

Electron Microscopic Study of the Polymyxin Treated Goat Erythrocytes

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Polymyxin B produced dose dependent changes in the surface topography of the goat erythrocyte cells. Transformation from the normal biconcave discs through crenated structures to the final rounded or spherical shape was recorded by scanning electron microscopy. A maximum of three to four crenations per cell was recorded corresponding to a polymyxin dose of 15.62 µg/ml. Transmission electron microscopy of the ultrathin sections of treated or untreated erythrocytes indicated that the crenations were formed by protrusions of the plasma membrane, occurring presumably because of the local increase of membrane fluidity after polymyxin treatment. Changes in the shape of the erythrocytes to the ultimate rounded forms were also indicated by the transmission electron microscopy.

Introduction

Polymyxin B was shown to cause membrane damage leading to leakage of intracellular or intravesicular solutes. Such membrane damage and leakage were studied with various types of membrane preparations, e.g., liposomes prepared from different combinations of phospholipids including both natural (extracted from different biological systems) and synthetic ones, membranes of different bacterial cells, e.g., *Escherichia coli*, *Vibrio cholerae*, etc., membranes of erythrocytes, and planar lipid bilayers [1–5]. It was noted that phosphatidyl ethanolamine represented a target molecule responsible for the polymyxin sensitivity of biological membrane [1]. A mixture of phosphatidyl ethanolamine, cardiolipin and phosphatidyl glycerol was found very sensitive to the polymyxin action [2]. In fact, polymyxin sensitivity was found to increase in proportion to the molar percentage of phosphatidyl ethanolamine present in the membrane [1, 4]. It was further shown that the expression of polymyxin action required a threshold quantity of phosphatidyl ethanolamine [4].

Polymyxin B was shown to bind with the negatively charged lipid in liposomal membrane [6]. The binding resulted in the formation of domains within the lipid bilayer, the polymyxin bound portions

exhibiting a lower phase transition temperature than the unbound ones [6]. The domain structures were visualized by electron microscopy using freeze-etching technique [6]. Whether polymyxin binding can produce any change in the surface topography of artificial or natural membranes has not been reported so far. This communication reports, in this respect, the polymyxin induced changes in the surface topography of erythrocytes by the scanning as well as transmission electron microscopy.

Materials and Methods

Chemicals

Polymyxin B was purchased from Sigma Chemical Co., USA, osmium tetroxide, glutaraldehyde and epon embedding materials from Electron Microscopy Sciences, USA, and uranyl acetate, lead nitrate and sodium citrate from BDH, England. All other chemicals used were of analytical reagent grade.

Erythrocytes

Erythrocyte suspension in 0.15 M saline was prepared from fresh goat blood anticoagulated with 3.8% sodium citrate by differential centrifugation as described previously [7, 8].

Abbreviations: PBS, phosphate buffered saline.

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Polymyxin treatment

A 0.5 ml aliquot of erythrocytes in 0.15 M saline was mixed with appropriate amount of polymyxin B and incubated for one hour at 37 °C.

Hemolysis estimation

After the desired period of antibiotic treatment, the erythrocytes were sedimented by centrifugation (4 °C; 14000 × *g*; 30 min). The supernatant was carefully aspirated and the hemoglobin content was determined by the measurement of its absorbance at 540 nm. The supernatant fluid prepared identically from untreated erythrocyte suspensions was used as control. In a parallel experiment, a 1.0 ml aliquot of the same stock of untreated cells was diluted to 30 volumes with glass-distilled water and hemolysis due to osmotic shock was allowed to continue for 1 h, after which the stroma were separated by centrifugation in the cold at 14000 × *g* for 30 min. The hemoglobin content in the supernatant fluid, as determined from its absorbance at 540 nm, was used as reference (100% leakage) for determination of the percentage of hemolysis after antibiotic treatment.

Scanning electron microscopy

Erythrocytes were fixed in 1.0% glutaraldehyde in PBS (0.10 M phosphate buffer, pH 7.4 containing 0.15 M saline) for about 20 h at 4 °C, washed twice in deionized water, spread on a glass slide (1 × 1 cm) and air dried. The dried smear was then coated with a thin layer of gold and photographed by the Philips PSEM 500 model scanning electron microscope operated at 25 KV [9].

Transmission electron microscopy

Erythrocytes were fixed in 1.0% glutaraldehyde in PBS containing 0.2 M sucrose for about 20 h at 4 °C, centrifuged at 400 × *g* for 10 min, washed in the buffer and subsequently postfixated in osmium tetroxide solution (1%) in PBS containing 0.2 M sucrose for 2 h at 4 °C in dark [8, 10, 11]. The cells were again washed in PBS containing 0.2 M sucrose, dehydrated in graded alcohol and finally in propylene oxide. Embedding was done in Epon 812 following in general the method of Luft [12]. Sections were stained with an alcoholic solution of uranyl acetate (1–2%) followed by lead hydroxide [8, 11]. Electron

micrographs were taken by a Siemens electron microscope ELMISKOP-I operating at 60 KV at an instrumental magnification of 8000 ×.

Results

Polymyxin B produced a dose dependent leakage of the hemoglobin from the erythrocytes. A 7.5% hemolysis was produced by treatment of the erythrocytes with 500 µg/ml of polymyxin for one hour at 37 °C (Fig. 1).

Scanning electron microscopy revealed progressive changes in the surface topography of the erythrocyte with increasing concentrations of the antibiotic (Fig. 2). The biconcave disc shaped structures of untreated erythrocytes were finally transformed into more or less rounded forms at high concentration (250 µg/ml) of polymyxin (Fig. 2, a–d). At intermediate polymyxin concentrations, crenated structures appeared with a maximum of three to four crenations per cell. The frequency distribution of crenations in the treated erythrocyte population is shown in Fig. 3. A transformation of the shape of the erythrocyte from the normal biconcave disc (discocyte) through the crenated disc, crenated sphere (echinocyte) into the final smooth sphere (spherocyte) was revealed by scanning microscopy.

Transmission electron microscopy of the ultrathin sections of treated and untreated erythrocytes

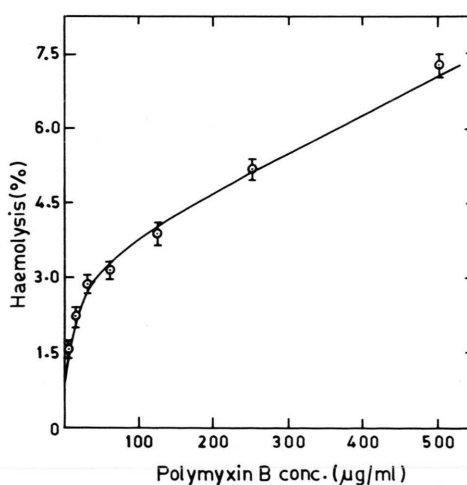


Fig. 1. Polymyxin B dose dependent hemolysis of goat erythrocytes.

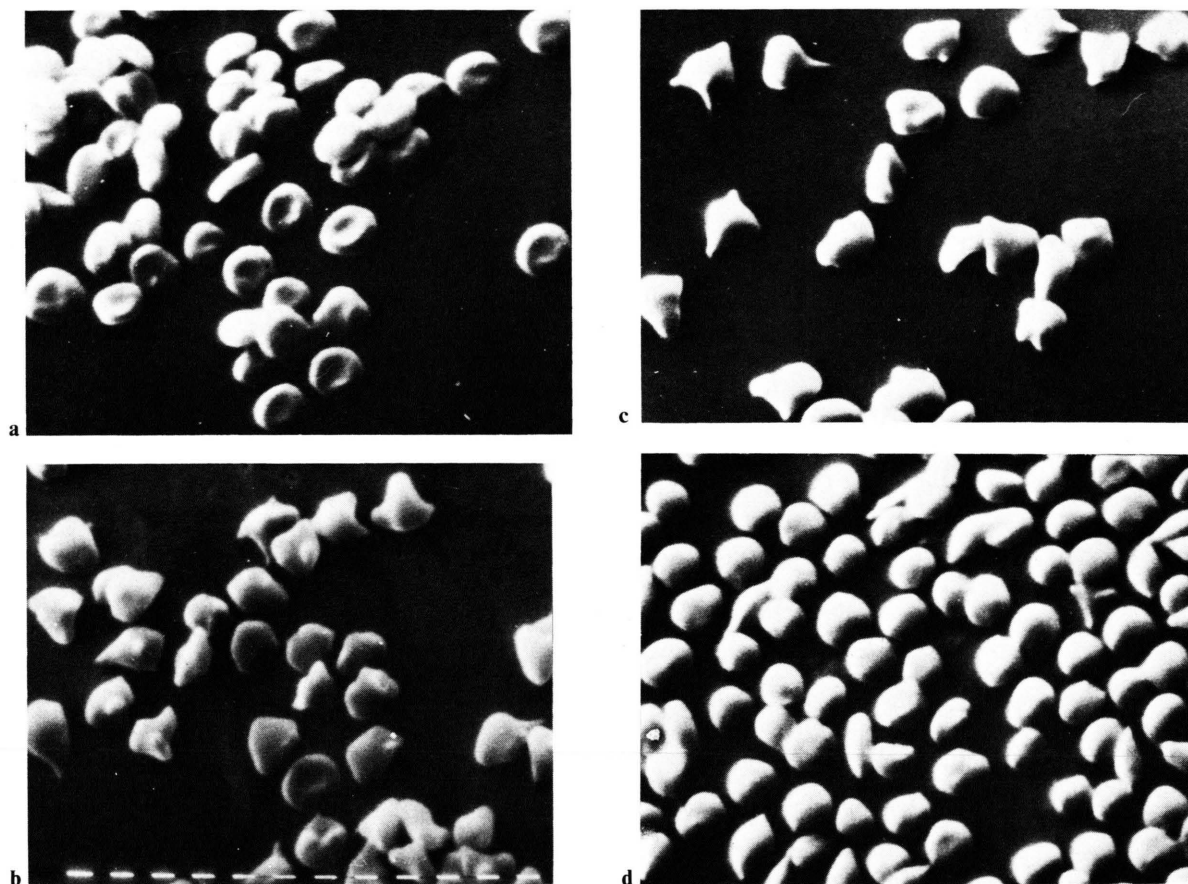


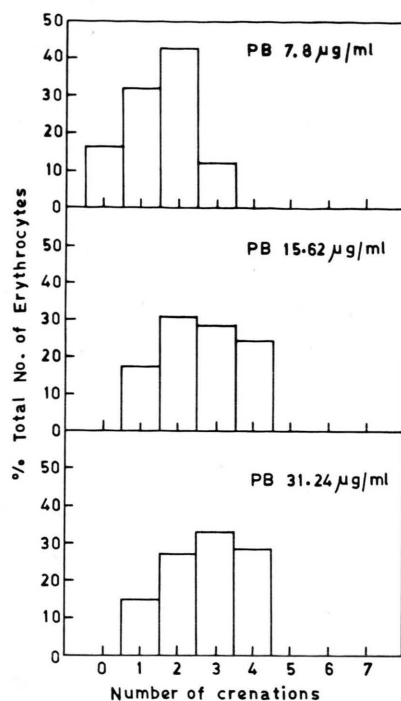
Fig. 2. Scanning electron micrographs of the goat erythrocytes treated with different amounts of polymyxin B. a) 0 $\mu\text{g/ml}$; b) 7.8 $\mu\text{g/ml}$; c) 31.24 $\mu\text{g/ml}$; d) 250 $\mu\text{g/ml}$. White bar represents 1 μm in all photographs.

revealed interesting changes in the surface structure of the cells (Fig. 4, a–d). Polymyxin treatment produced protrusion or outfolding (arrow in Fig. 4b) of the plasma membrane. But no significant lysis or clearing of the internal electron density of the cells could be detected even when the polymyxin concentration was 250 $\mu\text{g/ml}$. However, even in ultrathin sections, gradual transformation of the cells towards spherical forms was indicated and many completely rounded structures could be detected particularly when the polymyxin concentration was high (Fig. 4d).

Discussion

Transformation of the biconcave disc shaped structure of normal erythrocytes through various types of crenated structure to the ultimate rounded or spherical forms appears to be a rather non-

specific effect which can be induced by various agents, e.g., 1-anilino-8-naphthalene sulphonate [13], lyso-lecithin, bile acids [14], tunicamycin [15], ionophores with CaCl_2 [16], lectin [17] etc. That similar transformations could also be produced by peptide antibiotic, polymyxin B was not reported beforehand. However, compared to the action of many of the aforesaid agents, polymyxin B induced crenations are fewer in number. It appears that irrespective of diverse nature of these interacting agents, there is a common underlying mechanism responsible for such shape transformations. The present studies on the ultrathin sections of polymyxin treated erythrocytes have indicated that the crenations are formed as a result of protrusions or outfoldings of the cell membrane. The protruded portions are significantly long and could result by extra turnover or synthesis of the membrane components or because of enhanced fluidity of the



membrane structure. Since interaction of polymyxin B or other agents leading to such shape transformation was found to occur *in vitro* with the erythrocytes suspended in 0.15 M saline, extra synthesis of the membrane components during the period of interaction appears to be unlikely. It is significant to note in this respect that polymyxin B was shown to produce domain like structures within the membrane leading to a lowering of the lipid phase transition temperature [6].

Scanning and transmission electron microscopic studies were in reasonable agreement in indicating that the leakage of materials from the polymyxin treated erythrocytes, if any, was small and agreed reasonably with the observations that a maximum of only 7.5% hemolysis could be detected after the treatment of erythrocyte with 500 µg/ml of polymyxin B. The smaller extent of leakage and the fewer number of crenations in the treated erythro-

Fig. 3. Frequency distribution of the number of crenations per polymyxin B (PB) treated erythrocyte.

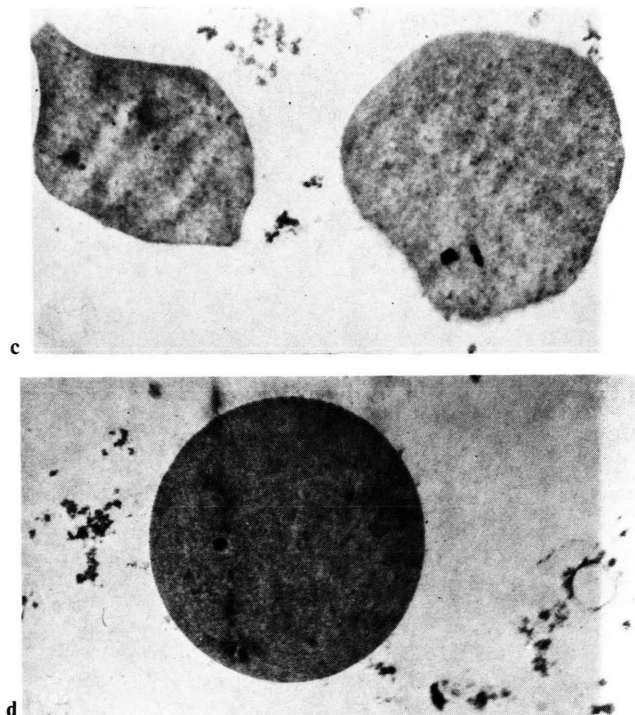
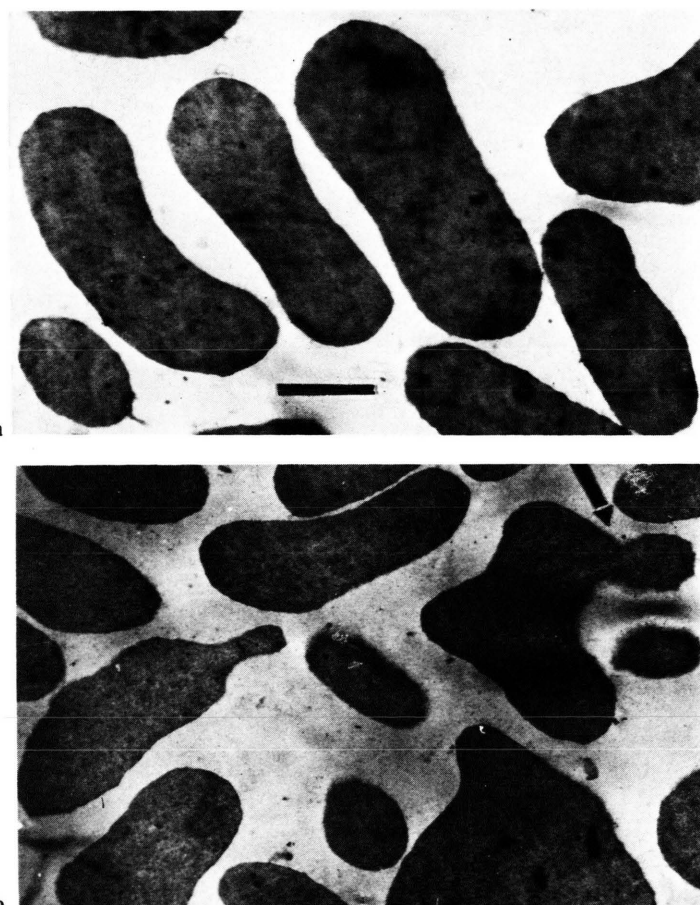


Fig. 4. Transmission electron micrograph of the ultrathin sections of goat erythrocytes treated with different amounts of polymyxin B: a) 0 µg/ml; b) 15.62 µg/ml; c and d) 250 µg/ml. Black bar represents 1 µm in all photographs.

cytes could probably be accounted for as due to the smaller amount of phosphatidyl ethanolamine content of the erythrocyte membrane. In fact a quantitative lipid analysis of the goat erythrocyte membrane revealed the presence of the following components: phosphatidyl ethanolamine (27.9%), phosphatidyl serine (20.8%), phosphatidyl inositol

(4.6%), sphingomyelin (45.9%), other phospholipids (as traces) and phosphatidyl choline (absent) [18].

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