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RADIOMETRIC DETECTION OF METABOLIC ACTIVITY OF PARACOCCIDIOIDES BRASILIENSIS AND ITS SUSCEPTIBILITY TO AMPHOTERICIN B AND DIETHYLSTILBESTROL

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SUMMARY

Paracoccidioidomycosis (South American blastomycosis) is a systemic disease, strikingly more frequent in males, caused by the dimorphic fungus **Paracoccidioides brasiliensis.** A radiometric assay system has been applied to study the metabolic activity and the effect of drugs on this fungus "in vitro". The Y form of the yeast, grown in liquid Sabouraud medium was inoculated into sterile reaction vials containing the 6B aerobic medium along with 2.0 μCi of ^{14}C-substrates. Control vials, prepared in the same way, contained autoclaved fungi. To study the effects of amphotericin B (AB) (0.1 and 10 μg/ml) and diethylstilbestrol (DSB) (1.0, 5.0 and 10 μg/ml) extra controls with live fungi and no drug were used. All vials were incubated at 35°C and metabolism measured daily with a Bactec instrument. $^{14}CO_2$ production by **P. brasiliensis** was slow and could be followed for as long as 50 days. AB at 10μg/ml and DSB at 5 μg/ml inhibited the metabolism and had a cidal effect on this fungus. The results with DSB might explain the low incidence of the disease in females. This technique shows promise for studying metabolic pathways, investigating more convenient ^{14}C-substrates to expedite radiometric detection and for monitoring the effects of other drugs and factors on the metabolism of **P. brasiliensis** "in vitro".

KEY WORDS: Paracoccidioides brasiliensis; **Radiometric assay; ^{14}C-Substrates; Amphotericin B; Diethylstilbestrol**

INTRODUCTION

Paracoccidioidomycosis is a systemic disease, strikingly more frequent in males than females, caused by the dimorphic fungus **Paracoccidioides brasiliensis.** Despite the various studies on the biochemical composition of both its Y and M forms (12) and several attempts at improving culture media (15), **P. brasiliensis** grows very slowly in all media so far used for its cultivation "in vitro".

Since the early 1970's, a radiometric method, which measures $^{14}CO_2$ evolved from a convenient reaction system, has been used for rapid detection of bacterial growth in clinical microbiology (7). Today the method is widely used as a clinical tool in many hospitals for fast detection and drug susceptibility testing of most common clinical pathogens (8). The method was extended to study the fastidious organisms of ge-

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nus **Mycobacterium** with very promising results (4). This led, in turn, to clinical application with mycobacteria, which are now becoming routine in several centers (18). However, the radiometric method has not been used with **P. brasiliensis.** Because of its high sensitivity, this method might be a useful tool to better understand metabolic requirements of this organism, as it did with mycobacteria (3, 6).

The purpose of this investigation was to determine the feasibility of detecting the metabolic activity of **P. brasiliensis** with the radiometric method using a commercially available radioactive medium, and the effect of factors that might interfere with is metabolism "in vitro".

MATERIALS AND METHODS

Preparation of Fungi:

The yeast form (Y) of **P. brasiliensis,** strain 18, was obtained on solid Sabouraud medium (Difco) from Laboratório de Micologia Médica do Instituto de Medicina Tropical de São Paulo. The fungi were transferred to liquid Sabouraud medium (Difco), and incubated at 35°C for two weeks. The fungal suspension was centrifuged at 1,200 xg and the supernatant discarded. The pellet was ressuspended with 10 ml of sterile saline and the centrifugation procedure repeated 3 times. The microorganisms were counted directly using a Neubauer chamber. The final suspension was diluted with sterile saline to yield 2×10^7 microorganisms per ml.

Reaction System:

The reaction system for $^{14}CO_2$ detection consisted of 30 ml of the 6B aerobic medium in a 50 ml multidose sterile vial along with 2.0 uCi of ^{14}C-substrates (Johnston Laboratories) and 1.0 ml of fungal suspension. All vials were prepared at least in triplicate. Control vials were prepared in the same way, but with autoclaved fungi added. When studying the effect of drugs on the fungal metabolism, extra controls with live fungi and no drug were always prepared for comparison.

Drugs:

The effects of amphotericin B (Squibb) and diethylstilbestrol (Sigma) on the metabolism of **P. brasiliensis** were studied. Amphotericin B was diluted with sterile saline so that the desired concentrations of 0.1 μg/ml and 10 μg/ml were delivered in 0.6 ml. Similarly, diethylstilbestrol was diluted with methanol so that concentrations of 1.0, 5.0 and 10 μg/ml were delivered in 75 μl. Control vials for diethylstilbestrol contained the same volume of methanol.

At the end of the incubation period of the experiments, the fungi from vials containing 10 μg/ml amphotericin B and from vials containing 5.0 μg/ml diethylstilbestrol were recovered after centrifugation at 1,200 xg, washed 3 times with sterile saline, inoculated separately into new 6B medium vials and incubated at 35°C for two weeks.

Radiometric Measurement:

All vials were incubated at 35°C and the $^{14}CO_2$ produced by fungal metabolism was measured radiometrically. The measurement device, a Bactec 301 B (Johnston Laboratories), consisted of an ionization chamber, a vacuum pump, a set of sterile sampling needles and a logarithmic scale up to 1,000 index units (100 index units = 25 nanocuries of ^{14}C activity). The needles penetrated the rubber stopper of the multidose sterile vial and the $^{14}CO_2$ was aspirated into the ionization chamber under vacuum. The atmosphere in the vials was replaced with room air filtered through a 0.22 nm pore size filter membrane. Radioactivity was measured by the ionization chamber as index units. More details of the measurement procedure have been published elsewhere (5). The vials were sampled daily for as long as 50 days. Results were expressed either as index units for the metabolic activity experiments or as percent activity of control vials for the drug experiments. Background readings of the Bactec ranged from zero to 8 index units. All readings above 12 units were considered as positive for growth.

Pilot Experiments:

Pilot experiments with the M form of this fungus were performed as describe above. The

colonies were scrapped from solid Sabouraud medium, suspended in sterile saline, diluted to approximately 2 x 10^7 organisms per ml, inoculated into the 6B aerobic medium and incubated at room temperature. The vials were sampled daily for 7 days.

RESULTS

Metabolic activity of **P. brasiliensis** in 6B aerobic medium was slow. After 2 days incubation, $^{14}CO_2$ production was fairly constant at about 40 index units per day up to 20 days. Then, it fell off to reach background levels by 50 days. Figure 1 represents the differential weekly $^{14}CO_2$ production, that is, the amount of $^{14}CO_2$ produced within a given week. As shown in the figure, differential metabolism peaked at 2 weeks. In every week, $^{14}CO_2$ sampling was performed daily for 5 days, starting over again at the beginning of the next week. Figure 2 represents the cumulative $^{14}CO_2$ production, that is, the addition of the differential $^{14}CO_2$ production within a given time interval.

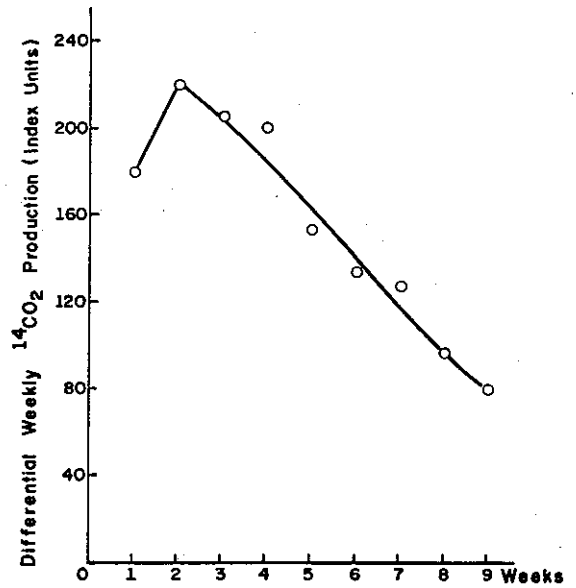

Fig. 1 — Differential weekly $^{14}CO_2$ production by the Y form of **P. brasiliensis** in 6B aerobic medium. There was a peak activity at two weeks, with a progressive decline to background levels. Index units difference from one experimental vial to another was negligible. Each experiment was repeated at least twice.

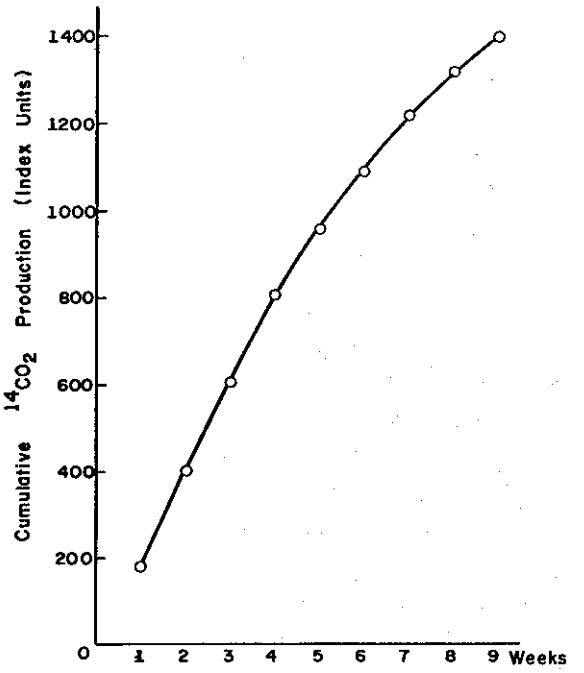

Fig. 2 — Cumulative weekly $^{14}CO_2$ production by the Y form of **P. brasiliensis** in 6B aerobic medium. Index units difference from one experimental vial to another was negligible. Each experiment was repeated at least twice.

Amphotericin B at 0.1 μg/ml was unable to block the metabolic activity of **P. brasiliensis.** After the first week, vials with the drug displayed about 60% of the $^{14}CO_2$ production of control vials, increasing to about 80% at two weeks and 95% at three weeks. At 10 μg/ml, however, metabolism was inhibited and dropped to 35% of the control vials by the end of the first week and to less than 10% at two weeks (Figure 3).

Diethylstilbestrol at 1.0 μg/ml was unable to inhibit the metabolism of **P. brasiliensis;** there was no difference between control vials and hormone vials. At 5.0 μg/ml, the metabolism dropped to 35% of control vials after one week, and to about 5% at two weeks. There was no metabolism at all when hormonal concentration was 10 μg/ml (Figure 4).

Fungi recovered from vials containing 10 μg/ml amphotericin B and from vials containing 5.0 μg/ml diethylstilbestrol showed no metabolic activity after two weeks incubation.

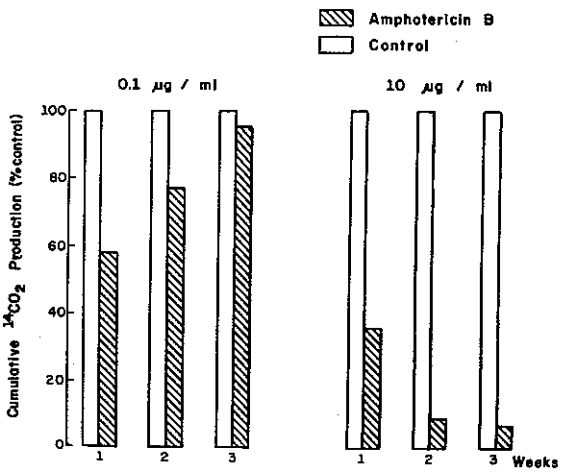

Fig. 3 — Effect of amphotericin B on the $^{14}CO_2$ production of the Y form of **P. brasiliensis** in 6B aerobic medium. At 0.1 µg/ml, there was no inhibition of metabolic activity of the fungus. At 10 µg/ml three was marked inhibition of metabolism, specially after 2 and 3 weeks incubation. Index units difference from one experimental vial to another was negligible. Each experiment was repeated at least twice.

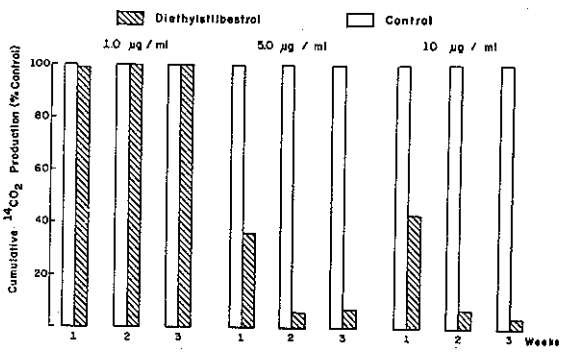

Fig. 4 — Effect of diethylstilbestrol on $^{14}CO_2$ production by the Y form of **P. brasiliensis** in 6B aerobic medium. At 1.0 µg/ml there was no effect. At 5.0 and 10 µg/ml there was marked inhibition of metabolic activity, more pronounced after 2 and 3 weeks incubation. Index units difference from one experimental vial to another was negligible. Each experiment was repeated at least twice.

There was no $^{14}CO_2$ production from vials containing the M form of **P. brasiliensis.**

DISCUSSION

Radiometric studies on metabolic activity of **P. brasiliensis** have not been reported. The present investigation has demonstrated that by using the commercially available 6B aerobic medium, which contins ^{14}C-substrates, radiometric detection of $^{14}CO_2$ production by this fungus is feasible within a few days. In contrast, the conventional culture technique in Sabouraud-agar takes 20 to 30 days for growth detection. Therefore, the radiometric method has the potential for fast, effective screening of new ^{14}C-substrates, more adequate growth media and in the investigation of other factors that might interfere with the metabolic activity of this fungus "in vitro". However, when compared to other fastidious microorganisms such as **Candida albicans** (11), **Mycobacterium tuberculosis** (6) and **Mycobacterium lepraemurium** (2-4), metabolic activity of **P. brasiliensis** as measured by $^{14}CO_2$ evolved from 6B aerobic medium was considerably slower. It is conceivable that the 6B medium, although adequate to support growth of a broad spectrum of the most common clinical pathogens, is not entirely suited for this fungus. Also, the unlabeled substrates in the medium might compete with the ^{14}C-substrates for oxidation. Or, the ^{14}C-substrates provided by the manufacturer and which most likely include (U-^{14}C) glucose, ^{14}C-formate and one of the simpler (U-^{14}C) L-amino acids, may not be adequate for the metabolic requirements of **P. brasiliensis.**

The search for the best ^{14}C-substrate to monitor the metabolism of a given microorganism may be a difficult process. Ideally, the simplest medium able to support its growth, and free of each of the unlabeled substrates to be tested in radioactive form, should be used. Different ^{14}C-substrates would then be added separately to the medium along with the microorganism and a simple yes-or-no answer obtained after incubation. This was the procedure used to determine that (U-^{14}C) glycerol, (U-^{14}C) acetate, (1-^{14}C) fatty acids and ^{14}C-formate were more effective than (U-^{14}C) glucose, (U-^{14}C) pyruvate and (U-^{14}C) glycine with **M. tuberculosis** and **M. lepraemurium** (except ^{14}C-formate) (2-4, 6). Biochemical studies have shown that **P. brasiliensis** utilizes fatty acids such as oleic, palmitic, and linoleic (12) and that growth can be stimulated by amino acids such as asparagine and glycine (9). One can then speculate that perhaps by using separately these substrates labeled with carbon-14, higher $^{14}CO_2$ outputs and faster results would be obtained.

The effect of steroid hormones on the growth of **P. brasiliensis** was reported by MUCHMORE et al. (16) who found that at 25°C and 37°C estradiol and diethylstilbestrol in concentrations of 10 μg/ml completely inhibited the growth of this fungus. However, testosterone and cholesterol in the same concentration showed no effect on any of the isolates tested. The rationale for their investigation was the striking preponderance of male patients with the disease: in our institution (13) and in other series (1, 17), ratios male/female of 13:1 have been found. Such a phenomenon cannot be explainded by different exposure rates to the fungus, since the incidence of delayed hypersensitivity to intradermal paracoccidioidin is the same in both sexes. MUCHMORE et al. (16) did not mean to imply that the simple inhibitory effects of natural estrogen were responsible for the low incidence of the disease in females, since the concentrations used in their investigation were much higher than those that occur in the human body. The present study has confirmed their findings, even at a lower concentration of 5 μg/ml of diethylstilbestrol. Because of the cidal effect of 5 μg/ml of diethylstilbestrol, it is conceivable that, not taking into account other defense mechanisms, this hormone alone would provide a basis for explanation of a low incidence of the disease in females. It is worthy of mention the study of STADALNIK et al. (19) on a very similar disease, coccidioidomycosis. After the 1977 dust storm in the Sacramento Valley, which randomly exposed an entire population to spores of **Coccidioides immitis,** an epidemic of the disease occurred and 12 patients were referred to their hospital. The striking fact was that 11 of these patients were males and only one female. In an randomized exposure such as this, a 1:1 ratio would be expected, and a 11:1 ratio could only be understood if the female population was protected, possibly by estrogens.

Effective levels of amphotericin B range from 2.0 to 4.0 μg/ml of blood (10, 14). The cidal effect of 10 μg/ml described in the present investigation represents less than the clinically effective dose, when one considers the heavy inoculum of 2×10^7 fungi/ml, a concentration not found under clinical conditions. This suggests that either the 6B medium, not being completely suited for **P. brasiliensis,** makes the organism more susceptible to drug action or that its "in vivo" effect may be hindered by partial inactivation of some sort (10).

This investigation has demonstrated that the metabolic activity of **P. brasiliensis** and the effects of factors that might interfere with its metabolism can be studied with a radiometric system. However, the slow $^{14}CO_2$ production using the commercially available 6B aerobic medium strongly suggests that the reaction system here utilized is not optimal. Investigation of other ^{14}C-substrates, such as fatty acids and amino acids, that might be more adequate for the metabolic requirements of this organism, seems to be warranted in future experiments.

RESUMO

Detecção radiométrica da atividade metabólica do Paracoccidioides brasiliensis e da sua sensibilidade à Anfotericina B e ao Dietilestilbestrol

A paracoccidiodomicose (blastomicose sul-americana) é uma doença sistêmica muito mais freqüente no sexo masculino, causada pelo fungo dimórfico **Paracoccidioides brasiliensis.** Um sistema radiométrico foi utilizado para estudar a atividade metabólica e o efeito de drogas sobre este fungo "in vitro". A forma Y do fungo, cultivada em Sabouraud líquido, foi inoculada em frascos estéreis contendo o meio aeróbio 6B, juntamente com 2,0 uCi de substâncias marcadas com carbono-14. Frascos-controle, preparados da mesma forma, foram inoculados com fungos autoclavados. Para estudar os efeitos da anfotericina B (AB) (0,1 e 10 μg/ml) e do dietilestilbestrol (DEB) (1, 5 e 10 μg/ml), controles adicionais foram preparados, contendo fungos viáveis mas não a droga. Todos os frascos foram incubados a 35°C e o metabolismo medido diariamente com uma máquina Bactec. A produção de $^{14}CO_2$ pelo **P. brasiliensis** foi lenta e pôde ser acompanhada por 50 dias. Concentrações de 10 μg/ml de AB e 5 μg/ml de DEB inibiram o metabolismo e tiveram efeito fungicida. Os resultados com DEB poderiam explicar a baixa incidência da doença em mulheres. Esta técnica é promissora para estudar as vias metabólicas, investigar substâncias marcadas mais adequadas para tornar mais rápida a detecção radiométrica do fungo e para acompanhar os efeitos de outras drogas

CAMARGO, E. E.; SATO, M. K.; DEL NEGRO, G. M. B. & LACAZ, C. da S. — Radiometric detection of metabolic activity of **Paracoccidioides brasiliensis** and its susceptibility to amphotericin B and diethylstilbestrol. **Rev. Inst. Med. trop. São Paulo, 29:289-294, 1987.**

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