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Measurement of Polyene Antibiotic-Mediated Erythrocyte Damage by Release of Hemoglobin and Radioactive Chromium

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Polyene antifungal antibiotics produce various degrees of membrane damage in sheep erythrocytes in vitro. Mediocidin, filipin, amphotericin B, and candicidin were found to result in greater damage than nystatin, pimaricin, and amphotericin B methyl ester. The degree of sensitivity of the cells varied by 100-fold for mediocidin verus amphotericin B methyl ester as measured by curves of hemoglobin release versus drug concentration. In erythrocytes prelabeled with radioactive chromium, release of the isotope through polyene-damaged cell membranes was found to occur at lower drug concentrations than measurable hemoglobin release, and the percentage of isotope released at the highest drug dose was consistently greater than the percentage of hemoglobin released. Thus, the isotope assay is a more sensitive indicator of polyene-induced membrane damage in the test system. These significant differences in release of molecules through polyene-induced membrane lesions indicate the complex nature of the binding and further interactions of this class of drugs with the plasma membrane.

Polyene antifungal antibiotics have been shown to induce the rapid lysis of rat and human erythrocytes in the presence of isotonic saline (6). The extent of hemolysis was dependent on the antibiotic/cell concentration ratio. Further, a critical threshold ratio of antibiotic molecules to cell surface area was established (7). It has been shown that the mode of action of the polyene antifungals involves the binding of drug molecules to cell membrane sterols, with the attendant distortion of the membrane and leakage of essential metabolites (9). Eventually, loss of protein occurs through the altered cell membrane. It has also been observed that heptaenes containing an aromatic moiety, such as candicidin (CAND), are generally more active than those without such a moiety, such as amphotericin B (AB) (11).

Minor changes in structure can radically affect the biological activity of a polyene. Amphotericin B methyl ester (AME) is a recently synthesized compound (1), possessing equal antifungal activity to the parent compound, AB, but with a greatly increased solubility in aqueous media and decreased in vivo toxicity (5).

In homeothermic animals, the selectivity of membrane transport of K^+ over Na^+ ions into the cell is on the order of 25:1 (2). This figure cannot be taken as the real rest-state selectivity since the absolute membrane selectivity for K^+ over Na^+ is much higher. This ratio refers to the presence of the ions in the cell cytoplasm and the suspending medium outside the plasma membrane. Chromium-51 (^{51}Cr) is a 0.33-MeV gamma radiation-emitting isotope with a half-life of 28.7 days. It is taken up, in the form of sodium chromate ($Na_2{}^{51}CrO_4$), by animal cells and erythrocytes in much the same way as potassium and is selectively concentrated, by active transport, against the normal-concentration gradient in aqueous solutions. Since the cell's sodium-potassium pump is involved in this process, any damage to the cell membrane will result in release of the isotope, thus rendering it available to scintillation counting in the supernatant of a pelleted cell suspension. ^{51}Cr isotope assays for cell toxicity have been used in recent years in tumor cell-antibody systems (10, 13), virus-cell antigen-antibody systems (3, 8), and lymphocyte-cell systems (4, 12, 14). In the studies described below, sheep erythrocytes were labeled with the gamma-emitting isotope ^{51}Cr in the form of radioactive sodium chromate, and the release of the isotope and/or hemoglobin was studied after damage to the cell membrane with filipin (FIL), mediocidin

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(MED), AB, AME, CAND, PIM, and NYS. I hoped to further illuminate the mechanism of membrane damage by polyenes by comparing the release of ^{51}Cr and hemoglobin in drug-treated erthrocytes.

MATERIALS AND METHODS

Erythrocytes. Fresh sheep erythrocytes were obtained as a 20% suspension in Alsever solution from Microbiological Associates, Inc., 100 ml at a time. They were washed in Dulbecco phosphate-buffered saline by centrifugation at 1,000 × *g* and 4°C, followed by resuspension. This procedure was repeated three times. Cell density was adjusted so that 0.2 ml of suspension, lysed with 2.2 ml of distilled water, gave an optical density of 0.700 at 541 nm. The pH of all suspensions was kept rigidly at pH 6.8.

Polyene antifungal antibiotics. Polyenes were obtained in crystalline form, purified by liquid chromatography, from Daniel P. Bonner and Wietold Mechlinski of Carl Schaffner's laboratory, Waksman Institute of Microbiology, Rutgers University. The drugs were stored at −20°C until use. After use they were stored as frozen solutions at −20°C for up to 7 days; after this time they were not reused. AME, AB, FIL, MED, NYS, and CAND were dissolved in highly purified dimethyl sulfoxide. PIM was dissolved in dimethyl fluoride.

Treatment of erythrocytes. Aliquots of a freshly prepared erythrocyte suspension were treated with polyene drugs in the following manner: 0.2 ml of the cell suspension was added to each of a number of glass disposable culture tubes (12 by 75 mm) and allowed to equilibrate at 24°C. Freshly prepared solutions of polyene antibiotic diluted in dimethyl sulfoxide or dimethyl fluoride were added by Hamilton microsyringe in 2-μl volumes. The drugs were diluted in such a manner that a 2-μl volume would contain a quantity of drug equivalent to the final concentration (in micrograms per milliliter) desired. (Thus, if the drug dose required was 10 μl, 2 μg of drug would be added in 2 μl of solution to the 0.2 ml of erythrocyte suspension.) The tubes were agitated and allowed to incubate at 24°C for 30 min.

Hemoglobin release assay. Tubes of treated erythrocytes were filled with 2.2 ml of Dulbecco phosphate-buffered saline except the distilled-water blanks, which received 2.2 ml of deionized, distilled water. All tubes were centrifuged at 1,000 × *g* and 4°C for 10 min, and the supernatant was analyzed for optical density at 541 nm. The distilled-water blank was considered to represent 100% hemoglobin release; a no-drug blank, treated only with dimethyl sulfoxide or dimethyl fluoride, represented 0% hemoglobin release. The following formula was utilized to calculate percentage of hemoglobin release:

$$\%\ \text{hemoglobin release} = \frac{OD_2\ (\text{drug sample}) - OD_1\ (\text{blank})}{OD_3\ (\text{distilled-}H_2O\ \text{blank})}$$

where OD is optical density. Results for each experiment were then graphed linearly. The concentration of a drug resulting in release of 50% of the value obtained after treatment of the cell suspension in distilled water was defined as the 50% release value of the drug.

Combined ^{51}Cr and hemoglobin release assay for erythrocytes. An assay, utilizing prelabeled erythrocytes, was developed in order to examine the comparative sensitivities of ^{51}Cr and hemoglobin release as a measure of erythrocyte membrane damage by the polyene drugs. A washed suspension of 10^8 to 2×10^9 cells/ml was incubated with an equal volume of balanced salt solution containing 100 to 300 μCi of ^{51}Cr per ml at 37°C, with gentle agitation every 5 min for the first 30 min. The cells were then washed five times by centrifugation and resuspension and used immediately. Ten million (4×10^8) erythrocytes/0.2 ml were dispensed into glass culture tubes.

After dispensing, the erythrocytes were treated, as described above, with various doses of polyenes. After resuspension and centrifugation, 0.4 ml out of the total 2.2 ml of each sample was assayed in a Packard Tri-Carb liquid scintillation counter, and the rest was assayed for optical density at 541 nm. The results were graphed as percentage of hemoglobin release and percentage of ^{51}Cr release versus drug concentration.

RESULTS

The standard time of incubation was 30 min from the moment of addition of drug to the erythrocyte suspension. This period of time was found to be sufficient for virtually 100% of the maximum damage to be imposed on the cell membrane by any of the polyenes utilized in these experiments. This experimental finding did not vary from drug to drug or from concentration to concentration for any particular drug, regardless of the specific hemolytic capability of a particular polyene. In other words, any drug dose of any polyene was found to exert its maximum degree of damage within 30 min of the time of application, whatever the final percent release value attained.

The drug concentrations resulting in 50% total hemoglobin release for sheep erythrocytes treated with seven polyene antifungal antibiotics under controlled conditions are given in Table 1. It is evident that MED, FIL, AB, and CAND were the most damaging agents to the cell membrane, followed by NYS, PIM, and AME. These 50% values were taken from the curves of percent release versus drug concentration made after each experiment (Fig. 1, lower curve). They represent the means of five separate determinations each; it was found that, under the same conditions of temperature, pH, buffer, and time of incubation, response to drug treatment agreed within a few percentage points. Since the drugs were obtained from the same source each time, batch variation was eliminated (although with AB, NYS, AME, and FIL it was found that different batches of

drug from the same sources differed only slightly in their hemolytic ability). Different erythrocyte batches were found to be quite similar in membrane fragility and sensitivity to a particular series of polyenes; in any case, all erythrocytes were obtained from the same source.

Comparative release of ^{51}Cr and hemoglobin from erythrocytes. The comparative release of isotope from prelabeled erythrocytes and that of hemoglobin from the same cells was examined after treatment with AME, AB, CAND, and PIM. Drug concentrations producing 50% release are shown in Table 2. An approximately twofold-lower concentration of drug was required for 50% release of the isotope in the cases of AME, AB, and PIM than for 50% release of hemoglobin. For CAND the ^{51}Cr release technique was found to be approximately threefold more sensitive than that of hemoglobin release. Figure 1 represents the curves of the release of the two indicators in sheep erythrocytes treated with CAND: at 2.5 μg/ml the difference between the two curves was significant at the 0.001 level. For PIM (Fig. 2) the significance was at the 0.001 level at drug concentrations of 75 μg/ml.

DISCUSSION

The twofold difference in drug concentration needed to give a 50% release of isotope versus

TABLE 1. *Release of hemoglobin from sheep erythrocytes damaged by seven polyene antifungal antibiotics*

Drug	Drug concn (μg/ml) leading to 50% hemoglobin release
AME	200
FIL	2.3
AB	3.2
MED	2.0
PIM	100
NYS	50
CAND	4.0

TABLE 2. *Release of chromium-51 and hemoglobin from sheep erythrocytes damaged by four polyene antifungal antibiotics*

Drug	Drug concn (μg/ml) leading to:	
	50% ^{51}Cr release	50% hemoglobin release
AME	95	200
AB	1.8	3.2
CAND	1.25	4.0
PIM	50	100

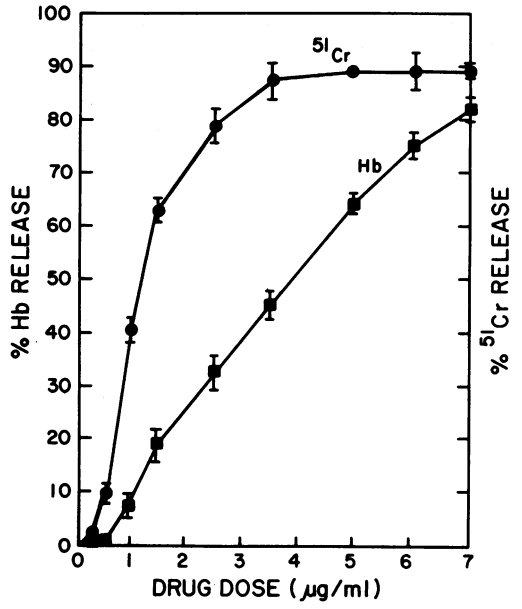

FIG. 1. *Graph of percentage of hemoglobin and ^{51}Cr release versus drug concentration for sheep erythrocytes treated with candicidin. Results are for sheep erythrocytes after treatment with graduated doses of the polyene. Symbols: (●) ^{51}Cr release; (■) hemoglobin release.*

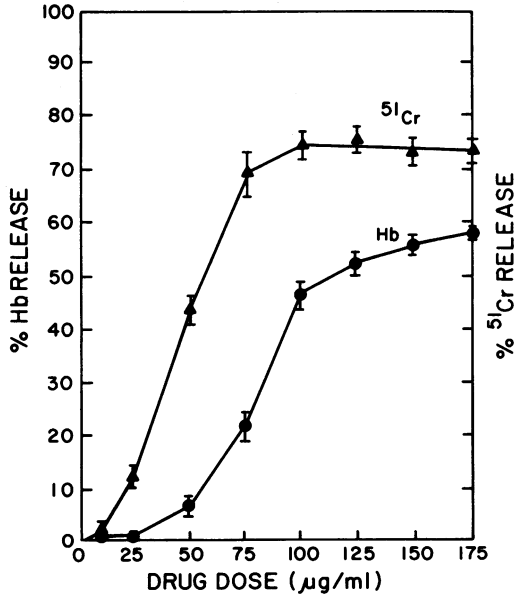

FIG. 2. *Graph of percentage of hemoglobin and ^{51}Cr release versus drug concentration for sheep erythrocytes treated with pimaricin. Results are for the sheep erythrocytes after treatment with graduated doses of the polyene. Symbols: (▲) ^{51}Cr release; (●) hemoglobin release.*

hemoglobin from polyene-treated sheep erythrocytes indicates the more sensitive nature of the isotopic method in quantifying membrane damage in treated cells (Table 2, Fig. 1 and 2). The significantly greater release of labeled chromium (and, by implication, that of K^+ ions) as compared with the much larger hemoglobin molecule indicates the complex nature of the lesions produced and the membrane damage and disruption incurred by the binding of polyene antifungal antibiotics to erythrocytes. The use of labeled compounds in this manner, combined with assays of endogenous release of such molecules as hemoglobin, could be a powerful tool for the study of membrane dynamics and mechanisms of cytotoxicity in animal cells.

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