Interaction of Penicillin G with the Human Erythrocyte Membrane and Models

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Penicillin G (PEN) is a widely used antibiotic whose mechanism of action is related to the interference with the synthesis of bacteria cell wall. In order to evaluate its perturbing effect upon human cell membranes PEN was made to interact with human erythrocytes, isolated resealed human erythrocyte membranes and molecular models. The latter were multibilayers of the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) as well as DMPC large unilamellar vesicles. These studies were performed by scanning electron microscopy, fluorescence spectroscopy and X-ray diffraction methods. The observed results coincide in that PEN did not exert any significant effect upon the structures of the red cell membrane neither on its molecular models. This is in agreement with its reported lack of major toxicity and hematological reactions.

Introduction

Penicillin G (PEN) corresponds to benzylpenicillin, whose structural formula is shown in Fig. 1a. It was the first antibiotic to be used in therapy (Martin, 1982) and still remains as the agent of choice for the treatment of infections caused by gram-positive bacteria and gram-negative cocci. Its mechanism of action, like penicillins in general, is based on the inhibition of the synthesis of peptidoglycans (Ved *et al.,* 1990). These units form a multidimensional structure that serves as the backbone of the bacterial cell wall (Ghuysen, 1990). Interference with such basic structural synthesis results in lysis and cell death. Because peptidoglycans are irrelevant to mammalian cell structures PEN supposedly has little direct effect on human cell membranes. However, it has been reported that penicillins may use the hydrocarbon chain region of the outer membrane of gram-negative bacteria as a secondary permeation route in order to reach their targets in the cytoplasmic membrane (Yamaguchi *et al.,* 1983). In fact PEN, despite its moderate hydrophobicity, would present a very high permeability in phospholipid liposomes (Yamaguchi *et al.,* 1983). Additionally, the hydrophobicity of penicillins has been related to neurotoxic effects(Snavely and Hodges, 1984).

In order to test the validity of these hypotheses, PEN was made to interact with human erythrocytes, isolated resealed human erythrocyte membranes and models constituted by phospholipid multilayers and large unilamellar vesicles. These systems have been previously used in our laboratories to determine the interaction and perturbing effects on membranes by antibiotics such as chlortetracycline (Suwalsky *et al.,* 1991a), chloramphenicol (Suwalsky *et al.,* 1991b) and gentamicin (Suwalsky and Frías, 1993) as well as other relevant therapeutical drugs (Suwalsky *et al.,* 1994; Suwalsky and Villena, 1995). The multilayers consisted in the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), which represent phospholipids that are respectively located in the outer and inner monolayers of the human erythrocyte membrane (Devaux and Zachowsky, 1994). Given the amphiphilic nature of PEN and of both phospholipids, they were made to interact in a hydrophobic and a hydrophilic medium under a wide range of concentrations. The capacity of PEN to perturb the mutilayer structure of DMPC and DMPE was determined by X-ray methods.

Fluorescent steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were determined on

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DMPC large unilamellar vesicles and isolated human erythrocyte membranes. DPH is one of the most used probe for hydrophobic regions of phospholipid bilayers. Its fluorescence steady-state anisotropy provides a measure of the rotational diffusion of the fluorophore restricted within a certain region due to the phospholipid acyl chain order. On the other hand, Laurdan has a high sensitivity of excitation and emission spectra to the physical state of membranes (Parasassi *et al.*, 1986). With the fluorophore moiety located in a shallow position of the bilayer normal, in the phospholipid polar head group environment, Laurdan provides information of dynamic properties in this zone of the bilayer (Chong, 1988; Chong, 1990). Laurdan spectral shifts quantification is done using the general polarization (GP) concept (Parasassi *et al.*, 1990).

Finally, PEN was incubated with human erythrocytes. The red cells were later observed by scanning electron microscopy to detect shape changes induced by PEN.

Materials and Methods

Chemicals

Synthetic DMPC (Lot 88F-8365, A grade, MW 677.9) and DMPE (Lot 12F-8360, A grade, MW 635.9) were from Sigma; PEN G Sodium (Lots 89061123 and 91061620, MW 356.4) from Lab. Chile, DPH and Laurdan from Molecular Probes and ethanol (Uvasol grade) from Merck were used without further purification.

X- ray diffraction analysis of phospholipid multilayers

About 3 mg of each phospholipid were mixed with the corresponding weight of PEN in order to attain DMPC:PEN and DMPE:PEN powder mixtures in the molar ratios of 10:1, 5:1 and 1:1. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry very carefully. The resulting samples, under the form of crystalline powders, were introduced into special glass capillaries 0.7 mm diameter. They were X-ray diffracted in Debye-Scherrer cameras of 114.6 mm diameter and flat-plate cameras with 0.25 mm diameter glass collimators provided with rotating devices. The same procedure was also followed with samples of each phospholipid and PEN. The hydrated specimens were prepared in 1.5 mm diameter glass capillaries, containing 2–3 mg of DMPC or DMPE. Each capillary was then filled with about 200 μL of a) distilled water, and b)10^{-5}M, 10^{-4}M, 10^{-3}M and 10^{-2}M PEN aqueous solutions. They were X-ray diffracted 2 and 14 days after preparation in flat-plate cameras. Specimen-to-film distances were 8 or 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Philips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak-integration in a Joyce-Loebl MKIIICS microdensitometer connected to an Acer 915 computer. No correction factors were applied. The experiments with aqueous solutions were performed at 17 ± 2 °C, which is below the main transition temperatures of both DMPC and DMPE.

Fluorescence measurements of large unilamellar vesicles (LUV) and isolated resealed human erythrocyte membranes (IRM)

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposomes suspension through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp.), employing nitrogen pressure at 10 °C over the lipid transition temperature, to a final concentration of 0.3 mM. Erythrocytes were separated from heparinized venous blood samples, obtained from normal casual donors, by centrifugation and washing procedures. IRM were prepared by lysis of the cells and their resealing in the presence of Mg^{2+}.

DPH and Laurdan were incorporated into LUV and IRM by addition of small aliquots of concentrated solutions of the probe in ethanol to LUV suspension in water and to IRM (protein concentration 0.25 mg/ml) suspended in phosphate buffered saline and gently shaken by ca. 30 min. The final probe concentrations were 0.5 μM. Fluorescence spectra and anisotropy measurements were performed on a Fluorolog spectrofluorometer from SPEX and on a phase shift and modulation GREG-200 spectrofluorometer from ISS respectively, both interfaced to personal computers employing ISS software. Measurements of LUV suspensions were made at 18 °C using 10 mm path-

length square quartz cuvettes. Measurements of IRM suspensions were made at 20 °C using 3 mm path-length square quartz cuvettes. Sample temperature was controlled by an external Cole Parmer bath circulator and measured prior and after each measurement using an Omega digital thermometer. Anisotropy measurements were done in the "L" configuration using Glan Thompson prism polarizers in both exciting and emitting beams. The emission was measured using a WG-420 Schott high pass filter which showed negligible fluorescence. GP was evaluated by $GP = (I_B - I_R)/(I_B + I_R)$, where I_B and I_R are the intensities at the blue and red edges of the emission spectrum respectively. These intensities were measured at 440 and 500 nm corresponding to the emission maxima of Laurdan in gel and liquid-crystalline lipid phases respectively (Parasassi *et al.*, 1990). For both probes the excitation wavelength was set at 360 nm. Blanck suspensions without probe were used to correct background light scattering. PEN was incorporated as small aliquots from a concentrated aqueous solution.

Scanning electron microscope (SEM) studies on human erythrocytes

The interaction of PEN with human erythrocytes was achieved by incubating blood samples from clinically healthy male adult donors not being treated with any pharmacological agent by puncture of the ear lobule disinfected with 70% ethanol. Two drops were received in a plastic tube containing 10 ml of saline solution (0.9% NaCl) at 5 °C. This blood solution was used to prepare the following samples: a) control, by mixing 1 ml with 9 ml of saline solution, and b)16 μg PEN/ml (which corresponds to the highest plasma therapeutical concentration (Norris *et al.*, 1991) and 32 μg PEN/ml by mixing 1 ml of blood solution plus 9 ml of PEN saline solutions of adequate concentrations. These samples were incubated at 37 °C for 1 h in an oven. They were then fixed with glutaraldehyde by adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. After resting overnight at 5 °C, the fixed samples were placed directly on Al stubs, air dried in an oven at 37 °C for half to one hour and gold coated for 3 min at 10^{-1} Torr in a S150 Edwards sputter device. The observations and photographic records were performed in a Etec Autoscan SEM .

Results

X-ray studies on lipid multilayers

The molecular interactions of PEN with DMPC and DMPE multilayers were studied in hydrophobic and hydrophilic media. The observed results are presented in Fig. 1 (b-e). Fig. 1(b) shows the diffractograms of DMPC, PEN and their 10:1, 5:1 and 1:1 molar mixtures after interacting and being recrystallized from chloroform:methanol 3:1 v/v solutions. As it can be observed, the perturbing effect of PEN upon DMPC structure was very mild. In fact, while the interplanar spacings of DMPC remained practically constant within experimental error, the intensities decreased moderately as the PEN concentration increased. However, no relevant reflections from DMPC disappeared neither a new one showed up. The results observed from their interaction in an aqueous medium are presented in Fig. 1(c). DMPC, when exposed to pure water, expanded its bilayer width from 55.2 Å when dry to 64.5 Å. At the same time, the observed reflections were considerably reduced. However, a new and strong reflection of 4.2 Å showed up. Practically the same X-ray pattern remained when DMPC was allowed to interact with PEN solutions.

The results of the interaction of PEN with DMPE in the hydrophobic medium are shown in Fig. 1(d). As it has been reported elsewhere (Suwalsky *et al.*, 1991a) DMPE presents two polymorphic forms when it is recrystallized from chloroform:methanol. One phase (Lc_1), obtained when these solvents are in a 3:1 v/v ratio, is characterized because the hydrocarbon chains are extended and parallel to the bilayer normal. The Lc_2 phase, obtained from a 1:3 solvent ratio, presents the hydrocarbon chains tilted by about 30° and a bilayer width of nearly 44 Å compared with the about 52 Å of the Lc_1 form. PEN produced the following effects: a) DMPE underwent a Lc_1 to Lc_2 phase transition when its molar ratio with PEN was 10:1; most of the reflections slightly increased their spacing as compared to those of single DMPE Lc_2 phase whereas their intensities remained unchanged; b) about the same results were observed

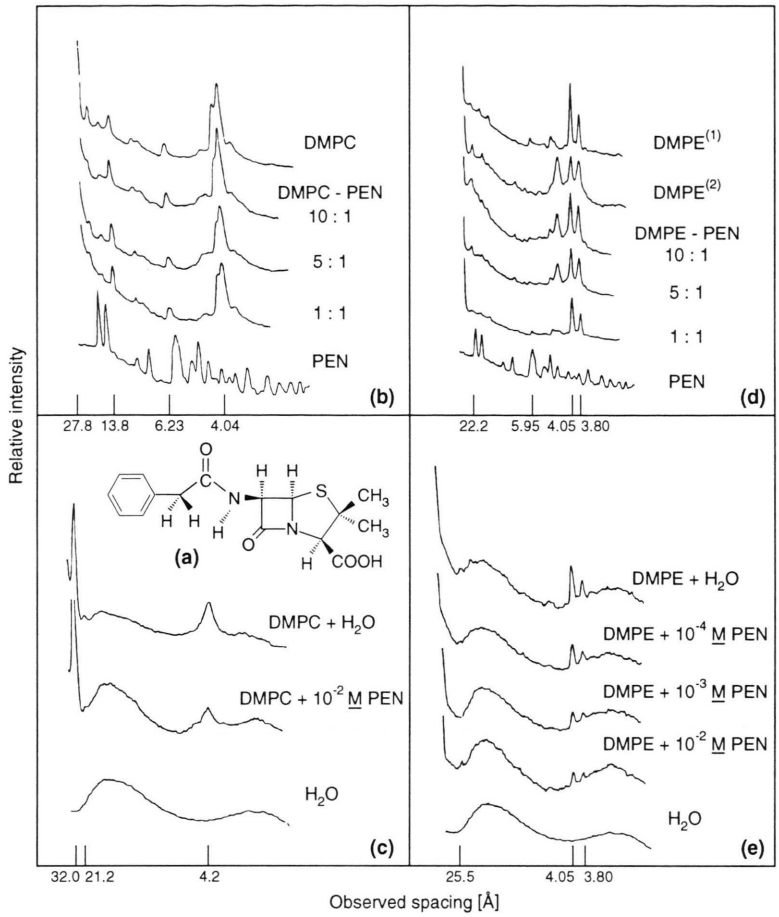


Fig. 1. (a) Structural formula of PEN; (b and d) microdensitograms from X-ray diagrams of specimens recrystallized from $CHCl_3$:CH_3OH 3:1; (1) phase Lc_1, (2) phase Lc2; (c and e) microdensitograms from DMPC and DMPE in PEN aqueous solutions. Flat-plate cameras; specimen-to-film distance 8 cm.

at a 5:1 molar ratio. However, when this ratio was 1:1, DMPE reverted to the Lc_1 phase. These phase transitions are most likely due to changes in the physicochemical properties of the solutions rather than to molecular interactions between PEN and DMPE. Finally, as it can be observed in Fig. 1(e), increasing PEN concentration induced a diminishing of DMPE reflection intensities. However, water neither PEN affected the bilayer parameters of DMPE as its reflection spacings remained unchanged.

Those results indicated that PEN was unable to insert into DMPC neither DMPE bilayers as it did not produce a significant perturbation to any of these phospholipids even at concentrations several times higher than those found in the plasma when it is therapeutically used (Norris *et al.*, 1991).

Fluorescence studies on LUV and IRM

The steady-state fluorescence anisotropy of DPH incorporated to DMPC LUV and to IRM was measured in the absence and in the presence of increasing concentrations of PEN. The GP of Laurdan incorporated in both systems was also

Table I. Effect of PEN on the anisotropy (r) of DPH and the general polarization (GP) of Laurdan embedded in DMPC large unilamellar vesicles (LUV) and in isolated resealed human erythroyte membranes (IRM).

PEN concn. [mM]	r(DPH) LUV	r(DPH) IRM	GP (Laurdan) LUV	GP (Laurdan) IRM
0.01	0.321	0.258	0.527	0.357
0.01	0.320	0.256	0.552	0.361
0.1	0.321	0.255	0.542	0.366
1.0	0.320	0.256	0.533	0.359

evaluated in the presence of the same concentrations of PEN. The results are given in Table I. As shown, when the concentration of PEN is increased up to 1 mM no change was observed in the fluorescence anisotropy of DPH nor in Laurdan GP embedded both in DMPC vesicles and in IRM. These results confirmed that PEN produced no perturbation to the acyl packing organization of the lamella neither to the molecular dynamics in the phospholipid polar group environment.

The values of DPH anisotropy and Laurdan general polarization embedded in IRM in the absence of PEN are lower than the corresponding figures of both probes embedded in DMPC LUV. These differences can be explained considering that the DMPC bilayer is in the gel state; therefore, the packing order of the phospholipid acyl chains are higher than those in a natural membrane which is in a liquid crystalline state. Consequently, the dynamics of water molecules relaxation in the environment of the excited fluorophore of Laurdan at the hydrophobic-hydrophilic interface of the bilayer must be faster in the membrane than in the LUV.

Morphological studies on erythrocytes

The observations made by SEM did not show any significant shape change induced by PEN to the red blood cells. In fact, the erythrocytes incubated with 16 μg PEN/ml and 32 μg PEN/ml, which respectively correspond to approximately 10^{-5} M and 10^{-4} M, have the normal discoid biconcave shape, the same shown by the untreated erythrocytes (Fig. 2). The finding that PEN was unable to change the shape of the erythrocytes was another indication that it did not interact with the membrane phospholipid bilayer. In fact, reports have shown that several amphiphilic drugs induce a transformation from the discoid shape of the erythrocytes to speculated (echinocyte) or cupped (stomatocyte) forms (Suwalsky and Villena, 1995; Nwafor and Coakley, 1985; Isomaa *et al.*, 1987). These shape alterations have been explained by the "bilayer couple hypothesis". Accordingly to it, the shape changes arise from the differential expansion of the two mono-

(a)

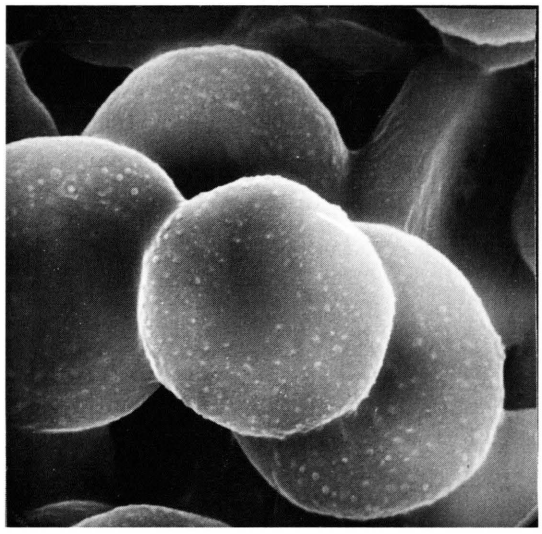
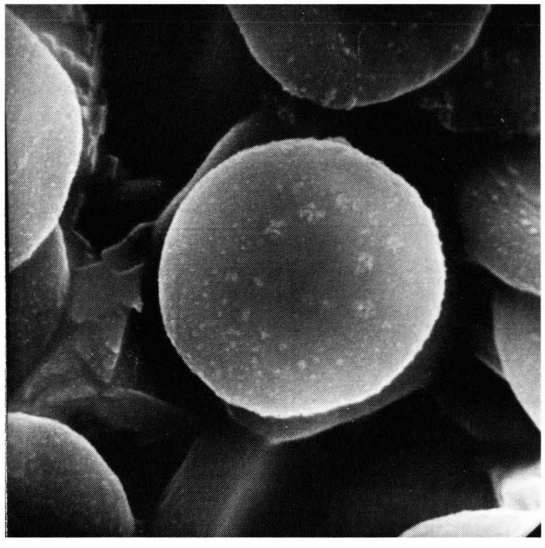


Fig. 2. SEM images of human erythrocytes: (a) PEN-free; (b) incubated with PEN (32 mg/ml). Magn. x 4,400.



(b)

layers of the erythrocyte membrane due to the insertion of the drug in one of them.

From these experimental results it can be concluded that PEN lacks major toxicity towards cell membranes.

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