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Rifampin: Inhibition of Ribonucleic Acid Synthesis After Potentiation by Amphotericin B in *Saccharomyces cerevisiae*

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The inhibition of *Saccharomyces cerevisiae* growth by amphotericin B and rifampin was studied. Rifampin alone had no effect on growth or macromolecular syntheses. Lethal amounts of amphotericin B produced a late inhibition of ribonucleic acid (RNA) synthesis simultaneous with the arrest of growth and protein synthesis. In contrast, low doses of amphotericin B along with rifampin caused an early arrest of RNA synthesis, followed by a later arrest of growth and protein synthesis. Used with rifampin, amphotericin B thus appears to increase cell permeability for rifampin, which in turn inhibits RNA synthesis; such results are consistent with some reports of inhibition of yeast RNA polymerase function by rifampin. Experiments with petite mutants ruled out any special effect of the antibiotics on mitochondrial RNA synthesis, so that nuclear RNA synthesis is affected. Acrylamide gel analyses of RNA pulse-labeled after addition of the two antibiotics in synergy showed that synthesis of all major classes of RNA was progressively and uniformly inhibited.

The effectiveness of polyene antibiotics against eukaryotic cells is related to the presence of sterols in their membranes (see review in reference 7). It is not known how the interaction between the polyene antibiotics and the sterols leads to lethality, but at high doses, ions and macromolecular constituents leak out of the cell, ultimately causing death (7).

At low concentrations, polyenes do not kill cells but can still alter the cell membrane sufficiently so that it becomes more permeable to a variety of substances. As a result, synergism based on increased uptake into the cell of second antibiotics has already been shown to occur when low concentrations of amphotericin B, a polyene, are used with rifampin, fusidic acid, mycophenolic acid glucuronide, or tetracycline against fungi and animal cells (9, 11).

In the earlier studies (9, 11), specificity of the second agents was retained so that fusidic acid, for example, inhibited protein synthesis rather than ribonucleic acid (RNA) synthesis. However, the observed inhibitions were only partial, and the results were achieved only under very specialized conditions. To make the technique easier to use, we have studied and defined here the conditions for efficient potentiation by amphotericin B. We have found that to permit easy reproducibility of the experiments, the ratio of concentration of cell to antibiotics as well as the time of treatment must be controlled. Under these conditions, inhibition by rifampin can be total though reversible.

The synergism of amphotericin B and second agents is of interest for three reasons. First, it has obvious clinical implications. Second, the nature of the polyene-induced permeability can be studied at nonlethal levels. Third, it provides a way to study in whole cells the effects of agents to which fungal cells are normally impermeable. In the detailed example reported here, the effects of amphotericin B itself are compared with the effects of amphotericin B in potentiating rifampin. Documentation of this case is especially interesting because the effects of rifampin were unexpected, in that the in vivo inhibition of RNA formation is in contrast to some published reports of the insensitivity to rifampin of isolated yeast RNA polymerases (1, 8; see Discussion).

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MATERIALS AND METHODS

Yeast strains. In most of our experiments, we used *Saccharomyces cerevisiae* strain S288c (*mal, gal*-2). In experiments involving petite mutants we used the strain 11-1-40, which does not appear to have any mitochondrial deoxyribonucleic acid (5), and its respiratory competent parental A664a/18a (*mal, ura*-2).

Both were kindly provided from the stocks of J. Marmur. A664a/18a shows a behavior towards rifampin and amphotericin B entirely similar to that of S288c.

Culture conditions. Minimal medium contained, per liter, 1.7 g of yeast nitrogen base (Difco, without amino acids and ammonium sulfate), 5 g of ammonium sulfate, and 20 g of glucose; CNB medium contained the same ingredients plus 2 g of Casamino Acids (Difco, technical) per liter. As a rich medium we used YEPD (4) which contained, per liter, 10 g of yeast extract (Difco), 20 g of peptone (Difco), and 20 g of glucose. Cultures were incubated at 30 C with moderate shaking; the growth was followed either in a Klett colorimeter or as optical density at 750 nm. Cell counts were performed in a hemocytometer or by plating over solid YEPD (containing 15 g per liter of agar).

Drugs and radioactive chemicals. Rifampin was obtained from Gruppo Lepetit, Milano, Italy. Amphotericin B was used in the form of the solubilized commercial preparation Fungozone (Squibb). In addition to the antibiotic, this preparation contained sodium deoxycholate and sodium phosphate. Neither of these two components seemed to interfere with the synergistic response, as shown by appropriate controls. [^{14}C]uracil (52 mCi/mmol), [^{3}H]uracil (20 Ci/mmol), and [^{3}H]leucine (18 Ci/mmol) were from Schwartz BioResearch.

Other methods. For RNA extraction, the cells were rapidly chilled to 0 C, treated at this temperature with 1% (vol/vol) of commercial snail-gut enzyme (Glusulase, Endo Laboratories) for 10 min, and extracted with 2 volumes of phenol saturated with 2% sodium acetate, pH 5.4, at 60 C. The extraction was repeated three times, and the RNA was precipitated by 2 volumes of cold ethanol (−20 C). These conditions are adequate to give quantitative extraction of cellular RNA. Polyacrylamide gel electrophoresis was performed in 8-cm gels made up with 1.8% acrylamide and 0.5% agarose, at a constant current of 5 mA/gel, for 2.5 h (11). Protein was estimated by the Lowry procedure (10); RNA was estimated by an adaptation of the orcinol method (3) to yeast cells.

RESULTS

Response of yeast cultures to amphotericin B and rifampin. Yeast strain S288c was able to grow normally at any rifampin concentration up to the limit of solubility of the antibiotic. In contrast, amphotericin B alone produced an inhibition of the growth and ultimately caused death of the cells at concentrations of 0.2 μg/ml or higher.

To quantitate the response of yeast growth to the addition of different amounts of amphotericin B and rifampin, 1.1 ml of CNB medium, containing amphotericin B and rifampin, were inoculated with 2.2×10^6 cells from an overnight culture of stationary-phase cells. Growth was monitored by turbidimetry in a Klett-Summerson colorimeter adapted to read 1.2-ml cultures. Doubling times were calculated from the Klett readings fitted to least-square lines (Table 1). Results are expressed as percent doubling time relative to the control without antibiotics. It can be seen that the depression of the rate of growth depended largely on the amounts of antibiotics added, the inhibition ranging from 0 to 100%. Appropriate controls in this experiment showed no inhibition by any of these concentrations of either of the two antibiotics added to the medium in the absence of the other.

In a second type of experiment, 10 ml of CNB medium was inoculated with 2.2×10^7 cells from an overnight culture and incubated with moderate shaking at 30 C. The antibiotics were added at different times after exponential growth was started. Growth was again followed as optical density, now at 750 nm. The response of exponentially growing cultures seemed to be different from that of stationary cells. Instead of the gradual, uniform depression of the growth rate, either 100% inhibition or no effect was obtained, depending on the amount and time of addition of amphotericin B. Nevertheless, conditions could be controlled to give reproducible results. In all cases the action was fungistatic.

Figure 1 shows the result of an experiment in which the antibiotics were added to exponentially growing cells at different stages of growth. The quantity of amphotericin B required to obtain growth inhibition was dependent on the number of cells present in the culture. Any

TABLE 1. *Growth of yeast cultures in the presence of different amounts of amphotericin B and rifampin*

Rifampin (μg/ml)	Amphotericin B (μg/ml)[a]			
	0.08	0.04	0.02	0.002
50	0	14	34	91
20	1	20	30	96
10	1	11	39	107
5	7	21	48	107
2	7	52	65	98
1	28	66	76	105

[a] Numbers given are the growth rates of cultures, stated as a percent of the growth rate of a control culture in the absence of the antibiotics. Cultures containing the indicated amounts of antibiotics were inoculated with 2.2×10^6 cells. Growth was followed in a Klett colorimeter, and the doubling times were calculated from a least-square fit of the Klett readings. The doubling time of the control culture was 120 min. All cultures containing only one antibiotic at the indicated levels showed doubling times in the range of 97 to 110% of the control culture.

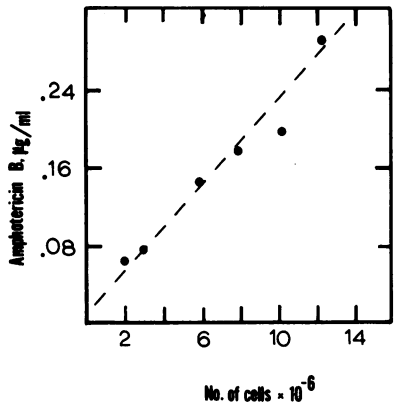

FIG. 1. *Minimal amounts of amphotericin B needed to inhibit the growth of cells in exponential culture. Exponential cultures (10 ml) in CNB medium contained 50 µg of rifampin per ml; they were divided into tubes containing different amounts of amphotericin B. Each point indicates the minimal amount of amphotericin B needed to block the growth of a culture containing that number of cells.*

amount of amphotericin B lower than those indicated failed to inhibit the growth. There was also a roughly linear relationship between cell input and amount of amphotericin B required to act synergistically with rifampin. Such a relationship is consistent with a requirement for many molecules bound on the surface of each cell.

The synergistic range of concentrations of amphotericin B also depends on the composition of the culture medium. For an input of 2.2 $\times 10^6$ exponentially growing cells, the effective range in minimal medium is about 0.04 to 0.08 µg/ml; in minimal medium containing 0.2% Casamino Acids (CNB medium), the range is 0.04 to 0.12; for cells growing in a complex medium (YEPD), the action of rifampin required 0.04 to 0.20 µg/ml. In every case, doses higher than the upper limit were effective by themselves, without the addition of rifampin, whereas amounts lower than 0.04 µg/ml did not display any effect at all either with or without rifampin.

The concentration of rifampin showed a much broader effective range. In general, doses higher than 20 µg/ml were effective at any cell concentration, provided that the right amount of amphotericin B was present.

One feature of amphotericin B action in this system is unusual for antibiotics: the sharpness of the dose-response for the lethal effect. Below a critical level, amphotericin B itself neither eliminates colony-forming capacity nor affects growth; above the threshold value lethality, defined as loss of colony-forming capacity, is observed; but no large range of intermediate, partially inhibitory concentrations were detected. The threshold value for lethality, like the lower threshold value required for potentiation of rifampin, is proportional to the number of cells.

Effects of amphotericin B and rifampin on RNA and protein synthesis. Studies of the effects of these antibiotics on macromolecular synthesis were carried out with cells in the exponential phase, where synthesis was most active.

When a culture was treated along with low doses of amphotericin B, rifampin caused an inhibition of the growth of yeast after approximately 90 min. This inhibition of growth was paralleled by an arrest in protein synthesis, as measured by leucine incorporation or by the Lowry method (10) (Fig. 2 and 3); however, the incorporation of uracil and the orcinol reaction seem to be inhibited about 1 h earlier, being obvious 30 min after the addition of the antibiotics.

At higher (lethal) doses of amphotericin B, no such specificity was observed; the polyene itself shut down macromolecular syntheses and growth at comparable rates (Fig. 4). The nonspecific effect of high doses of amphotericin B

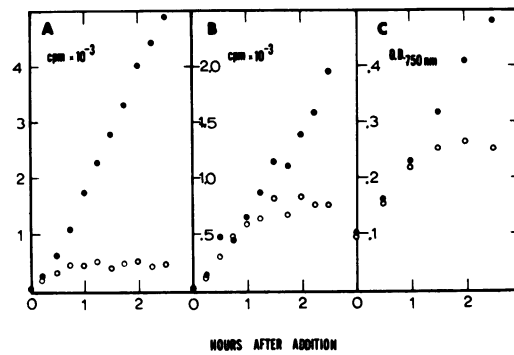

FIG. 2. *Effect of amphotericin B and rifampin on RNA and protein synthesis. A 90-ml sample of an exponentially growing culture of S288c in CNB medium, at an optical density (750 nm) of 0.1, was treated with 200 µg of rifampin and 0.08 µg of amphotericin B per ml. At the same time, the culture was labeled with 0.1 µCi of [^{14}C]uracil and 0.5 µCi of [^{3}H]leucine per ml. At the times indicated, 1-ml samples were removed and assayed for (A) cold acid-precipitable ^{14}C radioactivity; (B) hot acid-precipitable ^{3}H radioactivity; and (C) optical density at 750 nm. Symbols: ●, Control without antibiotics; ○, culture containing both antibiotics; controls containing only one of the two antibiotics (not shown) were identical to that without additions.*

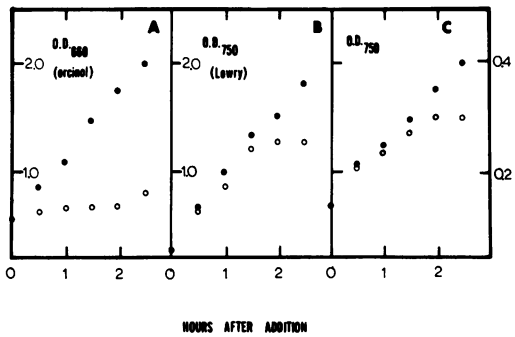

FIG. 3. *Effect of amphotericin B and rifampin on RNA and protein accumulation. An 80-ml sample of an exponential culture of S288c in CNB medium, at an optical density (750 nm) of 0.1, was treated with 200 μg of rifampin and 0.08 μg of amphotericin B per ml. At the times indicated, 1-ml samples were removed from the culture to determine (A) RNA content by the orcinol method; (B) protein by the Lowry method; (C) optical density at 750 nm. Symbols are as in Fig. 2. Controls containing either of these antibiotics alone (not shown) were identical to the control without antibiotics.*

(Fig. 4) are thus clearly distinguishable from the specific effects of rifampin (Fig. 2 and 3 and Discussion).

The early inhibition of RNA synthesis by rifampin does not seem to be based on a specific inhibition of mitochondrial RNA synthesis. Table 2 shows rates of growth of normal yeast cells and petite mutants in the presence of antibiotics. It can be seen that the inhibition rates were comparable in the two cases. Moreover, normal cells growing in conditions of mitochondrial depression (by using a nonfermentable carbon source such as glycerol) also showed the same inhibition levels by these two antibiotics.

Effect of amphotericin B and rifampin on the synthesis of different RNA species. Studies of yeast RNA polymerases in vitro (1) suggest differential effects of rifampin derivatives on the various enzyme fractions. We were interested in determining whether rifampin selectively inhibited the synthesis of a particular fraction of RNA in vivo. This might permit the assignment of distinct functions for some polymerases. To detect all of the stable RNA classes, a growing yeast culture was labeled for two generations with [^{14}C]uracil. Then the culture received 250 μg of rifampicin and 0.08 μg of amphotericin B per ml. At each of the times indicated in Fig. 5, the newly formed RNA was then pulse labeled with [^{3}H]uracil for 7 min. The RNA was extracted and analyzed by polyacrylamide electrophoresis. The pulse-labeled profile differed from the long-term label pattern in two main respects: it contained a peak probably identifiable with the 35*S* ribosomal precursor (12) and also a heterogeneous region running faster than the 18*S* ribosomal RNA, which probably contained messenger RNA sequences. The total amount of pulse-labeled RNA decreased about fivefold as the rifampin and amphotericin B treatment took effect (note change in scale for the tritium label), but the profile of rapidly labeled RNA remained unchanged (Fig. 5). In Fig. 6, the amounts of the different fractions are plotted as percentages of total radioactivity. These percentages remained constant during the experiment, suggesting that rifampin is not selective for any particular RNA fraction.

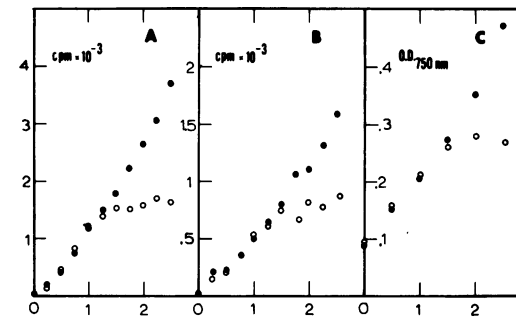

FIG. 4. *Effect of a lethal amount of amphotericin B on RNA and protein synthesis. Experimental conditions were as in Fig. 2, except that no rifampin was added and the amphotericin B was added at a concentration of 0.3 μg/ml.*

TABLE 2. *Growth of petite mutants and glucose-repressed cells in the presence of rifampin and amphotericin B*

Rifampin (μg/ml)	Amphotericin B (μg/ml)[a]			
	A[b]		B[c]	
	0.08	0.04	0.08	0.04
50	0	21	0	16
10	8	46	11	26

[a] Numbers are given as in Table 1. In each case, 1.1 ml of CNB medium in the indicated conditions was inoculated with 2.2 × 10^6 cells, and growth was followed on a Klett colorimeter. The control doubling time was 145 min for (A) and 130 min for (B).

[b] Strain 11-1-40, CNB medium.

[c] Strain s288c growing in CNB medium without glucose and with 2% glycerol.

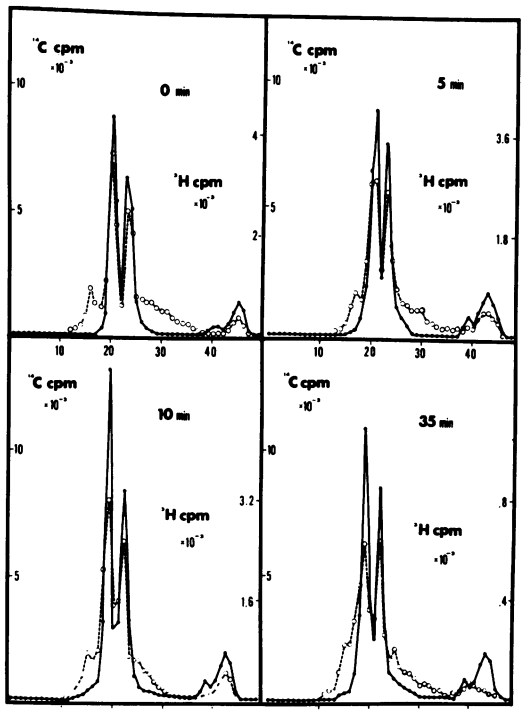

FIG. 5. *Polyacrylamide gel electrophoresis of RNA synthesized in the presence of rifampin and amphotericin B. An exponentially growing culture was labeled with [^{14}C]uracil during two generations. Then synergistic levels of amphotericin B and rifampin were added. At the time indicated in the figure, 10-ml samples were pulse labeled with [^{3}H]uracil for 7 min. The RNA was extracted from the cells and analyzed in polyacrylamide gels as in Materials and Methods, and the gels were sliced and counted in a Packard scintillation spectrometer. Symbols: ●, Long-term-labeled [^{14}C]RNA; ○, pulse-labeled [^{3}H]RNA.*

DISCUSSION

From other work we know that polyenes act synergistically with many inhibitors, unrelated to rifampin both in structure and mode of action (9). Furthermore, where the potentiated agents have had a specific action in vitro, the specificity was retained in the amphotericin-treated cells; for example, fusidic acid and tetracycline selectively inhibited protein synthesis, whereas actinomycin D inhibited RNA formation (9). One would infer that in the case of rifampin as well, the polyene has the same basic effect—to increase permeability without changing specificity. At higher levels the polyene is lethal; that is, treated cells can no longer form colonies. Whether the sharp threshold of concentration required for lethality results from the same process that permits potentiation of second agents is unknown. However, it is suggestive that the potentiation and lethal effects both show a minimal threshold of requirements for amphotericin, and the amount is proportional to the number of cells in the culture.

All of the results here are consistent with the notion that rifampin was affecting cell RNA synthesis: amphotericin at high concentrations was fungicidal, with no specificity for the inhibition of RNA or protein synthesis. By contrast, rifampin potentiated by amphotericin B was fungistatic and inhibited RNA synthesis as an early event, well before growth was inhibited. It may be worthwhile to note that at the levels of amphotericin B usually used clinically (2), it is usually fungistatic. The combination with rifampin may be useful by lowering the concentration of amphotericin B required for this effect.

Specific effects of rifampin on polymerase function, and specifically on RNA formation, were first observed with bacteria, and it was believed for some time that RNA synthesis in eukaryotic cells was completely resistant to this antibiotic. Our results suggest that at least part of the resistance of the yeast cells and animal cells is based on a permeability barrier.

The data cannot exclude that the reduction in RNA formation may result from an accelerated rate of RNA breakdown; but an increased rate of breakdown that would apply to all fractions of RNA (Fig. 5) seems unlikely. Further support for an inhibitory effect on RNA formation in

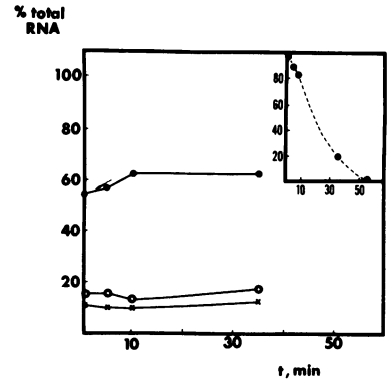

FIG. 6. *^{3}H radioactivity in different regions of the gels of Fig. 5, expressed as a percentage of total ^{3}H radioactivity. Symbols: ●, Radioactivity in the ribosomal RNA peaks; ○, radioactivity in the 18S to 10S region; ×, radioactivity in the 35S ribosomal precursor region. Insert: Total incorporation of [^{3}H]uracil as a function of time after addition of the antibiotics, expressed as percent of the pulse-labeling incorporation at time zero.*

vivo comes from studies of Venkov et al. (13), who have worked with sorbitol-dependent variants of yeast that are themselves sensitive to high levels of rifampin. Strong inhibition of RNA formation was observed in cells and in isolated nuclei of the sorbitol-dependent strain (P. Venkov, personal communication). In this case, the effects of rifampin are both clearly on RNA synthesis and clearly independent of the action of amphotericin B.

However, the uniform inhibition of nuclear transcription may not be a direct effect on yeast polymerases. In vitro data have shown that most RNA polymerase fractions do not show sensitivity to rifampin (1, 8). Adman et al. (1) have studied the effect of rifampin on RNA-polymerizing fractions isolated from yeast, and determined that it had no effect. Kuo et al. (8) have reported that rifampin completely inhibits one of three peaks of yeast polymerase activity. The apparent conflict of the in vivo and in vitro data is of interest, and its basis remains to be resolved. The degree of inhibition of isolated polymerase may reflect losses or changes in fractions during purification. Alternatively, the inhibition of RNA synthesis may occur by an effect on a component other than RNA polymerases.

The major deficiency in using amphotericin B to potentiate rifampin action is that the conditions must be clearly controlled, and inhibition of RNA synthesis in growing cells occurs only after a 30-min lag. Very likely, the lag is caused by the need for adequate binding of polyene to membrane sites, which may vary in kinetics from stationary-phase to exponential-phase cells. The resolution of the technique is thereby limited.

The advantage of the technique is that once conditions are controlled, agents like rifampin can be introduced into cells to study their physiological effects. For example, when RNA synthesis has been blocked in yeast, protein formation continues for about 60 min more (Fig. 2). This suggests that messenger RNA can function with a half-life of about 20 min in these cells, an estimate similar to that obtained by other indirect means (6). It should be possible to extend these studies to measurement of polysome breakdown and to the use of differential inhibitors of one of another RNA polymerase.

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