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Development of Resistance to Amphotericin B in *Candida lusitaniae* Infecting a Human

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Candida lusitaniae associated with infection in a patient with acute myelogenous leukemia developed resistance to amphotericin B during systemic treatment of the patient. The organism, when isolated initially, was inhibited by 0.31 μg of amphotericin B per ml in yeast nitrogen base agar, but when isolated (20 days later) just antemortem and postmortem, required 100 and 50 μg/ml, respectively, for complete inhibition at 48 h.

Despite widespread and frequent use of amphotericin B in the treatment of mycotic infections, development of resistance by pathogenic fungi to this polyene antibiotic has not posed a broadly significant problem. As emphasized by Safe et al. (10), under present clinical procedures there has been no selection for antibiotic-resistant candida, except for that recorded for a strain of *Candida tropicalis* by Woods et al. (12) and for a strain of *C. albicans* by Bodenhoff (2). Drutz and Lehrer (6) have reviewed the emergence of increased resistance to amphotericin B during therapy; and although the examples were few and in several instances of questionable magnitude, the authors suggest that recognition of more such resistant forms would result if tests of susceptibility to amphotericin B were more frequently carried out during therapy.

In the present report we describe the occurrence of *C. lusitaniae* in a human patient and its development of marked resistance to amphotericin B while the patient was being treated systemically with this polyene.

MATERIALS AND METHODS

Case report. The patient was a 47-year-old white male who was well until 1969 when he was found to have multiple myeloma. Subsequently, after toxic exposure to multiple organic substances, he developed abnormal liver function studies and a progressive decrease in erythrocytes and leukocytes. Therapy for the multiple myeloma was stopped except for periodic prednisone therapy. His pancytopenia progressed, and in October 1976 a diagnosis of acute myelogenous leukemia was made on the basis of findings in the peripheral blood and bone marrow.

Induction chemotherapy was begun on 14 October, using several doses of cytarabine, 6-thioguanine, and daunorubicin. The last dose of the latter was administered 30 days before the death of the patient. The total leukocyte count was less than 1,000 cells per mm^3 after this chemotherapy, remaining essentially unchanged by blood and platelet transfusion until his death. He was intermittently febrile, and on day 20 (2 November) multiple blood cultures yielded a yeast identified at that time as *C. parapsilosis* but later identified as *C. lusitaniae*. Similar isolates of the yeast were recovered from his blood and stool on 6 November. The candidemia occurred in the absence of an indwelling catheter. Intravenous amphotericin B was begun on 7 November. The initial dose was 1 mg, and on the following day 5 mg was administered. A 20-mg amount was given on day 3 of treatment, then 70 mg on days 4, 6, 7, 8, 9, 10, and 11. The dosage was then reduced such that 30, 35, or 50 mg was administered, usually daily but with occasional days off. At various times, cefazolin, gentamicin, carbenicillin, and trimethoprim-sulfamethoxazole were also administered. The single determination of the serum level of amphotericin B, on 11 November, yielded a figure of 0.98 μg/ml. Treatment with amphotericin B was continued until 23 doses totaling 981 mg had been completed 2 days before his death.

C. lusitaniae were recovered from his sputum on 7 and 11 November 1976, from a lesion of the buccal mucosa on 7 and 8 November and from his blood on 27 November, again in the absence of an indwelling catheter. A fragment of necrotic tissue expelled by coughing before his death yielded a similar organism. Multiple tests by counterimmunoelectrophoresis with the serum of the patient yielded no reaction for antibodies to *C. albicans*. On day 32 (14 November), a cellulitis developed in the left arm, and this yielded a coagulase-negative staphylococcus. A right middle lobe infiltrate developed. This was thought to be due to *Pneumocystis carinii*, and trimethoprim-sulfamethoxazole therapy was begun. Four days later, increasing dyspnea, hemoptysis, and a new left upper lobe infiltrate were noted. On day 38 after induction therapy and 18 days after amphotericin B were begun, the patient became disoriented, complained of abdominal tenderness, and showed abdominal distension. The pulmonary infiltrate and respiratory failure increased, and apparent congestive heart failure and shock supervened.

The patient died 46 days after initiation of induction therapy for leukemia, and postmortem examination was carried out 23 h later. The diagnoses at the time of death included acute myeloblastic leukemia, necrotizing pneumonitis, and cardiorespiratory arrest, possibly secondary to the necrotizing pneumonitis. "Recent Candida sepsis" and "probable Pneumocystis carinii pneumonia" were included as final diagnoses. *C. lusitaniae* was recovered from the lungs postmortem, but was not noted in the histopathological studies of the extensive necrotizing bronchopneumonia. Yeast cells were observed microscopically in blood vessels of the muscular layer of the distal esophagus and in the mucosa of the duodenum. Broad septate hyphae (not further identified) were found in an infarct and in blood vessels of the upper lobe of the left lung. Septate hyphae were also found invading the necrotic wall of the jejunum and duodenum, the mesenteric blood vessels, and the vessels and parenchyma of the uretero-pelvic area of the left kidney. No mycelial organism was recovered on culture. Clusters of gram-positive cocci were observed postmortem in the area of necrotizing pneumonitis and deep subcutaneous tissue of the forearm.

Susceptibility testing. The isolates of *C. lusitaniae* were tested for susceptibility to various concentrations of amphotericin B on two culture media, the complex organic Sabouraud glucose agar, and the defined yeast nitrogen base (YNB; Difco Laboratories) agar. These were solidified by using 2% ion agar. The latter was utilized to obviate interference with antimicrobial activity by components of the usual agar (5).

Amphotericin B was added in appropriate concentrations (0.0157 to 10 μg/ml) to melted and cooled agar medium. Portions (25 ml each) containing a given concentration of amphotericin B were poured into petri dishes.

The inocula were prepared by culture of the isolates on slants of Sabouraud glucose agar at 34°C for 24 h. The growth was rinsed off with sterile distilled water, and the concentration was adjusted to yield approximately 10^4 cells per 5 μl of inoculum. Duplicate plates containing a given concentration of amphotericin B were each inoculated with a 5 μl of each of the suspensions representing the different isolates. The plates were incubated at 34°C and read daily for visible growth. When it was determined that some isolates (7, 8, and 9) grew in the presence of 10 μg of amphotericin B per ml, these were tested further at doses of 1 to 100 μg/ml.

Virulence of the yeast isolates was examined by intravenous (tail vein) injection of 10^6 to 10^8 cells in 8- to 10-week-old male or female mice. In one experiment, tissues were obtained after sacrificing survivors 7 days after inoculation for counting of viable yeast cells and for histological examination. Tissue sections were stained with hematoxylin and eosin, and separate sections were stained with periodic acid-Schiff stain.

RESULTS

Susceptibility to amphotericin B in vitro. Isolates obtained before and 4 days after amphotericin B therapy was begun were inhibited by low concentrations of amphotericin B in Sabouraud glucose agar and in YNB agar (Table 1). However, a marked (approximately 100-fold) increase in resistance was noted for the next isolate obtained from the blood 3 weeks after the start of therapy with amphotericin B. Thus, isolates 7 (from blood 2 days antemortem) and 9 (from lung postmortem) were not inhibited by 10 μg of amphotericin B per ml in either YNB or Sabouraud glucose agar but were inhibited by 100 and 50 μg/ml, respectively, in YNB at 48 h. Isolate 8, obtained 1 day antemortem, was less resistant than culture 7 isolated immediately before it and less resistant than culture 9 isolated immediately after it (also derived from the lung).

The resistance to amphotericin B has been sustained for over 2 years during which these isolates have been maintained on Sabouraud glucose agar with periodic transfers.

It was felt important, in view of the marked change in susceptibility to amphotericin B, to determine whether in fact the yeast isolated

TABLE 1. *Susceptibility of isolates of C. lusitaniae to amphotericin B in vitro*

Isolate		MIC[b]			
Source	Time of culture[a]	24 h		48 h	
		Sabouraud agar	YNB agar	Sabouraud agar	YNB agar
Blood	−1	1.25	0.31	2.5	0.63
Stool	−1	1.25	0.31	2.5	0.63
Sputum	0	1.25	0.31	2.5	0.63
Buccal lesion	0	1.25	0.31	2.5	0.63
Buccal lesion	+1	1.25	0.31	2.5	0.63
Sputum	+4	1.25	0.31	2.5	0.63
Blood	+20	30	30	80	100
Lung	+22 (Antemortem)	1.25	1.25	2.5	2.5
Lung	+22 (Postmortem)	30	30	30	50

[a] Each value represents days in relation to start of amphotericin therapy.

[b] Each value represents the minimal inhibitory concentration (MIC) of amphotericin B, i.e., the concentration (micrograms of the drug per milliliter of medium) necessary to completely inhibit growth, at the incubation time indicated.

from the initial positive blood cultures was the same in other respects as the later resistant isolates from blood and lung. This comparison, carried out by D. L. Holzschu, H. L. Presley, M. Miranda, and H. J. Phaff (7a) involved assimilation and fermentation of various compounds, and growth in the presence of various concentrations of NaCl. The results indicated that the organisms isolated at different times ante- and postmortem were of the same species, i.e., *C. lusitaniae* (4). DNA homology studies by Holzschu et al. (submitted for publication) confirmed that the isolates correspond to *C. lusitaniae*.

Intravenous injection of 10^8 yeast cells in mice produced no deaths in 40 days. However, some neurological manifestations in the form of ataxia were noted in mice receiving a dose of 10^7 to 10^8 cells. Only yeast cells—no pseudohyphae—were observed in tissues of mice sacrificed at 7 days.

DISCUSSION

Infection with yeasts of immunologically or physiologically impaired human hosts has been recognized as a substantial problem in recent years (8, 9). The patient discussed herein, initially affected by multiple myeloma, was later assailed by acute myelogenous leukemia. The latter, and perhaps the chemotherapy applied thereto, set the stage for systemic infection. *C. lusitaniae* appeared to contribute to the pathogenesis, in view of its causation of candidemia in the absence of an indwelling intravenous catheter and its penetration into the media of esophageal blood vessels and mucosa of the duodenum. Its contribution to the death of the patient remains uncertain. That other infectious agent(s) were present was indicated by the presence of gram-positive cocci associated with cellulitis of the forearm ante- and postmortem and their presence postmortem in the sites of pneumonitis. Septate hyphae representing another fungus were observed in multiple tissues postmortem, although they were not recovered on culture. The terminal cardiorespiratory events in part may have been related to the daunorubicin therapy (which may yield cardiotoxic effects).

C. lusitaniae lacked virulence for normal laboratory white mice at doses up to 10^8 yeast cells given intravenously. This is similar to earlier findings with *C. parapsilosis*, which in doses up to 10^7 cells given intravenously caused no deaths of normal mice, although a dose of 10^8 *C. parapsilosis* caused rapid deaths (within 1 day) in 40% of normal mice (1). Only yeast cells were observed in tissues of the infected mice, and thus the hyphal fungus observed in the tissues of the patient at autopsy was not identified.

On day 4 after therapy was begun, amphotericin B was detected in the serum at a concentration of 0.98 μg/ml, and on that same day the yeast *C. lusitaniae* isolated from the sputum showed the same susceptibility as the pretreatment isolate, i.e., the minimal inhibitory concentrations of amphotericin B ranged from 0.313 to 0.625 μg/ml. Twenty days later, fungemia yielded *C. lusitaniae* resistant to >30 μg of amphotericin B per ml.

The emergence of resistance to amphotericin B in the present study occurred in a 20-day interval, rapid by comparison with the development of resistance in *C. tropicalis* reported by Woods et al. (12). In the latter study, the organism isolated after 2 months of treatment with amphotericin B showed a modest increase in resistance, the minimal inhibitory concentration increasing from 0.098 μg/ml in serum to 0.39 μg/ml; but 3 months later this minimal inhibitory concentration had risen to greater than 500 μg/ml.

Several species of fungi under laboratory conditions have been selected for resistance to polyenes (7). Sorensen et al. (11) demonstrated an increase in resistance to amphotericin B by *C. albicans* in vitro, and Bodenhoff (3) also demonstrated in the laboratory development of resistance to amphotericin B among several species of candida. Some alteration in sterol of the yeast cytoplasmic membrane accompanies and may account for increasing resistance to polyenes such as amphotericin B (7, 12). Safe et al. (10) reported similar findings in *C. krusei*, *C. tropicalis*, and "*C. parakrusei*" (presumably *C. parapsilosis*). We have not completed a comparison of sterols of susceptible and resistant isolates of our *Candida* species.

Although it has appeared to be a rare clinical phenomenon, development of resistance to amphotericin B by yeasts should be considered, particularly in patients whose clinical status continues to deteriorate in the face of presumably adequate therapy.

What influence such cytotoxic (and possibly mutagenic) agents as daunorubicin, cytarabine, and 6-thioguanine, used to treat the leukemia of the present patient, have on the development of polyene-resistant mutants of yeasts deserves consideration as a contributing factor.

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