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An Investigation on Adherence of *Cryptococcus neoformans* Serotypes (A, B, C and D) in Rat Lung Cell Culture

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Abstract: Cryptococcus neoformans causes an opportunistic infection known as cryptococcosis principally in immunocompromised individuals. It is not clearly known whether the serotype of the infecting strain of Cryptococcus neoformans has its own properties related to its adherence to host tissues in disease process. Here, the adherence of each serotype (A, B, C, and D) was investigated using primary rat lung cell culture in a time-dependent manner. After the confluent monolayer of primary rat lung cell culture was obtained, the adherence assay was performed. The mean binding index (BI) value of serotype D was statistically significant ($p<0.05$) when compared with those of remaining sertoypes at second hour. At fourth hour, the difference between any two means and also the difference between the values obtained at both period for all serotypes were not statistically significant ($p>0.05$). These findings suggest that the adherence of each serotype may not be increased in a time-dependent manner, but the adherence of serotype D seems to be significantly increased at early time period (at second vs. fourth hour).

Key Words: Cryptococcus neoformans, serotype, adherence, cell culture.

Introduction

Cryptococcus neoformans, a basidiomycetous yeast, causes an opportunistic infection known as cryptococcosis principally in immunocompromised individuals, with the primary infection usually beginning in the lungs following inhalation of aerosolized yeasts (1). The incidence of disseminated cryptococcal infection has risen in recent years and appears most prevalent in population with AIDS, various malignancies, and those undergoing corticosteroid therapy (1). Indeed, AIDS patients have become as major group susceptible to disseminated cryptococcosis, being the fourth most common opportunistic infection in this patient population (1, 2),

C. neoformans exists in two varieties, Cryptococcus neoformans var. neoformans and Cryptococcus neoformans var. gattii, defining four serotypes. Serotypes A and D belong to Cryptococcus neoformans var. neoformans, and serotypes B and C belong to Cryptococcus neoformans var. gattii (2). Serotyping C. neoformans strains does not help diagnosis, but it is mainly used for epidemiological studies (2). The serotype of the infecting strain is not related to antifungal drug susceptibility. However, Cryptococcus neoformans var. gatti has been associated with more severe infections in humans (2).

While the adherence of pathogenic fungi to host tissues is now considered to be an important virulence factor associated with colonization and pathogenesis, the adherence of C. neoformans to cells derived from typical sites of infection (e.g. lungs and central nervous system) has been recently started to be examined by some researchers (1, 3, 4). Does the serotype of the infecting strain have its own properties, which is differing from remaining serotypes, related to its adherence to host tissues? Here, it is presented in this study. With known four serotypes (A, B, C and D), the adherence of each serotype was investigated using primary rat lung cell culture.

Materials and Methods

A. Yeast Strains and Culture Conditions

In this study, the strains of C. neoformans belonging to serotype A (CDC 236), serotype B (CDC B237), serotype C (NIH 18C) and serotype D (NIH 52D) were used. The yeasts were grown on Sabouraud dextrose agar, then colonies were picked up and suspended in yeast nitrogen base (YNB) containing 1% glucose and 0.5% $(NH_4)_2SO_4$. These suspensions prepared for each serotype were incubated for 24 hours on a shaker at 28°C and then stored at 5°C no longer than a week (4).

The yeasts to be used for adherence assays in cell culture were prepared from refrigerated stock culture by taking $4x10^7$ yeast cells, suspending them again in YNB containing 1% glucose and 0.5% $(NH_4)_2SO_4$ and incubating at 37°C for 48 hours. The number of yeast cells was determined by hemocytometer (1).

B. Preparing Rat Lung Cell Culture

1. Cell Suspension: In the preparation of primary lung cell culture, neonatal Sprague-Dawley rats were used (4). Their lungs, removed aseptically, were put into Minimal Essential Medium (MEM; Gibco BRL, U.K.) containing 2-3% Fetal Calf Serum (FCS; Biological Industries, Israel), 200 µg/ml of streptomycin and 200 units/ml of penicillin. After the lungs were minced to small pieces, they were washed by PBS (Phosphate Buffered Saline, pH: 7.4) 5-6 times, put into 0.25% tripsine solution, and allowed to stand in water bath at 37°C for 30-45 minutes (5). After the tyripsinisation, a highly turbid cell suspension was obtained.

After this cell suspension was filtered through a gauze, the filtrate was centrifuged at 200 x g for 10 minutes. The supernatant was discarded and the sediment was suspended in MEM (pH:7.2) containing 0.37% $NaHCO_3$, 200 units/ml of penicillin, 200 µg/ml of streptomycin, and 15% heat inactivated FCS (6,7).

2. Cell Culture: We modified the procedure used in a similar study of Lucho et al (3). In brief, a sterilized coverslip (Marienfield, Germany) was placed in each well (growth area:$2cm^2$/well) of 24-well tissue culture plate (Costar, Cambridge, MA). Then, 1 ml of cell suspension was dispensed to each well and incubated in a humidified incubator containing 5-10% CO_2. The development of confluent monolayer was monitored by an inverted microscope once in a day. The confluent monolayer was observed in days 5-7. Monolayer cells were confirmed to be predominantly epithelial cells with their typical microscopic morphology. The number of cells was determined by hemocytometer and found to be $3x10^5$/well.

C. Adherence Assay

The procedure used for adherence assay was modified from the method of Merkel et al (1,4). The wells the confluent monolayer obtained was washed two times with EBS (Earle's Balanced Solution) containing 0.37% $NaHCO_3$ and 5% heat inactivated FCS (5). The yeasts prepared for assay at indicated growth conditions were washed two times with PBS. Then, the yeast cell concentration was adjusted to $3.8x10^6$/ml in MEM and 1ml of this suspension was dispensed to each well ($3.8x10^6$/well). The yeast inoculated plate was incubated in a humidified incubator containing 5-10% CO_2 at 37°C for two and four hours. After the end of indicated incubation periods, the wells were washed three times with EBS. With this washing step, the yeasts not adhering to cells were removed. The adherence assays were performed using three wells (triplicate) for each serotype.

D. Microscopic Examination

After washing the wells, the cells in each well were fixed by adding 250 µl of methanol for four minutes. Followed by adding 2-3 drops of Giemsa stain diluted 1/20 by its buffer solution to each well and allowed to stain for 30 minutes. After washing the wells with distilled water, the round coverslips were removed out, and placed on a slide, and examined by x 1000 magnification. The same procedure was applied after four-hour incubation. In microscopic evaluation, totally 90 randomly selected immersion fields from three coverslips for each serotype [3 coverslips for a given serotype (triplicate) x 30 immmersion fields = 90 immersion fields] were examined for the presence of peristoplasmic or intrastoplasmic interaction of yeast cells to lung cells. This interaction was expressed as binding index (BI) using following formula (8):

$$\text{Binding Index (BI)} = \frac{\text{Total number of yeast cells associated with lung cells}}{\text{Total number of lung cells associated with one or more yeast cells}}$$

E. Statistics

Mean BI values calculated for each serotype at second and fourth hours were statistically analyzed using one-way analysis of variance (ANOVA), Wilcoxon matched-pairs signed-ranks test and t-test for paired samples, and the degree of significance was chosen as $p<0.05$.

Results

When the coverslips stained by Giemsa were examined microscopically, the monolayer of lung cell culture was observed with its typical morphology (Figure 1). The very low concentration of yeast cells among epithelial cells was a distinctive finding for each serotype of C. neoformans both at the end of two and four-hour incubation.

In randomly selected 90 immersion fields for each serotype, the presence of peristoplasmic or intra-

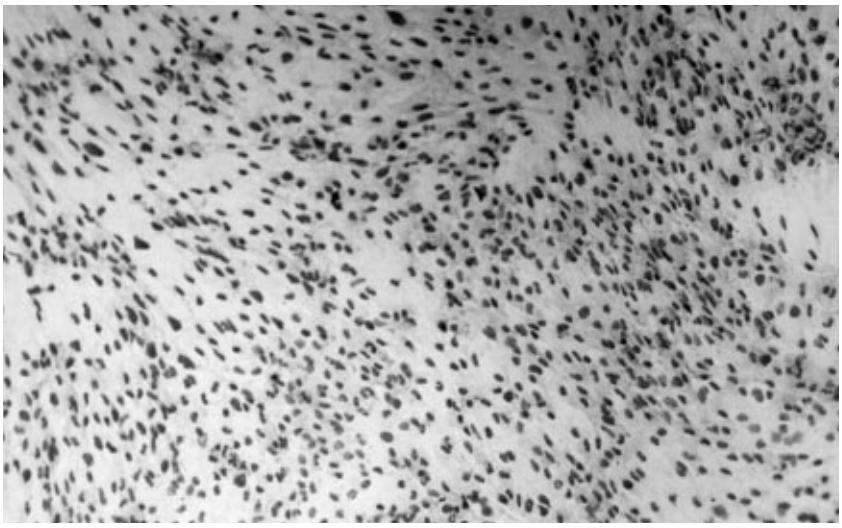

Figure 1. Epithelial cell monolayer of rat lung cell culture on coverslips (before yeast inoculation) (x400, Giemsa).

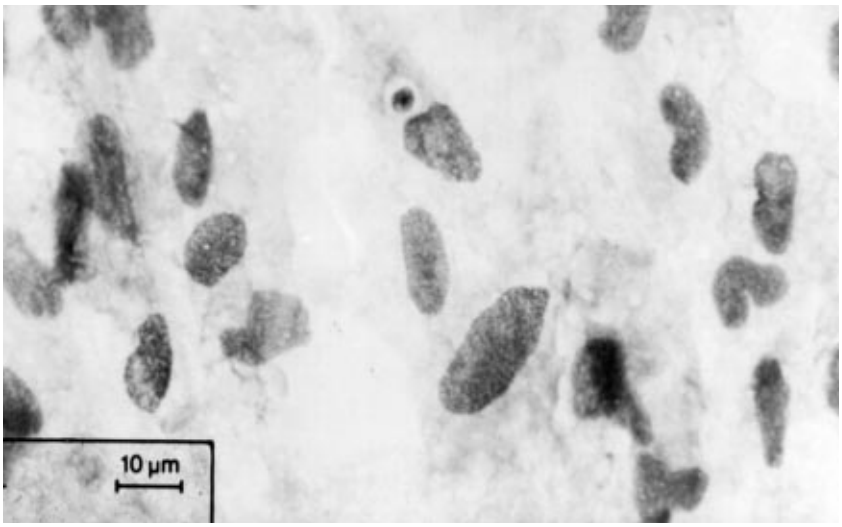

Figure 2. An encapsulated yeast cell (Cryptococcus neoformans) in peristoplasmic area of an epithelial cell (x400, Giemsa).

sloplasmic interaction of yeast cells with lung epithelial cells were observed using x1000 magnification (Figure 2 and 3), and the mean BI values were calculated using above formula. In Table-1, it was shown that the biggest BI value was 2.11. This value indicated that the number of yeast cells interacting with a epithelial cell was not more than two in fact.

A. BI Values at the End of Two-Hour Incubation

At the end of two-hour incubation, BI values of each serotype were calculated and it was observed that the adherence was lowest for serotype A and highest for serotype D by means of BI values (serotype D > serotype C > serotype B > serotype A) (Table-I). The highest BI value (2.11, of serotype D) was found to be statistically significant when compared with those of remaining serotypes at this period ($p<0.05$).

Table 1. The mean binding index (BI) values of four serotypes of Cryptococcus neoformans.

Serotype	Binding Index (BI)[a]	
	At 2-hour incubation[d]	At 4-hour incubation[d]
A	1.09±0.12	1.11±0.06
B	1.18±0.18	1.38±0.15
C	1.36±0.16	1.24±0.06
D	2.11±0.25[b]	1.46±0.23[c]

a= Mean ± standard error.

b= This mean was significant ($p<0.05$) when compared to other means separately at this period.

c= This mean was not significant ($p>0.05$) when compared to other means separately at this period.

d= The difference between two means calculated after 2-hour and 4-hour incubations for each serotype was not significant ($p>0.05$).

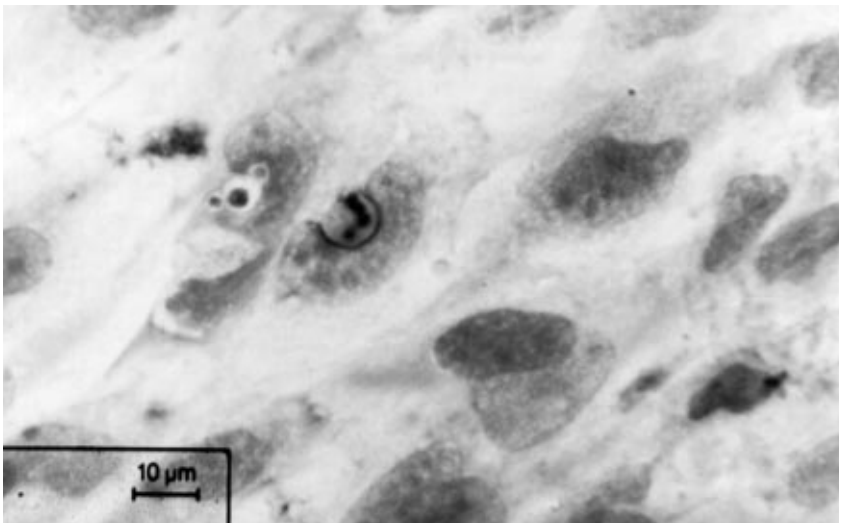

Figure 3. An encapsultad and budding yeast cell (Cryptococcus neoformans) about to be internalized by an epithelial cell (x1000, Giemsa).

B. BI Values at the End of Four-Hour Incubation

At the end of four-hour incubation, the order of BI values of serotypes D and A (serotype D > serotype B > serotype C > serotype A) was similar, but serotypes B and C as observed at the end of two-hour incubation (Table-I). For all serotypes, the increasing and decreasing BI values as compared with those of two-hour incubation were not found to be significant ($p>0.05$). At this time period, the difference between any two BI values also was not statistically significant ($p>0.05$).

Discussion

The adherence of pathogenic fungi to the host tissues is considered as an important virulence factor related to their colonization and pathogenesis (1). It is a critical step first in yeast colonization in tissues and subsequent development of infection in these sites (3). At present, the adhesin-receptor relationship of most microorganisms has been largely studied (9, 10). Candida albicans is the most studied fungus on fungal adherence (11, 12, 13, 14).

There is no other study aiming to detect the probable differences among four serotypes of C. neoformans as a matter of adherence according to our literature search. However, in a few studies, it was tested how the adherence of serotype A was affected by some indicated parameters such as growth phase, growth temperature, and at presence of some chemicals and sugars (1, 3, 4).

The present findings which was obtained at the end of second hour have indicated that the adherence of serotype D was significantly higher than the others. At the end of four-hour incubation, we expected their adherence was affected as the time passed, but no significant differences were obtained for all serotypes. We could not explain the decrease in BI value of serotype D at fourth hour as compared with value obtained at second hour (2.11 → 1.46) and this decrease was not significant too. Although this value was the highest at fourth hour, it was not significant. However, the serotype D, having the highest BI values both at the end of second and fourth hour, might have the feature of "heavily colonizing serotype". The BI value of serotype B, the only increasing value (1.18 → 1.38) at fourth hour as compared with previous period, was not statistically significant.

Although serotype A has the lowest BI values at the end of both time period (1.09 and 1.11), the value at second hour was significant when compared with serotype D, but to serotype B and C. At the end of fourth hour, the low fourth hour value of serotype A was not significant when compared with other serotypes. Merkel et al. (4), in one of their study on adherence with C. neoformans serotype A, grown the organism in a medium with 0.1% glucose and incubated at 28°C, and found no difference between two and five hour values. Besides, in the same study, authors showed that the adherence was increased time-dependently when they used parameters such as growth in a medium with 1% and 5% glucose, and incubation at 37°C (4). Although we performed an incubation at 37°C and used a medium with 1% glu-

cose, we could not observe a time-dependently increase in adherence of serotype A. As a constant condition for all serotypes, we chose a medium with 1% glucose, an incubation at 37°C, and yeasts pre-incubated for 48 hours (late stationary phase), since these parameters were reported to be optimal conditions (1, 3).

It has been known that serotype A and serotype D -a lesser extent-have been responsible from most of the human cases, especially from AIDS population (2, 10, 15, 16). In addition, it was reported that C. neoformans var. gattii (serotype B and C) was associated with more serious infections in humans (2, 10, 15, 16).

Consequently, our findings did not indicate a time-dependently significant increase for any serotype when tested their adherence to cultured rat lung cells. However, a significant and high adherence of serotype D after two-hour incubation may indicate its own special feature if any.

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