Enhanced Liposome-Mediated Activity of Piperacillin Against Staphylococci

MARCELO C. NACUCCHIO,[1] MAURICIO J. GATTO BELLORA,[1] DANIEL O. SORDELLI,[2]* AND MIGUEL D'AQUINO[1]

Departamento de Toxicología, Cátedra de Higiene y Sanidad, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina,[1] and International Center for Interdisciplinary Studies of Immunology, Georgetown University Medical Center, Washington, D.C.[2]

This study showed that encapsulation of the β-lactam antibiotic piperacillin (PIP) by liposomes prepared with phosphatidylcholine and cholesterol (1:1) protected the drug from hydrolysis by staphylococcal β-lactamase. This was demonstrated by growth inhibition of *Staphylococcus aureus* in the presence of the liposomal preparation containing PIP at a 50% MIC. Growth inhibition was also seen when exogenous β-lactamase was added. Furthermore, adsorption of PIP onto the surface of liposomes containing buffer conferred a significant degree of protection against enzymatic hydrolysis of the drug, thus enhancing its antistaphylococcal activity.

Bacterial resistance to antibiotics poses frequent and often serious problems in the treatment of bacterial infections. Piperacillin (PIP), although a wide-spectrum antibiotic (5), is inactive against β-lactamase-producing *Staphylococcus aureus*. Different laboratories have investigated the action of antibacterial (7, 10) and antimycotic agents (6, 9, 15) in liposome-antibiotic mixtures, and there are several reports concerning the evaluation of the biological activity of liposome-encapsulated antibiotics (1, 3, 4, 8, 12, 13). In one of these studies, enhancement of the antibacterial activity of three β-lactam compounds was achieved by encapsulating the antibiotics into liposomes (3). The authors, however, did not investigate in detail the mechanisms involved in this enhancement. The present study was undertaken to determine whether encapsulation in liposomes protects PIP from hydrolysis by β-lactamases, thus enhancing its antistaphylococcal activity.

Chemicals. Phosphatidylcholine (PC) was isolated from a soy extract for industrial use, generously provided by Natterman Chemie GMBH (Cologne, Federal Republic of Germany), and purified by chromatography on an alumina column. The purity of the PC was tested by thin-layer chromatography against a standard of egg yolk PC (Sigma Chemical Co., St. Louis, Mo.); PC was titrated by the method described by Chen et al. (2). Cholesterol was purchased from Sigma and tested for purity by thin-layer chromatography. PIP and nitrocephin were gifts from Laboratorio Lederle (Cyanamid of Argentina, Buenos Aires) and Laboratorio Glaxo (Buenos Aires), respectively. All chemicals were of analytical reagent grade.

Liposomes. Large unilamellar liposomes were prepared by the method described by Szoka and Papahadjopoulos (14). Briefly, a mixture of 30 μmol of PC and 30 μmol of cholesterol was prepared in ethanol; the solvent was evaporated to leave a thin film of lipids distributed over the walls of the test tube. PIP (1 ml of a 5-mg/ml concentration) in phosphate buffer, pH 7.2, was then added, and the tube was sonicated for 4 min under nitrogen. Liposome formation was confirmed by transmission electron microscopy of the mixture negatively stained with uranyl acetate, using standard

procedures. Based on a preliminary study, drug entrapment with this procedure varies from 35 to 60% (ca. 50% for PIP) (M. C. Nacucchio, unpublished data). Intraliposomal PIP was calculated by measuring the concentration of antibiotic detected in a suspension of liposomes and subtracting this from the concentration present after rupturing the liposomes, as described by Morgan and Williams (10). The PIP assay was performed by radial diffusion from wells cut in agar with a lawn of *Bacillus subtilis* ATCC 6633.

Bacteria. *S. aureus* was isolated from a clinical specimen and identified by standard bacteriological procedures. Production of β-lactamase was tested by hydrolysis of the chromogenic cephalosporin nitrocephin in culture medium containing 0.1 μg of potassium penicillin G per ml, by the method of O'Callaghan et al. (11). The PIP MIC was determined by the dilution susceptibility test as described by Washington and Sutter (16). The test for β-lactamase production was positive, and the MIC was ≥64 μg/ml.

Assay to quantitate the effect of PIP on *S. aureus* growth. To study the effect of liposome-entrapped PIP on *S. aureus* growth, 4.5 ml of broth (antibiotic medium no. 3, Oxoid, Chicago Heights, Ill.) was inoculated with 2.5×10^8 CFU of *S. aureus*, obtained from an early stationary-phase culture in broth at 37°C. Different liposome-PIP preparations were added to the tubes to a final volume of 5 ml per tube, and the tubes were incubated with constant agitation at 37°C; bacterial growth was monitored spectrophotometrically at 580 nm. The effect on staphylococcal growth of (i) PIP, (ii) liposomes containing phosphate buffer, (iii) liposome-encapsulated PIP, and (iv) liposomes containing buffer that were incubated with PIP were investigated. Control tubes contained phosphate buffer at pH 7.2. PIP was added to a final concentration equivalent to a 50% MIC (32 μg/ml). Preliminary experiments from our laboratory showed that the addition of PIP to a final concentration equivalent to a 25% MIC, either free or encapsulated in liposomes, did not modify the growth curves of *S. aureus*; the addition of a 75% MIC of free PIP, on the other hand, produced a degree of inhibition that would not have allowed sensitive detection of the effect of PIP associated with the liposomes. Lipid concentration was kept constant in all tubes containing liposomes. In another set of experiments of similar design,

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* Corresponding author.

0.15 ml of an extract of penicillinase (penase, Difco Laboratories, Detroit, Mich.) per tube was added to tubes containing *S. aureus* plus PIP or liposomal PIP and was incubated for 10 min at 37°C, to simulate the conditions for effective PIP hydrolysis, similar to that induced by *S. aureus* once it synthesizes and releases its own β-lactamase. Previous titration of the penicillinase extract activity against PIP was made by testing the remaining antibiotic activity of PIP against *B. subtilis* ATCC 6633 with a test of diffusion in agar. Based on the results of the test, the exogenous β-lactamase was then added in excess to the assay tubes.

The enhancement of the antibacterial activity of PIP against *S. aureus* by liposome encapsulation of the drug is detailed in Fig. 1. The results, expressed as the percentage of bacterial growth inhibition at a 50% MIC of PIP, demonstrated that growth inhibition was highest when PIP was encapsulated into liposomes. Interestingly, adsorption of PIP to liposomes containing buffer produced a certain degree of protection for the drug, rendering significant enhancement of the antistaphylococcal capacity when compared with the effect of PIP alone. To confirm whether PIP was actually protected from hydrolysis by β-lactamase, we performed a series of experiments of similar design but added exogenous staphylococcal β-lactamase. The results of these experiments are presented in Fig. 2. Exogenous β-lactamase hydrolyzed PIP, and the growth of *S. aureus* was no different from that of controls. Encapsulation of PIP within liposomes conferred the highest degree of protection against hydrolysis; consequently, bacterial growth was lowest after this treatment. Adsorption of PIP to liposomes containing buffer significantly protected the antibiotic from hydrolysis, although not as efficiently as liposomal entrapment of the drug.

In this report, we present evidence that liposome encapsulation enhanced the in vitro antibacterial activity of PIP
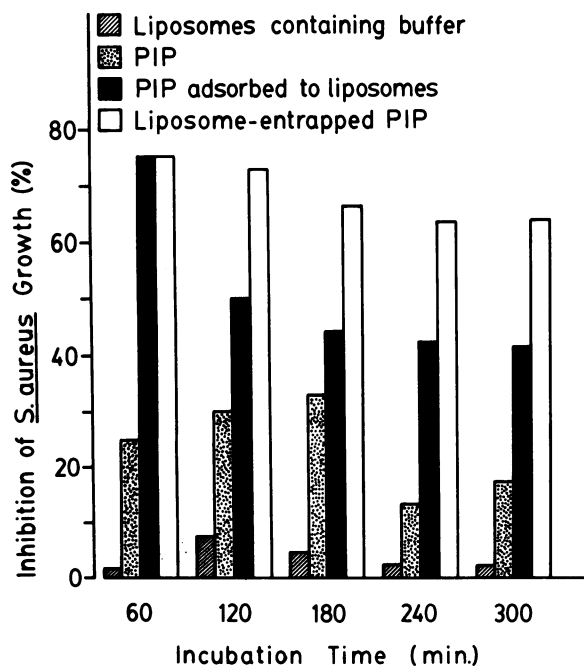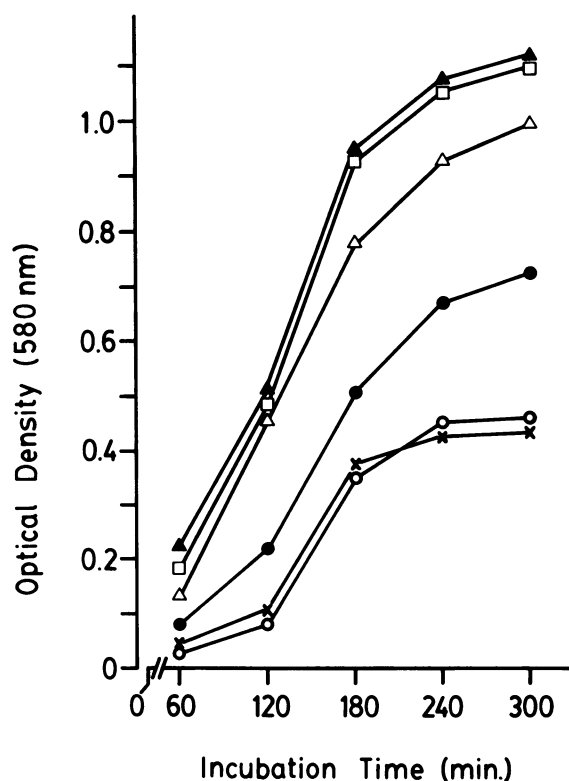

FIG. 2. *S. aureus* growth, measured as optical density of the cultures at different time points. *S. aureus* was grown in the following media: ▲, medium alone; □, PIP (50% MIC) + exogenous β-lactamase; △, PIP (50% MIC); ●, PIP adsorbed to liposomes; ○, liposome-encapsulated PIP + exogenous β-lactamase; and ×, liposome-encapsulated PIP. Each point represents the arithmetic mean of triplicates from a typical experiment.

FIG. 1. Percentages of *S. aureus* growth inhibition, expressed as [(optical density of control minus optical density of sample)/(optical density of control)] × 100, determined at different time points. Each bar represents the arithmetic mean of triplicates from a typical experiment.

against β-lactamase-producing *S. aureus*. Recent work by others has shown that encapsulation of methicillin, penicillin G, and cloxacillin in liposomes potentiates their antibiotic activity against *S. aureus*, *Bacillus licheniformis*, and *Escherichia coli*, respectively (3). Those authors used liposomes of different lipid composition and found that maximum inhibition of *S. aureus* growth was achieved with positively charged liposomes containing stearylamine. Enhancement of antistaphylococcal activity was attributed to facilitated diffusion of the antibiotics due to the electrostatic attraction of positively charged liposomes and the negatively charged surface of the bacteria. Besides facilitated diffusion of the drug, association with liposomes protects the antibiotic molecule from hydrolysis by enzymes released by the microorganism, and we showed that there are at least two other mechanisms involved. The first is the intrinsic protection of the drug by isolation inside the liposome microenvironment, where the β-lactamase cannot penetrate. The other mechanism was seen after preadsorption of PIP to liposomes, before incubation with the bacteria. The antibiotic adsorbed to the lipid surface had an enhanced antistaphylococcal activity when compared with the activity of PIP alone. Although there is no direct evidence of the mechanism involved, a likely explanation for this phenomenom is that the lipid surface of the liposome produced steric hindrance to the action of the β-lactamase, thus protecting PIP from hydrolysis by the enzyme.

In summary, we suggest that all three mechanisms detailed above are operating simultaneously. In light of this information, we believe that encapsulation of antibiotics within liposomes may be a successful way to prevent degradation of antibiotics and thus enhance their effect. Further experimental studies in vivo are needed to ascertain whether treatment with liposome-encapsulated antibiotics is an adequate method to overcome some of the clinical problems resulting from bacterial resistance to antibiotics.

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