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Killing of Intraphagocytic *Staphylococcus aureus* by Dihydrostreptomycin Entrapped Within Liposomes

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Liposomes (phospholipid vesicles) may serve as vehicles of antibiotic transport to intracellular sites. Liposomes containing entrapped dihydrostreptomycin enhanced killing of *Staphylococcus aureus* contained within phagocytic vacuoles.

Chemotherapy of bacterial infections of humans may be complicated by the intracellular residence of infectious agents. This applies for pathogens such as *Myobacterium tuberculosis* and other facultative intracellular parasites (11), as well as for extracellular agents of disease such as *Staphylococcus aureus* (12). In spite of the availability of many antibiotics for therapeutic use, efficacy is usually reduced when bacteria are located intracellularly (1, 11). Any means which might serve to increase intracellular concentration of antibiotics within infected phagocytic (and nonphagocytic) cells would enhance bacterial killing and elimination of infections. Liposomes (phospholipid vesicles) have been shown to transport antibiotics (5) and other drugs (5, 6) into intracellular sites. Evidence presented here suggests that phospholipid vesicles containing dihydrostreptomycin (DHS) sulfate enhance killing of *S. aureus* previously engulfed by mouse peritoneal macrophages.

Macrophages were harvested (2) from the unstimulated peritoneum of randomly bred mice (Animal Division, Clinical Research Centre) and cultured (3×10^6 cells per plastic petri dish, 60-mm diameter) in a CO_2 incubator for 24 h at 37°C in Parker medium 199 containing 10% heat-inactivated porcine serum. Nonadherent cells were removed by washing the monolayers with phosphate-buffered saline, and cell viability was ascertained by trypan blue exclusion. Streptomycin-susceptible *S. aureus* 337 (obtained from E. S. Anderson, Central Public Health Labs, Colindale, London) was maintained on Todd-Hewitt agar slants. For each experiment, 16-h broth cultures were standardized to contain 3×10^8 staphylococci per ml. Bacteria in complete tissue culture medium were added to the macrophage monolayers at a multiplicity of infection of 100 bacteria per cell. After 45 to 60 min at 37°C, the majority of cells contained one or more bacteria, as judged by examination of Giemsa-stained preparations.

Liposomes were prepared as described by Gregoriadis (7). In brief, a thin film is deposited on the walls of a round-bottomed flask by evaporation of a mixture of lipids dissolved in chloroform. The lipid film spontaneously forms liposomes when dispersed in aqueous medium. In these studies, the liposomes were formed with a mixture of egg phosphatidylcholine, cholesterol, and phosphatidic acid in molar ratios of 7:2:1. The lipid film was dispersed in 2.0 ml of 5 mM sodium phosphate buffer (pH 7.0) and sonically treated for 4 min at 4°C; sonic treatment for this period of time results in formation of small liposomes, many of which are unilamellar. Empty liposomes were concentrated by passage through a column of Sepharose 6B beads (Pharmacia Co., Uppsala, Sweden). DHS (British Drug House, Poole, United Kingdom)-containing liposomes were prepared in similar fashion except that DHS at a concentration of 125 to 150 mg/ml and tracer amounts of the tritiated DHS derivative (3 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom) were added to the phosphate buffer used to disperse the lipid layer. After sonic treatment, liposomes were separated from free DHS by molecular sieve chromatography. Drug entrapment was between 1.1 and 2.7% of DHS in solution.

Infected macrophage monolayers were washed thoroughly with tissue culture medium, and residual surface-associated staphylococci were killed by treatment with 10 U of lysostaphin per ml (Schwarz/Mann, Orangeburg, N.Y.) for 20 min at 37°C (10). The number of intracellular staphylococci recovered from infected monolayers after exposure to the staphylolytic enzyme was approximately 2×10^6/dish. Infection rates averaged greater than one viable unit per macrophage. Cell cultures were then exposed either to free DHS (1 to 2 mg/ml; i.e., at the same concentration as calculated to be

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TABLE 1. *Fate of phagocytized S. aureus within macrophages treated with DHS, DHS liposomes, and empty liposomes*[a]

Time of incubation (h)	No. of staphylococci after treatment of macrophage cultures:			
	Lysostaphin (control)	DHS liposomes	Empty liposomes	Free DHS
0	2.0×10^6	2.0×10^6	2.0×10^6	2.0×10^6
2.5			1.0×10^6	1.1×10^6
6.0		1.0×10^4		1.0×10^5
16.0		5.0×10^2	2.0×10^4	2.0×10^4

[a] All macrophage monolayers were treated with lysostaphin to establish intracellular, viable staphylococci at the initiation of the experiments. Therefore, values at zero time are identical since they represent the base line values for all groups. Values are the mean of four separate experiments.

entrapped within the liposome preparation used in each experiment), to liposome-entrapped DHS (1 to 2 mg of DHS per ml of liposome suspension), or to liposomes devoid of DHS. The DHS or liposome preparation was incorporated into NCTC-135 tissue culture medium before addition to the infected macrophages. In four different experiments, cells in four to six dishes in each of the three groups were washed extensively and processed for the enumeration of surviving intracellular staphylococci (2).

After incubation of the infected cells with free DHS or liposomes devoid of DHS for 2.5 h, relatively little (50%) bacterial killing occurred (Table 1); this is consistent with the extent of killing within 2 to 3 h attributable to the innate staphylocidal action of mouse macrophages. In previous studies (2, 3), we established that mouse macrophages in culture killed less than 50% of ingested *S. aureus* within 2.5 h. After 6.0 h the number of bacteria surviving in normal, untreated controls was less than 1.0 $\log_{10}$ unit (i.e., <90%). This is consistent with the extent of killing observed in the macrophage exposed to free DHS for 6.0 h (Table 1). Thus, the antibacterial activity of the free drug appeared to be negligible during this 6.0-h interval. Bonventre et al. (4) showed that tritiated DHS does not readily enter mouse macrophages; intracellular, biologically active DHS could not be detected by autoradiography or by bioassay after incubation of cells with the free antibiotic for several hours. In contrast, with cells exposed to liposomal DHS for 6 h, a reduction of two orders of magnitude in viable bacteria was observed; this is considerably greater than the rate of bacterial killing by normal mouse macrophages, which effect a similar reduction in viable staphylococci only after 24 h (2). The efficacy of the antibacterial action of the liposomal DHS was most apparent after 16 h of incubation, when only 5×10^2 viable bacteria per dish remained. When compared with the number of surviving bacteria after exposure to the antibiotic in solution or to liposomes devoid of DHS, this represents a 40-fold difference and suggests that the liposomal DHS was delivered to relevant intracellular sites in concentrations sufficient to enhance normal antibacterial action of the phagocyte.

The mechanism by which liposomal DHS enhances killing of intracellular bacteria is unknown. The absence of any effect of empty liposomes above that expected from the bactericidal action of the phagocytes suggests that liposome-cell interaction per se did not contribute to bacterial killing by nonspecific activation of phagocyte bactericidal mechanisms (9). Liposomes which enter macrophages by endocytosis may deliver the antibiotic by lysosomal fusion with phagocytic vacuoles. The data also suggest that intralysosomal inactivation of streptomycin (13) may be circumvented or diminished when the liposome is used as a drug carrier. Electron microscopic observations of infected macrophages provided suggestive evidence that after a 3-h exposure to liposomal DHS intracellular staphylococci were in a more advanced state of degradation than intracellular bacteria exposed to the free antibiotic or to empty liposomes.

The possibility that liposomes could enhance the antimicrobial action of a variety of drugs for treatment of intracellular infection is obviously attractive. Manipulation of the liposomal surface (i.e., attachment of recognition ligands) (8) could enhance selectivity for cellular targets. Although this report is concerned with only a single pathogenic species, appropriate systems can be devised to test the efficacy of liposomal entrapped drugs against a variety of intracellular bacterial and protozoal agents.

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LITERATURE CITED

1. **Alexander, J. W., and R. A. Good.** 1968. Effect of antibiotics on the bactericidal activity of human leukocytes. J. Lab. Clin. Med. **71**:971–983.
2. **Baughn, R. E., and P. F. Bonventre.** 1975. Phagocytosis and intracellular killing of *Staphylococcus aureus* by normal mouse peritoneal macrophages. Infect. Immun. **12**:346–352.
3. **Baughn, R. E., and P. F. Bonventre.** 1975. Cell-mediated immune phenomena induced by lymphokines from splenic lymphocytes of mice with chronic staphylococcal infection. Infect. Immun. **11**:313–319.
4. **Bonventre, P. F., R. Hayes, and J. Imhoff.** 1967. Autoradiographic evidence for the impermeability of mouse peritoneal macrophages to tritiated streptomycin. J. Bacteriol. **93**:445–450.
5. **Gregoriadis, G.** 1973. Drug entrapment in liposomes. FEBS Lett. **36**:292–296.
6. **Gregoriadis, G.** 1976. The carrier potential of liposomes in biology and medicine. N. Engl. J. Med. **295**:765–770.
7. **Gregoriadis, G.** 1977. Enzyme entrapment in liposomes. Methods Enzymol. **44**:218–227.
8. **Gregoriadis, G.** 1977. Targeting of drugs. Nature (London) **265**:407–411.
9. **Hawiger, J., R. D. Collin, R. G. Horn, and M. G. Koenig.** 1969. Interaction of artificial phospholipid membranes with isolated polymorphonuclear leucocytic granules. Nature (London) **222**:276–278.
10. **Hirt, W. E., and P. F. Bonventre.** 1973. Cultural, phagocytic and bactericidal characteristics of peritoneal macrophages. RES J. Reticuloendothel. Soc. **13**:27–46.
11. **Mackaness, G. B.** 1954. The growth of tubercule bacilli in monocytes from normal and vaccinated rabbits. Am. Rev. Tuberc. **69**:495–505.
12. **Mandell, G. L., and T. K. Vest.** 1972. Killing of intraleukocytic *Staphylococcus aureus* by rifampin: *in vitro* and *in vivo* studies. J. Infect. Dis. **125**:486–490.
13. **Tulkens, P., and A. Trouet.** 1972. Uptake and intracellular localization of streptomycin in the lysosomes of cultured fibroblasts. Arch. Int. Physiol. Biochim. **80**:623.