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LYSOSTAPHIN: AN ENZYMATIC APPROACH TO STAPHYLOCOCCAL DISEASE. I. IN VITRO STUDIES¶

Since 1960, Schindler and Schuhardt have reported several studies on a unique substance which rapidly and specifically lyses staphylococci but has no effect on bacteria of other genera.[1-3] This agent, termed lysostaphin, contains at least two enzymes, a peptidase and an N-acetyl-glucosaminidase.[4] The specific antistaphylococcal effect is thought to be due to the peptidase which cleaves genus-specific polyglycine bridges in the staphylococcal cell wall.[4] The enzyme can lyse heat-killed staphylococci and isolated cell wall preparations as well as living microorganisms.[3] Thus it appeared that lysostaphin might be active against metabolically quiescent staphylococci in abscess lesions where penicillin and other antimicrobials are largely ineffective. This study describes the *in vitro* activity of lysostaphin. The paper that follows evaluates its use as a therapeutic agent in experimental staphylococcal infections.

METHODS

The following coagulase positive strains of *Staphylococcus aureus* isolated from human sources were employed. *Wild-Type Staphylococci*: a) Giorgio strain.—This strain was used as the standard test organism in the majority of experiments and has been previously described.[5] This strain produces penicillinase and is inhibited by 25 μg/ml. of penicillin G and 0.39 μg/ml. of oxacillin. b) Smith Compact

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variant.—This strain, which does not produce penicillinase and is inhibited by 0.016 μg/ml. of penicillin G, has been described extensively.[6-8] The Driver and 502A staphylococcal strains also employed in these studies possess characteristics of "wild-type" strains.[9,10] *Encapsulated Staphylococci*: a) Smith Diffuse variant.—This strain is mouse virulent (ip), possesses an antiphagocytic surface antigen, and grows characteristically in streaming diffuse colonies in serum soft agar.[6,7] Two other staphylococcal strains used in the present studies, the Welwood and Shiver strains have similar characteristics.[7,8]

Stock cultures were maintained on agar slants stored at 4°C., and 18 hour transplants were made in trypticase soy broth. Bacteria in the exponential phase of growth were grown on a shaker for five hours. Large populations of organisms were obtained by concentrating liter volumes of shaker-grown cultures. Microorganisms were washed once and resuspended to appropriate concentrations in Tris Buffer.

Lysostaphin-resistant staphylococci were isolated from survivors of enzyme-treated cultures. These survivors were subjected to a series of tests designed to compare them with the parent strain: 1) Tube coagulase and clumping factor ("slide coagulase") assays were carried out using pooled human plasma.[11] 2) Phage typing was performed by the Tennessee Department of Public Health, Division of Laboratories, Nashville, Tennessee. 3) Antibiotic sensitivities were determined using serial twofold dilutions in trypticase soy broth.[7] 4) Two methods were employed to determine penicillinase production: (a) A modification of the rapid starch-iodide assay.[12] Starch-iodide was prepared as 0.0013 NI_2 in 0.005 M KI with 0.4% soluble starch.* Organisms were scraped from an agar slant and resuspended in M/10 phosphate buffer (pH 5.9) to give a cloudy suspension. To 1 ml. of this suspension was added 0.1 ml. penicillin G (2 mg/ml.) and 1 ml. starch-iodide solution. Decolorization of the blue starch-iodide mixture indicated penicillinase production. (b) Modified Gots test.[13] Trypticase soy agar plates containing a 1:100 dilution of a broth culture of *Sarcina lutea* (inhibited by 0.005 μg/ml. of penicillin G) and 0.008 μg/ml. penicillin G were poured and allowed to solidify. Eighteen-hour broth cultures of the strains to be tested were streaked in a circle on the surface of the plates. Strains producing penicillinase permitted a zone of satellite Sarcina colonies to appear. 5) Virulence was tested by employing two mouse infections previously studied:[5-7,14] (a) Animals were injected intraperitoneally with 0.4 ml. of an 18-hour culture in trypticase soy broth. Mortality rates were determined at 24 hours. (b) 0.2 ml. of an 18-hour culture was injected via a tail vein. Mice were observed for 28 days and cumulative mortality rates recorded.

Lysostaphin* and Trypsin** were prepared in sterile 0.05M Tris buffer, pH 7.5, which was 0.145M with respect to NaCl. The buffer was sterilized by Millipore filtration (0.45μ). The *Chalaropsis B* enzyme, a β-1, 4-N,O-diacetylmuramidase, used in solubilizing the staphylococcal cell wall, was prepared as previously described.[15]

Lysostaphin activity was measured by determining killing curves using standard pour plate techniques. Mixtures of enzyme and staphylococci in Tris buffer were incubated in a water bath at 37°C. Aliquots were removed at intervals, diluted appropriately in 0.9% saline, and cultured in trypticase soy agar. As trypsin destroys

* Fisher Scientific Co.

* Lot number 142-123-1, kindly supplied by Dr. Peter Tavormina of the Mead Johnson Research Center, Evansville, Indiana.

** C. E. Boehringer and Soehne, Manheim, Germany.

lysostaphin activity,[2] residual lysostaphin was inactivated by adding trypsin (0.1 to 0.5% final concentration) to the pour plates.

Human leukocytes were obtained by dextran sedimentation of whole blood as previously described.[9] Washed leukocytes were suspended in 10% human serum in Hank's solution. Staphylococci were added in a ratio of one microorganism to one polymorphonuclear leukocyte and these mixtures were rotated on a drum at 37°C.

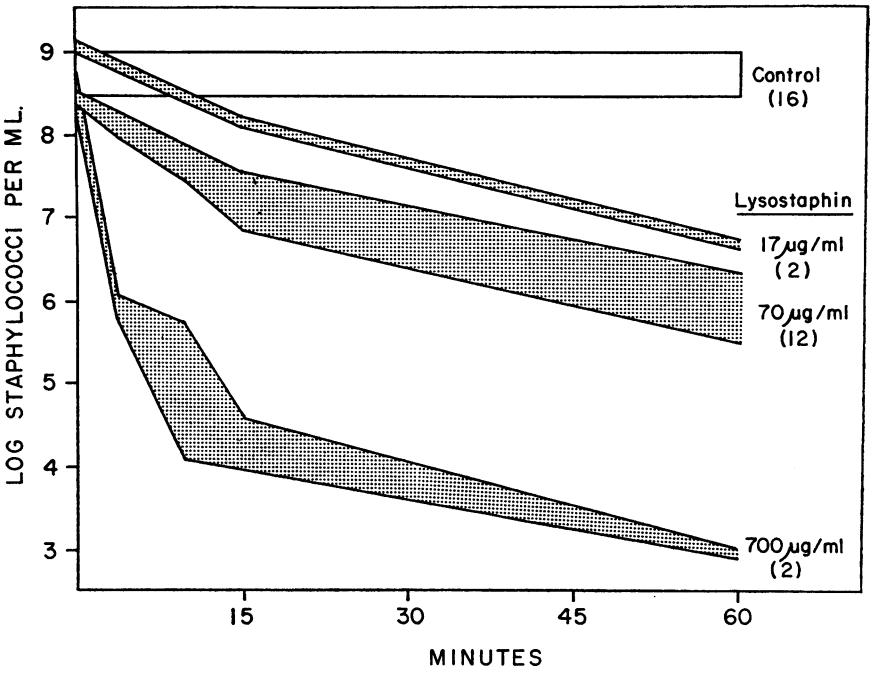

FIG. 1. The effects of increasing concentrations of lysostaphin on the Giorgio staphylococcus. The numbers in parentheses indicate the number of experiments in each group.

The incorporation of labeled glycine* by lysostaphin-resistant staphylococci and the subsequent fractionation of the microorganism were performed as previously described.[16] Washed cells (12.8 mg. dry wt.) from the log phase of growth were suspended in 20 ml. of synthetic media containing 2.0 µc of ^{14}C-glycine. Samples were incubated with shaking at 37°C. and 5 ml. aliquots were withdrawn and fractionated as described.[16]

After solubilization of the cell wall with the *Chalaropsis* N-acetylmuramidase, aliquots corresponding to 0.8 mg. dry weight cells were placed on a Sephadex G-25 column (1 x 25 cm.) and eluted with water. Fractions (0.7 ml.) were collected and added to 10 ml. of scintillation fluid and counted.[16]

* Purchased from New England Nuclear Corporation as ^{14}C-glycine, specific activity 74 mc/mmole.

RESULTS

In vitro activity of lysostaphin

Lysostaphin rapidly kills *Staphylococcus aureus in vitro*. As is demonstrated in Figure 1, increasing doses of lysostaphin killed increasing numbers of staphylococci. Using 70 μg/ml. of lysostaphin, the bacterial population fell approximately two log units in one hour. Ten times as much

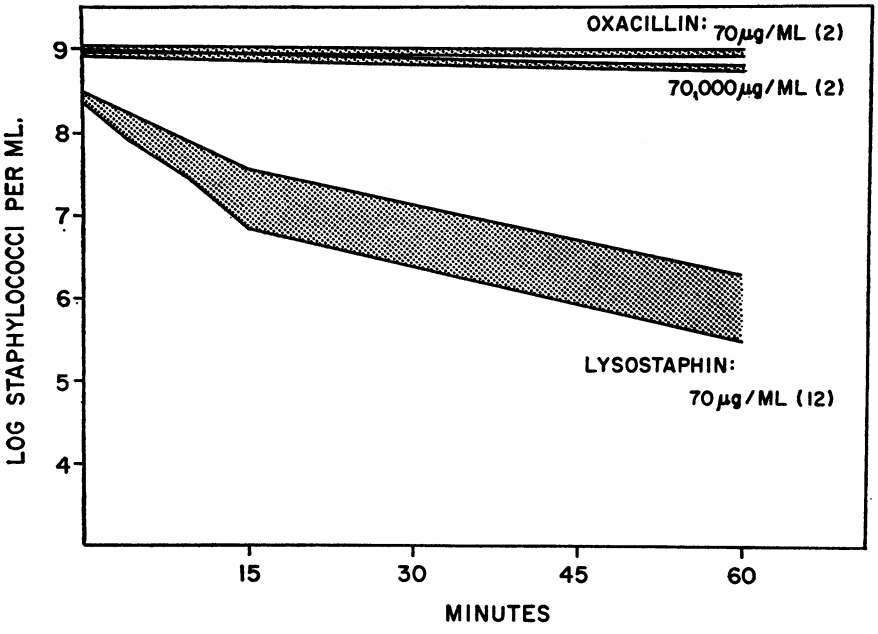

FIG. 2. The effects of lysostaphin or oxacillin on the Giorgio staphylococcus. This microorganism is inhibited by 0.39 μg/ml. of oxacillin. The numbers in parentheses indicate the number of experiments in each group.

lysostaphin reduced the viable count by five log units. Oxacillin, even in amounts one thousand times as large (70,000 μg/ml.), did not alter the population of a resting oxacillin-sensitive staphylococcal strain during this time period (Fig. 2).

Temperatures between 22°C. and 56°C. had relatively little influence on the speed of bacterial killing. At room temperature (22°C.), initial killing rates were less than those observed at 37°C., but at the end of an hour similar degrees of killing had occurred. At 56°C. lysostaphin remained actively lytic, but such temperatures alone killed staphylococci.

A number of staphylococcal strains have been described which produce diffuse streaming colonies in serum or plasma soft agar, have increased

virulence for mice, possess antiphagocytic surface antigens, and appear encapsulated.[6-8] Although only a few of these strains have been isolated from patients with active staphylococcal infection,[8] it has been proposed that such strains represent the biological form of the disease-producing staphylococcus *in vivo* and may actually be responsible for more disease than is currently suspected.[17] Studies were thus performed to determine whether

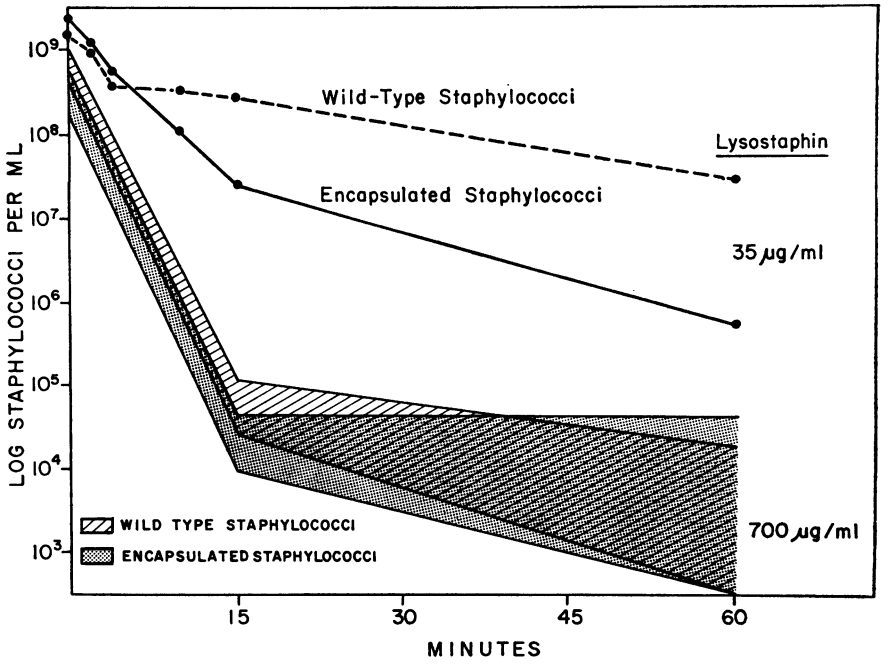

FIG. 3. *In vitro* lysostaphin susceptibility of "wild-type" and encapsulated staphylococci. The upper curves show lysostaphin activity (35 μg/ml.) against the diffuse encapsulated and the compact "wild-type" variants of the Smith strain of staphylococcus. The lower curves show the range of lysostaphin activity (700 μg/ml.) against several encapsulated (Diffuse, Welwood, and Shiver) and "wild-type" (Compact, Driver, and 502A) staphylococcal strains.

the surface structures of such encapsulated strains would impair the action of lysostaphin. As noted in Figure 3, this was not the case. Small quantities of lysostaphin (35 μg/ml.) were more active against an encapsulated strain than against a usual wild-type strain, while larger amounts of lysostaphin lysed encapsulated and wild-type staphylococci at similar rates.

Studies performed both in experimental animals[18,19] and *in vitro*[19,20] have clearly demonstrated that bacteria which have reached maximal titers in tissue or in the test tube are relatively insusceptible to penicillin despite

rapid killing of smaller numbers of these same bacteria during a phase of active multiplication. As Figure 4 demonstrates, lysostaphin was active against very large populations of staphylococci that were penicillin insusceptible. In this instance lysostaphin produced over a hundredfold reduction in a large population of microorganisms when penicillin G showed

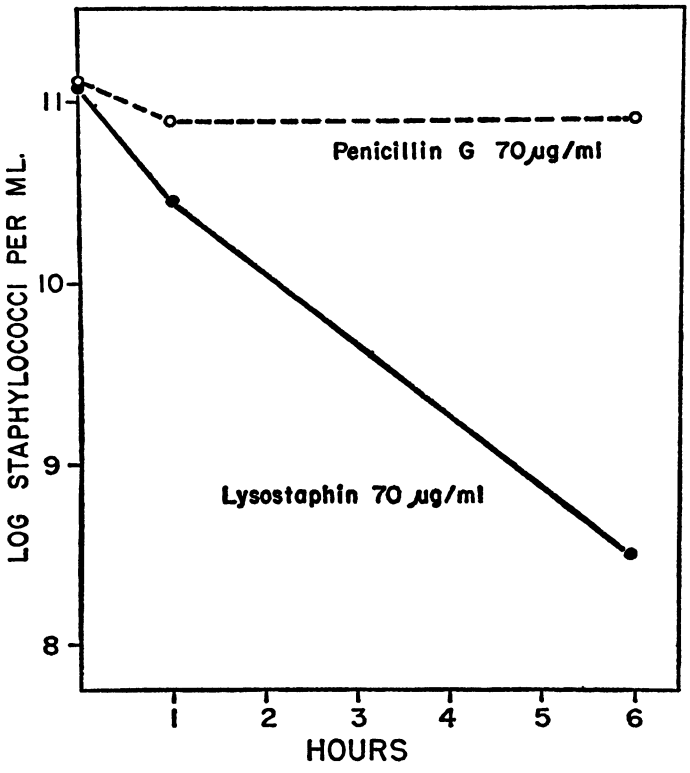

FIG. 4. Comparison of penicillin G and lysostaphin activities against large populations of the Smith Compact staphylococcus. This strain does not produce penicillinase and is inhibited by 0.016 µg/ml. of penicillin G.

no activity over the six-hour period of study even though over 4,000 times the standard minimum inhibitory concentration of the drug was employed.

Lysostaphin was relatively stable at 37°C. and maintained its activity in human serum. Over a 36-day period of incubation at 37°C. lysostaphin was slowly inactivated (Fig. 5A), but the enzyme was still able to kill more than 99% of the staphylococci in one hour. In a system containing

50% serum no alteration of lysostaphin activity could be detected (Fig. 5B).

Lysostaphin activity was only slowly reduced after repeated exposure to staphylococci. As shown in Figure 6, the addition of fresh populations of staphylococci at hourly intervals to buffer containing lysostaphin produced some reduction in staphylococcal killing after each successive staphylococcal

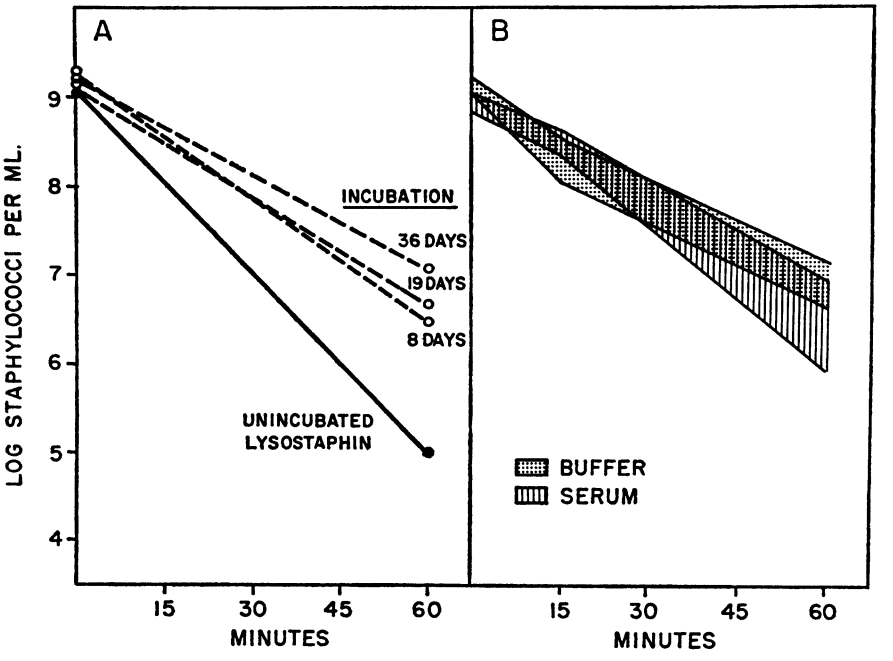

FIG. 5A. Effect of prolonged incubation at 37°C. on the activity of lysostaphin (70 μg/ml.). The Giorgio staphylococcus was used as the test organism.
FIG. 5B. Effect of human serum on lysostaphin activity (35 μg/ml.) against the Giorgio staphylococcus. Two separate experiments are represented in each group.

challenge. However, after three additions of fresh staphylococci, over 91% of the microorganisms were still killed during the fourth hour. The accumulation of inhibitory and toxic end products consequent to bacterial lysis provides the most likely explanation for the observed progressive decrease in lysostaphin activity. Lysostaphin controls incubated alone maintained full activity for the duration of the experiment.

Effect of intracellular residence on lysostaphin activity

Coagulase-positive staphylococci are able to survive for a prolonged time within polymorphonuclear leukocytes.[21,22] Studies were thus undertaken to

determine whether lysostaphin was active against such phagocytized staphylococci. These studies are represented in Figure 7. When human leukocytes were allowed to phagocytize the Giorgio staphylococcus (a non-encapsulated strain) the intracellular population of staphylococci re-

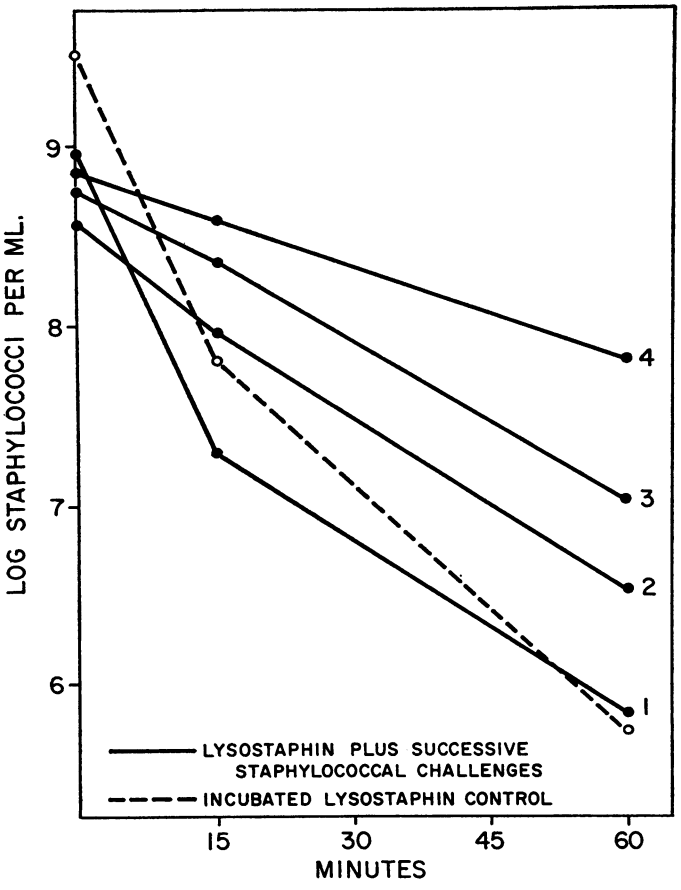

FIG. 6. Sustained lytic activity of lysostaphin (70 μg/ml.). The numbers refer to successive additions of Giorgio staphylococci at hourly intervals. The dashed line indicates the activity of lysostaphin incubated for 3 hours and exposed to staphylococci during the fourth hour.

mained constant over the one-hour period of study. Lysostaphin (30 μg/ml.) had no effect on such intracellular staphylococci, while staphylococci alone were rapidly killed on exposure to lysostaphin. The intracellular position had protected the bacteria from lysostaphin action. Furthermore, if fresh leukocytes, extracellular staphylococci, and lysostaphin

were simultaneously mixed together, rapid staphylococcal killing was initially observed. As phagocytosis proceeded and more staphylococci were ingested, killing slowed and then stopped.

In order to demonstrate that leukocytes did not inactivate the lysostaphin and thus, account for the observed impairment in killing, leukocytes

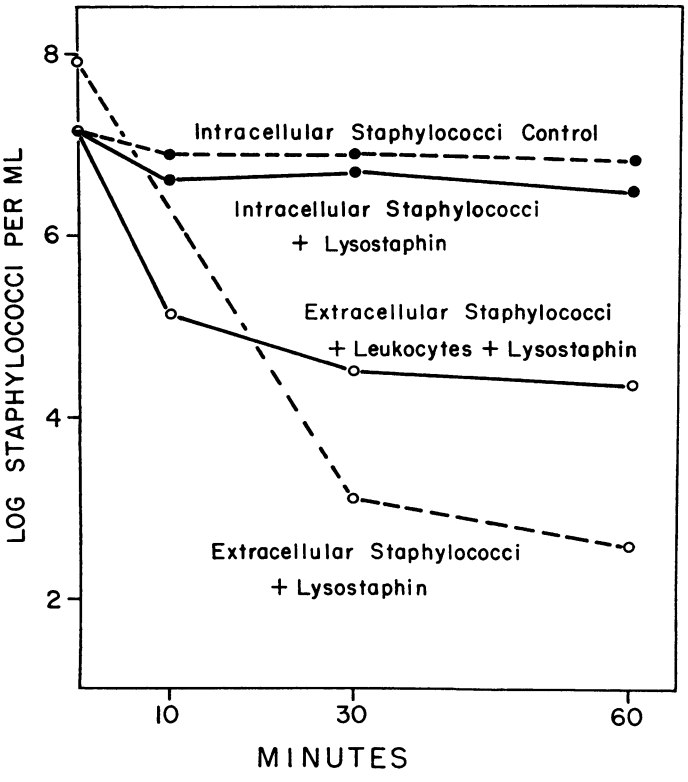

FIG. 7. The effects of lysostaphin (30 μg/ml.) on intracellular and extracellular staphylococci. The Giorgio staphylococcus and human polymorphonuclear leukocytes were employed.

were suspended in lysostaphin (35 μg/ml.) at 37°C. for one hour. The leukocytes were then removed by centrifugation, staphylococci were added to the lysostaphin-containing supernate, and no impairment of lysostaphin activity was observed.

Leukocytes that had been exposed to high concentrations of lysostaphin (1,400 μg/ml.) for 30 minutes at 37°C. were twice washed in buffer and homogenized in a teflon grinder. No evidence of lysostaphin activity was

obtained in such leukocyte homogenates. This finding suggests that, in the system described, lysostaphin did not enter leukocytes in appreciable amounts or was promptly inactivated by intracellular residence.

In vitro resistance to lysostaphin

Lysostaphin-resistant microorganisms were easily recovered *in vitro*. The Giorgio strain of *Staphylococcus aureus* was exposed to 3,500 μg/ml.

TABLE. 1. BIOLOGIC CHARACTERISTICS OF PARENT STAPHYLOCOCCUS AND LYSOSTAPHIN-RESISTANT VARIANTS

	Giorgio parent	*Variant no. 1*	*Variant no. 2*
Morphology	Yellow-white opaque colony	Pearly translucent colony	Pearly translucent colony
Growth rate	Normal	Prolonged lag phase	Prolonged lag phase
Phage type	47/53/54/75/77	47/53/54	47/54
Coagulase	+	+	+
Clumping factor	+	+	0
Morphology in plasma soft agar	Compact with a few diffuse colonies	Diffuse with occasional compact colony	Compact with rare diffuse colony
Mouse mortality ip (Dead/Total)	0/10	0/4	0/4
Mouse mortality iv (Dead/Total)	17/20 (85%)	11/20 (55%)	19/23 (83%)
Penicillin sensitivity	25 μg/ml.	0.195 μg/ml.	1.56 μg/ml.
Penicillinase production	+	+	+
Lysostaphin sensitivity: per cent staphylococci killed in 1 hour			
70 μg/ml.	> 99.9	0	52
700 μg/ml.	——	0	> 99

of lysostaphin at 37°C. Aliquots were taken at intervals, plated without trypsin, and the few surviving colonies were isolated. Two chosen at random were studied further, as indicated in Table 1.

The colonial morphology of both variants differed from the parent strain. They grew in pearly translucent colonies rather than in the yellow-white opaque colony of the parent staphylococcus. The variants had a prolonged lag phase of growth as compared to the parent strain, although identical titers were reached by 18 hours. Both variants and the parent

strain were lysed by Group III phages; however, the variants were lysed by fewer typing phages. Both variants produced tube coagulase. Variant no. 1 produced primarily diffuse streaming colonies in plasma soft agar. Variant no. 2 did not produce clumping factor ("bound coagulase"). Neither of the two variant strains were mouse virulent when injected intraperitoneally. However, when mice were infected intravenously, the parent strain and variant no. 2 produced equivalent mortality rates while variant no. 1 killed significantly fewer mice ($0.05 > p > 0.01$).

While both lysostaphin-resistant variants and the parent strain produced penicillinase, the varients were significantly more sensitive to peni-

TABLE 2. INCORPORATION OF ^{14}C-GLYCINE INTO LYSOSTAPHIN-RESISTANT VARIANTS OF STAPHYLOCOCCUS AUREUS

Organism	*Time (min)*	*mμmoles ^{14}C-glycine/mg. dry weight*	
		Protein	*Cell wall*
Parent	10	56	120
	20	105	315
	30	160	530
Variant no. 1	10	37	30
	20	66	71
	30	123	129
Variant no. 2	10	45	106
	20	90	258
	30	158	436

cillin. The parent strain was sensitive to 70 μg/ml. of lysostaphin while both variant strains were resistant to this quantity of lysostaphin. When 700 μg/ml. of lysostaphin was employed, variant no. 1 remained completely resistant while variant no. 2 was sensitive to this quantity of enzyme.

Studies on the basis of lysostaphin resistance in Staphylococcus aureus.

The peptidase in lysostaphin releases N-terminal glycine and alanine, thus cleaving the cross-linking polyglycine bridges which are unique to the cell wall of *Staphylococcus aureus*.[28] Resistance to lysostaphin might result from the absence of polyglycine structures in resistant variants or might be due to the inaccessibility of susceptible bonds through an alteration in the chemical or physical structure of the cell wall. The first possibility was tested by determining whether ^{14}C-glycine could be incorporated into the cell wall. As shown in Table 2, both variant strains were able to incorprate ^{14}C-glycine into both cell wall and protein. Variant no. 2 incor-

porated glycine into both cell wall and protein at a rate similar to that of the parent, whereas variant no. 1 incorporated considerably less label into its cell wall than did the control. Nevertheless, both resistant variants incorporated glycine into their cell wall structures and their insensitivity to lysostaphin could not be ascribed to a lack of cell wall glycine.

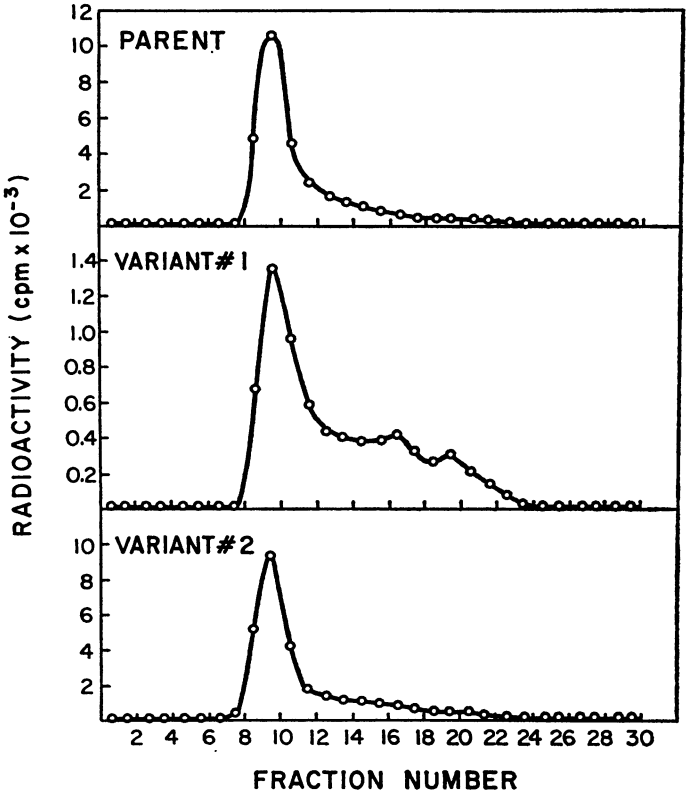

FIG. 8. Gel filtration of cell wall fractions of the parent Giorgio staphylococcus and of two lysostaphin-resistant variants. The column was calibrated with dextran blue which emerged in fractions 8-12 and dinitrophenyl-glycine which emerged in fractions 18-26.

The cell wall fractions were further examined by subjecting them to gel filtration on Sephadex G-25 to determine if the cell wall polymer were cross-linked. The *Chalaropsis B* enzyme cleaves at every N-acetyl-muramic acid and if polyglycine bridges were not cross-linked the resulting disaccharide-peptide product would be eluted with low molecular-weight materials. The results, shown in Figure 8, indicated that the product

solubilized by the *Chalaropsis B* enzyme was still high molecular-weight material. Though there were some products of low molecular weight in variant No. 1, the resistance of these lysostaphin-resistant variants could not be ascribed to a lack of cross-linking in the cell wall. The mechanism of resistance is, therefore, more complex and possibly involves surface alterations that protect susceptible bonds.

DISCUSSION

The present *in vitro* studies confirm that lysostaphin is a potent bacteriocidal agent. It maintains its activity in human serum, is stable at body temperature for long periods of time, and is capable of lysing repeated challenge inocula of staphylococci. Unlike penicillin it is active against large populations of metabolically quiescent staphylococci analogous to those found within staphylococcal abscesses. Thus lysostaphin would appear to have potential therapeutic advantages in the management of staphylococcal disease.

The finding that staphylococci residing within polymorphonuclear leukocytes were protected from lysostaphin action resembled results of experiments reported with penicillin. Tompsett has demonstrated that the activity of penicillin is greatly diminshed against such intracellular staphylococci.[22]

Lysostaphin-resistant staphylococci were readily selected *in vitro*. However, they differed from the parent strain in several significant characteristics. While the two variants did not change their phage type, ability to produce coagulase, or mouse virulence when administered intraperitoneally, their colonial morphology was markedly altered, their lag phase of growth was greatly prolonged, and the variant most resistant to lysostaphin was significantly less virulent for mice when given by the intravenous route.

The decreased mouse virulence resembles the situation reported with laboratory produced penicillin-resistant staphylococci. These also appear to have diminished disease-producing potential.[24] Furthermore, both lysostaphin-resistant variants, while retaining their ability to produce penicillinase, were found to be significantly more sensitive to penicillin *in vitro* than was the parent staphylococcus. This suggests that the varients either produced less penicillinase or that their cell wall structure was altered so that they had become more susceptible to penicillin. If the latter were correct these strains might have been killed before significant quantities of penicillinase were produced.

The present studies suggest that resistance to lysostaphin is not related to a basic change in the chemical composition of the staphylococcal cell wall. The observed resistance to lysostaphin could not be attributed to the

lack of glycine substrate or the absence of appropriate glycine cross-links in the cell walls of the resistant variants. These findings suggest that lysostaphin resistance may possibly relate to alterations in steric configurations at the surface of the cell wall preventing the apposition of lysostaphin and the susceptible bonds in the wall.

Because of its potent activity *in vitro,* its selectivity, and its effect on large populations of metabolically inactive staphylococci, it would appear that lysostaphin deserves study as a therapeutic agent in experimental staphylococcal infections.

SUMMARY

The *in vitro* characteristics of lysostaphin, an enzyme that lyses the cell walls of staphylococci, were studied. Unlike the penicillins, lysostaphin rapidly killed large inocula of resting microorganisms. It maintained its activity in serum and for prolonged periods at 37°C. The enzyme was equally active against encapsulated and non-encapsulated staphylococcal strains. Like penicillin, lysostaphin was inactive against intracellular staphylococci.

Lysostaphin-resistant staphylococci could be readily isolated *in vitro.* These resistant variants differed from the parent strain in their colonial morphology, slower growth rates, increased penicillin sensitivity, and, in one instance, diminished virulence for mice.

Studies of lysostaphin-resistant staphylococci suggested that resistance might possibly relate to configurational alterations on the surface of the cell wall rather than to changes in its chemical composition.

The impressive *in vitro* activity of lysostaphin indicates that this enzyme deserves further study as a therapeutic agent in experimental staphylococcal infections.

ACKNOWLEDGMENT

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