability of the AKM. cell walls is lost by treatment with cell extract of Newman-1. Because of its heat stability, coagulase would be one of the factors affecting the cell wall of AKM.

Similar effects of the concentrated supernatants of various strains of coagulase-positive staphylococci on the cell wall of AKM.

Table 5 The effects of cell extracts of coagulase-positive (Newman-1) and coagulase-negative (AKM.) strains on the adsorption of leucozyme A onto AKM. cell walls

	1	2	3	4	5	6	7
Cell extract (5.0 ml. respectively)	Newman-1 (C+)	Newman-1 100°C 15′(C+)	AKM. (C−)	AKM. 100°C 15′(C−)	—	—	—
Physiological saline	—	—	—	—	ml. 5.0	—	—
Cell wall suspension ($D_{5500}=0.4$ 1.0 ml. respectively)	AKM.	AKM.	AKM.	AKM.	AKM.	—	—
Toluene	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	—	—
Following incubation at 37°C for 16 hours and centrifugation, sedimented cell walls of the samples were washed twice with saline, resuspended and mixed with leucozyme A.							
Leucozyme A	0.5	0.5	0.5	0.5	0.5	0.5	—
Physiological saline	—	—	—	—	—	—	0.5
Following incubation at 37°C for 20 min. and centrifugation, the supernatants were added to each system described below.							
Guinea pig serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Bacterial suspension ($D_{5500}=0.1$ 0.25 ml. respectively)	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.
Incubated at 37°C for 2 hours							
Microscopical findings	+++	++	−	−	−	+++	−

In these experiments, concentrated supernatants of cultures of coagulase-positive staphylococci were used in place of cell extracts and their effect on the leucozyme A adsorbing ability of the cell walls of AKM. was examined.

The broth dialysate was prepared as described below. Fifty ml. of yeast-extract (2.5gr.) and Peptone (10.0gr.) was dialyzed against 950 ml. of a solution containing lactose (2.0 gr.), K_2HPO_4 (5.0 gr.), NaH_2PO_4 (2.0 gr.) and NaCl (5.0gr.)for 72 hours in a cold room. Two hundred ml. aliquots of this broth dialyzate containing only dialysable components was seeded with the strains listed in Table 6 and shaken for 16 hours at 37°C. The shaken cultures were centrifuged and cell free culture supernatants were dialyzed against distilled water for 72 hours in a cold room to remove the components of the media. The dialyzed culture supernatants were lyophilized.

The dried material of each sample was dissolved in saline at a concentration of 2mg. per ml. and used as the concentrated culture supernatants.

Table 6. Effect of concentrated culture supernatants of various strains of coagulase-positive staphylococci on the cell walls of AKM

	1	2	3	4	5	6	7	8	9
Concentrated culture supernatant (5.0 ml. respectively)	Newman-1	Newman-1 100°C15′	Newman-2	Newman-2 100°C15′	209-P	209-P 100°C 15′	AKM.	AKM.	—
Coagulase activity	+++	+++	—	—	+++	+++	—	—	—
Physiological saline	—	—	—	—	—	—	—	—	ml. 5.0
Cell wall suspension (D_{5500}=0.4, 1.0 ml. respectively)	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.
Toluene	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1

Following incubation at 37°C for 16 hours, the leucozyme A adsorbing ability of the treated cell walls of AKM was tested.

Adsorption of leucozyme A	—	—	—	+	—	—	+	+	+

As shown in Table 6, concentrated culture supernatants of free coagulase-positive staphylococci (Newman-1, 209-P) were able to destroy the leucozyme A adsorbing ability of the cell walls of AKM. (tube 1 and 5). A similar effect was observed with boiled preparations (tube 2 and 6). However, a native preparations of a free coagulase-negative and bound coagulase-positive strain (Newman-2) exerted a similar effects on the cell walls of AKM. (tube 3), while the boiled preparation had no effect (tube 4).

It is suggested that the culture supernatant of coagulase-positive staphylococci (Newman-1) contains at least two active substances affecting the leucozyme A adsorbing ability of the cell walls of AKM. One may be the free coagulase and the other a heat labile factor as seen in the case of Newman-2.

The effects of coagulase prpearations on the cell walls of coagulase-negative strains.

As shown above the concentrated supernatants of cultures of coagulase-positive staphylococci were able to destroy the leucozyme A adsorbing ability of the cell walls of AKM. To identify the active principle contained in the concentrated culture supernatant, a partially purified coagulase preparations from culture supernatant were used. This effect on the cell walls of coagulase-negative staphylococci was examined.

Coagulase preparations from culture supernatant were concentrated and partially purified by the method of Duthie (1952), and their effects on the cell walls of AKM. were observed.

As shown in Table 7, coagulase preparations of Newman-1, 209-P, KAT. and KAT.-W affected the adsorption of leucozyme A by the cell walls of AKM.

Table 7. Effect of coagulase preparations on the cell walls of AKM

	1	2	3	4	5	6	7	8	9
Coagulase preparation (2 mg/ml. 5.0 ml. respectively)	Newman-1	Newman-1 100°C15′	209-P	209-P 100°C15′	KAT.	KAT. 100°C 15′	KAT.-W	KAT.-W 100°C 15′	—
Cell wall suspension (D_{5500}=0.4, 1.0 ml. respectively)	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.	AKM.
Physiological saline	—	—	—	—	—	—	—	—	ml. 5.0
Toluene	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1	ml. 0.1
Incubated at 37°C for 16 hours, and washed twice by centrifugation. The sedimented cell walls of the samples were examined for adsorption of leucozyme A.									
Adsorption of leucozyme A	—	—	—	—	—	—	—	—	+

Table 8. Effect of coagulase preparations of Newman-1 on the cell walls of coagulase-negative staphylococci (Air-1 and Air-2)

	1	2	3	4	5	6
Cell wall suspension (D_{5500}=0.4, 1.0 ml. respectively)	Air-1	Air-1	Air-1	Air-2	Air-2	Air-2
Coagulase preparation (2mg/ml., 5.0 ml. respectively)	Newman-1	Newman-1 100°C 15′	—	Newman-1	Newman-1 100°C 15′	—
Physiological saline	—	—	ml. 5.0	—	—	ml. 5.0
Incubated at 37°C for 16 hours, and washed twice by centrifugation. The sedimented cell walls of each sample were examined for adsorption of leucozyme A.						
Adsorption of leucozyme A	—	—	+	—	—	+

Furthermore as shown in Table 8, under the action of coagulase preparation of Newman-1 the cell walls of Air-1 and Air-2 lost their ability to adsorb the leucozyme A.

It is obvious from this experiment that the coagulase preparations from the culture supernatant of coagulase-positive staphylococci as well as the concentrated culture supernatant are effective in destroying the leucozyme A adsorbing ability of cell walls of coagulase-negative strains.

It is suggested that free coagulase is one of the most important factors affecting the leucozyme A adsorbing ability of cell walls of coagulase-negative staphylococci.

DISCUSSION

It is reported in this paper that coagulase-negative and leucozyme A sensitive

strains of staphylococci could adsorb leucozyme A, whereas coagulase-positive and leucozyme A resistant strains did not adsorb it. These results are comparable to those of Few and Schulman (1953) who found that polymyxin sensitive bacteria were capable of adsorbing much greater quantities of the antibiotics than resistant organisms.

It was generally considered that the ability to produce coagulase is one of the most reliable indices of pathogenicity of staphylococci, even if it is not the determinant factor of pathogenicity.

It may be probable that coagulase affects the leucozyme A adsorbing sites of cell walls of pathogenic staphylococci and subsequently renders them resistant to leucozyme A. However, it may be also explained to be independent from the production of coagulase, because coagulase production and the lack of leucozyme A adsorbing sites are controlled by different genes.

With regard to the first possibility, it can be stated that coagulase enables pathogenic staphylcocci to survive in phagocytic cells and initiate staphylococcal infections.

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