EFFECT OF IRON ON CAPSULE BIOSYNTHESIS OF CRYPTOCOCCUS NEOFORMANS

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Abstract–In this study several tests showed that high level of iron either in sera from patients with rheumatoid arthritis, thalassaemia major or in standard iron solutions, have inhibitory effect on capsule production of Cryptococcus neoformans. This work is concerned with the probable role of iron in pigeon's natural resistance against cryptococcal infection. No protease activity was detected in either capsular or acapsular yeasts whereas high activity of β-glucuronidase was demonstrated in the only experimentally produced acapsular one. This suggests the participation of iron in demonstrating pathogenic character of organism and related aspects. Acta Medica Iranica 33 (3&4): 83-87; 1995.

Key words: Cryptococcus neoformans; iron; capsular; acapsular

INTRODUCTION

It is now forty five years since Schad and Caroline (1) first discovered that specific iron-binding proteins, present in blood and in the white of egg, would inhibit the growth of certain bacteria. The ability of iron compounds to abolish the antibacterial effects of body fluids in vitro is now well established; only iron is found to act in this way. Transferrin has been shown to inhibit the growth of fungi such as Candida albicans, the mycelial and yeast forms of H. capsulatum in vitro (2,3,4). King identified transferrin as the dermatophyte inhibitory substance of serum (5). Inhibitory activity of transferrin was shown to correlate with its iron-binding capacity and is disrupted by the addition of iron. This led to the speculation that fungistatic activity of serum was mediated by the capacity of transferrin to bind iron and thus deprive dermatophytes of iron needed for growth.

There are extensive data indicating that microbial growth and secondary metabolism require the catalytic activity of transition metals (3,6,7,8,9). To our knowledge no significant information is available regarding the effect of iron on capsule biosynthesis of Cryptococcus neoformans. The purpose of the present study was to investigate the probable role of iron on capsule production which may cause alteration in enzymatic activities, and, therefore, in pathogenic process.

MATERIALS AND METHODS

Cryptococcus neoformans used in this investigation was isolated from a patient with cryptococcal meningitis and the culture was maintained on Brain Heart Infusion Agar (BHIA, Difco Laboratories Ltd. P.O. Box 148, UK) at 37° C. Capsule production was scored by use of the India ink technique. All the following tests were done in triplicate as follows:

Assay 1). Sera were obtained from four patients with thalassaemia major (TM) and five patients with rheumatoid arthritis (RA) who had more than 200 μg/dl serum iron (10). The yeast cells were treated with one ml of the sera for 2 hours at room temperature. A loopful of serum-treated yeast cells was streaked on BHIA and incubated at 37°C, for 5 days. Finally, the morphology of the colonies and capsule production was examined.

Assay 2). Crytococcus neoformans was introduced to 1 ml (200 μg/dl) of iron standard solution (Wako, Pure Chemicals Industries Ltd., Japan), prior to use; the capsule production and mucoid colonial characterization were examined as in assay 1.

Assay 3). As control, either serum from patients with TM and RA diseases, who had normal levels of serum iron (100-120 μg/dl), or normal saline, was used. Again the morphology of the cells and colonies were tested as in the above.

Assay 4). The small and dry-like colonies obtained in assay 1 were subcultured on BHIA and incubated at 37°C, for 5 days.

Assay 5). Non-mucoid dry-like colonies of Cryptococcus neoformans which were obtained after treatment of the yeast cells (with sera from TM and RA patients containing high iron level), were tested for sugar assimilation to see if any alteration occurred in the sugar assimilation pattern.

Assay 6). In themanners by which the enzymes may participate in the pathogenetic processes, the enzymatic

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activities of the either intact or iron-treated C.neoformans cells were detected by use of API Zym. The urea medium was used for detection of urease production.

Assay 7). Serum iron values of 40 pigeons were obtained as mentioned by Wako Industries.

Assay 8). The dried dropping samples from five different pigeons were examined for their iron content using Wako's kit.

RESULTS

The pre-treatment of Cryptococcus neoformans with sera containing high levels of iron caused an obvious alteration on the yeast cells either in direct examination with India ink or cultural morphology. The yeast cells were much thinner than those of wild-type and became acapsular or hypocapsular. Also the colonies were small, dry-like and rough in comparison with capsular yeast and mucoidal colonies of the controls in assay 3 (Figs. 1a and 1b and Fig. 2). Furthermore when the dry colonies were subcultured on BHIA, they maintained their characteristic feature. Later, when the yeast cells were introduced to 200μg/dl of iron standard solution for 2 hours at room temperature they lost completely their capsules in direct examination and did not grow on BHIA after 5 days at 37°C.

The sugar assimilation tests for both capsular and acapsular yeasts showed that only the capsular did not assimilate the arabinose, cellobiose, and trehalose.

Study of the enzymatic activities in both capsular and acapsular yeasts showed only differences in the amount of enzyme activity as summarized in Table 1. Capsular yeasts showed 10% activity for alkaline phosphatase whereas acapsular showed 30% activity for β-glucuronidase's only. Both forms of the yeast had urease activity whereas no protease activity was detected in either of them.

The amount of iron in one gram of pigeon's dried dropping was as low as 5.56±1.54μg/g whereas the pigeon's serum iron level was as high as 348.97 ±122.68μg/dl (Table 2).

DISCUSSION

Cryptococcus noeformans is the etiological agent of a life-threatening meningitis and the pigeon appears to be the chief factor in the distribution and maintenance of the organism. Cryptococcus neoformans has a characteristic polysaccharide capsule which represents its best understood virulence factor (12,13,14,15,16). Acapsular strains are of reduced virulence and are more readily ingested by leucocytes than are by capsular strains (17,18).

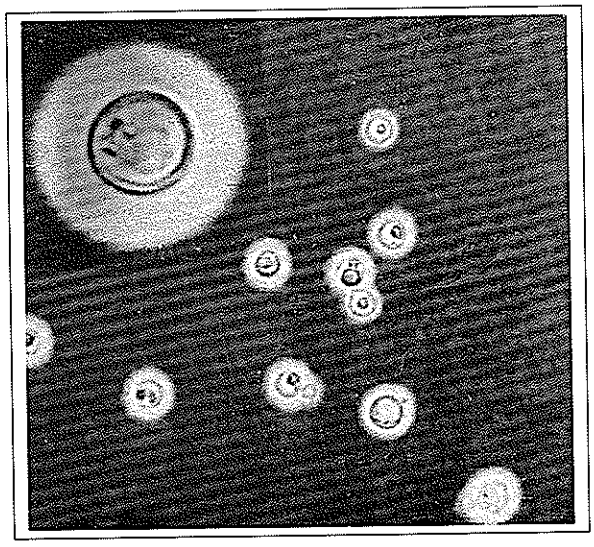
The present study demonstrated that iron had an obvious inhibitory effect on Cryptococcus neoformans capsule production. This process was quite convenient in assessing the lack of a capsule by means of India ink technique, and grossly observing the dry and small colonies.

It seems that there is physiochemical interaction between negative charge of capsule and iron cations which causes the separation of capsule from yeast. Therefore they lack the negative charge which is present on the surface of normal capsular and hypocapsular strains. However culturing of capsular cells after

Table 1. Enzymatic activity of capsular and acapsular Cryptococcus neoformans.

Enzymes	Capsular	Acapsular
Phosphatase alkaline	+	0
Esterase (C4)	+++	++
Esterase lipase (C82)	++++	++++
Lipase (C14)	+	+
Leucine arylamidase	++++	++++
Valine arylamidase	+	+
Phosphatase acid	++++	++++
β-Glucuronidase	0	+++
a-Glucosidase	+++	++++
β-Glucosidase	++++	++++

The approximate amount of the substrate hydrolysed in 4h at 37°C is shown by symbols: ++++ = 40%, +++ = 30%, ++ = 20%, + = 10%, and 0 = not detected.



Figs. 1a. Cryptococcus neoformans' capsular morphology by Indian ink (× 400).

Table 2. The amount of pigeon's serum and iron of dried dropping.

Sample	No. of sample	Amount of iron
Serum	40	348.97±122.68μg dl^{-1}
Dried dropping	5	5.26±1.54μg g^{-1}

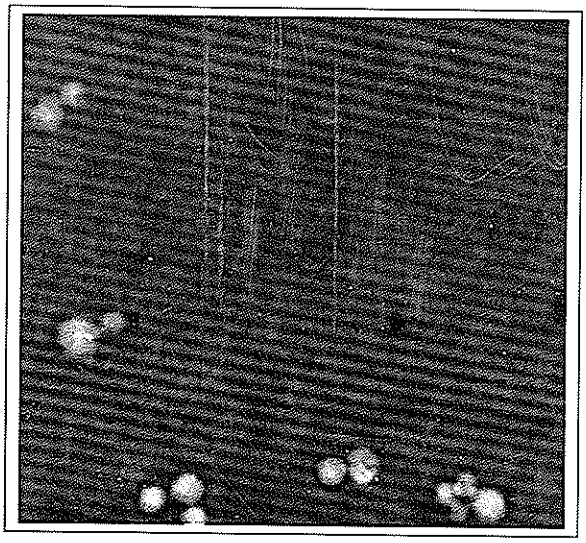


Fig. 1b. Cryptococcus neoformans capsular morphology after pretreatment with sera containing more than 200μg/dl (× 400).

treatment with either TM or RA patient sera which had normal level of iron or with normal saline only, showed glistening, mucoid colonial morphology and capsular form.

Contrary to the results obtained in Assays 1 and 4, all the yeast capsular cells lost their capsules and viability when they were introduced to the iron standard solution. Probably the direct toxic effect of high iron concentration and the absence of any other nutritional elements were responsible for this reaction.

Considering the 5 × 10^7 viable Cryptococcus neoformans cells per gram of pigeon's dropping (19), the competition for the host's iron stores which affects the outcome of many infections (20), and the results obtained in this study, the questions is raised as how a pigeon can be reservoir of Cryptococcus neoformans without itself suffering from any compli-cation? Does iron have a role in protecting the pigeon from cryptococosis? These questions have not yet been satisfactorily answered.

So far, it is presumed that the high average body temperature (up to 40° C) of the pigeon is the only factor in protecting the bird against the disease. But there seems to be no clear-cut answer (21).

In seeking an answer to the above question, we decided to study the amount of iron in sera and droppings of pigeons. As a result we detected high levels of iron in sera in contrast to low amount of iron in dried dropping. These findings suggest that the pigeon as a healthy carrier may have a natural resistance, in harboring Cryptococcus

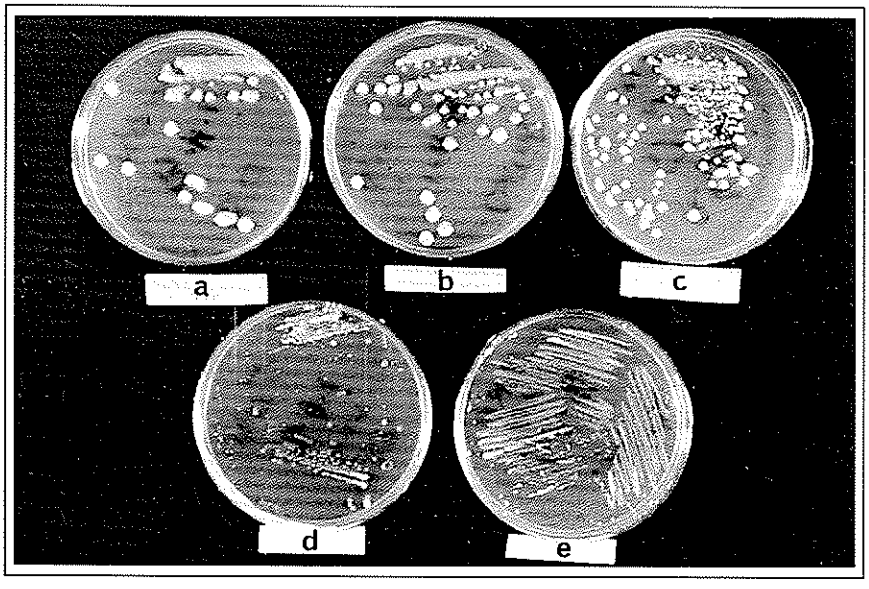


Fig 2. Cryptococcus neoformans colonies on BHIA at 37°C for 5 days. a) Normal feature, b) From normal saline suspension, c) After treatment with serum containing iron less than 200μg/dl, d) After treatment with RA serum containing iron less than 200μg/dl, and e) After treatment with TM serum containing iron less than 200 μg/dl.

neoformans in gut, due to high level of iron in serum. Once the yeast is shed in fecal pellets it may remain viable for extended period of time until it is inhaled as an aerosol by humans, specially by those with underlying disease.

Most workers believe that the primary portal entry of Cryptococcus neoformans is the respiratory tract and that the lung serves as a site where dissemination may occur (12). It can involve almost any organ, but there is extreme tropism for the central nervous system (CNS) particularly the meninges. Neurologic involvement has been initial presentation in approximately 80% of the patients and only a minority is free of signs and symptoms despite of CNS involvement; diagnosis however is nonetheless established by examination of cerebrospinal fluid (CSF).

In humans it was supposed that the organism probably encounters less cellular (phagocytic) response. It has been theorized that selective nutritional factors for the yeasts are present and the absence of inhibitory factors reputed to be in serum may play a role here, but conclusive evidences are lacking (21). Comparison of normal values of iron and transferrin in human plasma and CSF (22) shows that the amount of iron and transferrin CSF is quite low (iron= 1-2μg/dl and transferrin=14.4 mg/l) whereas they are high in plasma (iron= 50-150μg/dl, transferrin=20-40 mg/l). Transferrin, which is the major iron binding protein of the host is known to provide nonspecific immunity by restriction of microbial growth through deprivation of iron. The work done by previous investigators (4,5,18,23) and this study support the hypothesis of extreme neurotropism of the organism, and the reason why pigeon is a healthy carrier. However, the process is more complex and depends on multiple factors to clearly define the role of iron in this respect.

Study of enzymatic activities showed high β-glucuronidase activity in acapsular yeasts in contrast to capsular one. This enzyme is necessary in synthesizing the capsular, fundamental and major extracellular component as glucuronoxylomannan, from uridine diphosphoglucuronic acid (15,17). These data show that high β-glucuronidase activity enables the acapsular yeasts to maintain the capsule and revert to capsular state (virulent form) if they are subcultured on iron free medium. This work suggests clearly how this enzyme associates indirectly with pathogenesis of the organism.

We did not observe any protease activity in Cryptococcus neoformans as demonstrated by Muller and Sethi (24).

Both forms of the yeasts had urease activity. The presence of urease in Cryptococcus neoformans was first reported by Seelinger (25) but as yet has not been implicated in the pathogenesis of the disease. It is more likely that the urease-induced release of ammonia, destroys the host's complement function and which in turn affects the pathogenetic processes involved in cryptococcosis (26).

Since there were some differences between capsular and acapsular forms in sugar assimilation, a new field of study in biotyping strain is probably needed in future. It is also recommended that the rate of iron in treatment of cryptococcal infection be further studied.

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