Aggregation-Dependent Changes in Susceptibility of *Dictyostelium discoideum* to Amphotericin B

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In this study, the polyene antibiotic amphotericin B was used to induce changes in permeability and viability of the eucaryotic microorganism *Dictyostelium discoideum*. The results show that as the cells progress from the growth phase through stationary phase to eventual aggregation, they become increasingly resistant to both permeability changes and lysis. Plasma membranes were prepared from cells harvested at exponential growth, stationary phase, and the aggregation-competent stage, and both the neutral lipid and phospholipid content were determined. An increase in the neutral lipid and a decrease in the phospholipid were observed as the cells progressed from growth into aggregation, with the overall result that the phospholipid-sterol ratio decreased during this period. The extent of amphotericin B binding by cells from the different stages was also determined. Aggregation cells exhibited a small but significant increase in binding compared to cells from either of the other two stages. The permeability changes produced by the drug were measured as a function of temperature. Exponentially growing cells showed a marked temperature dependence for the drug effect, whereas aggregating cells did not. Rates of inactivation by the drug were also determined over a range of temperatures. With exponentially growing cells, the rate of inactivation was temperature dependent, whereas with aggregating cells it was not. Finally, drug binding for both growing and aggregating cells was temperature dependent. Thus, no binding was observed for cells of either type at 4°C. This finding suggests that the differences in the temperature dependence of the permeability changes and inactivation between the cells involve parameters other than drug binding.

Polyene antibiotics, such as amphotericin B, have been shown to produce striking increases in water and solute permeability in both lipid bilayers (4, 6) and intact cells (8), which often leads to cell death by lysis. Although the action of these drugs is dependent upon the binding of the polyene to sterol residues in the membrane (13), the mechanisms underlying the permeability changes and lysis remain to be elucidated. In a previous study in this laboratory, amphotericin B together with the eucaryotic microorganism *Dictyostelium discoideum* were used to study this question, and we showed that lysis is preceded by the formation of an osmotically fragile cell (22). In addition, the results of the previous study suggested that growing cells were more susceptible to the drug than those from the stationary phase (22).

To explore the relationship between growth and drug sensitivity in more detail, we have taken advantage of the fact that *D. discoideum* begins to aggregate only after the cessation of growth. This means that cell populations can

be obtained from three separate growth stages: i.e., exponential growth, the stationary growth phase, and the stage of no growth that is found at aggregation. In the present study, cells from all three stages were exposed to amphotericin B, and the effects of the drug on viability and permeability were determined.

Part of the present study necessitated a comparison of the extent of drug binding by cells from the three different stages. Although several chemical procedures for this determination have been reported (10, 14), a bioassay technique of a type similar to one previously described using erythrocyte hemolysis as the indicator was developed for the present study. (12). In the present assay, *D. discoideum* cell lysis is the indicator.

The assay used in the present study is based on the observation that, although cell lysis by the polyenes occurs above a threshold value (11), *D. discoideum* can be induced to lyse below its threshold by dilution into distilled water (22). This bioassay has enabled us to compare

the extent of drug binding by cells from the different stages.

MATERIALS AND METHODS

Organisms and growth conditions. Cells of the axenic strain of *D. discoideum* AX-3, a derivative of NC-4 (haploid), were grown as previously described (22). Under these conditions, the cells double in about 10 to 12 h.

Materials. Amphotericin B (Fungizone, E. R. Squibb & Sons) solutions were prepared by the addition of a low-phosphate saline (LPS) buffer (22, 23). [^{14}C]thiourea (25.9 mCi/mmol) was purchased from New England Nuclear Corp. and diluted with water before use. Stigmasterol was purchased from the Sigma Chemical Co.

Aggregation and disaggregation conditions. The synchronous formation of aggregates was accomplished by dispensing cells, at a final concentration of 7×10^7 to 8×10^7 cells/ml, on 42-mm Whatman no. 50 filters resting on absorbant pads in 60-mm plastic petri dishes as previously described (19, 23). Cells, harvested from filter pads, were resuspended in LPS buffer and disaggregated by repeatedly pipetting the solution with a fine-tipped 10-ml pipette (1, 18). This procedure was continued until over 99% of the aggregates had been disrupted to single cells, as confirmed by microscopic examination. No evidence of cell lysis was obtained using the clonal assay procedure.

Preparation of plasma membranes. Plasma membranes were isolated and purified from *D. discoideum* as previously described (21).

Cell viability. The effect of amphotericin B on cell viability was determined using the clonal assay procedure as previously described (22). Cells were harvested from the required stage of growth or development and resuspended in LPS at a density of 1×10^7 cells/ml, and they were further incubated with amphotericin B at 22°C on a gyratory shaker. After 2 h, the drug reaction was terminated by diluting a portion of the incubation solution with protease peptone broth (1:100; 22). The number of viable cells present in portions of the culture was determined. The cells were appropriately diluted and spread on an agar surface together with *Aerobacter aerogenes* as food source. Clones, observed for 3 to 4 days after incubation at 22°C, were counted, and the number was used to calculate the number of viable cells (22).

Thiourea incorporation. Thiourea incorporation into intact cells was determined as previously described (22). [^{14}C]thiourea (2×10^5 cpm) was added to cell cultures harvested from the appropriate stages. After the addition of amphotericin B, the culture was incubated at 22°C with shaking. After 2 h, a 0.2- to 0.5-ml portion was transferred to 2 ml of water, shaken vigorously, and filtered using a glass-fiber circle (Whatman, GF/C.) The filter was dried, and the amount of radioactivity retained on the filter was determined by liquid scintillation counting (22).

Binding of amphotericin B to cells. Cells were harvested from the required stage and resuspended in 10 ml of LPS at a final concentration of 1×10^6 cells/ml in a Corex tube (30 ml). Amphotericin B

was added to a final concentration of 400 $\mu\text{g/ml}$, and the tube was shaken for 5 min at room temperature. The control tubes contained cells but no drug. After the incubation, the cell titer was determined by counting a portion in a hemocytometer (Neubauer Bright Line) under $\times 200$ total magnification. No cell lysis occurred at the drug-cell ratio used under these incubation conditions in this part of the experiment. In separate experiments, it was shown that, although incubation for as long as 1 h did not influence the extent of binding, some (about 10%) of the cells did lyse. After the 5-min incubation, the cells were removed from the solution by centrifugation ($5,000 \times g$ for 5 min), and the supernatant (S-1) was recovered. In experiments performed without cells, it could be shown that all the drug remained in the S-1 fraction. In addition, to determine if any unbound drug remained with the pellet, it was resuspended in 10 ml of LPS, the centrifugation was repeated, and the supernatant (S-2) was recovered. This washing procedure was repeated twice, yielding supernatant fractions S-3 and S-4. Determinations of the drug concentration in each of the supernatants showed that over 95% of the drug was recovered in the S-1 fraction. The procedure used to measure the concentration of drug in the supernatants is the bioassay technique described below.

Bioassay procedure. The bioassay procedure employed cells from stationary growth phase as the indicator cells. Indicator cells were resuspended at a final concentration of 10^8 cells/ml in 1 ml of LPS in a 10-ml test tube. A 0.1-ml portion was transferred to 0.9 ml of S-1 in a 25-ml beaker, and the solution was incubated at room temperature. After 30 min, a 0.1-ml portion was transferred to 0.9 ml of glass-distilled water, and the number of cells present was determined by counting a portion of this suspension in a hemocytometer.

In the absence of drug, about 10% of the indicator cells were lysed by the dilution into distilled water. To relate the titer of indicator cells to drug concentration, a calibration curve was constructed by incubating indicator cells with drug over a concentration range of 0 to 400 $\mu\text{g/ml}$. After the 30-min incubation, the indicator cells were diluted into distilled water, and the cell titer was determined as described above. About 90% of the cells lysed at 400 $\mu\text{g/ml}$, whereas proportional numbers of the cells survived at the intermediate drug concentrations. The log of the cell concentration observed was plotted as a function of drug concentration, and a straight line was obtained. This curve was used to determine the drug concentration present in S-1 (S-2, -3, and -4; see above) after the addition of cells from various stages.

Chemical determinations. Proteins were determined by the Lowry procedure (16), using bovine serum albumin as standard. Neutral lipid content of purified membranes was determined as described (22, 24), using stigmasterol as standard. Control experiments showed that amphotericin B did not interfere with the neutral lipid determination using this procedure. Phospholipids were extracted as described by Bligh and Dyer (5), and phosphorous was determined according to the procedure of Ames (2).

RESULTS

Changes in susceptibility of cells during growth. To determine the effect of amphotericin B on cells during growth, cells were harvested from either the exponential or stationary phase and resuspended at 10^7 cells/ml in LPS buffer together with amphotericin B. After a 2-h incubation, portions were removed, and the concentration of viable cells was determined by the clonal assay. The data obtained shows that a greater number of viable cells are present in the stationary-phase culture (Table 1). In addition, cells harvested after 5 h on filters showed an even greater resistance to drug action (Table 1). These results suggest that growing cells are more susceptible to the drug and are consistent with a previous observation, which suggested that the susceptibility of the cells to the drug decreases with the growth rate (22).

Changes in susceptibility of cells during aggregation. The susceptibility of cells to the drug during aggregation was determined using both viability and thiourea incorporation. For these experiments, cells were harvested from filter pads during the initial 5 h of development and disaggregated by rapid pipetting, and the single cells were resuspended in LPS buffer together with amphotericin B. After a 2-h incubation with the drug, the concentration of viable cells and the amount of thiourea incorporation were determined. In Fig. 1, the fraction of cells remaining viable in the population is shown as a function of developmental time. The results indicate that, as aggregation proceeds, there is an increase in the concentration of viable cells. These data also show that, in the absence of the drug, the number of viable cells detected at each developmental time point remains constant. This indicates that the extent of disaggregation at each stage is the same. Therefore, the observed effect is not due to some

FIG. 1. Effect of amphotericin B on the viability of cells from different stages of aggregation. Cells were harvested from liquid culture at 2×10^6 to 4×10^6 cells/ml, washed in LPS, and resuspended at a final titer of 1.8×10^8 cells/ml. Portions (0.5 ml) were dispensed on Whatman no. 50 filters (presoaked in LPS) resting on absorbant pads (also soaked in LPS) in 60-mm plastic petri dishes. Cells were harvested from filters at intervals, washed and resuspended in LPS, and dispersed by repeated pipetting as described in Materials and Methods. Disaggregation to single cells could be achieved after a few passes through the pipette. The cells were diluted to 10^7 cells/ml and incubated with 200 μ g of amphotericin B per ml. After 2 h, the reaction was terminated by dilution, and the number of viable cells was determined by the clonal assay. Each point represents the results of three determinations. Symbols: survivors with ($\times$) and without ($\circ$) amphotericin B.

TABLE 1. Susceptibility of cells from different stages of growth and development to inactivation by amphotericin B

Stage	Survivors (%) ^a
Exponential growth, 1×10^6 to 2×10^6 cells/ml . . .	2
Stationary phase, 1×10^7 to 2×10^7 cells/ml	10
Preaggregation, 5 h on filters	90

^a Cells harvested from the required stage were resuspended at 1×10^7 cells/ml in LPS buffer with amphotericin B at a final concentration of 100 μ g/ml. After incubation at room temperature (22°C) for 2 h, a portion was diluted 1:100 into broth, and portions were transferred to an agar plate and spread together with *A. aerogenes*. Plates were incubated 3 days at 22°C, and the number of viable cells was determined by counting clones that were visible on plates.

effect of aggregation. In Fig. 2, the rate of uptake of thiourea is also shown as a function of developmental time. These results show that during aggregation, there is also a decrease in the uptake of thiourea. Thus, the results of both assays suggest that as cells proceed through the aggregation stage of development, there is an increase in resistance of the cells to the drug.

Lipid content of membranes from growing and aggregating cells. Although the exact mechanism by which amphotericin B induces changes in osmotic permeability has not been determined, available data suggest that the process requires the binding to sterols in the membrane as the first step (13). To determine if

the increased drug resistance could be accounted for by a decrease in membrane sterols, the total sterol content of plasma membranes, prepared from cells harvested from exponential growth, stationary phase, and after 5 h on filters, was determined. Values of approximately 7, 9, and 11 μg of protein per ml were obtained

FIG. 2. Effect of amphotericin B on thiourea (TU) incorporation of cells from different stages of development. Cells were allowed to develop on filters, harvested, and incubated at 22°C with amphotericin B as described in legend to Fig. 1. Thiourea (2×10^5 cpm) was added, and after 2 h, the cell-associated radioactivity was determined as described in Materials and Methods. Symbols: incorporation with (○) and without (Δ) amphotericin B.

for cells from exponential growth, stationary growth, and the aggregation stage, respectively (Table 2). These data show that there is a small, but significant, increase in the neutral lipid content of the plasma membrane as the cells progress from exponential growth to aggregation. This finding suggests that the increase in drug resistance observed for aggregating cells is not due to a decrease in the sterol content of the membrane.

Since it has been suggested that increased resistance of cells to polyenes is related to an increase in the phospholipid-sterol ratio (12, 25), both the phospholipid content of *D. discoideum* membranes and this ratio were determined. Table 2 shows these values. In contrast to the neutral lipid values, the phospholipid content of the membrane decreased while the phospholipid-neutral lipid ratio decreased from 8.3 for exponentially growing cells to 4.4 for aggregation-competent cells. This finding suggests that the increased resistance of aggregating cells cannot be explained by this mechanism.

Effect of aggregation on amphotericin B binding. Since polyene action requires the binding of the drug as the first step (13), the extent of binding by cells from the three representative stages was determined using the bioassay procedure described in Materials and Methods.

Table 3 shows the results of this comparison. The titer of indicator cells as well as the concentration of free drug, as determined from a calibration curve (see Materials and Methods), are

TABLE 2. Lipid content of *D. discoideum* plasma membranes from different stages

Stage	Lipid content ($\mu\text{g}/\text{mg}$ of protein, $\pm$ S.D.) ^a		
	Phospholipid	Neutral lipid	P/S ^b
Exponential growth, 1×10^6 to 2×10^6 cells/ml	64 ± 10.1	7.7 ± 1.2	8.3
Stationary phase, 1×10^7 to 2×10^7 cells/ml	54 ± 8.5	8.9 ± 1.6	6.1
Aggregation competent (5 h)	46 ± 11.2	10.5 ± 1.5	4.4

^a Lipid determinations were performed as described in Materials and Methods.

^b Ratio of phospholipid to neutral lipid.

TABLE 3. Extent of amphotericin B binding by cells from different stages

Stage	Titer of indicator cells ($\times 10^6$ cells/ml)	Concn of amphotericin B ($\mu\text{g}/\text{ml}$)	
		Free ^a	Bound ^b
Exponential growth, 1×10^6 to 2×10^6 cells/ml	$1.5 \pm 10\%$	200	200
Stationary phase, 1×10^7 to 2×10^7 cells/ml	$2.5 \pm 10\%$	115	285
Aggregation competent (5 h)	$3.1 \pm 10\%$	75	325

^a The concentration of free drug was determined from the indicator cells titer, and the calibration curve was constructed as described in Materials and Methods.

^b The bound drug concentration was calculated as the difference between the original (400 $\mu\text{g}/\text{ml}$) and free drug concentrations.

presented in Table 3. Also shown are the values obtained for the concentration of bound drug. Taken together, these data show no decrease in the extent of drug binding as cells progress from growth to aggregation. In fact, there appears to be an increase in the extent of binding, a result which might be explained by the increase in sterol content of the membrane shown in Table 2.

Effect of temperature on permeability changes of cells from different stages. Temperature has been implicated as a factor in polyene action (14, 15). Therefore, the permeability changes produced by the drug were studied as a function of temperature for cells from different stages. For these experiments, exponentially growing and aggregating cells were incubated with amphotericin B over a range of temperatures, and the permeability changes were determined by the amount of thiourea incorporated (23). The results shown in Fig. 3 indicate that for cells from the exponential growth phase, no permeability change occurs until about 6°C. Above this temperature, the permeability increases with increasing temperature until it reaches approximately 28°C, when no further increase occurs. In contrast, with aggregating cells, the increase in temperature results in only a minor increase in a permeability (Fig. 3). These data show that the permeability changes in growing cells are temperature dependent, whereas in aggregating cells they are not.

FIG. 3. Effect of temperature on rate of thiourea (TU) incorporation. Cells were incubated at indicated temperatures with amphotericin B and TU for 2 h as described in the legend to Fig. 2. Amount of cell-associated radioactivity was determined after a 2-h incubation as described in Materials and Methods. Symbols: TU uptake in cells harvested from exponential growth (Δ) and cells after 5 h on filters (□).

Effect of temperature on inactivation of cells from different stages. The effect of temperature on the ability of the drug to lyse cells from different stages was also studied. The rate of cell lysis, determined at each temperature, normalized to the rate obtained at 30°C (the maximum rate), is shown in Fig. 4 as a function of the reciprocal of temperature. These data show that, for growing cells, the rate of inactivation is temperature dependent, but that the increase in rate between 18 and 22°C is greater than that between 22 and 30°C. In contrast, aggregating cells show only a minor temperature dependence on the effect of the drug. Thus, at 30°C there is only a slight increase in the rate of inactivation as compared to the rate at 18°C, and there is no change in slope. These results show that temperature affects the rate of inactivation of growing cells but not aggregating cells.

Effect of temperature on drug binding. Since we showed above that the extent of drug binding by cells from the different stages at 22°C was similar, it was of interest to compare the binding values at 4°C. Consistent with previous observations (15), we found that the extent of binding at 4°C was about 1% the value obtained at 22°C and, in addition, there were no differences between the cells from the different stages.

DISCUSSION

The results of a previous study on the effects of amphotericin B on *D. discoideum* suggested that exponentially growing cells were one order of magnitude more susceptible to the drug than were cells from the stationary phase (22). The results of the present study confirm this observation and, in addition, show that during the aggregation process the cells become completely resistant to the drug.

Polyene action requires, as a first step, binding of the drug to sterols in the plasma membrane (13). Therefore, one possible explanation for the resistance of the aggregating cells to drug action might be a decrease in the accessibility or number of binding sites. However, direct measurements of amphotericin B binding showed no decrease in drug binding by aggregation-competent cells. In fact, a small, but significant, increase in binding by these cells was observed. This finding is consistent with the results of direct determinations of the sterol content of plasma membranes from the three stages. These results showed a small, but significant, increase in sterol content. These findings suggest that the acquisition of resist-

FIG. 4. Effect of temperature on rate of cell death. Cells were incubated with amphotericin B for 2 h at the indicated temperature as described in legend to Fig. 1. Symbols: number of survivors determined with exponential-phase cells (○) and with cells after 5 h on filters (□).

ance is not due to changes in accessibility; nor is the number of binding sites due to the extent of drug binding.

Although polyene action requires sterols for drug action, some types of sterol-containing cells (20) and organelles (12) are not lysed by this class of drugs. In a previous study, a correlation between cell lysis and the ratio of phospholipids to sterol was found (12). For example, it was shown that erythrocytes with a ratio of approximately 1 were lysed, whereas mitochondria with a ratio of approximately 40 were not (12). On the basis of this correlation, it was suggested that this ratio might be a factor regulating the effect of the drug (12, 25). As part of the present study, this ratio was determined for plasma membranes from *D. discoideum* cells at the three stages. Since we found that this ratio

decreased as the cells entered the aggregation stage, we conclude that the increased resistance of *D. discoideum* cannot be explained by changes in the phospholipid-sterol ratio. The results of a study on a lecithin membrane model system are consistent with the idea that polyene-induced permeability alteration is affected by the composition of the membrane phospholipid fatty acyl chains (9). Since we have not analyzed the compositional differences between growing and aggregating cells, we cannot rule out this explanation.

As part of the present study, we examined the effect of temperature on the drug-induced permeability changes using cells from two stages. With cells from the exponential growth stage, we find that decreasing the temperature produces a decrease in permeability. However, since a reduction in temperature also produces a decrease in drug binding, the effect of temperature on permeability might be explained by this result. In contrast, when aggregation-competent cells were studied, we found that temperature produced no significant change in permeability, even though it produces the same change in the extent of binding. This finding suggests that the temperature-dependent changes in permeability are mediated by events occurring after drug binding and that it is these steps at which the aggregation-competent cells are unable to complete. Based on previous studies which have implicated temperature as a factor affecting the rearrangement of sterol residues (7), the present results are consistent with the possibility that the sterol residues in the aggregating cell surface are more restricted in their mobility within the plane of the membrane than are those on the surface of growing cells. This interpretation is similar to one previously suggested to explain permeability changes in lipid bilayers (3).

The effect of temperature on the lytic effects of the drug was also studied. With exponentially growing cells, when the rates obtained are plotted as a function of the reciprocal of the temperature, an abrupt change in slope is observed at 22°C. Since a change in slope, such as that observed here, has been interpreted in terms of phase transitions of membrane components (17), this change could reflect alterations in the physical state of the membrane of exponentially growing cells, a change which apparently cannot occur in aggregating cells.

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