Amphotericin B is the main chemotherapeutic agent for the treatment of systemic mycoses (1). Its action depends on its relatively selective binding to the fungal cell membrane in preference to animal cell membranes (2, 3). Inspite of well described events which occur after amphotericin B is bound to fungal cell membranes, such as potassium and amino acid efflux from the cells (4, 5), the kinetics of amphotericin B killing has not been well described. There is some evidence that after brief exposures of Candida cells to 50 mcg/ml of amphotericin B, the oxygen consumption decrement is not reversed after a two hour period of observation (4). Whether the organisms eventually recovered from this effect is not known. Furthermore, the kinetics of amphotericin B damage to many other pathogenic yeast species is unknown.

An understanding of amphotericin B kinetics of cell damage is less important in the therapy of systemic mycoses since the intravenous use of the drug provides a fairly constant and acceptable blood level after intermittent infusions (6, 7). However, the nature of the kinetics may have considerable clinical relevance because amphotericin B and nystatin (a compound with a similar mechanism of action) have been advocated in intermittent oral therapy of gastrointestinal luminal fungal infections (8) and in urinary bladder infections (9) where the drug's contact with the yeasts may be only a few seconds. For these reasons we studied the rate of amphotericin B killing of yeast cells, the reversibility of amphotericin B effects on the yeast cells, and the effect of amphotericin B concentration on the kinetics of yeast cell death.

Materials and Methods

**Yeast strains:** Torulopsis glabrata was a urine isolate from an infected patient. Candida albicans SC 5314 was obtained from Squibb Pharmaceutical. The two strains of Cryptococcus neoformans were cerebrospinal fluid isolates from two patients with meningitis. The minimum inhibitory concentrations of the yeasts studied were determined according to the method of Shadomy and Espinal-Ingraff (10, 11).

**Yeast killing kinetics:** Amphotericin B powder was obtained from Squibb Pharmaceutical and dissolved in N', N'-Dimethylformamide at a concentration of 1,000 mcg/ml and stored at -70°C for a maximum of one month before use. Overnight cultures of the yeasts in brain heart infusion broth (BHI) at 30°C were adjusted to a final concentration of 1.5 x 10⁵ cells/mm³. Amphotericin B was added to the cells, and aliquot samples were removed after 0, 60, 120, and 180 minutes of incubation to determine the number of viable organisms by surface streaking on Sabouraud dextrose agar plates. One ml aliquots from various times were also washed on a 25G Milli-

**Summary:** Little is known of the kinetics of yeast cell killing by amphotericin B. This is important in intermittent topical therapies using the polyene antibiotics. Our studies demonstrated that higher concentrations of amphotericin B gave higher killing rates for one strain of Candida albicans, Torulopsis glabrata, and two strains of Cryptococcus neoformans. Washing the amphotericin B-treated yeast cells numerous times did not nullify the effects of the drug. Morphological changes were not demonstrated by electron microscopy when most of the cells were nonviable and efflux of essential nutrients from the yeast cells had occurred. These observations explain the success of intermittent topical treatment of yeasts with the polyene antibiotics, situations where brief exposure of the yeast cells to high concentrations of the drug can be effective.

pore filter by suction filtration with 20 ml of 4°C distilled water and resuspended in one ml of the same. All procedures were performed aseptically using 12×75 millimeter plastic tubes (Falcon).

**Electron microscopy:** Candida albicans and Cryptococcus neoformans from this experiment were examined by electron microscopy after exposure to amphotericin B for various periods of time. The yeast cells were prepared by suction filtration washing on a Millipore filter with 20 ml of 0.01 molar pH 7.2 phosphate buffer. The cells were then fixed with 4% glutaraldehyde and electron microscopy was performed by glutaraldehyde and osmium tetroxid fixation (12).

**Nutrient efflux:** Extracellular leakage of amino acids was measured by first labeling yeast cells in the log phase of growth with 10 microcuries of tritiated mixed amino acids (Amersham) for 30 minutes and then washing the cells with 5 ml of distilled water five times. After exposure of these labeled cells to amphotericin B, equal aliquots were taken at various times and put on a Millipore filter and washed by suction filtration using 20 ml of 4°C distilled water. The radioactivity remaining on the filter represented amino acids and newly synthesized proteins that still were within the yeast cells. The filters were counted in a Parker Tricarb Model 3390 liquid scintillation counter.

**Results**

The yeast strains studied showed variable susceptibility to amphotericin B. Torulopsis glabrata, Candida albicans SC 5314, and the two Cryptococcus neoformans were inhibited by 0.125, 0.03, 0.06 and 0.06 mcg/ml of amphotericin B respectively.

The generation times for the four yeasts studied in BHI at 35°C were approximately one hour. With the addition of amphotericin B, the initial killing of the yeast cells is logarithmic with 50% reduction in viable cells approximately every 20 minutes. An increased rate of killing was found when a higher concentration of amphotericin B was used or when the yeasts tested had a lower MIC to amphotericin B.

The percentage of viable cells lost from the Millipore filter and the wash procedure was individually determined for each yeast at approximately 10^3 cells per ml and 10^5 cells per ml. The loss of yeast cells was reproducible at both concentrations of yeasts for all the species and was determined to be between 21% and 23% (Table 1).

**Table 1: Yeast cell loss by washing technique.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Yeast per ml</th>
<th>Before filter</th>
<th>After filter</th>
<th>Percent loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>1.5×10^1</td>
<td>1.47×10^1</td>
<td>1.17×10^1</td>
<td>21</td>
</tr>
<tr>
<td>Torulopsis glabrata</td>
<td>1.2×10^2</td>
<td>1.22×10^2</td>
<td>0.95×10^2</td>
<td>22</td>
</tr>
<tr>
<td>Cryptococcus</td>
<td>6×10^3</td>
<td>5.43×10^3</td>
<td>4.30×10^3</td>
<td>21</td>
</tr>
<tr>
<td>neoformans</td>
<td>6×10^3</td>
<td>5.76×10^3</td>
<td>4.44×10^3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>4×10^3</td>
<td>3.70×10^3</td>
<td>2.86×10^3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>3×10^6</td>
<td>3.00×10^6</td>
<td>2.35×10^6</td>
<td>22</td>
</tr>
</tbody>
</table>

In all instances, washing the cells with cold distilled water with the thought of washing off the amphotericin B to rescue the cells was unsuccessful. The washed cells showed the same rate of survival as the unwashed cells exposed to the same concentration of amphotericin B for the same period of time. The organisms unexposed to amphotericin B showed growth on Sabouraud dextrose agar after 18 hours, but the exposed organisms that survived did not show growth until 42 hours and the morphology and size of the colonies were very variable.

**Figure 1:** Torulopsis glabrata killing using 2.0 µg/ml of amphotericin B: Control without amphotericin B (A), no washing after exposure (B), washed after exposure (C).

**Figure 2:** Cryptococcus neoformans strain 1 killing by amphotericin B 2.0 µg/ml: Control without amphotericin B (A), no washing after exposure (B), washed after exposure (C).

Figure 1–4: Figure 1, 2, 3 and 4 illustrate the kinetics of yeast cell growth and killing by amphotericin B, and the ineffectiveness of washing in rescue of the yeast cells. After the viable yeast cells were exposed to tritiated amino acids 75% of the radioactivity was taken up intracellularly. When these labeled cells were exposed to amphotericin B, there was rapid loss of the radioactivity from the cells (Figure 4). At the end of two hours of amphotericin B exposure nearly all of the amino acids and newly synthesized proteins (measured by radioactivity) were no longer intracellular.