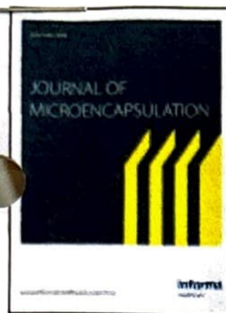

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Enhanced liposome-mediated antibacterial activity of piperacillin and gentamicin against gram-negative bacilli *in vitro*

MARCELO C. NACUCCHIO, MAURICIO J. GATTO BELLORA, DANIEL O. SORDELLI† and MIGUEL D'AQUINO

Departamento de Toxicología, Cátedra de Higiene y Sanidad, Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, Argentina, and Department of Pediatrics, Georgetown University, Washington, DC

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This study showed that encapsulation of piperacillin (PIP) and gentamicin (GE) by liposomes prepared with phosphatidylcholine and cholesterol (1 : 1) enhanced the antibiotic activity against gram negative bacilli. This was demonstrated by growth inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* in the presence of the liposomal preparations containing either PIP or GE at 50 per cent MIC. Mixtures of liposomes containing buffer and either PIP or GE also exhibited an enhanced activity of the drug against the micro-organisms.

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Introduction

Resistance to antibiotics is a major obstacle to the treatment of bacterial infections. Beta-lactamase production is an important resistance mechanism (Sykes and Matthew 1976). Bacterial envelopes are, on the other hand, a physical barrier that decreases cell wall permeability to the drug, thus preventing the interaction of the drug and its target site (Richmond 1978). Different laboratories have investigated the action of antibacterial (Gregoriadis 1973, Morgan and Williams 1980, Nacucchio *et al.* 1985) and antimycotic agents (Graybill *et al.* 1982, Lopez-Berenstein 1983) entrapped in liposomes, and there are several reports concerning the evaluation of the biological activity of liposome-encapsulated antibiotics (Bonventre and Gregoriadis 1978, Stevenson *et al.* 1983, Tadakuma *et al.* 1985). In a previous work we have shown that the antibacterial activity *in vitro* of piperacillin against *Staphylococcus aureus* is enhanced by encapsulation of the antibiotic in large, unilamellar liposomes (Nacucchio *et al.* 1985). The present study was undertaken to determine whether liposomal entrapment of PIP and GE enhanced their antibacterial activity against clinically isolated strains of *E. coli* and *P. aeruginosa* resistant to both antibiotics.

Materials and methods

Chemicals

Phosphatidylcholine (PC) was isolated from a soy extract provided by Natterman Chemie GMBH (Cologne, F.R. Germany), and purified by chromatography on an

† Present address: Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina de Buenos Aires, Argentina.

Address for correspondence: Marcelo C. Nacucchio, PhD, Cátedras de Farmacotecnia I y II, Junin 956, 1113 Buenos Aires, Argentina.

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.* (1956). Cholesterol was purchased from Sigma and tested for purity by thin layer chromatography. Gentamicin and PIP were gifts from Laboratorios Schering–Essex (Buenos Aires) and Laboratorios Lederle (Buenos Aires) respectively. All chemicals were of analytical grade.

Liposomes

Large unilamellar and oligolamellar liposomes were prepared by the method described by Szoka and Papahadjopoulos (1978). 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 films of lipids distributed over the walls of the test tubes. The antibiotic (1 ml of either 5 mg/ml PIP or 0·62 mg/ml GE) 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. Intraliposomal PIP and GE 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 (Morgan and Williams 1980). The PIP assay was performed by radial diffusion from wells cut in agar with a lawn of *Bacillus subtilis* ATCC 6633. A similar procedure was performed for GE, using a lawn of *Staphylococcus epidermidis* ATCC 12228. Liposomes were ruptured by treatment with 0·1 per cent sodium deoxycholate for 5 min at room temperature. Mixtures containing either antibiotic diluted in 0·1 per cent sodium deoxycholate were added to additional wells in order to ascertain whether the detergent affected bacterial growth. Liposome encapsulation, as determined by this procedure, ranged from 35 to 50 per cent.

Bacteria

E. coli and *P. aeruginosa* were isolated from clinical specimens and identified by standard bacteriological procedures. The PIP and GE MICs were determined by the dilution susceptibility test as described by Washington and Sutter (1980). Piperacillin MIC for *E. coli* and *P. aeruginosa* was >64 μg/ml whereas GE MIC was >8 μg/ml for both bacterial strains.

Quantitation of the effect of antibiotics on bacterial growth

To study the effect of liposome-entrapped PIP and GE on *E. coli* and *P. aeruginosa* growth, 4·5 ml of broth (Antibiotic Medium no. 3, Oxoid, Chicago Heights, IL) was inoculated with $2{\cdot}5 \times 10^8$ CFU of the microorganism, obtained from an early stationary-phase culture in broth at 37°C. Different liposome–antibiotic mixtures 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 530 nm. The effect on bacterial growth of (1) free PIP or GE, (2) liposomes containing phosphate buffer (control tubes), (3) GE or PIP adsorbed onto liposomes containing buffer, and (4) liposome-associated PIP or GE was investigated. The increase in optical density caused by addition of liposomes (final total lipid concentration in culture medium was 0·36 μM) was not significantly different, at any time, from that of tubes containing medium alone. The tubes containing liposomes and culture media were included in order to ascertain whether

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lipid peroxidation products or any other residual component, if present, contributed to the bactericidal action exhibited by the liposome mixtures. PIP and GE were added to the tubes to a final concentration equivalent to a 50 per cent MIC (32 μg/ml and 4 μg/ml, respectively).

Results

The enhancement of GE antibacterial activity against *E. coli* and *P. aeruginosa* is shown in figures 1 and 2 respectively. The results, expressed as optical density at 530 nm against time at 50 per cent MIC of GE, demonstrated that bacterial growth was inhibited by the antibiotic encapsulated within liposomes, when compared with the action of free GE alone. Interestingly, mixtures of GE and liposomes containing buffer also produced a significant increase of the antibacterial activity, when compared with the action of free GE alone. Although GE encapsulated within liposomes seemed to be more active against *E. coli* than GE mixed with liposomes containing buffer, the changes in growth inhibition were not significantly different. Either preparations of liposome-associated GE were highly active against *P. aeruginosa*, and produced 100 per cent growth inhibition after incubation for 120 min. Association of PIP to liposomes also induced an enhancement of antibacterial activity against *E. coli* and *P. aeruginosa*. Figures 3 and 4 show that PIP, either encapsulated within or mixed with liposomes, induced a significant enhance-

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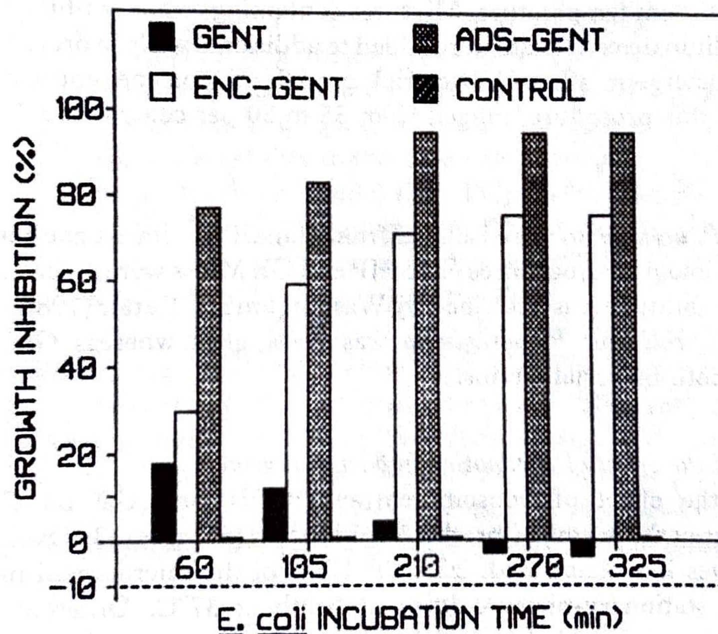

Figure 1. Inhibition of *E. coli* growth (per cent) by liposome-associated gentamicin. Each bar represents the median of the percentage of bacterial growth inhibition from triplicates of a representative experiment. Growth inhibition was calculated as the ratio of the optical density of a given test mixture against that of tubes containing *E. coli* alone. The control tubes contained bacteria and liposomes filled with buffer. Bacterial growth was significantly inhibited ($P<0{\cdot}01$) by both liposome-GE preparations when compared with the effect of the free antibiotic alone (dark bars). Levels of significance were determined by means of the Rank Sum Test (Epistat Statistical Package, Tracy Gustafson, Round Rock, Texas).

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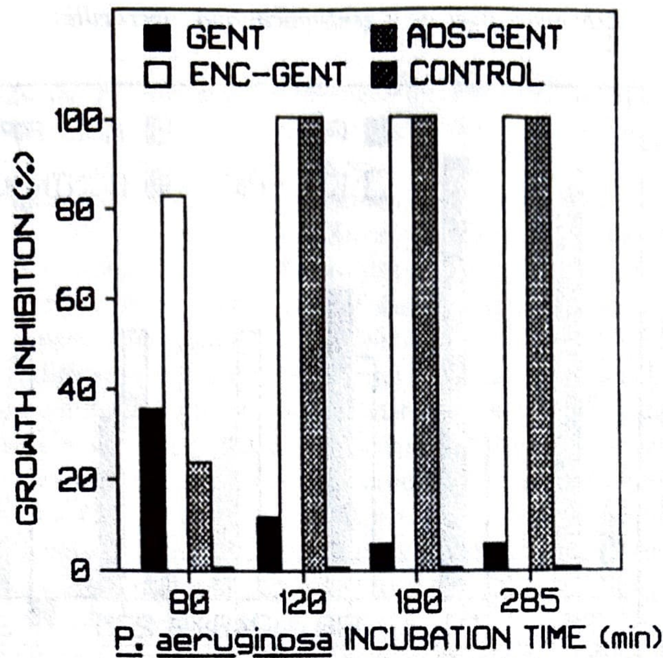

Figure 2. Inhibition of *P. aeruginosa* growth (per cent) by liposome-associated gentamicin. Each bar represents the median of the percentage of bacterial growth inhibition from triplicates of a representative experiment. Growth inhibition was calculated as the ratio of the optical density of a given test mixture against that of tubes containing *P. aeruginosa* alone. Bacterial growth was significantly inhibited ($P<0{\cdot}01$) by both liposome-GE preparations when compared with the effect of the free antibiotic alone (dark bars), after incubation for 120 min. Levels of significance were determined as explained in the legend to figure 1.

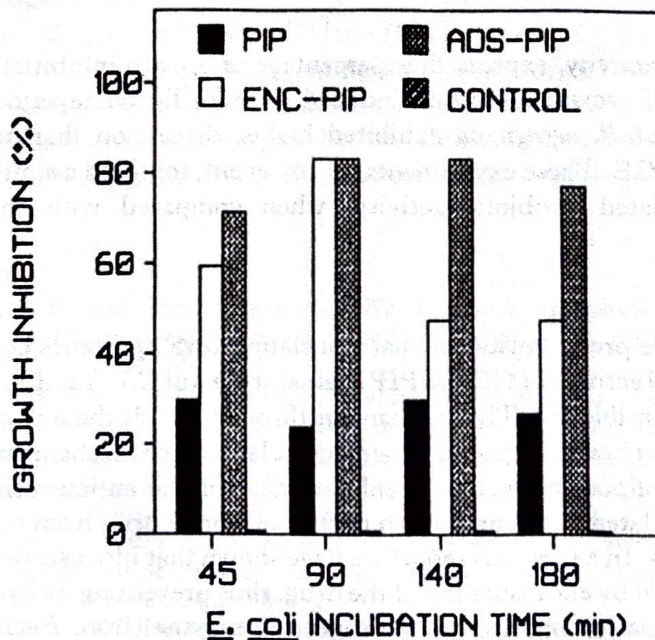

Figure 3. Inhibition of *E. coli* growth (per cent) by liposome-associated piperacillin. Each bar represents the median of the percentage of bacterial growth inhibition from triplicates of a representative experiment. Bacterial growth was significantly inhibited ($P<0{\cdot}01$) by PIP-adsorbed to liposomes, when compared with the effect of the free antibiotic alone (dark bars). Bacterial growth was significantly inhibited by encapsulated PIP ($P<0{\cdot}01$ at 90 min, $P<0{\cdot}05$ at 45 min, 140 and 180 min). Levels of significance were determined as explained in the legend to figure 1.

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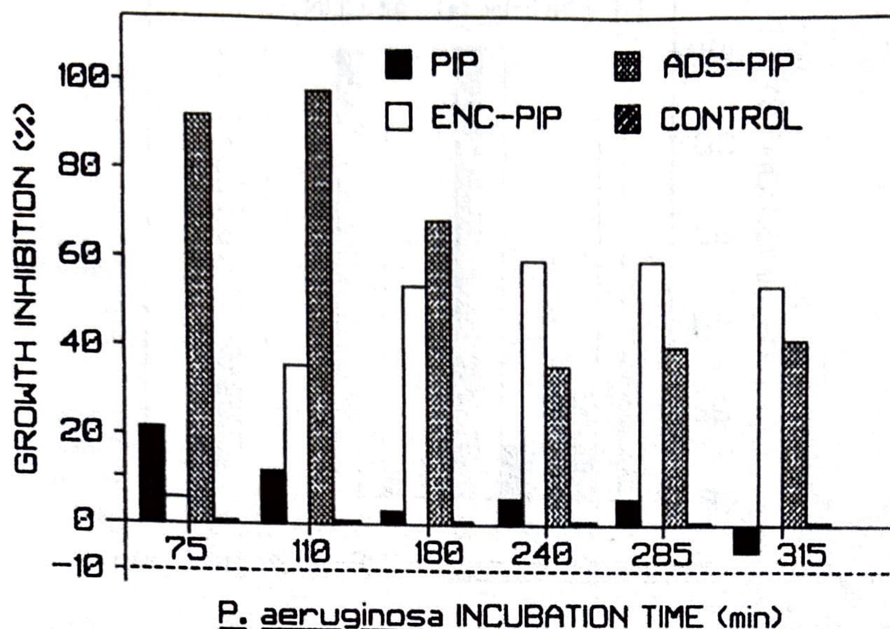

Figure 4. Inhibition of *P. aeruginosa* growth (per cent) by liposome-associated piperacillin. Each bar represents the median of the percentage of bacterial growth inhibition from triplicates of a representative experiment. Bacterial growth was significantly inhibited ($P<0{\cdot}01$) by PIP-adsorbed to liposomes, when compared with the effect of the free antibiotic alone (dark bars). Bacterial growth was significantly inhibited by encapsulated PIP ($P<0{\cdot}05$ at 110 min, $P<0{\cdot}01$ after 180 min). Levels of significance were determined as explained in the legend to figure 1.

ment of antibiotic activity, expressed as percentage of growth inhibition at 50 per cent MIC. Data of growth inhibition induced by both liposome-associated PIP preparations against *P. aeruginosa* exhibited higher dispersion than those from experiments using GE. These experiments, in any event, followed a similar pattern of liposome-associated antibiotic activity, when compared with that of the antibiotics alone.

Discussion

In this report we present evidence that association with liposomes enhances the *in vitro* antibacterial activity of GE and PIP against strains of *E. coli* and *P. aeruginosa* resistant to those antibiotics. The mechanism through which the enhancement is produced has not yet been elucidated. There are at least three mechanisms that may be involved in the liposome-mediated enhancement of the antibacterial activity. Two of them are related to the protection of the entrapped drug from the action of hydrolytic enzymes. In a previous report we have shown that intrinsic protection of PIP may be obtained by encapsulation of the drug, thus preventing its hydrolysis by either *S. aureus* beta-lactamases or the exogenous enzyme (from *Bacillus cereus*) (Nacucchio *et al.* 1985). In the same report we have shown that not only encapsulation within but also adsorption to liposomes may produce an enhancement in the antibiotic activity. A likely explanation for this phenomenon is that the liposome produces steric hindrance to the action of the beta-lactamase. Protection of the antibiotic molecule, however, may not explain satisfactorily the enhancement of

GE activity against *E. coli* and *P. aeruginosa*. Changes of the cell-envelope permeability, rather than enzymic inactivation, is the resistance mechanism more often displayed by gram-negatives. In this regard, it has been suggested that all GE resistance may ultimately be due to impermeability of the bacterial envelope to the antibiotic (Bryan 1979). Furthermore, Zimmermann and Rosselet (1977) have demonstrated that changes in the permeability of the outer membrane play an important role in the resistance of *E. coli* to several beta-lactamase antibiotics.

Our results suggest that association of piperacillin and gentamicin to liposomes may facilitate the diffusion of the drug across the bacterial envelope. This hypothesis is in accordance with the findings of Sekeri-Pataryas *et al.* (1985), who demonstrated that non-permeable substances can permeate through *P. aeruginosa* external envelopes by the use of liposomes. Our results are further supported by the studies of Jones and Osborn (1977 a, b) who investigated the interaction of *Salmonella typhimurium* with phospholipid vesicles and concluded that a direct fusion of vesicles with the outer membrane of the cell and a translocation of phospholipids between the outer and inner membrane takes place.

In conclusion, we suggest that the enhancement of the antibacterial activity of antibiotics is by any or all of the mechanisms explained operating either singly or together. In the light of this information we believe that encapsulation of antibiotics within liposomes may be a successful way to overcome bacterial resistance mechanisms. Further experimental studies *in vivo* are needed in order to ascertain whether treatment with liposome-encapsulated agents is an adequate method to address some of the clinical problems resulting from bacterial resistance to antibiotics.

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