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THERAPEUTIC ACTION OF AMPHOTERICIN B IN EXPERIMENTAL BLASTOMYCOSIS

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Thy mycosis caused by Blastomyces dermatitidis (North American blastomycosis or Gilchrist's disease), characterized by granulomatous and suppurating lesions harboring the parasite, can evolve in a fatal, widespread (visceral and cutaneous) form, as well as in a benign localized form.

This mycosis is considerably improved by use of aromatic amidines but prolonged use of these products is limited by toxic neural incidents.

Nystatin (3) was the first of the antifungal polyenes to be used clinically in infections of Candida; the discovery of these fungicides opened a new era in the therapy of these mycoses and gave hope of a cure for these often fatal deep mycoses. But, although nystatin (tetraene) and candicidine (heptaene) are active in vitro against Blastomyces dermatitidis and in vivo parenterally in the experimental blastomycosis of the mouse, these antibiotics have not found therapeutic application in human blastomycosis. Actually, these products are hardly tolerated parenterally and their weak oral resorption in deep mycosis does not permit a cure.

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Finally, amphotericin B, described by Gold et al. (2) and by Vandeputte et al. (10), showed itself remarkably active orally or subcutaneously in the following experimental mycoses: candidiasis, (7,8), cryptococcosis (5,7,8), histoplasmosis (1,5), coccidioidomycosis (8), and even in a case of widespread human histoplasmosis (4).

In this article on the treatment of experimental blastomycosis, we will compare the action of amphotericin B (heptaene) with that of amphotericin A and of nystatin (both tetraenes): for this study we used both the mouse and the golden hamster, the experimental interest of which has been emphasized previously (6).

Equipment and Methods

Antibiotics: The batches of antibiotics used were as follows: amphotericin A: batch HA 674-A (titrating 1,000 μg/mg); amphotericin B: batch Diam 2 CR (titrating 818 μg/mg) and batch Diam 8-12-24 CR (titrating 850 μg/mg); amphotericin A and B: batch St 691-11-15J (product containing the two antibiotics in equal proportions); nystatin: batch 5J 4649. These antibiotics were kindly supplied by Drs. G. Rake and L. B. Hobson of the Squibb Institute for Medical Research, New Brunswick, N. J. The antibiotics were placed into a water suspension at the moment of use.

Strains: In the experiments on animals we used the Blastomyces dermatitidis strain number 1P60 of our mycologic collection, a strain which originated from the mycologic service of the Oswaldo Cruz Institute. In the in vitro studies three other strains were used: A 639, A 640 and A 825, which came from the collection of Dr. L. Ajello (Center of Communicable Diseases, Atlanta, Georgia). These strains were maintained in the mycelian phase on Sabouraud's glucose gel, at normal temperature. The fermented phase used for animal inoculation was obtained at 37° by successive passage in the blood-cysteine-brain-heart medium.

In vitro study: The sensitivity of the mycelian phase was studied either in a gel (Sabouraud glucose medium poured into 10 cc. Petri dishes) or in a liquid medium (Sabouraud liquid medium). The sensitivity of the fermented phase was studied at 37° in the blood-cysteine-brain-heart gel medium poured into Petri dishes. For the gelled media we transferred a suspension of fungic elements in rows using a tapered pipette (4 strains per Petri dish). The fungic suspension

was made from ferments to study the ferment phase and from spores to study the mycelian phase.

In vivo study: The animals used were whitemice weighing about 20g and young golden hamsters of the same weight. For incoulations, in every instance, we used 0.5cc of a fermented suspension of Blastomyces dermatitidis IP 50 containing about 1,000,000 cells per cc. Upon autopsy of dead or sacrificed animals, fragments of spleen, livor and lungs were spread upon the Sabouraud medium and held at a temperature of 27°. In some animals, histologic examinations of organs was carried out.

Subcutaneously administered antibiotics were suspended in a physiological solution. Per os administration was performed with an antibiotic suspension in one series of experiments; 1 or 2 drops of the suspension, containing 1 mg. of the product per drop, were administered using a drop-counting pipette twice daily throughout the experiment. In another series the antibiotic was placed into the drinking water at the level of 10 mg. per 20 cc., this volume being available to a group of 5 mice and renewed daily. At the end of the experiment the treatment was stopped 3 days before sacrificing the animals in order to permit elimination of antibiotics possibly fixed in the tissues.

1. Action of Amphotericines in Vitro

The result of the action of amphotericins A and B upon the growth of the mycelian phase (Sabouraud gel or liquid medium at 25°) and upon the ferment phase (blood medium at 37]) of 4 strains of Blastomyces dermatitidis is summarized in Table 1.

It can be seen from the table that amphotericin B has a powerful fungicidal action upon the two phases of Blastomyces dermatitidis, especially in a liquid medium where a concentration of 0.1 µg/cc inhibits the growth of the 4 strains of Blastomyces dermatitidis for a ten day period. Amphotericin A is very much less active especially in the blood medium where one strain in four is inhibited. Both antibiotics diffuse poorly in the gel.

Table 1

In vitro action of amphotericins A and B upon 4 strains of Blastomyces dermatitidis

				n° 60	n° 639	n° 640	n° 625
				12	50	50	50
				0,7	0,7	0,7	1,5
				1,5	1,5	1,5	1,5
				3	3	3	3
				0,1	0,1	0,1	0,1

*Read ten days after spreading

[Legend]: 1) Phase tested; 2) Medium; 3) Antibiotic; 4) Minimum inhibiting concentration* (μg/cc) Strains of Blastomyces dermatitidis; 5) Ferment; 6) Blood-cystein-heart-brain gel 37°; 7) Amphotericin A; 8) Mycelian; 9) Sabouraud gel; 10) Sabouraud liquid.

Action of Amphotericins in Vivo

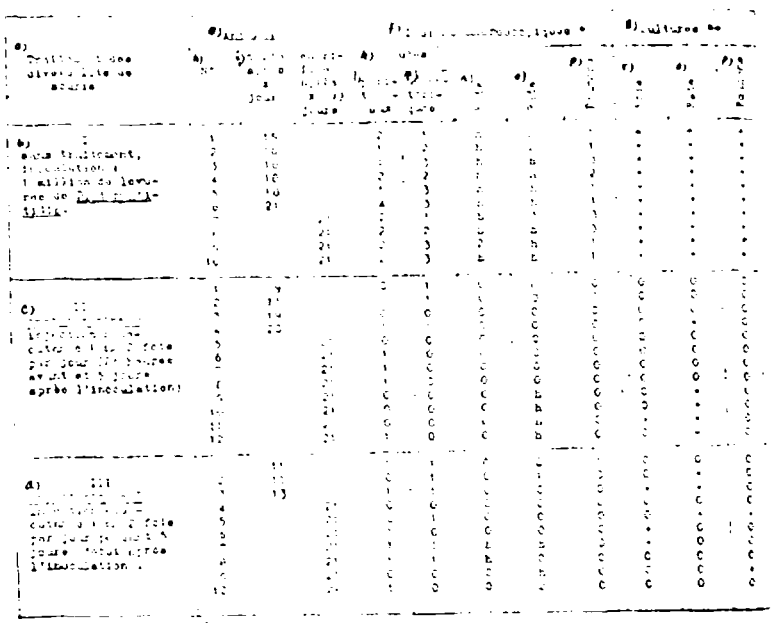
1. Subcutaneous action of amphotericin B

EXPERIMENT I - Three groups each of 10 mice were inoculated intraperitoneally. An untreated group served as control (Group I), another (Group II) was treated with amphotericin B administered subcutaneously for six consecutive days starting twenty-four hours before the inoculation; lastly, another group (Group III) was treated immediately after inoculation for five consecutive days. The daily doses of amphotericin B used were of 2 mg. administered in two injections of 1 mg. morning and evening. A group of mice which were not inoculated but were treated in the same manner as Group II served as controls for the toxicity of the antibiotic. Twenty-one days after inoculation the animals which remained alive were sacrificed and their main organs cultivated in Sabouraud's medium.

RESULTS - Mortality - The administration of amphotericin B considerably reduced the mortality of infected animals.

Table II

Action of subcutaneous amphotericin B in the experimental blastomycosis of the mouse (Experiment I)



*0: no lesions; h: hypertrophy of the organ; 1: very few nodular lesions; 2: few nodular lesions; 4: very many nodular lesions; - **0: culture negative; + culture positive

[Legend]: a) Treatment of the various groups of mice; b) I. untreated, inoculation: 1 million ferments of Blastomyces dermatitidis; c) II. Amphotericin B. Subcutaneous injection of 1 mg. twice daily (24 hours before and 5 days after the inoculation); d) III. Amphotericin B. Subcutaneous injection of 1 mg. twice daily for 5 days (beginning after inoculation); e) Animals; f) Macroscopic lesions*; g) Cultures**; h) Number; i) Dead after X days; j) Sacrificed after X days; k) Nodules; l) Peritoneal; m) Mesenteric; n) Liver; o) Spleen; p) Lungs.

Whereas in the control group 6 of the 10 inoculated animals died by the twenty-first day, in Group II only 4 animals out of 12 were dead and in Group III, 3 out of 10.

Anatomic pathologic lesions-- Upon autopsy of the spontaneously dead or sacrificed animals of Group Number I (untreated) lesions of generalized blastomycosis were noted, in particular numerous peritoneal and mesenteric nodules, and hepatic, spleenic, pulmonary and testicular lesions. In contrast, the majority of animals in Groups II and III showed no macroscopic lesions, but in several animals a few peritoneal or mesenteric nodules and slight splenic hypertrophy could be found.

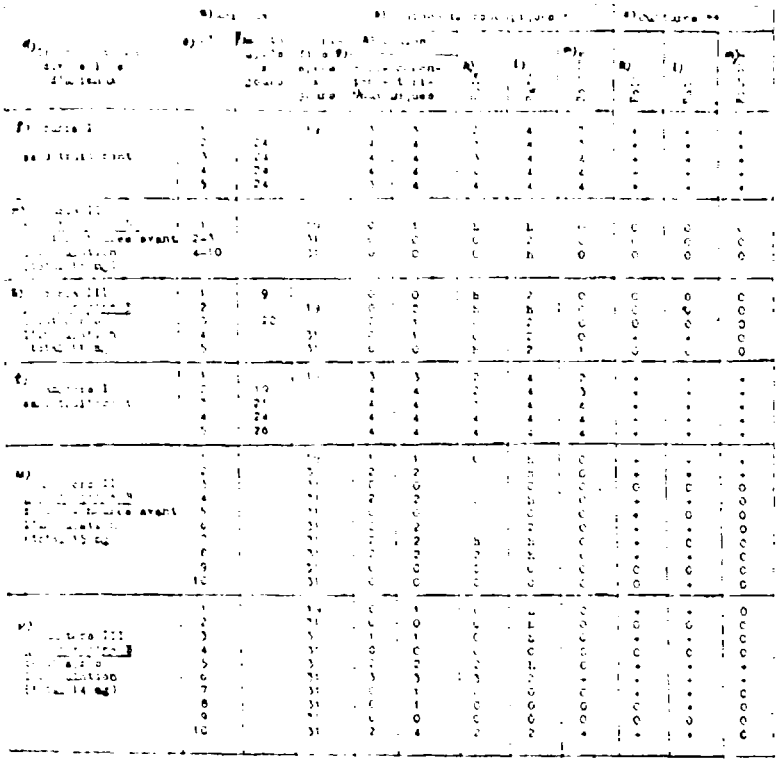
Cultures - The spleen, liver and lung cultures were positive in all animals of the control group; in contrast, the majority of those from animals of the two other groups were negative. Tables II and III summarize the findings from the autopsies and organ cultures.

Several animals from Groups II and III died somewhat earlier than those of the control group. The small number of inflamed lesions and the low percentage of positive cultures from organs of these animals leads one to suppose that a sudden liberation of toxic products coming from the dead Blastomyces occurred at the beginning of treatment.

EXPERIMENT II - This experiment was carried out simultaneously in mice and hamsters treated with amphotericin B under the following conditions: a group of animals (Group II: 10 mice, 10 hamsters) was injected subcutaneously with amphotericin B twenty-four hours before the incoulation; another group (Group III: 5 mice and 10 hamsters) was treated immediately after the inoculation, and lastly a group (Group I: 5 mice and 5 hamsters), inoculated but not treated, served as control. The amphotericin B was given subcutaneously via one injection of 1 mg. per day, in accordance with the following plan: during the first week five injections were given, four the second week and then one injection every three days thereafter. Total amphotericin B dose in the mouse was 11 mg., in the hamsters of Group II it was 15 mg. and in those of Group III, 14 mg. On the nineteenth day, one animal from each group was sacrificed, and on the twenty-seventh day of the experiment the remainder of the living animals were sacrificed. A fourth group which was not inoculated served as control for study of product toxicity.

Table III

Action of subcutaneously administered amphotericin B in experimentally induced blastomycosis of the mouse and golden hamster (Experiment II)



* and ** Same legend as in Table II

[Legend]: a) Animals; b) Macroscopic lesions*; c) Cultures**; d) Treatment of the various groups of animals; e) Number; f) Dead after X days; g) Sacrificed after X days; h) Nodules; i) Peritoneal; j) Mesenteric; k) Liver; l) Spleen; m) Lungs; q) Mouse I, without treatment; r) Mouse II, amphotericin B beginning 24 hours before inoculation (total 11 mg.); s) Mouse III, amphotericin B beginning after inoculation (total 11 mg.); t) Hamsters I, untreated; u) Hamsters II,

amphotericin B beginning 24 hours before inoculation (total 15 mg.); v) Hamsters III, amphotericin B beginning after inoculation (total 14 mg.).

RESULTS - Using amphotericin B subcutaneously we obtained a survival rate of 100 percent in hamsters and 50 to 60 percent in mice inoculated with Blastomyces dermatitidis. The mortality rate in untreated animals reached 100 percent.

Except for slight splenic hypertrophy, no macroscopic lesions were observed in mice of Group II treated twenty-four hours prior to inoculation: hypertrophy of the spleen was also noted in the animals of Group III -- treatment of this group had begun after the inoculation -- but no other lesion was observed except for a few mesenteric nodules in certain animals. The organ cultures of treated animals were negative for all animals of Group II and for 4 of the 5 animals of Group III.

In the treated hamsters greatly reduced macroscopic lesions contrasted with the multiple nodular lesions observed in the controls (Table III); however, they were nonetheless evident than in mice which had undergone the same treatment. The high percentage of positive cultures coming from the hamster organs contrasts with the absence of positive mouse cultures. This difference is probably due to the insufficient quantity of amphotericin administered to the hamsters, whose initial weights of 24-30 gm. doubled during the experiment.

Toxicity - Amphotericin B administered to uninoculated animals showed itself to be devoid of toxicity, but a local reaction was noted at the site of the injection where a yellow deposit of amphotericin B was found; this deposit was larger in the mouse than in the hamster, the quantity of amphotericin administered per gram of body weight being slightly less in the latter.

In conclusion, parenteral amphotericin B prevents the death of mice and hamsters inoculated with Blastomyces dermatitidis and causes the sterilization of the majority of animals treated. Some local reaction occurs at the site of the subcutaneous injection of this product, thus limiting therapeutic use of the method.

EXPERIMENT III - Thirty-five mice were inoculated intraperitoneally with Blastomyces dermatitidis. Twenty-five were treated with amphotericin B added to their drinking water. The quantity absorbed daily per mouse was about 2 mg. The treated mice were separated into two groups. For Group I (15 mice) treatment began three days before inoculation, for Group II (10 mice) treatment commenced at the same time as the inoculation. A third group (Group III: 5 mice) remained untreated as a control for study and a fourth group, uninoculated (Group IV; 5 mice), served as control for study of the toxicity of the antibiotic. One animal for each group was sacrificed nineteen days after inoculation and animals which survived were sacrificed on the thirty-first day.

RESULTS - Mortality -- All untreated animals suffered from generalized blastomycosic lesions and died between the twenty-fourth and twenty-ninth days. Only three mice from among the fourteen of Group I treated prophylactically died between the twenty-fifth and twenty-sixth days, 5 of 9 mice from Group II treated at the same time as the inoculation died between the fifteenth and twenty-sixth days after inoculation.

Pathologic findings. Certain of the treated animals, which died spontaneously, showed a few peritoneal lesions and slight splenic hypertrophy; the others had no signs of infection; in contrast, all untreated animals died with multiple peritoneal, hepatic, splenic and pulmonary lesions (Figs. 1 and 2). The sacrificed animals from the prophylactically treated group showed no blastomycosic visceral lesions; only slight hypertrophy of the spleen was observed.

Cultures - with the exception of one case, all the organ cultures of the animals of the group treated prophylactically remained negative. In the group of mice treated after inoculation all cultures were negative, even in two animals with peritoneal and hepato-splenic lesions.

EXPERIMENT IV - Seventy mice and 30 hamsters, divided into groups of 10, were inoculated with Blastomyces dermatitidis. Ten mice and 6 inoculated but untreated hamsters made up Group I, control; the other groups of animals were treated orally. For three groups of mice (lots II, V and VII) the fungicidal antibiotics (amphotericin A, amphotericin AB and nystatin) were placed into the drinking water; the dose administered daily was 10 mg. in 20 cc. of water for 5 mice using the same bottle. For three other groups of mice (Groups III, IV and VI) and for the two groups of hamsters (Groups II

Fig. 1. Appearance of lesions of generalized blastomycosis in a golden hamster. According to the legend used for Tables II, III, IV and V, one notes: peritoneal nodules, 4; mesenteric nodules, 4; liver, 3; spleen, 2; lungs, 2.

and III) the antibiotics studied (nystatin, amphotericin B and amphotericin AB) were administered orally with a drop-counting pipette. The dose per mouse was one drop containing 2 mg. of antibiotic twice daily, morning and evening, during the first three days, then two drops of 2 mg. twice daily. For hamsters the same routine was followed but each drop contained 4 mg. of antibiotic. The antibiotics were administered for four weeks, 6 days a week with one day of rest. Treatment commenced one day before inoculation. On the

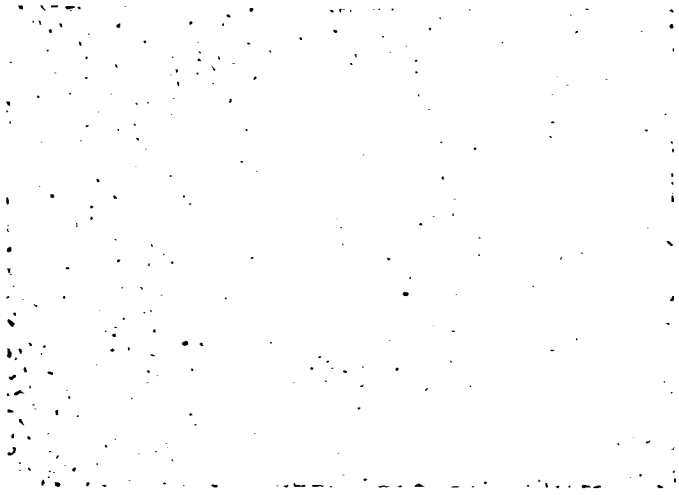


Fig. 2. Histologic section of a peritoneal nodule from an inoculated but untreated hamster. Note the large number of different sized ferments. Hotchkiss -- MacManus stain.

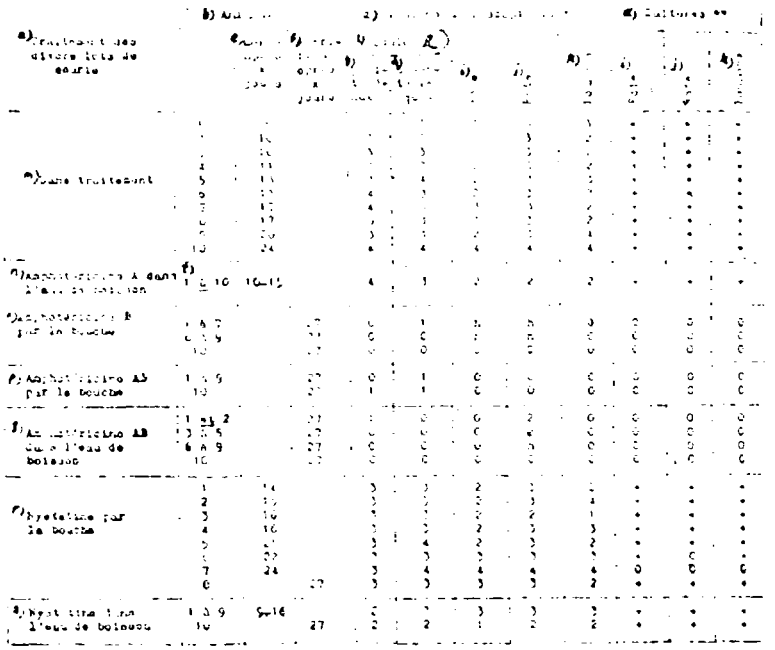
twenty-seventh day after inoculation the animals were sacrificed and the main organs dispersed into Sabouraud's medium as in the previous experiments.

RESULTS - The results of the experiment summarized in Tables IV and V show animal mortaility rates, the anatomic-pathologic lesions observed at autopsy and the results of the cultures of the organs. According to these tables it appears that oral administration of amphotericin B and amphotericin AB (either via pipette or in drinking water) allows survival of all the infected mice and hamsters: the mortality of untreated animals reached 100 percent; no visceral spread of Blastomyces dermatitidis was observed in animals treated with these antibiotics; the only visible lesions were hypertrophy of the spleen and, at times, hypertrophy of the liver. All organ cultures (liver, spleen, lungs) were found to be negative.

In contrast, oral amphotericin A and nystatin showed no therapeutic activity in the blastomycosis of mice and

Table IV

Comparison of the action of orally administered amphotericins A, B, AB and of nystatin in the blastomycosis of the mouse (Experiment IV)

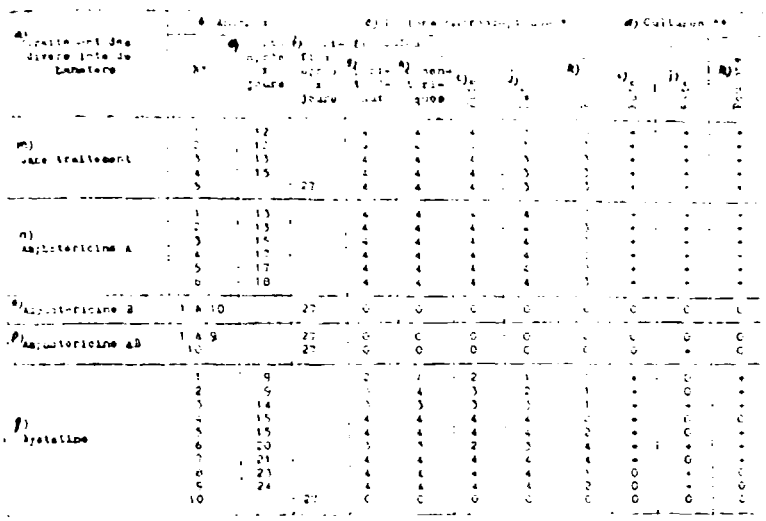


* and ** Same legend as in Table II

[Legend]: a) Treatment of the various groups of mice; b) Animals; c) Macroscopic lesions*; d) Cultures**; e) Dead after X days; f) Sacrificed after X days; g) Peritoneal; h) Mesenteric; i) Liber; j) Spleen; k) Lungs; l) Nodules; m) Without treatment; n) Amphotericin A in drinking water; o) Amphotericin B orally; p) Amphotericin AB orally; q) Amphotericin AB in drinking water; r) Nystatin orally; s) Nystatin in drinking water; t) N.B. a'- to, et - and

Table V

Comparison of the action of orally administered amphotericins A, B, AB and of Nystatin in experimental blastomycosis of the hamster (Experiment IV)



* and ** Same legend as in Table II

[Legend]: a) Treatment of the various groups of hamsters; b) Animals; c) Macroscopic lesions*; d) Cultures**; e) Dead after X days; f) Sacrificed after X days; g) Peritoneal; h) Mesenteric; i) Liver; j) Spleen; k) Lungs; l) Nodules; m) Without treatment; n) Amphotericin A; o) Amphotericin B; p) Amphotericin AB; q) Nystatin; t) N.B. a' - to.

hamsters. Mortality rates with amphotericin A were 100 percent, the same as for the untreated group; the mortality of the nystatin group varied between 87 and 90 percent. The visceral lesions of these animals were identical to those observed in untreated animals.

Discussion

According to our experiments, orally administered amphotericin B has remarkable therapeutic actic . in the experimental blastomycosis of the mouse and golden hamster. Results recently obtained upon various experimental mycoses such as histoplasmosis (1, 5), coccidioidomycosis (8), cryptococcosis (5,7,8), candidosis (7,8) may permit therapeutic application of this antibiotic in severe mycoses of man and animals; at the present time no specific treatment exists. Nystatin, the first fungicidal antibiotic to be found particularly effective orally or locally in Dandida-type infections, has proven ineffective orally against visceral mycoses such as histoplasmosis, coccidioidomycosis, cryptococcosis, etc. because of its low concentration in the blood. Parenterally, nystatin has proven active against numerous experimental mycoses including blastomycosis; but the difficulty of obtaining pure, active and parenterally well-tolerated preparations still precludes therapeutic application via this means. Subcutaneously administered amphotericin B also provokes certain local reactions, but its oral tolerance is perfect and its activity, as we shall show, is remarkable. In contrast, amphotericin A, another antibiotic extracted from the same Streptomyces as amphotericin B, less active in vitro upon Blastomyces dermatitidis, has proven ineffective against blastomycosis. From our experiments, the favorable action of the product containing amphotericins A and B is due solely to the B fraction. The physical-chemical properties of these products as studied by paper chromatography and by bio-auto-radiography in the ultraviolet indicate that amphotericin A is a nystatin-like tetraene while amphotericin B is a heptaene similar but not identical to heptaene of the trichomycin and candicidin type.

According to Louria et al. (5), daily administration to mice of 75 to 150 μg of amphotericin B per kilogram leads to an antibiotic level of 0.70 to 1.5 μg/cc. in the blood; in man, administration of 2.4 to 5 g. per day gives a blood amphotericin level of 0.12 to 0.21 μg/cc. As the sensitivity in vitro of Blastomyces dermatitidis to amphotericin B is considerable (0.1 $\mu g/cm^3$) and as in animals average doses of 100 μg/kg. per day have proven effective in experimental blastomycosis, therapeutic application of this antibiotic has every possibility of being effective in cases of widespread blastomycosis.

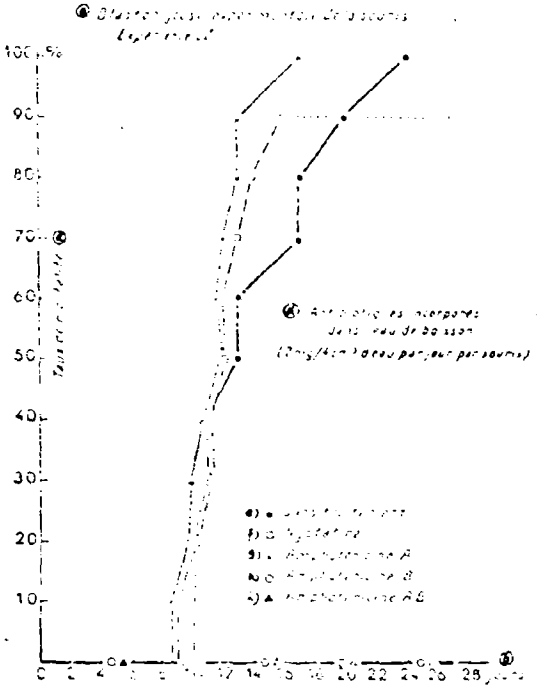


Fig. 3. Mortality rate of mice inoculated with Blastomyces dermatitidis and treated with fungicidal antibiotics placed in the drinking water. (Experment IV)

[Legend]: a) Mortality rate; b) Days; c) Experimental blastomycosis in the mouse Experiment IV; d) Antibiotic placed in drinking water (2 mg/4 cc. of water per day per mouse); e) Untreated; f) Nystatin; g) Amphotericin A; h) Amphotericin B; i) Amphotericin AB.

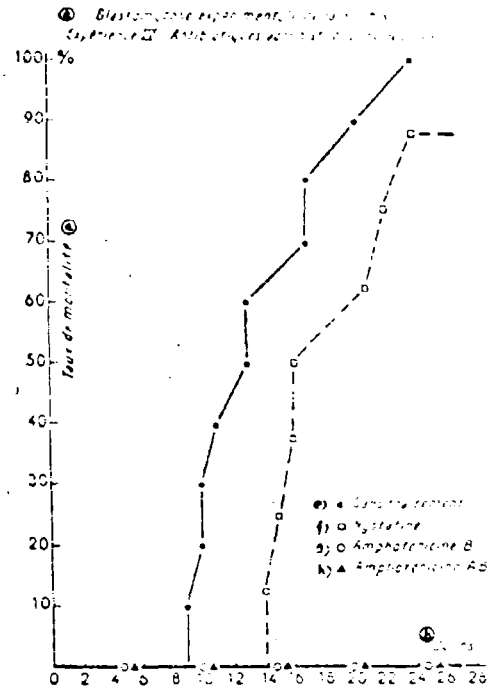


Fig. 4. Mortality rate of mice inoculated with Blastomyces dermatitidis and treated with oral antibiotics (Experiment IV)

[Legend]: a) Mortality rate; b) Days; c) Experimental blastomycosis in mouse Experiment IV - orally administered antibiotics; e) Untreated; f) Nystatin; g) Amphotericin B; h) Amphotericin AB.

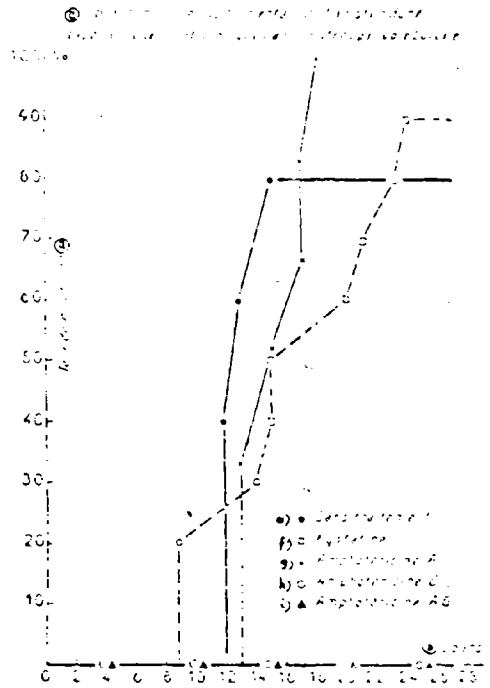


Fig. 5. Mortality rate of golden hamsters inoculated with Blastomyces dermatitidis and treated orally with fungicidal antibiotics. (Experiment IV)

[Legend]: a) Mortality rate; b) Days; c) Experimental blastomycosis in the golden hamster Experiment IV - orally administered antibiotics; e) Untreated; f) Nystatin; g) Amphotericin A; h) Amphotericin B; i) Amphotericin AB.

SUMMARY

Therapeutic Activity of Amphotericin B in Experimental Blastomycosis

Amphotericin B is a heptaene isolated from a Streptomyces. It has a marked inhibitory activity on Blastomyces dermatitidis in vitro and a curative activity on experimental blastomycosis in mice and hamsters.

In vitro, the growth of the mycelium phase (experiment on four strains of B. dermatitidis) is inhibited during 10 days by 0.1 $\mu g/cm^3$ (Sabouraud liquid medium). The growth of the yeast phase is inhibited, by 0.7 $\mu g/cm^3$ (blood-cysteine-brain-heart medium).

In vivo, 100% of the mice and hamsters inoculated intraperitoneally with 1,000,000 B. dermatitidis yeasts survive when they receive amphotericin B, orally in dosages from 2 to 8 μg per animal. The death-rate of the controls is 100% within 4 weeks. But for a few exceptions, the treated animals do not present any anatomo-pathological lesions and the cultures of spleens, livers and lungs are negative.

Amphotericin A and nystatin (both tetraenes), administered orally, are inactive on experimental blastomycosis in mice and hamsters.

The effect of subcutaneous injection of amphotericin B is similar to that of ingestion, but provokes a local reaction at the site of injection.

Amphotericin B is not toxic at the dosages used in the experiments.

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