Toxic Effect of Puromycin on Erythrocyte Membranes Which Is Unrelated to Inhibition of Protein Synthesis

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Exposure of rabbit or human erythrocytes to concentrations of puromycin as low as $7 \times 10^{-4} M$ for 2 hr causes damage to the cell membrane, as evidenced by increased susceptibility of the cells to hypotonic lysis, increased cell rigidity, and ultrastructural changes consistent with severe membrane damage. Puromycin causes a concentration-dependent internalization of the erythrocyte membrane, resulting in vacuolization of the cells, at concentrations between $7 \times 10^{-4} M$ and $10^{-2} M$. Since the erythrocyte does not synthesize protein, the data indicate that puromycin has a direct toxic effect on erythroid cell membranes which is unrelated to its action in inhibiting the synthesis of protein.

Puromycin, an antibiotic which is a structural analogue of phenylalanyl-transfer RNA, is a potent inhibitor of protein synthesis in mammalian cells. It has been widely used as a tool in the study of conditions such as the hemoglobinopathies, in which protein synthesis is abnormal. Although puromycin has been reported to inhibit lipid synthesis in chick aortic cells and nucleic acid synthesis in HeLa cells, these effects have not been shown to be independent of the effects of the agent on protein synthesis. An observation that rabbit reticulocyte suspensions showed hemolysis after prolonged exposure to puromycin suggested that this agent might have a direct toxic effect on the membrane. Since reticulocytes actively synthesize protein, however, such an effect could not be divorced from the action of puromycin in inhibiting protein synthesis. In order to study this phenomenon further and to strictly separate this effect from the action of puromycin in inhibiting protein synthesis, further experiments were carried out with erythrocytes, cells which during maturation have lost the capacity to synthesize protein. The data indicate that puromycin has a toxic effect on the cell membrane which is clearly unrelated to its effects in inhibiting the synthesis of protein.

MATERIALS AND METHODS

Freshly drawn washed heparinized rabbit or human erythrocytes were incubated in a buffered salt medium without or in the presence of concentrations of puromycin between $5 \times 10^{-4} M$ and $1 \times 10^{-2} M$. The effect of puromycin on the erythrocyte membrane was assessed by determining the susceptibility of the cells to hypotonic lysis, by studying the deformability of the cells, by determining permeability of the cell membranes, and by direct examination of cell ultrastructure by transmission electron microscopy.

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Fig. 1. The change in osmotic fragility of human and rabbit erythrocytes after exposure to puromycin. A 30% suspension of washed erythrocytes in phosphate-buffered normal saline, pH 7.4, was incubated at 37°C without or in the presence of $5 \times 10^{-3} M$ puromycin for 2 hr. One-tenth milliliter of the suspension was then added to 1.0 ml of a solution of NaCl of the strength shown on the abscissa, and the proportion of cells hemolyzed was determined from the amount of hemoglobin present in the supernatant after separation of the unlysed cells by centrifugation. •--•, control cells; ---○, puromycin-treated cells.

RESULTS

Osmotic Fragility of Erythrocytes.

The initial studies determined the effect of puromycin on the osmotic fragility of erythrocytes. The resistance of a 30% suspension of human or rabbit erythrocytes to lysis in a hypotonic medium was decreased after exposure to $5 \times 10^{-3} M$ puromycin for 2 hr at 37°C (Fig. 1). This effect was more pronounced if the cells were incubated with puromycin for 24 hr. Although this concentration of puromycin did not cause spontaneous lysis of erythrocytes, exposure of a 30% suspension of rabbit or human erythrocytes in phosphate buffer to higher concentrations of puromycin for 2 hr at 37°C caused spontaneous hemolysis (Fig. 2).
2) The proportion of cells hemolyzed increased in a concentration-dependent manner at levels of puromycin greater than $5 \times 10^{-3} M$. Rabbit erythrocytes were approximately ten times more susceptible to the lytic action of puromycin than human erythrocytes, being almost totally lysed at the highest concentration used.

**Deformability of Erythrocytes.**

The capacity of the erythrocyte to become deformed while passing through small passages is another criterion of membrane damage. The effect of puromycin on erythrocyte rigidity was assessed by the ability of the cells to pass through a previously moistened filter paper (Schleicher and Schull white ribbon, No. 589) supported by a glass funnel. The rate of filtration was measured by monitoring the volume of filtrate collected at 1 min intervals. The time required for half of a given volume of an 80% suspension of human erythrocytes to pass through the filter was increased from 4.25 to 11 min after exposure to $5 \times 10^{-3} M$ puromycin for 2 hr at 37°C. This finding is consistent with an alteration of the cell resulting in excessive rigidity of the cell membrane.

**Membrane Permeability**

Further indirect evidence that puromycin caused erythrocyte membrane damage was obtained by studying the influx of cations into the cell during exposure to different concentrations of puromycin. Washed human erythrocytes were exposed to $10^{-3} M$ puromycin while being incubated in a medium containing $^{22}$Na. The amount of radioactivity within the cell was increased at concentrations of puromycin as low as $7 \times 10^{-4} M$ and was concentration dependent between $7 \times 10^{-4} M$ and $6 \times 10^{-3} M$. This effect was evident after 1 hr of incubation at 37°C (Fig. 3). After 4 hr of incubation with $10^{-3} M$ puromycin, erythrocytes contained almost twice the amount of $^{22}$Na as did untreated cells (Fig. 3). Since these data adequately confirm that erythrocytes exposed to puromycin have increased membrane permeability, further studies to precisely quantitate cation flux were not carried out.

**Fig. 3.** The flux of radioactive sodium across human erythrocyte membranes exposed to $10^{-3} M$ puromycin. Thirty per cent cell suspensions of erythrocytes were incubated in a glucose-containing media in the absence or presence of $10^{-3} M$ puromycin. The amount of $^{22}$Na remaining in the cells following these washings in isotonic magnesium chloride was determined.
Ultrastructure of Puromycin-treated Erythrocytes

In order to obtain direct evidence that puromycin damaged the erythroid cell membrane glutaraldehyde-fixed sections of untreated and puromycin-treated human erythrocytes were examined by transmission electron microscopy (Fig. 4). Sections of untreated human erythrocytes incubated in phos-
phosphate-buffered saline for 2 hr at 37°C showed that the normal biconcave disc configuration was maintained (Fig. 4, upper left). Incubation in the presence of $5 \times 10^{-3} M$ puromycin produced a striking alteration in erythrocyte shape (Fig. 4, upper right), and most of the puromycin-treated erythrocytes contained intracellular vacuoles ranging in size between 0.60 and 1.25 μ. These vacuoles were bounded by well-formed membranes which were grossly similar in structure and dimensions to the erythrocyte cell membrane (Fig. 4, lower, arrows). The appearance of the membrane-bound vacuoles suggested their derivation from the erythrocyte surface. This concept was supported by the frequent occurrence of invaginations of the external membrane into the cell interior (Fig. 4, lower, insert).

The concept that the intraerythrocytic vacuoles induced by puromycin arose from internalization of the cell membrane was confirmed by studies in which the exterior surface of the cell membrane was tagged with a complex of $^{57}$Co-vitamin B$_{12}$, as described by Ben-Bassat and co-workers. Briefly, heparinized washed normal human erythrocytes were externally labeled by incubation with plasma previously equilibrated with a subsaturating dose of $^{57}$Co-B$_{12}$. Different concentrations of puromycin were then added to aliquots of the cell suspension, and incubation was continued at 37°C for up to 4 hr. With internalization of the membrane, the $^{57}$Co-B$_{12}$-protein complex on the external surface is transferred to the interior of the cell and cannot be removed when the cells are subsequently treated by mild tryptic digestion. Figure 5 shows that there was a linear relationship between the radioactivity within the cells and the concentration of puromycin at levels between $7 \times 10^{-4}$ and $10^{-2} M$. Higher concentrations of puromycin caused hemolysis, thereby interfering with the assay. Membrane internalization was time dependent, plateauing after 2 hr of incubation at 37°C, and temperature dependent, being optimal at 37°C. The membrane effect was not reversible if the treated cells were further incubated in

![Fig. 5. The internalization of erythrocyte membranes in the presence of puromycin.](image)

Following tagging of the external cell membrane with $^{57}$Co-vitamin B$_{12}$, the cells were exposed for 2 hr at 37°C to concentrations of puromycin shown on the abscissa. Internalization of the plasma membrane was assessed by determining the radioactivity remaining within the cell following stripping of the labeled B$_{12}$-protein complexes by mild tryptic digestion. There was a linear relationship between radioactivity within the cell and concentrations of puromycin between 0.7 and 10 mM.
medium which did not contain puromycin, nor did the presence of $10^{-2}\, M$ NaF during the incubation with puromycin prevent membrane internalization. These findings confirm that the vacuoles observed by electron microscopy of puromycin-treated human erythrocytes are due to internalization of the cell membrane.

**DISCUSSION**

The experimental findings provide direct evidence that puromycin has a toxic effect on the erythrocyte membrane. Since mature erythrocytes do not synthesize protein, the damage to the cell membrane cannot be ascribed to the well-known action of puromycin in inhibiting protein synthesis. Other effects of puromycin have been shown to be independent of its action in inhibiting protein synthesis. These include increased glycogenolysis in mouse liver, inhibition of cyclic AMP phosphodiesterase in rat hemidiaphragms, and stimulation of rat liver ornithine decarboxylase activity. The structure of puromycin does not suggest the mode of action by which this agent affects membrane structures. The morphologic effects of puromycin are, however, similar to those of vinblastine, colchicine, and strychnine, all of which precipitate the microfilamentous protein of the erythrocyte membrane, thus making the cell more rigid. Since puromycin has been reported to enhance the vinblastine-induced precipitation of microtubular membrane protein in fertilized sea urchin eggs, it is possible that it also has a direct effect on membrane protein. It is likely that other chemicals may produce a similar effect on the red cell membrane.

Puromycin, considered solely as an inhibitor of protein synthesis, has been extensively used as a tool in investigating the mechanisms of mammalian protein synthesis. The concentration most often used in the reticulocyte system, $10^{-2}\, M$, virtually eliminates protein synthesis in the intact cell. The present finding that puromycin, in concentrations within this range, can affect aspects of metabolism in the intact erythroid cell other than protein synthesis is of particular significance in view of the widespread use of this cell line as a model system. The effect of puromycin on cell membranes must be taken into account when interpreting studies which involve structures bound to the cell membrane or metabolic activities in which membrane structures are implicated.

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