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Effect of amphotericin B on Candida albicans

Edward Carney

The University of Montana

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EFFECT OF AMPHOTERICIN B ON CANDIDA ALBICANS

By

Edward M. Carney

B. S., Southern Connecticut State College, 1965

Presented in partial fulfillment of the requirements for the degree of

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UNIVERSITY OF MONTANA

1968

Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

AUG 2, 1968

Date
ACKNOWLEDGMENT

The author wishes to express his sincere appreciation for the guidance and advice received from Dr. John J. Taylor, Associate Professor, Department of Microbiology, University of Montana, during the course of this investigation.
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CHAPTER I

INTRODUCTION

*Candida albicans* (Robin) Berkhout is a moniliaceous fungus of the subfamily Cryptococcoideae, which can exist as a budding yeast-like cell (blastospore) or a branched pseudomycelium with whorled blastospores and terminal chlamydospores. Under "normal" conditions, the organism exists as a saprophyte on the skin and mucous membranes of man. *C. albicans* can, however, under certain conditions, become mycelial in the host and assume the role of a pathogen.

The recognized pathogenicity of *C. albicans* dates back to the mid-nineteenth century at which time it was reported to be implicated in oral thrush (Langerbeck, 1839) and vaginitis (Wilkinson, 1849).

Since the early 1940's, clinical candidal infections have been reported on the increase (Kashkin et al., 1961) and systemic candidosis has become a recognized diagnostic and therapeutic problem (Wolfe and Henderson, 1951; Pearl and Sidransky, 1960; Hyun and Collier, 1960; Kay et al., 1961). From 1940 until April, 1966, there were 41 reported cases of candidal endocarditis with only five reported survivals among them (Utz, 1966). Although candidal infection of the brain, heart, lung, and other deep structures is not common, it is important because the infection is usually associated with a fatal outcome.

The increase in candidal infections may in part be attributed to the development of more accurate diagnostic techniques for the detection and identification of the organism (Rosenthal and Furnari, 1958;
Taschdjian et al., 1960; Walker et al., 1960). However, since 1940 antibiotics used in the treatment of bacterial infections and corticosteroids used in correcting metabolic disturbances have been shown to predispose an individual to a superimposed fungal infection (Kashkin et al., 1961; Woods et al., 1951; Young et al., 1956).

The mechanism of antibiotic predisposition of mucous membranes has been attributed to an inhibition of the normal bacterial flora (which normally maintains low numbers of C. albicans by competition, antibiosis, etc.) with a subsequent proliferation and invasion of the mucosa by C. albicans (Gorzczca and McCarty, 1959; Isenberg et al., 1960; Young et al., 1956; Pain, 1958).

In the case of the corticosteroids, the mechanism of predisposition has been attributed to their effect on the reticuloendothelial system resulting in decrease in antibody production, in the inflammatory response process, and in phagocytosis (Kass et al., 1955; Germuth and Ottinger, 1950; Crepea et al., 1951; Bjørneboe et al., 1951; Michael and Whorten, 1951). Corticosteroids may also affect nonspecific resistance (Seelig, 1966).

Certain disease processes and metabolic disorders which directly or indirectly decrease host resistance may predispose an individual to candidosis. A positive correlation of abnormalities of leukocyte metabolism with impaired phagocytic capacity has been demonstrated in cases of diabetes, leukemia, and corticosteroid therapy (Seelig, 1966).

The pathogenicity of C. albicans has been attributed to its ability to invade tissues in the mycelial form (Young, 1958) and to the production of toxic metabolites including an endotoxin (Salvin, 1952).
The production of chlamydospores by \textit{C. albicans} has also been associated with the pathogenicity of the organism in endocarditis (Heineman \textit{et al.}, 1961).

There have been a great many agents employed in the treatment of superficial candidal infections: undecylenic acid (Kendall, 1947), various fatty acids (Van Assen, 1950), caprylic acid (Lapan, 1959), gentian violet (Hesseltine, 1955), candidicidin (Fox, 1955), nystatin (Jennison and Llywelyn-Jones, 1957), and sodium caprylate (Watt \textit{et al.}, 1962). Some of these agents have been more successful and aesthetically more acceptable than others. However, there were two major problems which plagued the therapeutic use of the early antifungal agents in cases of systemic candidosis:

a. the antifungal agent produced serious side effects in the host;

b. the antifungal agent was toxic to the host in the concentration needed to exert its fungistatic or fungicidal effects on the fungus.

It was not until the discovery of the polyene antibiotics in 1949 (Hazen and Brown, 1950) and more specifically amphotericin B in 1956 (Gold \textit{et al.}, 1956) that there appeared an effective agent for the treatment of systemic candidosis with a minimal incidence of serious side effects in the afflicted host.

Amphotericin B was isolated from \textit{Streptomyces nodosus}, which was recovered from a soil sample in the area of the Orinoco River in Venezuela (Gold \textit{et al.}, 1956). The antibiotic was characterized physically and chemically in 1956 (Vandeputte \textit{et al.}, 1956; Donovick \textit{et al.}, 1956).
The mode of action of amphotericin B has been attributed to its ability to bind to the cell wall and cell membrane which subsequently results in an alteration in cell membrane permeability. Accompanying this alteration in cell membrane permeability is a loss of anions, cations and acids of the citric acid cycle. Terminally, cell respiration and glycolysis cease (Marini et al., 1961; Kinsky, 1961; Gale, 1960).

The primary problem which had to be overcome in order for amphotericin B to be used therapeutically was its insolubility in water. This problem was overcome without loss of fungicidal activity by complexing amphotericin B with sodium desoxycholate (Louria, 1958; Bartner et al., 1958).

Amphotericin B has been used successfully in the therapeutic treatment of candidal endocarditis (Prinsloo and Pretorius, 1966; Kroetz et al., 1962), meningitis (Parillo et al., 1962) and septicemia (Sweeney and Dineen, 1960; Louria and Dineen, 1960). However, in some cases where a concentration of the antibiotic which inhibited growth of C. albicans in vitro was reached, it appeared to be ineffective in combating the systemic candidal infection (Kay et al., 1961).

Concentrations of amphotericin B given intravenously in excess of 1.5 mg/kg generally produce serious side effects which may adversely affect the outcome of the treated infection.

The present research problem is addressed in part to the question: Has Candida albicans resistance to concentrations of amphotericin B which would be toxic in a systemic human infection?
Lones and Peacock (1959), Sorenson et al. (1959), Littman et al. (1958a), Lepper et al. (1959), Seabury and Dascomb (1958), Hebeeka and Solotorovsky (1965) and Utz et al. (1959) have investigated the inhibitory effects of amphotericin B on *C. albicans*. The concentrations which were reported to be fungistatic ranged from 0.2 to 3.7 μg/ml (Seabury and Dascomb, 1958; Littman et al., 1958a). Such wide variation may have been expected since most of the reports cited differed in the assay medium used for the determination of the minimal inhibitory concentration, the number of organisms used in the assay, the time after which the inoculated assay tubes were read, the method used to determine the per cent of inhibition of amphotericin B on *C. albicans* and the defined inhibitory point measured.

Littman was the only investigator of those cited who reported a fungicidal dose of amphotericin B for a strain of *C. albicans*. This observation formed the basis for the second part of the research problem: the determination of the fungicidal concentration of amphotericin B for 6 clinical isolates of *C. albicans*.

In the selection of naturally occurring resistant mutants to amphotericin B, Lones and Peacock (1959) reported that 12 strains of *C. albicans* grown for 52 serial transfers in culture media containing graded concentrations of amphotericin B all experienced an increase in tolerance to the antifungal agent. Accompanying this increase in tolerance to amphotericin B was a depressed growth rate among the derived strains and a decreased ability to form mycelium and chlamydomospores on corn meal and rice-polysorbate 80 medium. This observation formed the basis for a third area of research to be considered in this investigation:
Does tolerance to amphotericin B result in a decrease in the ability of *C. albicans* to produce the characteristics associated with the pathogenicity of the parent strain, i.e., yeast-mycelium transformation and chlamydospore production?

Finally, the author wished to determine the effect of amphotericin B on an immunologically depressed host and to determine if there is any correlation between immunological depressed, and the outcome of the amphotericin B-treated systemic candidal infection.
Candida albicans is a moniliaceous fungus which may assume the role of a systemic pathogen. This study was undertaken (1) to investigate the fungistatic and fungicidal properties of amphotericin B on C. albicans; (2) to determine if characteristics associated with the pathogenicity of C. albicans are affected by exposure of C. albicans to fungistatic amounts of amphotericin B, and (3) to determine if a decreased host resistance affects the outcome of an amphotericin B-treated candidal infection.
CHAPTER III

METHODS AND MATERIALS

(1) Organisms Employed

Six strains of Candida albicans which were isolated from clinical cases of vaginitis were employed in this investigation. The strains were designated 1B, 2A, 3A, 5A, 6C, and 7A, respectively.

(2) Isolation of the Organism

Primary isolation of the clinical isolates was made on Pagano-Levine medium (Pagano et al., 1958). Colonies which appeared creamy-white or faintly pink in color (Gillespie et al., 1960) were transferred to Sabouraud dextrose broth and then restreaked for isolation on Pagano-Levine medium.

(3) Identification of the Isolates as Candida albicans

The identification of the isolates as Candida albicans was based on the appearance of the organisms on Sabouraud agar, blood agar, and corn meal agar with 1% Tween 80 (Walker et al., 1960). The ability of the organisms to produce germ tubes in human serum within 4 hours (Taschdjian et al., 1960) and agglutinate in C. albicans antiserum (Rosenthal and Furnari, 1958) were also considered as criteria for the establishment of the identity of each isolate. If an isolate appeared as a white opaque budding yeast on Sabouraud and blood agar within 48 hours, produced a pseudomycelium with whorled blastospores and terminal chlamydospores on
corn meal agar with 1% Tween 80, produced germ tubes in human serum within 4 hours and agglutinated in C. albicans antiserum, the isolate was identified as C. albicans.

(4) Culture Media

Stock cultures of C. albicans were grown and maintained on modified Sabouraud agar (2% glucose, 1% peptone, 1.5% agar) which was buffered to pH 7 with 2N NaOH. After incubation of the inoculated Sabouraud slants at 37°C for 48 hours, they were refrigerated at 5°C and a duplicate frozen at -80°C. The refrigerated stock cultures were transferred monthly and periodically checked for contamination microscopically and by streaking on blood agar. Modified Sabouraud agar made up in 0.001 M potassium phosphate buffer to pH 6.7 was used as the medium for all viable cell counts.

Corn meal agar with 1% Tween 80 was prepared by adding 17 gm of (Difco) corn meal agar to 990 ml of distilled water. The medium was then heated until all components were completely dissolved. Before autoclaving the medium, 10 ml of Tween 80 were added to the flask.

Modified Sabouraud broth which was made up in 0.001 M potassium phosphate buffer to pH 6.7 was used as an assay medium.

Trypticase soy broth was prepared according to the BEL Manual (Baltimore Biological Laboratory, Inc., Baltimore, Maryland, 1956). Blood agar was prepared by adding 50 ml of sterile human blood aseptically to a liter of trypticase soy agar which, after having been autoclaved was cooled to 50°C in a water bath. The mixture
was swirled to obtain a uniform distribution of the blood and then dispensed into sterile petri plates and allowed to solidify. Pagano-Levine agar was prepared according to the Difco Literature Supplement (1964).

Human plasma was obtained by centrifugation and filter-sterilization of outdated unhemolyzed human blood obtained from the blood bank of Saint Patrick Hospital, Missoula, Montana.

(5) Chemicals

Hydrocortisone acetate was obtained as a sterile suspension from Towne, Paulsen and Company, Inc., Monrovia, California. The suspension which contained 25 mg/ml was used undiluted directly from the vial as recommended by the pharmaceutical company. Amphotericin B* was obtained as a sterile lyophilized powder from E. R. Squibb and Sons, New York. Each vial contained 50 mg of amphotericin B which was complexed with sodium desoxycholate (Bartner et al., 1958). The amphotericin B was refrigerated and reconstituted with sterile distilled water just prior to use. The hydrated unused amphotericin B was stored at 5°C for no longer than one week or frozen at -20°C and kept for no longer than three weeks.

Neomycin sulfate and triphenyl tetrazolium chloride were obtained in powder form from Nutritional Biochemical Corporation.

* The trade name of E. R. Squibb and Sons for amphotericin B Fungizone.
(6) **Mice**

Mice used in this investigation were male adult white mice of the RML strain obtained from the Rocky Mountain Laboratory, U.S.P.H.S., Hamilton, Montana. The average weight of the mice used in the investigation was 28.5 gm.

(7) **Counting Method**

Five 1:10 serial saline dilutions were made of a cell suspension standardized to 60% transmittance at a wave length of 660 mu. One-tenth ml aliquots of the standardized suspension were pipetted in duplicate from each dilution on to modified Sabouraud agar. The inoculum was uniformly spread over the surface of the agar with a sterile bent glass rod (hockey stick). The plates were inverted and incubated at 37°C for 48 hours. After incubation, colonies were counted with the aid of a Coulter colony counter and the number of organisms per ml calculated.
Experiment 1. Determination of the fungistatic and fungicidal dose of amphotericin B for six strains of C. albicans.

Forty-eight hours prior to the start of the investigative procedure, each of the 6 strains of C. albicans was inoculated on modified Sabouraud agar and incubated at 37°C.

Screw-capped tubes, 16 x 150 mm, were filled with 8.5 ml of modified Sabouraud broth. The tubes were autoclaved at 15 psi for 15 minutes, and then refrigerated until just prior to use.

Immediately before use, 1 ml volumes of the desired concentration of the amphotericin B were added to the test tube series bringing the total volume before inoculation of the test strain of C. albicans to 9.5 ml.

Each strain of C. albicans was washed from a 48-hour slant culture with physiological saline (0.85% NaCl). Each washed cell suspension was placed in a calibrated 16 x 150 mm screw-capped tube and inserted into a Coleman Model 6A spectrophotometer. The cell suspension was adjusted to 60% transmittance at a wave length of 660 nm. After adjustment of the cell suspension, 0.5 ml was used as inoculum. Each series was run in duplicate.

Viable cell counts of the standardized cell suspensions were made on modified Sabouraud agar.

The optical density of all tubes was recorded at time zero and
Figure 1. Inhibitory activity of different concentrations of amphotericin B on the isolates of *C. albicans*. The isolates represented above were designated as follows: 1A (•); 6C (△); 1B (■); 2A (○); 7A (○); 5A (△).
Table I. Effects of amphotericin B on growth of 6 isolates of *Candida albicans* in modified Sabouraud broth.

<table>
<thead>
<tr>
<th>Concentration of Amphotericin B (µg/ml)</th>
<th>Isolates examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1B</td>
</tr>
<tr>
<td>0.1</td>
<td>65.7*</td>
</tr>
<tr>
<td>0.2</td>
<td>10.9</td>
</tr>
<tr>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* O.D. experimental/O.D. control.

again in 48 hours. After 48 hours, samples from each tube were streaked onto blood agar which was then incubated at 37°C for 48 hours. Yeast phase cells from positive blood agar plates were inoculated into human serum, incubated at 37°C for 48 hours and then checked microscopically for the production of germ tubes.

The results of this investigation appear in Figure 1 and Tables 1 and 2. Concentrations of amphotericin B ranging from 0.1 to 0.5 µg/ml affected the growth of the 6 strains of *C. albicans* to different degrees; however, all 6 strains were inhibited by 0.5 µg/ml of amphotericin B.

With the method cited above and the concentrations of amphotericin B employed, a fungicidal concentration of amphotericin B could be demonstrated for only one of the 6 strains of *C. albicans*.
<table>
<thead>
<tr>
<th>Concentration of Amphotericin B (μg/ml)</th>
<th>Isolates examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1B</td>
</tr>
<tr>
<td>0.1</td>
<td>+</td>
</tr>
<tr>
<td>0.2</td>
<td>+</td>
</tr>
<tr>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>0.4</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>1.5</td>
<td>+</td>
</tr>
<tr>
<td>2.0</td>
<td>+</td>
</tr>
<tr>
<td>3.0</td>
<td>+</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>10.0**</td>
<td>+</td>
</tr>
<tr>
<td>20.0</td>
<td>+</td>
</tr>
<tr>
<td>30.0</td>
<td>+</td>
</tr>
<tr>
<td>40.0</td>
<td>+</td>
</tr>
<tr>
<td>50.0</td>
<td>+</td>
</tr>
</tbody>
</table>

* + = viable organisms recovered on subculture; - = no viable organisms recovered.

** Precipitates which appeared to be amphotericin B were noted at concentrations greater than 10 μg/ml.
Experiment 2. Determination of the effect of amphotericin B on C. albicans in different assay media.

Sabouraud broth was modified so that the assay broths contained 2% glucose, 1% peptone; 2% glucose, 0.1% peptone; 0.5% glucose, 0.1% peptone; and 0.5% glucose, 1% peptone, respectively. Except for the composition of the assay broth, the investigative procedure was the same as previously outlined (Experiment 1). The results of this experiment appear in Figure 2.

When the concentration of peptone was varied in the assay system, a marked decrease in growth of C. albicans was noted in the low-peptone media. This marked decrease in growth was present in both the antibiotic-free and antibiotic-containing media. Varying the concentration of glucose, however, did not appear to affect the growth of C. albicans in either medium significantly.

Experiment 3. Effect of human plasma and amphotericin B on 4 strains of C. albicans.

Outdated human blood was obtained from St. Patrick Hospital. The plasma was drawn off the sedimented cells, centrifuged and then filter-sterilized. After filtration, 3.4 ml of sterile plasma was pipetted into a series of 16 x 125 mm screw-capped tubes and then refrigerated until just prior to use. At the start of the investigative procedure, 0.4 ml of amphotericin B and 0.2 ml of a standardized cell suspension of C. albicans were added to the test system. All test cell suspensions were checked to determine if the organism was in the yeast phase. After the inoculated assay cultures had been incubating for 48 hours at 37°C,
Figure 2. Effects of peptone and glucose concentrations on the growth of *C. albicans* and the growth-inhibitory effects of amphotericin B.
slides were prepared from each assay tube and examined for the production of germ tubes from the initial yeast phase culture. The presence of germ tubes in the assay cultures was interpreted to mean cell growth in the presence of the cited concentration of amphotericin B. One ml samples were also removed from each tube in the series and transferred to a blood agar plate and Erlenmeyer flask containing 125 ml of sterile trypticase soy broth. The suspensions were spread over the surface of the blood agar plates with sterile bent glass rods. The blood plates and trypticase soy broth flasks were incubated for 48 hours at 37°C and then read for growth. Of the organisms recovered after exposure to various concentrations of amphotericin B, representative colonies were checked for their ability to form germ tubes in human serum within 4 hours. The results of this study appear in Tables III and IV and Figure 3.

The fungistatic concentration of amphotericin B in plasma was observed to be greater than in broth. However, viability of the organism was lost much more rapidly in plasma than in the modified Sabouraud broth system.
Figure 3. Effect of amphotericin B on *C. albicans* in human plasma:

(A) Control lacking amphotericin B

(B) 0.5 µg amphotericin B/ml

(C) 2.0 µg amphotericin B/ml
Table III. Effects of amphotericin B on growth of *Candida albicans* in human plasma 48 hr., 37°C.

<table>
<thead>
<tr>
<th>Concentration of Amphotericin B (µg/ml)</th>
<th>Isolates examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1B</td>
</tr>
<tr>
<td></td>
<td>2A</td>
</tr>
<tr>
<td></td>
<td>3A</td>
</tr>
<tr>
<td></td>
<td>6C</td>
</tr>
<tr>
<td>0.0</td>
<td>+++*</td>
</tr>
<tr>
<td>0.5</td>
<td>++</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
</tr>
</tbody>
</table>

* Degree or extent of filamentation was rated from extensive (+++) to none (-).

Table IV. Effects of amphotericin B on viability of *Candida albicans* in human plasma, 48 hr., 37°C.

<table>
<thead>
<tr>
<th>Concentration of Amphotericin B (µg/ml)</th>
<th>Isolates examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAP*</td>
</tr>
<tr>
<td></td>
<td>1B</td>
</tr>
<tr>
<td></td>
<td>2A</td>
</tr>
<tr>
<td></td>
<td>3A</td>
</tr>
<tr>
<td></td>
<td>6C</td>
</tr>
<tr>
<td>0.0</td>
<td>+**</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>2.0</td>
<td>+</td>
</tr>
<tr>
<td>3.0</td>
<td>+</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
</tr>
</tbody>
</table>

* BAP = blood agar plates; TSB = trypticase soy broth.

** + = growth in subcultures; - = no growth in subcultures; + = both viable and non-viable cultures among replicates.
Experiment 4. Determination of the effect of amphotericin B on mycelial formation and chlamydospor production in 5 strains of C. albicans.

Corn meal agar with 1% Tween 80, was autoclaved at 15 psi for 15 minutes in 500 ml screw-capped Erlenmeyer flasks. After autoclaving the flasks were cooled in a water bath to 50°C. After cooling, 0.6 ml of sterile distilled water were added to the control flask, while 0.6 ml of amphotericin B were added to the test flask resulting in a final amphotericin B concentration of 2 μg/ml in a fluid volume of 300 ml. The media were dispensed into trisected sterile petri plates and allowed to solidify. Three hours after solidification, the plates were inoculated in duplicate with 5 isolates of C. albicans which had been previously exposed to the antifungal agent. The plates were incubated at room temperature (24°C) and the results recorded 7 days after inoculation (Table 5).

There was no correlation observed between prior exposure to amphotericin B and the ability of the isolates to form mycelium and produce chlamydospores on corn meal-tween agar. However, there was a constant significant reduction in mycelium formation among the strains of C. albicans grown on corn meal-tween agar containing amphotericin B. The reduced mycelium formation by the strains did not appear to be dependent on their prior exposure to amphotericin B.
Table V. Effects of previous and concurrent exposure to amphotericin B on growth and chlamydomspore formation by *Candida albicans* on corn meal agar.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Concentration to which inoculum had been exposed*</th>
<th>Corn meal-Tween 80 agar</th>
<th>Corn meal-Tween 80 agar with amphotericin B</th>
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<tr>
<td></td>
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<td>Mycelial growth</td>
<td>Chlamydomspore production</td>
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* *µg amphotericin B/ml of modified Sabouraud broth, 48 hr., 37°C.*
Experiment 5. Effect of hydrocortisone acetate on an experimentally induced systemic candidosis.

Rocky Mountain Laboratory white mice were used in this study to determine the effects of hydrocortisone on the course of an experimentally induced systemic candidosis. The treated mice received 1.25 mg of hydrocortisone intraperitoneally daily for 4 days prior to being challenged with C. albicans isolate 3A. A saline suspension of the challenge dose, $1 \times 10^5$ cells, was given intravenously in 0.1 ml. The composition of the various groups in this experimental study were as follows: 13 cortisone-treated, challenged mice; 8 challenged control mice; 7 cortisone-treated, unchallenged mice; and 2 saline-treated, control mice. The results of this experiment (Figure 4) clearly show that hydrocortisone definitely decreased the animal's ability to control the systemic candidosis. Autopsies were randomly performed to verify the cause of death. In some animals kidney lesions were more evident than in others.

There was one death which occurred among the hydrocortisone-treated, unchallenged mice. It occurred on the 11th day after hydrocortisone treatment was discontinued and was attributed to cannibalism. Earlier in the experiment two mice in the same group had incurred inflicted injuries to their hind quarters and were removed and isolated. They recovered from their wounds and survived until the termination of the investigation.
Figure 1. Effect of hydrocortisone acetate on the course of a systemically...
Experiment 6. Determination of the effect of immunological depression on the outcome of an amphotericin B-treated systemic candidal infection.

Male F344 white mice weighing from 25 to 30 gm were used in this investigation. Each group consisted of 9 mice with the exception of 5 cortisone-treated control mice and 3 saline-treated control mice. Pretreatment of the mice with hydrocortisone acetate was carried out according to the procedure outlined in Experiment 5. The procedure was, however, modified in so far as the mice were challenged with C. albicans strain 3A shortly after receiving their final dose of hydrocortisone. The reason for this modification was to allow for the establishment of the systemic infection at a point of greatest immunological depression. The treated mice received 1 mg of amphotericin B 24 hours after challenge and then 0.5 mg every 48 hours for 6 days. On a mg/kg basis each mouse received approximately 40 and then 20 mg/kg, respectively. Autopsies were randomly performed and the kidneys examined for macroscopic lesions. When the cause of death was in doubt, cultures of the kidneys were made. The results of this investigation appear in Figures 5 and 6.

Hydrocortisone was again shown in this investigation to decrease the ability of the infected mice to control the systemically-induced candidal infection. Treatment of both the cortisone- and saline-pretreated infected mice with amphotericin B showed that the response of both groups to the antibiotic was an arrest of the induced candidal infection. Figure 6 shows that there was approximately a 4±% fatality due to the doses of amphotericin B employed among the control animals which may account for and/or contribute to the deaths observed in the infected-treated groups.
Figure 5. Effect of amphotericin B on the course of a systemically-induced candidal infection in mice pretreated with hydrocortisone acetate. Mice received the following injections: hydrocortisone acetate, cells, □; saline, cells, ●; hydrocortisone acetate, cells, amphotericin B, ■; saline, cells, amphotericin B, ▲. (See text for injection schedule).
Figure 6. Toxicity resulting from amphotericin B treatment in infected and uninfected mice. The mice received the following injections: hydrocortisone acetate, amphotericin B, ○; saline, amphotericin B, ●; hydrocortisone acetate, cells, amphotericin B, ■; saline, cells, amphotericin B, ▲.
CHAPTER IV

DISCUSSION

The results of the in vitro broth assays of the effect of amphotericin B on C. albicans, showed that the 6 isolates of C. albicans were inhibited to different degrees by a given concentration of amphotericin B. The inhibition was partial for all isolates at 0.1 µg/ml and complete for the 6 isolates at 0.5 µg/ml. These results are similar to those reported by Sorensen et al. (1959), Hald et al. (1957), and Littman et al. (1958a). Their method of determining fungistasis, however, was based on a visual comparison of the assay tubes to determine growth inhibition. The use of an increase in optical density as a criterion for growth inhibition was considered to be a more accurate method for determining fungistasis than that based on visual comparison.

There were other areas in the actual methods and materials employed by Littman and coworkers (1958a) and Sorensen and coworkers (1959) which also differed from those used in this investigation. Their use of pH 7 and inoculum size 5 x 10^4 - 1 x 10^5 cells compared with pH 6.7 and inoculum size 5 x 10^5 cells/ml used in this study did not appear to significantly alter the fungistatic concentration of amphotericin B. A comparison of the pH and inoculum size used by Hald et al. (1957) cannot be made since neither was reported.

Both Littman and coworkers (1958a) and Sorensen and coworkers (1959) read their results after a 48-hour inoculation at 37°C. When the assay tubes were read earlier or later, a significantly different result was
reported to have occurred. Littman et al. (1958a) demonstrated that the fungistatic concentration of the antibiotic varied from 0.09 μg/ml at 24 hours to 5.0 μg/ml at the end of 7 days. This alteration in the fungistatic concentration of amphotericin B was attributed to its deterioration in the hydrated state during prolonged incubation. Littman's observation may explain the low fungistatic value of 0.07 μg/ml reported by Utz and coworkers (1959) for 2 strains of *C. albicans* after a 24-hour exposure to the antibiotic.

Based upon the work of Gold et al. (1956), Vandeputte et al. (1956), Littman et al. (1958a) and Bartner et al. (1958) a 48-hour incubation period was chosen for the present studies. This period would allow the organism sufficient time in which to grow and would also give a more accurate picture of the fungistatic activity of amphotericin B without allowing an unnecessary deterioration of the antibiotic.

The assay media employed by previous investigators included Penasay broth with 1% glucose, asparagine broth and mycophil broth. Those media and that used for assays in the present investigation appeared to be rich enough so as not to introduce a growth limiting factor into the assay. However, studies employing a minimal medium (0.5% glucose, and 0.1% peptone) which are reported here, demonstrated a significant decrease in growth both in the presence and in the absence of the antibiotic when compared to an enriched medium containing 2% glucose and 1% peptone. The relative growth in the presence of 0.1 μg/ml of amphotericin B in the rich medium was 71.2% of the control as compared to 55.3% of the control in the minimal medium. Whether the greater
relative inhibition by the antibiotic in the low-peptone medium was a result of the reduced peptone concentration itself or whether there was a greater concentration of unbound antibiotic present in the minimal medium has not been shown. A reduced glucose concentration (0.5%) in the medium did not appear to have a significant effect on the growth of the organism. These observations may explain in part the low fungistatic values (0.029-0.052 μg/ml) reported by Lones and Peacock (1959) who also used a medium containing 0.5% glucose and 0.1% peptone in contrast to the high inhibitory value (2.5 μg/ml) reported by Quinn et al. (1960) who used a very rich medium for C. albicans.

An additional variable, which must be considered in an attempt to contrast the results of others with those of the present study, is the specific isolate or "strain" of C. albicans employed in the assays. That strains differ in their response to a given concentration of amphotericin B is demonstrated by the results of the present investigation as well as that of McNall and coworkers (1958) and Lepper and coworkers (1959).

The observation of fungicidal effects of amphotericin B on C. albicans in modified Sabouraud broth demonstrated qualitatively that cells from 5 to 6 isolates of C. albicans were able to survive in the presence of an initial concentration of 50 μg/ml of the antibiotic. Similar results were reported by Hald and coworkers (1957) who demonstrated that viable cells were recoverable from 3 strains of C. albicans after having been exposed to 100 μg/ml of amphotericin B for 3 days. The fungicidal concentration reported by Littman et al. (1958a), 0.4 μg/ml, however, appeared to be inconsistent with their observation that
the minimal inhibitory concentration of amphotericin B for the same strain of *C. albicans* varied from 0.09 μg/ml at 24 hours to 5 μg/ml at the end of 7 days. It must be considered that their fungicidal concentration may reflect the limitations of their sampling procedure and may not have represented a fungicidal concentration for the entire inoculum. The limitations of the sampling procedure used in the present broth assay studies also may be considered as a factor in the failure to recover viable cells from isolate 6C after it was exposed to concentrations greater than 10 μg/ml. However, it was possible to recover viable cells from the remaining 5 isolates at concentrations more than 100 times greater than the fungicidal concentration reported by Littman et al. (1958a).

Iones and Peacock (1959) reported a precipitation of amphotericin B at concentrations of 10 or 20 μg/ml of assay broth (4% glucose and 1% peptone). They demonstrated that each component of the assay medium was capable of causing flocculation of the antibiotic in the absence of the other. In an attempt to eliminate the precipitate, they modified their medium to contain 0.5% glucose and 0.1% peptone. With this alteration they were able to prevent precipitation of the antibiotic. When a similar medium was used in the present investigation, a precipitate was nevertheless observed at concentrations of 10 μg/ml or more of amphotericin B. The precipitate was not present initially but occurred during incubation of both inoculated and uninoculated cultures for 48 hours at 37°C. Other investigators who have employed similar media (Hebeka and Solotorovsky, 1965; Sorensen et al., 1959) have not reported a precipitate. Their failure to observe a precipitate may have resulted
from their use of shaken cultures which would tend to prevent any visible settling of the antibiotic. The use of stationary cultures by Lones and Peacock (1959) and in the present study was believed to be primarily responsible for the observation of the antibiotic precipitate. The precipitate could, however, be visibly eliminated by shaking the tubes and at no time altered the optical densities of the suspensions. Since amphotericin B occurs as a colloidal suspension (Bartner et al., 1958), the aggregation of micelles may not effectively reduce their fungistatic and/or fungicidal effects on the cell.

Other factors may influence the fungicidal activity of amphotericin B in Sabouraud broth. However, since the same factors may also operate in human plasma, they will be considered with the results of the assays of fungicidal activity of the antibiotic in human plasma.

The clinical significance of the recovery of viable cells from amphotericin B concentrations greater than 1 μg/ml is difficult to ascertain since the constituents and/or their concentrations in the Sabouraud broth assay system are not directly comparable to those present in the in vivo system.

Reports of decreased growth rates, decreased ability to form mycelium and chlamydospores (Lones and Peacock, 1959) and the observation of pleomorphic cells (Sorensen et al., 1959) in amphotericin B-resistant strains of \textit{C. albicans} prompted a reexamination of the organism's ability to produce mycelia and/or chlamydospores during or after exposure to amphotericin B. The inoculation of corn meal-tween agar with and without 2 μg/ml of amphotericin B with 5 strains of \textit{C. albicans} and antibiotic-tolerant isolates derived from them
demonstrated that previous exposure to amphotericin B did not affect the ability of the organism to produce mycelium and chlamydospores on normal corn meal-tween agar. Two strains did, however, show consistent significant differences in the amount of mycelial growth in the presence of 2 μg/ml of amphotericin B. Chlamydospore formation per given area of mycelium however appeared to be unaffected by the presence of the antibiotic. It appears, therefore, that exposure of C. albicans to high concentrations of amphotericin B does not affect the characteristics which are used clinically to identify C. albicans: mycelium formation, chlamydospore production and blastes in human plasma. It is unlikely that C. albicans recovered from an amphotericin B-treated case would go unrecognized in a routine cultural examination.

The ability of C. albicans to form hyphae (filaments or germ tubes) in human plasma or serum within 2 or 3 hours when incubated at 37°C has been reported by a number of investigators (Taschjian et al., 1960; Mackenzie, D.W.R., 1962; Griffin, 1964; Dabrowa et al., 1965; Landau et al., 1965; Bonfante and Barroeta, 1968) and is presently felt by some (Taschdjian et al., 1960; Bonfante and Barroeta, 1966) to be a rapid and reliable method for identifying C. albicans in the diagnostic laboratory. This ability was used as the criterion for determining growth of the organism in human plasma.

Landau and coworkers (1956) reported that as the inoculum size increases the per cent blastes or filamentation decreases. Therefore, it is essential that the inoculum size to be used in the assay be carefully considered. The inoculum (5 x 10⁵ cells/ml) which was employed in this study, has been reported to undergo approximately 70%
blastesis within 3 hours. It was therefore felt that the absence of blastesis for 48 hours represented a reliable indication of the fungistatic activity of amphotericin B in plasma. In the present investigation the relative occurrence of blastesis was not precisely quantified; however, if it occurred in greater than about 3 percent of the cells, which allows for a minimal carry over of germ tubes within the inoculum, the result was considered positive. Otherwise, it was recorded as negative.

The results of this investigation demonstrated that the fungistatic activity of amphotericin B when based on blastesis was higher (2.0 μg/ml) in plasma than in Sabouraud broth (0.5 μg/ml). This difference in fungistatic concentrations between the plasma and the broth systems may in part result from the use of a more sensitive index for determining growth in plasma. However, the possibility that some plasma components may also influence the activity of amphotericin B can not be excluded (Taschdjian and Kozinn, 1961; Heite et al., 1964; Roth et al., 1959; Roth and Goldstein, 1961; Louria and Brayton, 1964; Caroline et al., 1964; Landau et al., 1964).

Concurrent with the reduction in blastesis as the concentration of amphotericin B increased was a decrease in the length of the germ tube at antibiotic concentrations of 0.5 μg/ml or more and also a decrease in viability within the original inoculum.

Other investigators (Vogel and Crutcher, 1958; Littman et al., 1958b; McNall et al., 1958; Gerke and Madigan, 1961) have studied the fungistatic effects of amphotericin B on C. albicans and on other Candida species in serum and other body fluids. However, with one
exception, their results are not directly comparable with the present study since the criteria for growth, the assay medium diluent and other materials and procedures were significantly different such as to render the validity of a comparison questionable.

The only report found in the literature which included blastesis as an indicator of the effect of amphotericin B on C. albicans was that by Landau and coworkers (1965) who used a serum assay medium. They reported that blastesis in sera incubated for 3 hours was less than 5% when the amphotericin B concentration was 5 mg/ml or more, and 16% at a concentration of 1 mg/ml, as compared to the control with 85%. The variation which they observed in the percentage of germination in duplicate tests did not exceed 10% and an average was reported as the result.

The results reported by Landau and coworkers (1965) were determined after 3 hours at 37°C. A 3-hour incubation period, although it is sufficient time to demonstrate blastesis in the diagnostic laboratory, was not considered to be a realistic time from a clinical point of view to determine the activity of the antibiotic. In most clinical cases, 48 hours represents the maximum time which would elapse between infusions of the antibiotic. It was therefore decided that it would be of more value to measure the activity of a given concentration of amphotericin B at this clinical limit than at a time which may not be long enough to represent an accurate indication of its possible in vivo effectiveness. Secondarily, a 48-hour incubation period was considered since it would allow comparison of results with those obtained using the Sabouraud broth assay system.
The fungicidal activity of amphotericin B in human plasma was demonstrated in the present investigation to be greater than in Sabouraud broth. Viable cells were not recovered after exposure to greater than $3 \mu g/ml$ of amphotericin B when subcultured on blood agar. However, evidence of viability could still be demonstrated if the assay sample was diluted with sufficient trypticase soy broth to reduce the concentration of amphotericin B below the fungistatic level. At an amphotericin B concentration of $5 \mu g/ml$ even after dilution, viability was lost in 50% of the samples from 3 of the 4 isolates of \textit{C. albicans} tested.

In the bioassay studies previously mentioned, the fungicidal effects of amphotericin B on \textit{C. albicans} in human plasma or serum were not investigated.

In 1963, Louria and Brayton reported the presence of a candidicidal substance in human plasma and serum. The substance was reported (Louria and Brayton, 1964) to remain active 2 weeks when refrigerated at 4°C and indefinitely when frozen at -20°C. It was therefore not considered to influence the results of the present investigation. Other factors which may have influenced the fungistatic and/or fungicidal activity of amphotericin B in plasma include: the isolate of \textit{C. albicans} employed, the size of the inoculum, the growth phase of the inoculum (McNall \textit{et al.}, 1958), the duration of antibiotic contact with the organism, and in the case of plasma, the medium itself may contribute to the fungicidal activity observed.

The results presented here have shown that \textit{C. albicans} is able to grow in the presence of an amphotericin B concentration (1.0 $\mu g/ml$)
which approximates the highest \textit{in vivo} concentration which can safely be maintained for about 24 hours. However, there was a marked reduction in viability accompanying the limited growth at 1 \( \mu \text{g/ml} \) and, as demonstrated by failure to obtain subcultures on blood agar, viability was lost at an antibiotic concentration of 4 \( \mu \text{g/ml} \). Since the effects reported here represent a single contact with the antibiotic and not a repeated contact as would occur in a clinical infection, the growth and viability demonstrated in this study although they are of academic significance may not be of clinical significance.

In conclusion, the plasma assay system appeared to demonstrate a greater fungicidal activity of amphotericin B than did the Sabouraud broth system. Although there was apparent growth (blastesis) of \textit{C. albicans} in plasma at an amphotericin B concentration of 1 \( \mu \text{g/ml} \), the growth was not sufficient enough to impart visible turbidity to the plasma tubes containing 0.5 \( \mu \text{g/ml} \) of amphotericin B when visually compared to the control. It was therefore considered that the fungistatic activity of amphotericin B in both assay systems was similar.

The effects of amphotericin B on growth of the mycelial phase of \textit{C. albicans} in Sabouraud broth or human plasma were not considered in this investigation.

The steroid hormones ACTH, cortisone, and hydrocortisone have been shown by a number of investigators to depress host resistance to mycotic, bacterial and viral infections (Seligmann, 1953; Louria et al., 1960; Hellman, A., 1965; Foley et al., 1957; Batten and McCune, 1957). The mechanisms by which they lower host resistance include: a decrease in the inflammatory response process (Ketchel et al., 1958; Menkin, V.,
Michael and Whorton, 1951), decreased antibody production (Bjørneboe et al., 1951; Kass et al., 1955; Siedlicka et al., 1963; Germuth and Ottinger, 1950), lymphopenia (Paluska and Hamilton, 1963), and a decreased phagocytic activity (Crepea et al., 1951).

In 1961, Boyd and Chappell reported a systemic *C. albicans* infection which terminated fatally after combined steroid and antibiotic therapy. The present study was undertaken to determine if the immunological depressant effects produced by the steroid hormone, hydrocortisone, could be overcome by the administration of amphotericin B. To determine this, the effect of hydrocortisone acetate on the course of an induced systemic candidal infection was first investigated.

The results of this investigation showed that a significant increase in mortality rate occurred when mice were pretreated with hydrocortisone as compared to the control group. In Experiment 5 there was a 50% mortality in the hydrocortisone pretreated group on the sixth postinfectional day as compared to 12.5% in the control group. In a second experiment (Experiment 6), on the sixth postinfectional day there was a 77% mortality in the hydrocortisone pretreated group as compared to 22% in the control group. These studies very clearly demonstrate the adverse effects exerted by hydrocortisone on the course of systemic candidal infections.

Henry and Fahlberg in 1960 reported that hydrocortisone acetate (1 to 10 mg) given as a single intraperitoneal injection 24 hours prior to challenge with *C. albicans* by the same route did not produce an increase in the mortality rate. However, repeated daily doses of hydrocortisone acetate given intraperitoneally over a 2-week period
brought about an enhancement of the candidal infection and correlated directly with the amount of hydrocortisone acetate given. When 1.0 mg of hydrocortisone acetate was given daily for 2 weeks, the mortality rate in the hydrocortisone-treated group was 54% as compared to 13% for the control group. In the present study, 1.25 mg of hydrocortisone acetate given for 4 successive days before challenge with C. albicans was shown to produce a similar enhancement of the candidal infection and increase in the mortality rate.

Using the immunological depressant effects of hydrocortisone acetate to establish a rapidly fatal systemic candidal infection, the effect of amphotericin B on the course of this infection was investigated. The results at the termination of the experiment showed that amphotericin B in the dose regimen employed was able to arrest the systemic infection in both amphotericin B-treated groups. In the 18th postinfectional day, there was 100% mortality in both the hydrocortisone- and saline-treated infected groups as compared to a 44% mortality and a 55% mortality in the hydrocortisone and control groups receiving amphotericin B. In this study the effects of temporary immunological depression were overcome and the systemic candidal infection was arrested by the use of amphotericin B.

The dose of amphotericin B used in the present investigation may have accounted for the deaths observed in both infected-treated groups as is evidenced by the 44% mortality in both antibiotic control groups (Figure 6). The uncomplexed form of amphotericin B when given intraperitoneally in concentrations from 5 to 100 mg/kg was reported by Sternberg and coworkers (1956) not to produce chronic toxicity. Emmons
and Piggott (1959) did not report any fatal toxicity resulting from the administration of 56 mg/kg of crystalline amphotericin B to mice intraperitoneally. However, they did report that the same solubilized form of the antibiotic as was used in the present study when given intravenously produced fatal toxic effects in the control mice at a concentration of 2.2 mg/kg. In the present investigation the intraperitoneal route was used in an attempt to increase the concentration of amphotericin B which could be given and decrease its toxic effects. Although the intraperitoneal route was shown to increase the tolerable concentration of antibiotic, amphotericin B in the concentrations given still produced serious toxic side effects.

At the termination of this experiment, autopsies performed on the surviving normal and immune—suppressed amphotericin B—treated infected mice—revealed macroscopic cortical kidney lesions and yeast-like cells in a number of renal tubules. These observations together with the death which occurred in an amphotericin B—treated mouse on the 12th postinfectional day suggest that amphotericin B even in the high concentrations (almost an LD₅₀) given to animals with normal host defense mechanisms was not adequate to eliminate the infecting organism from the host.

Since _C. albicans_ is capable of invading and residing within the kidney of mice, it would appear to be capable of producing an acute infection in the untreated animal and a chronic infection in the treated animal. Further research, however, is required to demonstrate this chronic state.
The significance of this investigation is that amphotericin B was shown to be able to compensate when given early in the course of a systemic candidal infection for a temporary impairment of the defense mechanisms of the afflicted host. In a clinical systemic candidal infection, an impairment of the host's defense mechanisms may also be temporarily compensated for by the early administration of amphotericin B; however, ultimately amphotericin B may be dependent to some degree on the host's defense mechanisms, in particular the host's cellular defense mechanisms, to complete its antimicrobial effect.
CHAPTER VI

SUMMARY

1. Six isolates obtained from clinical infections were characterized and identified as *C. albicans*.

2. A fungistatic concentration of amphotericin B was determined for each of the 6 isolates of *C. albicans* in Sabouraud broth. The fungistatic concentrations of amphotericin B ranged from 0.4 µg/ml to 0.5 µg/ml.

3. A fungicidal concentration of amphotericin B (10 µg/ml) was demonstrated for only 1 (isolate 60) of the 6 isolates of *C. albicans* in Sabouraud broth. Viable cells were recovered from the remaining 5 isolates at amphotericin B concentrations as high as 50 µg/ml.

4. The ability of 5 isolates of *C. albicans* to produce mycelium and chlamydospires after exposure to 10 or 20 µg/ml of amphotericin B was unaltered. However, mycelium formation (but not chlamydospore production) was reduced in 2 isolates of *C. albicans* (1B and 5A) when grown on corn meal-tween agar in the presence of 2 µg/ml of amphotericin B.

5. The growth of *C. albicans* as well as the inhibitory activity of amphotericin B were shown to be influenced by the concentration of peptone in the assay medium. In a low peptone medium (0.1%) the growth of *C. albicans* was significantly reduced and the inhibitory activity of amphotericin B increased as compared to an assay medium
with a high peptone concentration (1.0%).

6. The concentrations of glucose used in the assay media did not appear significantly to affect the growth of \textit{C. albicans}, or the inhibitory activity of amphotericin B.

7. Using blastesis as a criterion for determining fungistasis, the fungistatic concentration of amphotericin B was shown to be greater in plasma (2.0 \(\mu g/ml\)) than in Sabouraud broth (0.4-0.5 \(\mu g/ml\)) for 4 isolates of \textit{C. albicans}.

8. A fungicidal concentration of amphotericin B in plasma as measured by the recovery of viable cells on blood agar plates was demonstrated for 4 isolates of \textit{C. albicans} to be 4 \(\mu g/ml\).

9. \textit{C. albicans}, isolate 3A, was shown to be pathogenic for male white mice.

10. Hydrocortisone acetate was shown to increase significantly the mortality rate of mice infected with \textit{C. albicans}.

11. The immunological depressant effects of hydrocortisone treatment of infected mice were overcome and the systemic candidal infection arrested by the use of amphotericin B.

12. There was some evidence to suggest that the concentrations of amphotericin B used to treat the systemic candidal infection, although toxic to some of the mice, were unable even in normal hosts to rid the animal of the infecting organism.
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