WILLIAM SCHAFFNER*
M. ANN MELLY†
M. GLENN KOENIG‡

George Hunter Laboratory, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

LYSOSTAPHIN: AN ENZYMATIC APPROACH TO STAPHYLOCOCCAL DISEASE. II. IN VIVO STUDIES¶

Lysostaphin is a potent enzymatic anti-staphylococcal agent that lyses the cell walls of staphylococci *in vitro*. Studies described in the preceding paper demonstrated that lysostaphin rapidly killed staphylococci, was unaffected by human serum, and maintained its activity after prolonged incubation at body temperature.[1] Unlike the penicillins, lysostaphin was active *in vitro* against very large populations of stationary phase staphylococci.

Serious staphylococcal infections continue to pose major therapeutic problems despite the introduction of the penicillinase-resistant penicillins and cephalosporins. Mortality rates from staphylococcal bacteremia approach 30%,[2] and may be higher in staphylococcal endocarditis, especially when prosthetic cardiac valves are infected. It is believed that the therapeutic failures often result from the relative impotence of currently available antimicrobials against staphylococci residing in high titers in abscess lesions. Thus the availability of a chemotherapeutic agent active against large numbers of staphylococci in such lesions might be of great importance. Lysostaphin was therefore further studied as a therapeutic agent in experimental staphylococcal infections. These studies show that lysostaphin is highly effective in the treatment of experimental staphylococcal disease.

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* U.S.P.H.S. Postdoctoral Fellow, National Institute of Allergy and Infectious Diseases. Instructor in Medicine.

† Instructor in Medical Research.

‡ Research Career Development Awardee, National Institute of Allergy and Infectious Diseases. Associate Professor of Medicine. Address reprint requests to Dr. M. Glenn Koenig, George Hunter Laboratory, Vanderbilt University School of Medicine, Nashville, Tennessee 37203.

¶ Presented in part at the national meeting of the American Federation for Clinical Research, Atlantic City, New Jersey, May 1, 1966. Supported by U.S.P.H.S. grant AI-03082 and a grant from the Mead Johnson Research Center, Evansville, Indiana.

Received for publication 10 November 1966.

METHODS

Two coagulase-positive strains of *Staphylococcus aureus* were utilized in these studies. The Giorgio strain (previously described[1, 3]) is inhibited by 0.39 μg/ml. of oxacillin. The Diffuse variant of the Smith strain has also been extensively characterized.[4, 5] It is coagulase positive, clumping factor negative, possesses an antiphagocytic surface antigen, and is virulent for mice when injected intraperitoneally. It is inhibited by 25 μg/ml. of penicillin G. Cultures were maintained on trypticase soy agar slants at 4° C. Eighteen-hour transplants in trypticase soy broth were used in all experiments.

In one group of experiments 20 g Swiss albino mice* were infected by intraperitoneal injections of 0.4 ml. of the Diffuse culture containing approximately 5 x 10^8 viable staphylococcal units. Animals were sacrificed at intervals for quantitative bacterial counts, as described previously.[4] One milliliter of sterile saline was used to wash the peritoneal cavity and was then diluted appropriately and plated in trypticase soy agar. Additional groups of infected mice were observed for 24 hours to determine mortality rates.

In another group of experiments, mice were infected via a tail vein with 0.2 ml. (approximately 2 x 10^8 viable staphylococcal units) of the Giorgio strain. Animals were sacrificed at intervals and kidneys removed aseptically. Each pair of kidneys was placed in 4 ml. of sterile 0.9% saline in a graduated centrifuge tube so that the fluid displaced indicated kidney volume. Specimens for staphylococcal L-form isolation were placed in 5% saline. The kidneys were then homogenized in the saline using a Teflon tissue grinder. Homogenates were diluted appropriately and plated in trypticase soy agar. Culturable units of staphylococci per milliliter of kidneys were calculated from kidney volumes. Additional groups of infected mice were observed for 28 days to determine cumulative mortality rates.

Crude alpha hemolysin was prepared from the Diffuse colonial variant of the Smith strain growth for six days in modified Burnet's broth into which 20% CO_2 was bubbled continuously.[6] Culture supernatants were Millipore filtered and assayed for hemolytic activity (hemolytic titer 1:64).

Media for the recovery of L-forms was prepared according to the method of Kagan,[7] and contained 6 g. of brain heart infusion agar, 6.7 g. of NaCl, 116 ml. of distilled water, and 30 ml. of sterile pooled human plasma, which had been inactivated at 56° C., frozen overnight at —20° C. and Zeitz filtered. Aliquots of kidney homogenates in 5% saline were streaked on the surface of agar plates and incubated at 37° C. for at least five days. Daily observations of the plates were performed using a binocular dissecting microscope. L-forms appeared as tiny "fried-egg" colonies which grew into the agar and could not be moved easily with a bacteriological loop. Serial transfers were made by cutting out agar blocks and streaking them on fresh plates.

A modification of the Ouchterlony agar gel diffusion technique was employed to demonstrate precipitin antibody.[8] The media contained 1.2% agar in 0.85% NaCl and 1:10,000 merthiolate. Wells were placed ¼ inch apart in agar plates. Plates were incubated overnight at 37° C., the wells filled with appropriate solutions, and the plates reincubated at 37° C. for 18 hours. An additional day at room temperature or at 4° C. allowed maximum production of precipitin bands.

* Dublin Laboratory Animals, Inc., Dublin, Virginia.

Neutralizing antibody was measured using 50% serum from mice or rabbits* immunized with lysostaphin.† The standard assay for enzyme activity was performed as previously described.[1]

Chi square tests were used in evaluating the statistical significance of differences in mortality rates. In assessing the significances of differences in the averages of log titer determinations, *t* tests were used except in certain cases where rank sum tests seemed to be indicated because of variance heterogeneity in the data. Two-tailed tests were used in all cases.

TABLE 1. THE EFFECT OF LYSOSTAPHIN AND PENICILLIN G ON THE INTRAPERITONEAL MOUSE INFECTION WITH THE SMITH DIFFUSE STAPHYLOCOCCUS. TREATMENT AT 4 HOURS

	Survivors/total	*Per cent surviving*
Control	2/33	6
Penicillin G 2.5 mg. IV	9/17	53
Lysostaphin 2.5 mg. IV	34/34	100

RESULTS

Efficacy of lysostaphin in an intraperitoneal staphylococcal infection in mice

Intraperitoneal infection with the diffuse colonial variant of the Smith strain of *Staphylococcus aureus* produces almost 100% mortality within 6 to 10 hours.[4,5] Staphylococcal alpha hemolysin produced by rapidly multiplying microorganisms is the cause of death.[4,6,9] In the present study mice were treated intravenously with a single 2.5 mg. dose of either lysostaphin or penicillin G four hours after intraperitoneal infection. Mice succumbing to the infection all died within 12 hours. Survivors were observed for 72 hours after challenge. Such an experiment is shown in Table 1. Only 6% of control animals survived. While 53% of the mice treated with penicillin survived, all the mice treated with lysostaphin lived. This is a significant difference in survival ($.01 > p > .001$). Cultures of intraperitoneal contents obtained six hours after infection showed no differences between intraperitoneal staphylococcal titers of control and

* Male albino rabbits (2.5 to 3 kg.) from H and M Rabbits Breeders, Louisville, Kentucky.

† Lot no. 142-123-1 kindly supplied by Dr. Peter Tavormina of the Mead Johnson Research Center, Evansville, Indiana.

penicillin-treated mice (Table 2). Lysostaphin significantly reduced the intraperitoneal staphylococcal titers when compared with the results in the penicillin-treated animals ($p > .01$). Staphylococci isolated from the peritoneal cavities of lysostaphin-treated mice were uniformly sensitive to lysostaphin.

TABLE 2. INTRAPERITONEAL INFECTION IN MICE WITH THE SMITH DIFFUSE STAPHYLOCOCCUS. TREATMENT AT 4 HOURS AND AT 6 HOURS

(*Intraperitoneal titers*)

	Onset of infection	*4 hours after infection*	*6 hours after infection*
Control	4.1×10^8	1.1×10^9	1.7×10^9
	3.3×10^8	1.1×10^9	1.4×10^9
	2.9×10^8	1.0×10^9	5.7×10^8
Penicillin G 2.5 mg. IV			2.1×10^8
			9.5×10^7
			3.1×10^7
Lysostaphin 2.5 mg. IV			2.7×10^6
			3.6×10^5
			3.2×10^4

TABLE 3. LYSOSTAPHIN-STAPHYLOCOCCAL ALPHA HEMOLYSIN INTERACTION

		Mortality of mice at 24 hours
Control	0.5 ml. of alpha hemolysin IP	7/8
Lysostaphin treatment	0.5 ml. of alpha hemolysin IP; 30 minutes later treated with lysostaphin, 2.5 mg. IV	7/10
Lysostaphin-alpha hemolysin mixture	0.5 ml. of alpha hemolysin + 2.5 mg. lysostaphin incubated at 37° C. for 30 minutes, then injected IP	5/5

Because death in this infection results from staphylococcal alpha hemolysin production, two studies were performed to determine whether lysostaphin interfered with the biological activity of alpha hemolysin. As shown in Table 3, 0.5 ml. of crude alpha hemolysin was lethal for mice. Lysostaphin administered 30 minutes after intraperitoneal injection of crude alpha hemolysin did not increase the survival of mice. Preincubation of lysostaphin with alpha hemolysin did not alter its mouse-lethal

properties. Hence, the survival of infected mice treated with lysostaphin appeared to represent a direct effect on the staphylococcal cell.

Efficacy of lysostaphin in an intravenous staphylococcal infection in mice

When injected intravenously into mice,[8, 10] the Giorgio strain of *Staphylococcus aureus* produces a progressive and usually fatal staphylococcal pyelonephritis with abscess formation. The Giorgio strain is sensitive

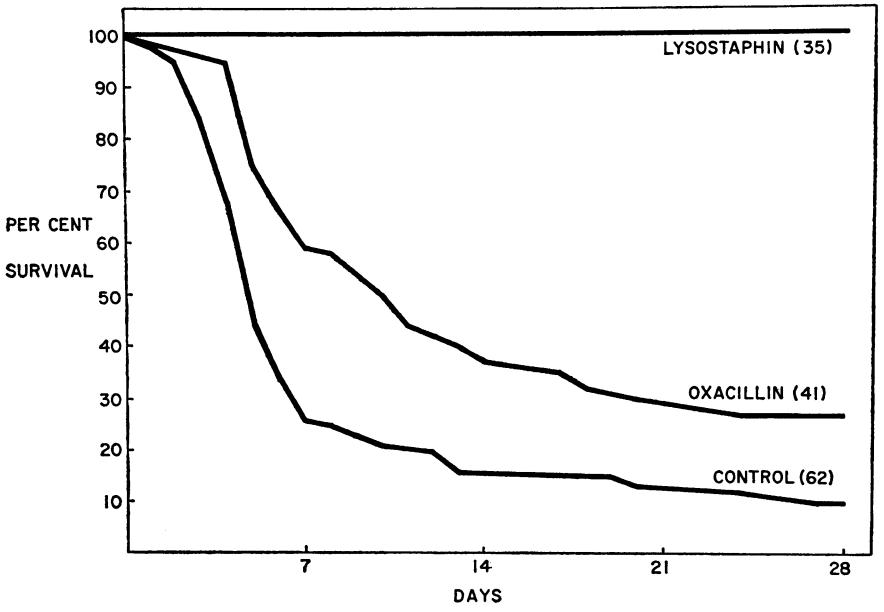

FIG. 1. Intravenous infection of mice with the Giorgio staphyloccus. Survival rates after intravenous treatment at *6 hours* after infection with 2.5 mg. of either lysostaphin or oxacillin. The numbers in parentheses indicate the number of mice in each group.

to oxacillin (M.I.C. 0.39 μg/ml.). In these studies mice were treated 6 or 24 hours after intravenous infection with a *single* intravenous dose of 2.5 mg. of either lysostaphin or oxacillin (approximately 100 mg/kg.). As shown in Figure 1, only 10% of untreated control mice were alive four weeks after infection. When the single intravenous treatment was given 6 hours after infection, oxacillin increased the survival rate of 27%. This was a significant increase in survival rate ($p > .01$). However, all of the mice treated with lysostaphin survived. The difference between the survival rates of the oxacillin and lysostaphin-treated animals was highly significant ($p > .001$).

When such single dose treatment was delayed until 24 hours after infection, the survival rates shown in Figure 2 were obtained. Treatment with oxacillin yielded a 30% survival rate, significantly greater than that among untreated mice ($p > .01$). Lysostaphin treatment protected 56% of the animals, a result significantly better than that observed with oxacillin ($p > .01$).

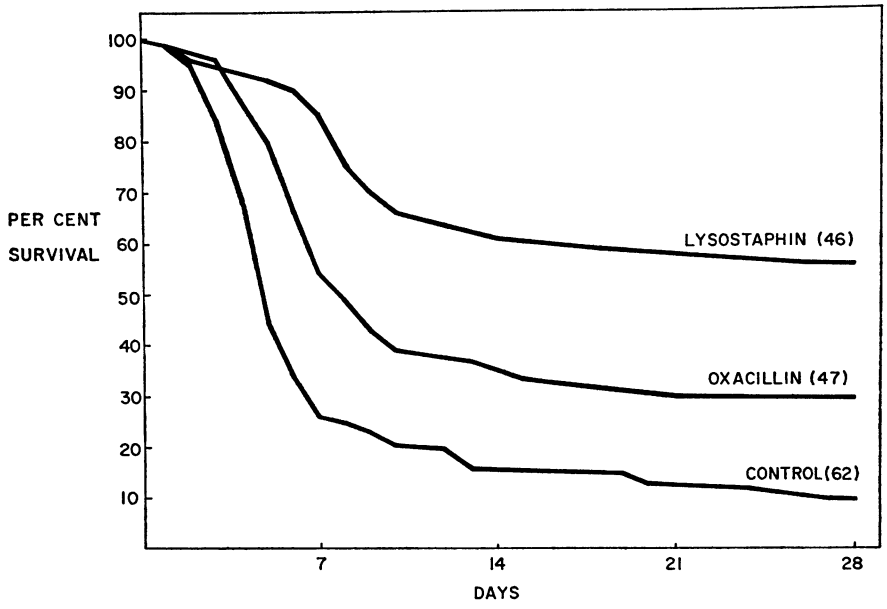

FIG. 2. Intravenous infection of mice with the Giorgio staphylococcus. Survival rates after intravenous treatment at *24 hours* after infection with 2.5 mg. of either lysostaphin or oxacillin. The numbers in parentheses indicate the number of mice in each group.

Maximum staphylococcal multiplication occurs in the kidneys when mice are infected intravenously.[10] The concentrations of microorganisms in the kidneys were thus determined to define more precisely the effects of therapy. As shown in Figure 3, kidneys contained approximately 10^5 to 10^6 staphylococci per milliliter 6 hours after infection. The staphylococcal populations in the kidneys of untreated mice gradually increased over the 7-day period studied, reaching a maximum titer of more than 10^9 microorganisms/ml. A single dose of oxacillin 6 hours after infection did not significantly alter kidney microbial populations. Lysostaphin, as compared with oxacillin, significantly reduced the concentration of staphylococci in the kidneys, 1 day, 3 days, and 7 days after infection. In some

kidneys of lysostaphin-treated mice, no culturable staphylococci could be demonstrated.

If treatment were delayed until 24 hours after infection (Fig. 4), kidneys from untreated mice contained between 10^6 and 10^8 staphylococci/ml. A single dose of oxacillin did not significantly alter these populations. In contrast, lysostaphin significantly lowered the bacterial titers when com-

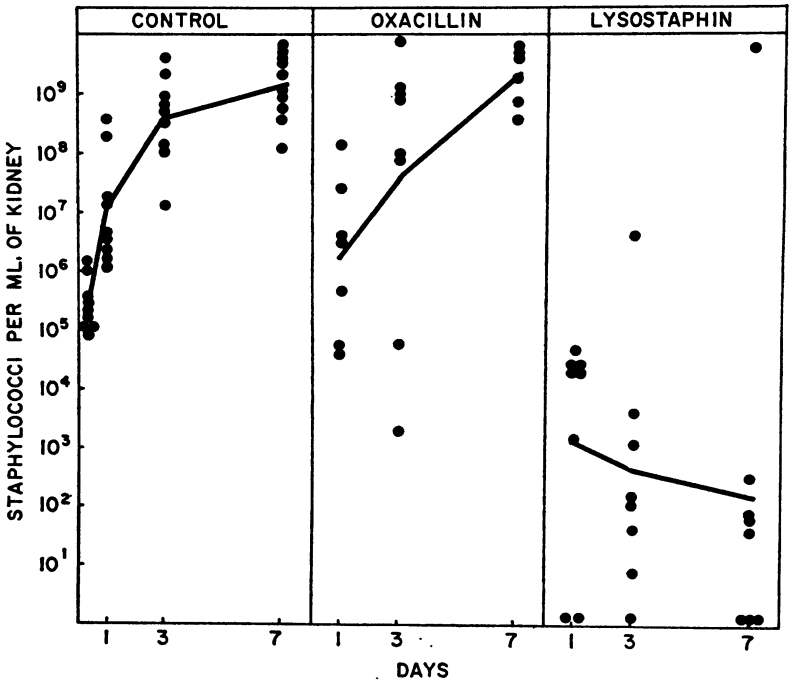

FIG. 3. Intravenous infection of mice with the Giorgio staphylococcus. Kidney titers after intravenous treatment at *6 hours* after infection with 2.5 mg. of either lysostaphin or oxacillin. The lines connect the means in each group.

pared with oxacillin 3 days and 7 days after infection. Staphylococci isolated from the kidneys of mice treated with lysostaphin all retained their sensitivity to the enzyme.

Another therapeutic program was then devised to approximate more closely the conditions of clinical practice. In this instance both single and multiple dose oxacillin regimens were compared with a single injection of lysostaphin. Six hours after intravenous infection with the Giorgio staphylococcus, mice were treated intravenously with 2.5 mg. of either lysostaphin or oxacillin. One group of oxacillin-treated mice then received

additional oxacillin, 2.5 mg. intramuscularly, for each of the next four days. The lysostaphin-treated mice received no further treatment. As shown in Figure 5, while the multiple-dose oxacillin regimen improved survival rates, a single lysostaphin treatment resulted in a 90% survival rate, significantly higher than either oxacillin treatment program ($p > .001$).

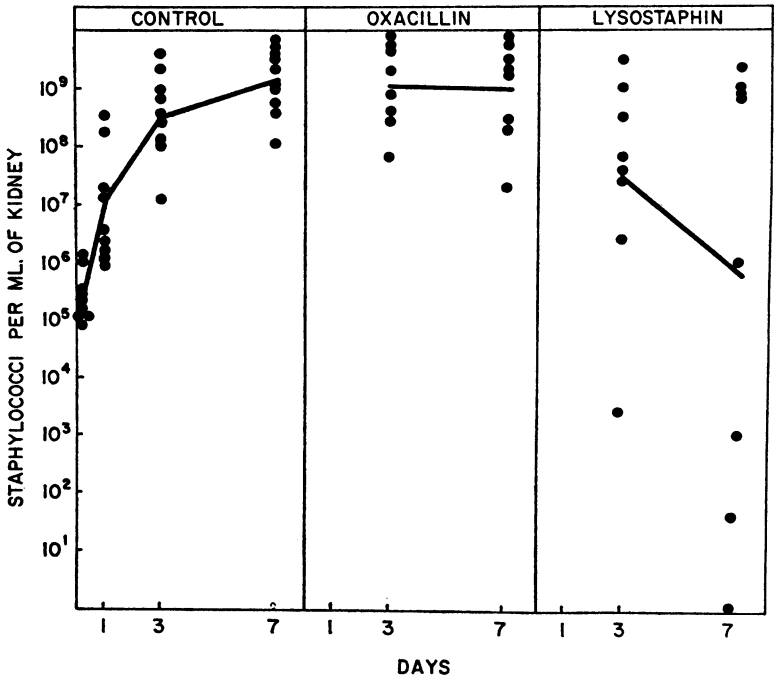

FIG. 4. Intravenous infection of mice with the Giorgio staphylococcus. Kidney titers after intravenous treatment at *24 hours* after infection with 2.5 mg. of either lysostaphin or oxacillin. The lines connect the means in each group.

Five days after intravenous infection with the Giorgio staphylococcus most mice have gross renal cortical abscesses (Fig. 6). To determine the effect of lysostaphin and oxacillin on such staphylococcal populations, mice were treated once intravenously with 5 mg. of either lysostaphin or oxacillin (approximately 200 mg/kg.) on the 5th day after infection. After 24 hours, kidney staphylococcal titers were determined. All kidneys had grossly visible cortical abscesses. As shown in Figure 7, kidneys from untreated mice contained greater than 10^9 microorganisms/ml. The kidneys from the oxacillin-treated group contained similar microbial popula-

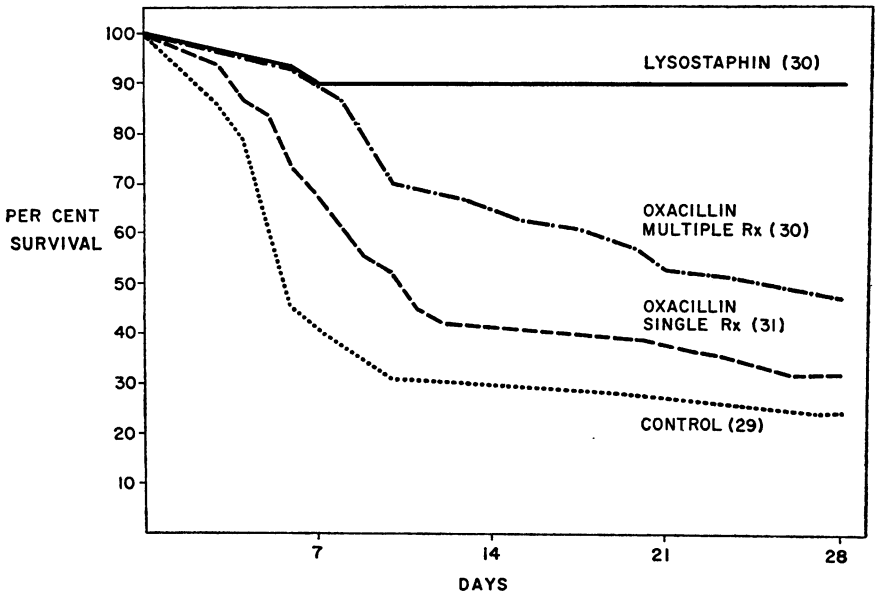

FIG. 5. Intravenous infection of mice with the Giorgio staphylococcus. Survival rates after treatment at *6 hours* after infection with single doses of lysostaphin or oxacillin or multiple doses of oxacillin. The numbers in parentheses indicate the number of mice in each group.

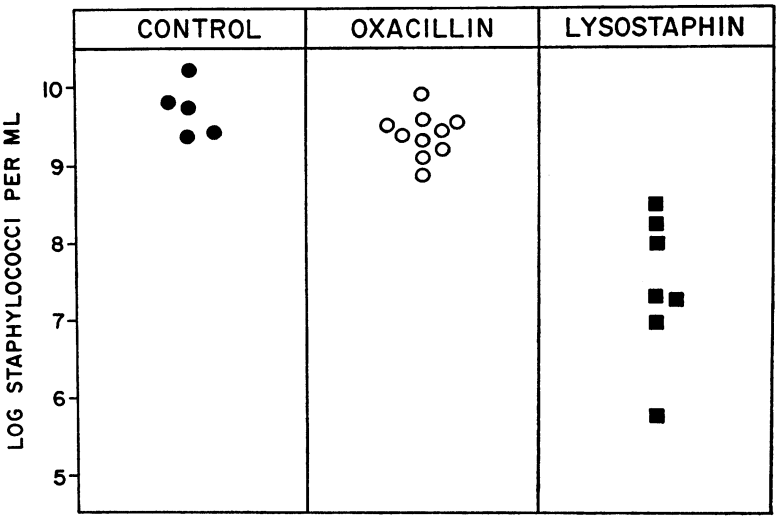

FIG. 7. Intravenous infection of mice with the Giorgio staphylococcus. Kidney staphylococcal titers after treatment of established renal abscesses. Five milligrams of either lysostaphin or oxacillin were given intravenously 5 days after infection.

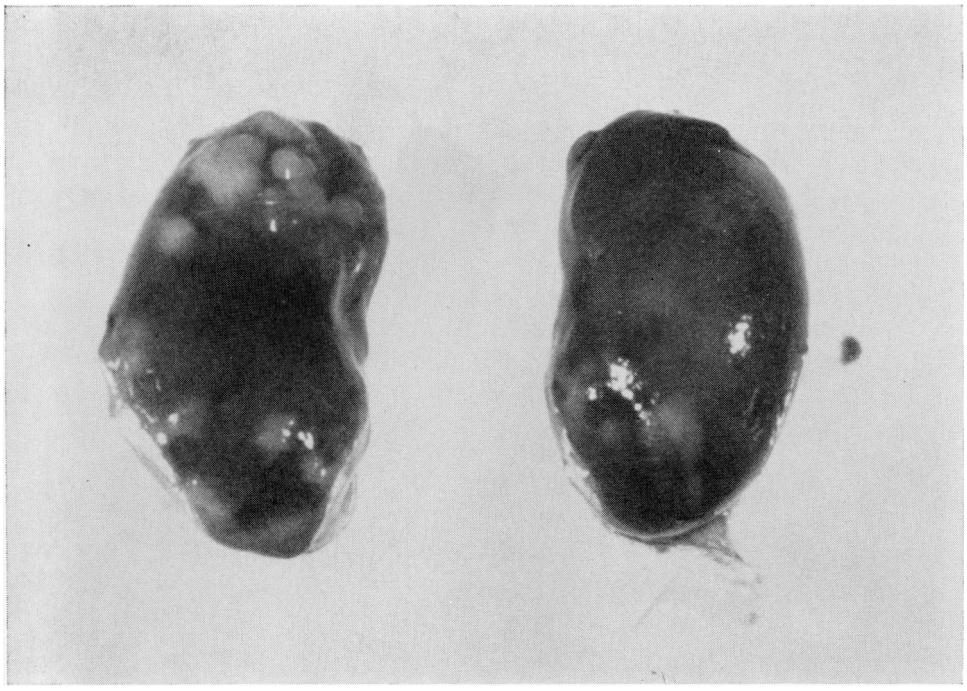

FIG. 6. The appearance of renal abscesses in mice infected with the Giorgio staphylococcus 5 days previously.

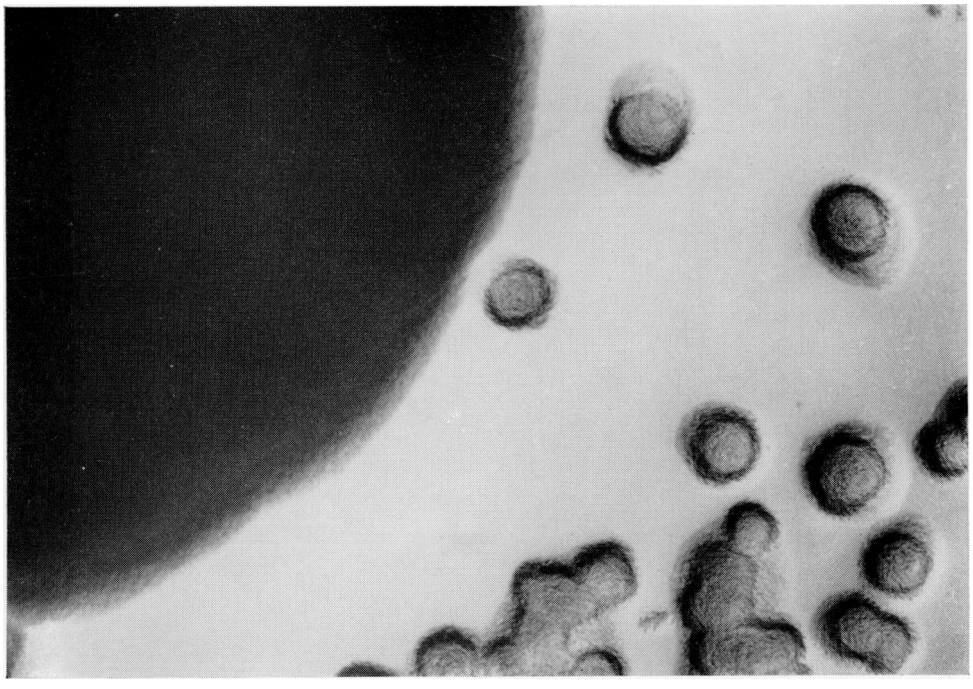

FIG. 8. Colonies of staphylococcal L-forms on solid media. The large shadow on the left is a portion of the parent staphylococcal colony. X 50.

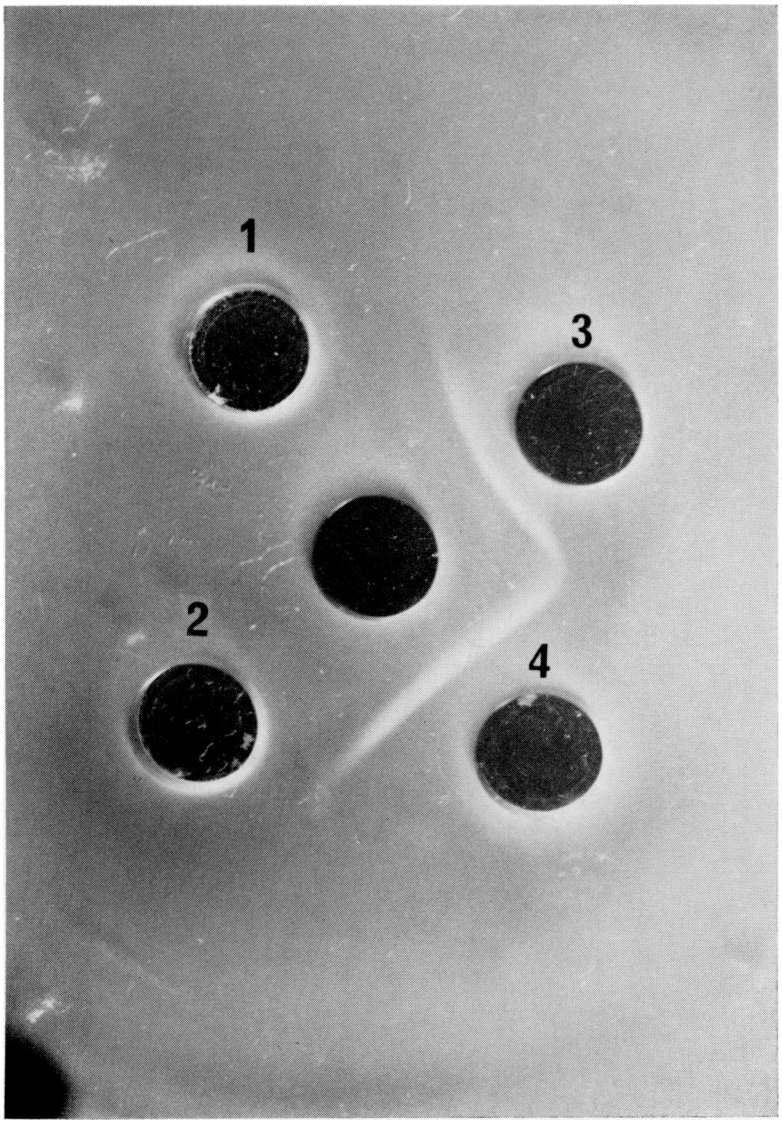

FIG. 10. Agar diffusion plate. Center well contains lysostaphin. Well 1 contains serum from mice immunized with multiple doses of lysostaphin in Freund's adjuvant. Well 2 contains serum from mice immunized with multiple doses of intravenous lysostaphin. Wells 3 and 4 contain serum from rabbits obtained 4 days after the 4th intravenous dose of lysostaphin.

tions. In contrast, lysostaphin had significantly reduced the staphylococcal population of the abscessed kidneys ($p > .01$). Thus, lysostaphin was active against high titers of staphylococci inside the abscess lesion.

Production of staphylococcal L-forms by lysostaphin

It has been suggested that the persistence of staphylococcal infection and the relapses of staphylococcal disease after conventional antimicrobial therapy are due to the formation of staphylococcal L-forms in tissues.[11]

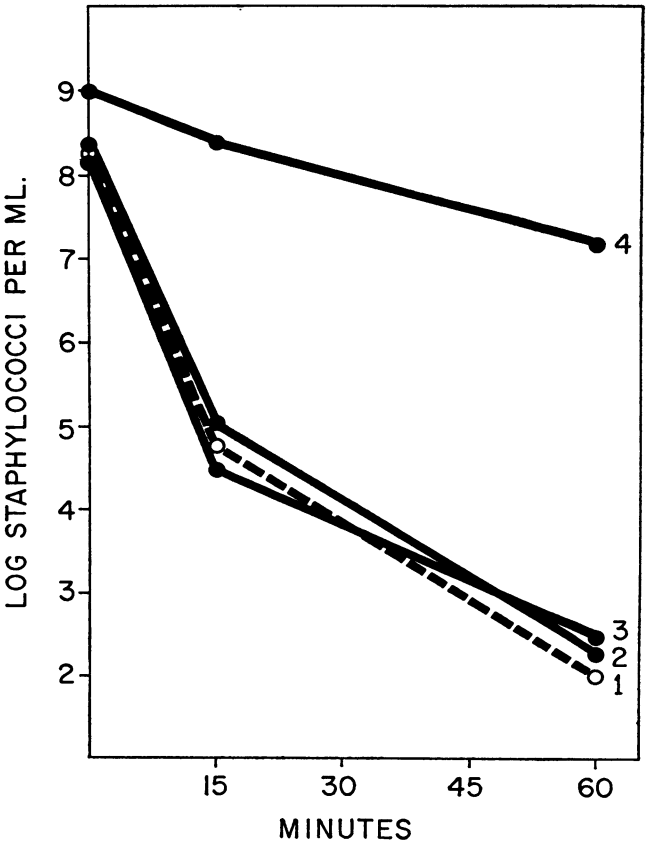

FIG. 9. Effect of 50% lysostaphin-immune rabbit serum on *in vitro* lysostaphin activity (70 μg/ml.): (1) Lysostaphin activity in normal rabbit serum. (2) Lysostaphin activity in serum obtained 14 days after a single intravenous injection of lysostaphin. (3) Lysostaphin activity in serum obtained 4 days after two intravenous injections of lysostaphin separated by 3 days. (4) Lysostaphin activity in serum obtained 4 days after the 4th intravenous injection of lysostaphin administered over 3 to 4-day intervals.

As lysostaphin lyses staphylococcal cell walls while, presumably, leaving cell membrane and bacterial cytoplasm intact, the *in vitro* and *in vivo* production of L-forms by lysostaphin was investigated.

Lysostaphin readily produced staphylococcal L-forms *in vitro*. After their production in hypertonic salt broth they grew as typical "fried-egg" colonies on agar containing salt and human serum (Fig. 8). These osmotically fragile L-forms could be maintained through several passages on solid media not containing lysostaphin or other antibiotics. Eventually they slowly reverted to the usual bacterial forms that had the same characteristics and phage type as the parent strain.

Attempts were then made to isolate L-forms from the kidneys of mice infected intravenously with the Giorgio staphylococcus and treated with lysostaphin or oxacillin. When mice were treated intravenously 6 hours after infection with 2.5 mg. of either lysostaphin or oxacillin, staphylococcal L-forms were isolated from their kidneys from ½ to 2 hours after treatment with either drug, while no L-forms could be isolated from kidneys of untreated control animals. As with the L-forms produced *in vitro,* these microorganisms reverted to typical staphylococci that appeared identical to the parent strain and possessed the same phage type. However, L-forms could not be cultured from any kidneys at 24 and 48 hours after treatment and at the four week termination of the experiment.

Observations on the immunology of lysostaphin

Lysostaphin is a protein with a molecular weight of approximately 30,000. Its antigenicity and the nature of the antibody produced in response to its parenteral administration in mice and rabbits were therefore studied.

Rabbits were immunized with single or multiple intravenous injections of 40 mg. of lysostaphin (approximately 13 mg/kg.). The multiple doses were spaced at intervals of 3 or 4 days for four doses, then every two weeks for two additional doses. There were no discernable adverse reactions to this repeated intravenous administration of the enzyme. As shown in Figure 9, serum obtained 14 days after the intravenous administration of a single dose of lysostaphin or 4 days after two intravenous doses did not interfere with *in vitro* lysostaphin activity. However, serum obtained 4 days after the fourth dose of the multiple-dose regimen did significantly interfere with the lytic activity of lysostaphin. When lysostaphin was added to this serum, a visible precipitate was observed and agar diffusion techniques demonstrated a single broad precipitin line (Fig. 10). Rabbits in which such precipitating and neutralizing antibody was demonstrated received two further intravenous injections of lysostaphin without observed ill effects.

Immunological studies in mice are summarized in Table 4. Mice were immunized at 3 day intervals with four intramuscular doses of 2.5 mg. of lysostaphin emulsified with incomplete Freund's adjuvant. Eleven days after the last immunization 10 mice were challenged with 2.5 mg. of lysostaphin intravenously. All 10 died a respiratory death within 30 to 40 minutes. Precipitating antibody could not be demonstrated in serum from such mice using agar diffusion techniques (Fig. 10).

Mice were also immunized intravenously with lysostaphin alone. Two schedules were employed. One group of animals received four doses of

TABLE 4. IMMUNOLOGICAL STUDIES IN MICE

Immunization schedule	*Mortality (killed/total)*
1. Lysostaphin in incomplete Freund's adjuvant Immunized IM Q3 days for 4 doses Challenged IV 11 days after last dose	10/10
2. Lysostaphin Immunized IV Q3 days for 4 doses Challenged IV 12 days after last dose	1/10
3. Lysostaphin Immunized IV every day for 5 days Challenged IV 12 days after last dose	1/10
4. Equine tetanus antitoxin Immunized IV Q3 days for 4 doses Challenged IV 12 days after last dose	4/10

2.5 mg. of lysostaphin administered over 10 days. A second group received five doses of 2.5 mg. of lysostaphin on five consecutive days. The first group was challenged intravenously with both Tris buffer and 2.5 mg. of lysostaphin 12 days after the last dose. The mice receiving Tris buffer alone were not affected. While the 10 mice receiving lysostaphin appeared transiently ill with ruffled hair and shallow respirations, only one mouse died 30 minutes after the challenge injection.

The second group, having received five daily intravenous doses of lysostaphin, was challenged with the same dose of lysostaphin 13 days after the last immunization. All appeared transiently ill but only one mouse died.

Attempts to demonstrate precipitin antibodies in sera from both lysostaphin-immunized groups of mice were unsuccessful (Fig. 10). Further-

more, in systems containing 50% serum from such mice, no inactivation of lysostaphin could be shown.

In order to compare the antigenicity of lysostaphin with a foreign protein that has been used clinically, a group of mice was immunized intravenously over a 10-day period with four doses of 0.2 ml. of tetanus antitoxin of equine origin. When these mice were challenged intravenously with 0.2 ml. of tetanus antitoxin 12 days after the last immunization, four of 10 mice died.

DISCUSSION

The present studies indicate that lysostaphin is superior to penicillin and oxacillin in the therapy of experimental staphylococcal infections in mice. This superiority was demonstrated by an increased survival rate among lysostaphin-treated animals and by greater killing of staphylococci *in vivo*. Lysostaphin lowered bacterial titers within well-established renal abscesses while oxacillin had no demonstrable effect against staphylococcal populations in such lesions. In mice infected intravenously, a single dose of lysostaphin produced significantly greater survival rates than did multiple doses of oxacillin. Thus, lysostaphin appears to be capable of killing staphylococci within abscess lesions, a situation in which conventional antimicrobials are relatively ineffective.

Staphylococcal L-forms were easily produced by lysostaphin *in vitro*. However, they could be recovered from the kidneys of animals infected intravenously only briefly after treatment with either lysostaphin or oxacillin and were not demonstrable 24 and 48 hours or 28 days after treatment. While Kagan has proposed that L-forms may be the cause of persisting or relapsing staphylococcal disease,[11] the present studies suggest that lysostaphin would pose no greater problem in this regard than penicillin or other antibiotics whose site of action involves cell wall synthesis.

The major area of concern regarding the possible use of lysostaphin in humans is its antigenicity. Lysostaphin is a protein with a molecular weight of about 30,000. It has not been completely purified. The present studies indicate that precipitin and neutralizing antibodies were evoked in rabbits, although subsequent intravenous challenge of rabbits with demonstrable antibody produced no discernible signs of illness.

In contrast, intravenous lysostaphin challenge produced apparent anaphylaxis in mice immunized with multiple injections of lysostaphin emulsified with incomplete Freund's adjuvant. Immunization with lysostaphin *alone* on five successive days or intermittently over 10 days resulted

in obvious illness, but reduced the death rate to only 10%. These deleterious effects in mice were noted only after multiple injections of the enzyme and when the challenge doses were given 11 and 12 days after the last immunizing dose.

In contrast to the results observed in rabbits, neutralizing or precipitating antibodies could not be demonstrated in serum obtained from mice at the time of anaphylaxis. Thus, while lysostaphin proved to be antigenic, there was a marked species difference in the reaction to this enzyme. Mice immunized with equine tetanus antitoxin had similar anaphylaxis, and mortality rates were greater than in mice immunized with lysostaphin on the same schedule. Thus, despite its antigenicity, lysostaphin like equine tetanus antitoxin might be administered in a single dose or in a series of injections over a short period of time without undue risk. The present animal studies indicate that such a dosage schedule is effective in the therapy of etablished staphylococcal infection.

While lysostaphin-resistant strains were easily selected *in vitro*,[1] no lysostaphin-resistant strains were recovered from infected animals treated with lysostaphin. Furthermore, the resistant strains recovered *in vitro* were unusual staphylococci which appeared in one instance less virulent for mice and, in both cases, more susceptible to penicillin than the parent strain.[1] It is possible that lysostaphin-resistant staphylococci may prove to have reduced disease producing potential, and increased sensitivity to currently available antimicrobial agents. The striking effectiveness of lysostaphin against high titers of staphylococci suggests that use of a single injection or short course of lysostaphin plus, or followed by, a conventional anti-staphylococcal antimicrobial may be worth exploring in serious staphylococcal disease.

SUMMARY

Lysostaphin was superior to the penicillins in the therapy of experimental staphylococcal infections in mice. In contrast to oxacillin, it was capable of killing staphylococci residing in high titers within abscess lesions. While staphylococcal L-forms were easily produced by lysostaphin *in vitro*, they could be recovered from the kidneys of infected animals only for a brief time after treatment.

Multiple doses of lysostaphin given over a two-week period evoked precipitins and neutralizing antibodies in rabbits. Such antibodies could not be demonstrated in mice but anaphylaxis occurred in mice on challenge. Single doses of lysostaphin produced no deleterious effects in either animal species.

The present studies suggest that lysostaphin, despite its antigenicity, may deserve evaluation in the therapy of life-threatening staphylococcal disease. Perhaps the problem of sensitization and lysostaphin-resistant microorganisms can be avoided by administering only a short course of lysostaphin, followed by conventional anti-staphylococcal antimicrobial therapy.

ACKNOWLEDGMENT

The technical assistance of Mrs. Sandra Wood is gratefully acknowledged.

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