Effects of the Antitumor Drug Adriamycin on Human Red Blood Cell Discocyte-Echinocyte Transitions

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ABSTRACT

The antitumor drug Adriamycin, when preincubated with human red blood cells (discocytes) for 10 min, prevented the formation of echinocytes induced by the calcium ionophore A23187 in the presence of 0.2 mM calcium. The degree of protection was concentration dependent and was >90% at 10 μM Adriamycin. Adriamycin did not interfere with the accumulation of calcium induced by a 5 μM concentration of the ionophore. Adriamycin reversed echinocyte morphology to the discocyte form in echinocytes which had been formed by adenosine triphosphate depletion but not those formed after treatment with A23187 and Ca²⁺. Its ability to protect against Ca²⁺-induced echinocyte formation contrasts with the failure of the local anesthetic procaine to exert such an effect, even at 45 mM (J. Palek et al., Blood, 50: 155-164, 1977), and this difference suggests that Adriamycin may not be acting simply as a chaperone agent. This hypothesis was supported by the observation that Adriamycin alone did not induce a cup-form morphology in discocytes (stomatocytosis). Wheat germ agglutinin protection of echinocyte formation induced by calcium loading was reversed by 30 mM N-acetylglucosamine, which partially reversed the Adriamycin protection of echinocyte formation. However, desialylation of human red blood cells with Clostridium perfringens type V neuraminidase, while preventing the protection of echinocyte formation by wheat germ agglutinin, had no effect on the protection afforded by Adriamycin. This suggests that Adriamycin does not prevent echinocyte formation via binding to the sialic acid residues of the transmembrane protein glycophorin and that another mechanism or mechanisms are involved in its action to modulate morphological transitions of the red blood cell membrane.

INTRODUCTION

Evidence to support the hypothesis that the antitumor drug Adriamycin may exert its cytotoxicity via interaction with the plasma membrane of tumor cells is accumulating (reviewed in Ref. 2). Among this evidence are the results of studies of the cytotoxicity of Adriamycin which has been immobilized on beads of cross-linked polymers (3-5). For example Tritton and Yee (5) showed that polymer-immobilized drug was incapable of penetrating murine L1210 leukemia cells but was cytotoxic to them.

Recent studies by us have suggested that cytotoxic concentrations (<10 μM) of Adriamycin had no early effect on the ionic homeostasis of human RBC or L1210 leukemia cells, measured in terms of changes in membrane potential, rubidium influx, and intracellular calcium pools. These results suggested that the function of many integral membrane proteins may thus be normal (1, 6). However, two studies have shown that antineoplastic anthracyclines alter certain properties of the erythrocyte membrane (7, 8). In a preliminary study Mikkelsen et al. (8) showed that low concentrations of Adriamycin were able to reverse the echinocyte morphology of human RBC, induced by ATP depletion, back to a discocyte morphology. Additionally their study suggested that Adriamycin interacted with elements of the RBC cytoskeleton, including spectrin. It is therefore possible that the interaction of the drug with plasma membranes may mediate changes in cytoskeletal components and that this may contribute to or be responsible for the cytotoxicity of the drug to nucleated cells.

The RBC is a commonly used model for studies of membrane and cytoskeleton function (9). We have chosen to reinvestigate the interaction of Adriamycin with this cell and in particular to study the effects of the drug on morphological discocyte-echinocyte transitions. The precise mechanism of control of this transition is controversial but a number of hypotheses have been forwarded to account for its modulation by amphiphatic drugs and other agents. These include the involvement of calcium-calmodulin-controlled kinase activity (10), changes in membrane potential (11), altered bilayer coupling after the differential expansion of one layer relative to another (12), changes in the coupling of cytoskeletal components mediated via the transmembrane lectin binding-protein glycophorin (13, 14), and changes in the metabolism of the inositol phospholipids (15). With regard to the protection of discocyte morphology afforded by wheat germ agglutinin (13), it was reported that this could be annulled through the displacement of the lectin from its binding site, the transmembrane protein glycophorin, by N-acetylglucosamine (8). In the present study on the basis of the effects of Adriamycin on discocyte to echinocyte morphological transitions, we report a similar finding but conclude that Adriamycin does not mediate its effects via the lectin-binding site or, apparently, by acting simply as a chaotropic agent which expands the inner membrane bilayer.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade. The Ca²⁺ ionophore A23187, GlcNAc, and neuraminidase (EC 3.2.1.18) were obtained from Sigma Chemical Company (United Kingdom). WGA was obtained from Boehringer/Mannheim (West Germany). 46Ca (116 μg/1.9 mM) was purchased from the Radiochemical Centre (Amersham, United Kingdom).

The abbreviations used are: GlcNac, N-acetylglucosamine; WGA, wheat germ agglutinin.
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Scintillation fluid was purchased from New England Nuclear (Southampton, United Kingdom). Adriamycin was a generous gift from Farmitalia. Human RBC were drawn from volunteers of various blood types 1–2 h before the experiment.

Modulation of RBC Morphology. Fresh RBC were washed three times in a buffer consisting of 10 mM KCl, 130 mM NaCl, 2 mM MgCl₂, and 15 mM Tris-HCl (pH 7.4). All compounds used were dissolved in the same Tris-saline buffer except A23187 which was dissolved in dimethyl sulfoxide (final concentration, 0.05%). The final number of RBC used was 1 x 10⁷/ml at 23°C and the sequences of addition of reagents were as described in the figure legends.

Electron Microscopy. The cells were fixed directly for scanning electron microscopy by the addition of 5 ml of 2% glutaraldehyde in Millonig’s (16) buffer at pH 7.3. The cells were then washed twice with deionized water, placed on grids which were dried at room temperature and then sputter coated with gold, and viewed in a JEOL JEM-100 CXII electron microscope. A minimum of 100 cells were scored for morphology in each experiment.

Neuraminidase Desialylation. Sialic acid residues (N-acetylneuraminic acid residues) were removed by neuraminidase (Clostridium perfringens type V). RBC at 10⁷/ml in the Tris-HCl buffer (pH 7.4) outlined above were incubated with 0.5 unit neuraminidase at 37°C for 1 h with gentle shaking. The total release of sialic acid residues was measured during incubation and was found to be maximal at 1 h (17). The cells were then centrifuged at 1200 x g for 5 min and washed twice with the Tris buffer.

⁴⁵Ca²⁺ Uptake by RBC in the Presence of A23187. The RBC (10⁷/ml) were incubated with or without drugs for 10 min. After this period 0.2 mM Ca²⁺ (plus ⁴⁵Ca²⁺, 10 µCi/ml) and 1 min later A23187 (5 µM) were added. At given times 200-µl samples of cells were removed and spun through 0.2 ml silicone oil (d = 1.05 g/ml) in a Beckman microfuge at 9000 x g for 1 min. The microfuge tubes were frozen in liquid nitrogen, cut just above the cell pellets, and placed in scintillation vials. The RBC pellets were lysed, hemolysates were decolorized with 0.9 ml of 30% (v/v) H₂O₂, and radioactivities were determined by liquid scintillation counting in the presence of 10 ml of NEN 260 scintillation fluid in a Packard Tri-Carb 2606 scintillation counter.

RESULTS

Fig. 1a shows that the biconcave discocyte form of the RBC was well preserved on fixing with 2% glutaraldehyde for scanning electron microscopy. RBC, when exposed to 0.2 mM Ca²⁺ and 5 µM A23187 for 5 min to increase intracellular Ca²⁺ levels, changed from a discocyte to an echinocyte morphology (Fig. 1b). The echinocytic form consisted of variable number of short, cone-shaped extrusions on the cells. Exposure of discocytes to a range of concentrations of Adriamycin for 10 min prior to the addition of 0.2 mM Ca²⁺ and 5 µM A23187 blocked the formation of echinocytes in a dose-dependent manner (Chart 1). Adriamycin alone had no effect on the morphology of discocytes, even at concentrations of 100 µM. At the lowest concentration of Adriamycin used (10⁻⁸ M) the drug slightly stimulated the formation of echinocytes above the level of the calcium-loaded controls. Anecdotal evidence from in vitro assays of the toxicity of Adriamycin suggests that low concentrations may actually stimulate cell growth in suspension culture. Whether these phenomena are related awaits proper experimentation.

When RBC were incubated with WGA (2 µg/ml) for 10 min and then exposed to 0.2 mM Ca²⁺ and 5 µM A23187 for 5 min, the cells completely retained their normal discocyte morphology (Fig. 2a). When the WGA-protected, Ca²⁺-loaded discocytes were exposed to 30 mM GlcNAc for 5 min the cells were converted to echinocytes (Fig. 2b). Similarly if Adriamycin-protected cells (10 µM Adriamycin) (Fig. 2c) were exposed to GlcNAc (30 mM), then a similar conversion to echinocyte form was seen, although the effect was not as dramatic as seen with WGA (Fig. 2d). Chart 2 summarizes the above scanning electron microscopy results quantitatively. WGA and Adriamycin both blocked echinocyte formation by 100% and GlcNAc released this block by 95 and 60%, respectively. GlcNAc alone had no effect on the morphology of RBC (data not shown).

In order to investigate whether the protection afforded by WGA or Adriamycin was due to the blockade of Ca²⁺ transport into the cell, uptake of ⁴⁵Ca²⁺ was determined. Chart 3, A and B, shows that the uptake of Ca²⁺ by the RBC is dependent on the presence of the ionophore A23187. The influx of Ca²⁺ in the presence of A23187 is rapid and is complete within minutes. If the RBC were first preincubated for 10 min with Adriamycin (Chart 3C) or WGA (Chart 3D) and then given 0.2 mM Ca²⁺ and
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Fig. 2. Scanning electron micrographs of RBC from stages indicated by the letters in the sequence. The RBC were exposed to WGA (2 mg/ml) or Adriamycin (10 μM) for 10 min, followed by 0.2 mM Ca²⁺ and 5 μM A23187 for 5 min and finally GlcNAc (NAG) (30 mM) for 5 min. × 1500.

Chart 2. Comparison between the effects of WGA and Adriamycin on percent age protection against echinocyte formation and of its reversal by GlcNAc (NAG). Cells were preincubated with WGA (2 μg/ml, □) or Adriamycin (10 μM, □□) for 10 min followed by 0.2 mM Ca²⁺ and 5 μM A23187 for 5 min and finally GlcNAc (NAG) (30 μM) for 5 min. × 1500.

Chart 3. Influx of ⁴⁶Ca²⁺ into the cell in the presence of A23187 added at the time indicated by the arrow. The cells in A and B were incubated with ⁴⁶Ca²⁺ in the absence and presence, respectively, of A23187. The cells in C (Adriamycin, 10 μM) and D (WGA, 2 μg/ml) were incubated for 10 min with the drug prior to ⁴⁶Ca²⁺ influx studies.

5 μM A23187, the rate of ⁴⁶Ca²⁺ influx and the final equilibrium level were the same as the control cells. GlcNAc (30 μM) had no effect on ionophore-induced calcium uptake (data not shown).

It is known that the receptor for WGA on the RBC is the major sialoglycoprotein glycophorin and that WGA binds to the sialic acid residues of glycophorin. WGA binding and blockade of the changes in cell morphology are eliminated when sialic acid residues are removed (14). In view of the release of the WGA and Adriamycin block by GlcNAc, we studied the effect of WGA and Adriamycin on desialylated cells. The desialylated cells were preincubated with WGA or Adriamycin (10 min) followed by Ca²⁺ and A23187 for 5 min and the percentage of echinocytes was scored. Chart 4 shows that WGA completely lost its ability to prevent echinocyte formation but that Adriamycin still protected desialylated cells from forming echinocytes.

DISCUSSION

In an initial study of the effects of Adriamycin on RBC morphology, Mikkelsen et al. (8) showed that Adriamycin was able to reverse the echinocyte morphology of ATP-depleted or nitroxide stearate-treated cells. We were able to confirm the results of Mikkelsen et al., using ATP-depleted echinocytes, to show that Adriamycin reversed echinocyte morphology to that of discocytes but we found that echinocyte morphology induced by A23187 and Ca²⁺ was not reversed (data not shown). Recently it has been suggested that the mechanism of the induction of echinocyte morphology by ATP depletion and by A23187 and Ca²⁺ may be different (15).

Adriamycin protected against echinocyte formation induced by A23187 and Ca²⁺ in a dose-dependent manner (Chart 1) and the drug is a potent modulator of the transition. This is an important
result with possible implications regarding the mode of action of Adriamycin in protecting against echinocyte formation. Palek et al. (18) have shown that 45 mM procaine was able to protect against the echinocyte formation induced by ATP depletion but not against that induced via an elevation of intracellular calcium by A23187. They proposed that the high concentration of procaine required was acting as a chaotropic agent, intercalating the inner membrane of the RBC and preventing morphological change according to the bilayer-couple hypothesis (12) rather than by bringing about changes in the coupling of cytoskeletal proteins. It thus appears that Adriamycin, because of its greater (100-fold) potency of action and its protective effect against echinocyte formation induced by calcium loading, may not be acting simply as a chaotropic agent but may instead be interfering with cytoskeletal protein interactions. Additional support for this hypothesis lies in the observation that Adriamycin itself does not alter RBC morphology (see also Ref. 7) whereas the chaotropic agents, such as procaine, induce an exaggerated cup-form morphology (stomatocyte).

Previous work had shown that Adriamycin was capable of binding to anionic mucopolysaccharide (19) and we wondered whether it was possible that the drug could bind to negatively charged parts of glycoproteins, such as the sialic acid residues of the transmembrane protein glycophorin, which is involved in the control of discocyte-echinocyte transition (13, 14). Preliminary results supported this hypothesis in that Adriamycin protection against A23187 and Ca$^{2+}$-induced echinocyte formation could be blocked by N-acetylgalactosamine (Fig. 2; Chart 2), a finding similar to that obtained with the lectin WGA which binds to glycoporin and can be displaced by GlcNAc (14). However, blockade of the protective effects of Adriamycin by GlcNAc was less efficient than its effects on WGA protection and this raised doubts as to whether it was a specific blockade. These doubts were confirmed by the finding that desialylated RBC were still protected from forming echinocytes by Adriamycin (Chart 4), whereas the effect of WGA was completely lost, as found by others (14). Adriamycin may interact with glycophorin independently of an interaction with sialic acid residues but such a possibility awaits a detailed analysis of the interaction of erythrocyte membrane components with the drug.

It is difficult to provide an explanation of the effects of GlcNAc on Adriamycin protection. The high concentration of GlcNAc required may have brought about the displacement of the drug from some binding site other than sialic acid residues or a change in the general distribution of the drug in erythrocytes, and we will be investigating this in future studies. 2-Deoxyglucose, which has been reported to block the cytotoxicity of Adriamycin (20), did not reverse the effect of Adriamycin on RBC under conditions where GlcNAc was effective (data not shown).

The mechanism whereby Adriamycin blocks the formation of echinocytes induced by both ATP depletion and calcium loading is also unclear. The effects of Adriamycin reported here show that this amphiphatic drug is a potent modulator of the discocyte-echinocyte transition but that it has properties which are distinctive from chaotropic agents such as procaine, namely the capability to block echinocytosis induced by calcium loading and the failure of high concentrations of the drug to induce a stomatocytic (cup-shaped) morphology (7). Several mechanisms have been proposed to account for the action of amphiphatic drugs on the morphological transition of RBC. Most prominent among these is the report of an excellent correlation between their effect on morphology and their potencies as calmodulin inhibitors (15).

Preliminary data show that Adriamycin is a poor inhibitor of the calmodulin-dependent calcium ATPase of RBC (21) and we will report on this and on other biochemical studies aimed at elucidating the precise locus of the action of Adriamycin in a future communication. By the use of the RBC we, like others, hope that this cell type will be a useful guide to events occurring in the membrane and cytoskeleton in other, less well defined cell types and thus to clarify the mechanism of action of Adriamycin at the level of the plasma membrane and cytoskeleton.

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REFERENCES

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