International Blood Research & Reviews

12(1): 41-51, 2021; Article no.IBRR.65830
ISSN: 2321–7219

Interaction between Phagocytic Cells with Antiphagocytic Properties of *Cryptococcus neoformans*: When Love and Hate Collide

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/IBRR/2021/v12i130144
Editor(s):
(1) Dr. Dharmesh Chandra Sharma, J. A. Groups of Hospital and G. R. Medical College, India.
Reviewers:
(1) Pritam S. Jain, R C Patel Institute of Pharmaceutical Education and Research, India.
(2) Arnold T. Luuse, University of Health and Allied Sciences, Hohoe, Ghana.
Complete Peer review History: http://www.sdiarticle4.com/review-history/65830

Systematic Review Article

Received 10 December 2020
Accepted 16 February 2021
Published 20 February 2021

ABSTRACT

Global systemic fungal infection, including meningeal cryptococcosis caused by the encapsulated yeast Cryptococcus spp, continue to rise in number, especially among HIV infected individuals. Infection occur through inhalation of spore which is abundant in the environment. Initially this fungus stay in the lungs for a certain time without causing any symptoms and when the host's cellular immune status is depleted, it can uses monocyte as a vehicle to take them to the brain, using a mechanism called Trojan Horse mechanism. Normal alveolar macrophage as the first line of innate immune system in the lungs are supposed to phagocytose, internalized and then destroy it inside an organelle named phagolysosome. But Cryptococcus spp seemed to have a built in antiphagocytic mechanism to avoid destruction and even can multiply therein. The interaction between this clever yeast and the host's phagocytic cells determine the course of the disease.

Keywords: Macrophage; monocyte; yeast; polysaccharide capsule; virulence.

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1. INTRODUCTION

The number of infection caused by pathogenic or opportunistic fungi is continously increasing globally, in terms of morbidity and mortality, including Cryptococcosis caused by the yeast *Cryptococcus neoformans*; an abundant environmental fungal pathogens that is the leading cause of global fungal origin of meningitis [1,2]. Approximately 278,000 cases and 181,000 deaths related to Cryptococcus happened globally each year and that number is 15% of AIDS-related deaths worldwide [3]. It is an opportunistic, facultative intracellular pathogen and beside HIV individuals, other risk groups are organ transplant recipients and chemotherapy recipients; although recently cases in immunocompetent individuals have also begun to be detected [1,3,4].

The course of the disease actually begins with inhalation of yeast particles, it can be in the form of spores or desiccated yeast cells into the host lung, as its initial predilection organ. In an immunocompetent host, this fungus can be efficiently eradicated through an immune response including phagocytosis by an innate immune cell called alveolar macrophages (AMs), an immune response that occured earliest [5]. Host AMs represent the first line of defense against the fungus. Once phagocytosed by AMs, fungal cells are killed by a concerted mechanism, involving the host-cellular response [6]. If the cellular response is impaired, phagocytosis of the fungus may be detrimental for the host, since *Cr. neoformans* can grow within macrophages. Usually there is usually a time lag since the first time this yeast entered its host until it caused a clear and definite clinical manifestations [7].

This yeast interacts closely with innate immune cells of the lungs, leading to various fates, including fungal persistence within cells, and possibilities of dissemination of the yeast cells to the brain [7,8] Unfortunately, in an immunocompromised host with poor immune response, the yeast manage to thrive and avoid destruction by the host's immune cells [9-11] The organism will then grow and then disseminate, with the aid of monocytes through the blood, a mechanism called the Trojan Horse Strategy. By using this approach, it can reach the central nervous system after crossing the blood brain barrier and resulting in meningioencephalitis [10]

When I first started to write this review paper, I remember an old rock song entitled "When love and hate collide", where to some extent reminded me to the interaction between macrophages and *Cr. neoformans*. There is a link between the 'love for foreign objects', namely the ability of macrophage to phagocytes, but unfortunately this clever encapsulated yeast has the ability to evade the host's immune armamentarium, especially with its antiphagocytic properties, its properties that we hate. The macrophage-yeast interaction is complex and related with the host's cellular immune status [6-10].

Chronic infection by *Cr. neoformans* results from combination of altered host immune responses and the existence of virulence factors [11] By adjusting their existence inside the host's inner condition, this microorganism showed us that they have mastered the bargaining of cellular vs parasite interaction [8,11] This minireview aimed to discuss how *Cr. neoformans* evade their host's immune armamentarium.

2. BIOLOGY OF *Cryptococcus neoformans*

Cryptococcus neoformans is a basidiomycetous opportunistic yeast, present abundantly in the environment [12,13]. *Cryptococcus neoformans* grows in vegetative state as budding yeast and can be frequently isolated from pigeon guano and tree hollows. During the reproductive sexual stage, *Cryptococcus* manage to change its form, from yeast growth to hyphal growth [14]. Eventhough this yeast undergoes amazing morphological transition during its life cycle, *Cryptococcus* is not classified to be dimorphic because the shape of yeast cells are always the predominant form in the environment, abundant in number and even when found inside the human host. The morphological transition (yeast to hyphal) is never found to be responsible in disease pathogenesis and progression [14].

The development of *Cryptococcus* is relevant to its pathogenicity, at least in three different development condition:

(1) Spores that result from hyphal development during mating are truly infectious propagules where upon inhalation, spores (in addition to desiccated yeast) can stay and colonize the lungs of a host for a certain period without producing any side effect [15-17]. But when the host's immune status is depleted, usually due to HIV infection, this resident *Cr. neoformans* might uses the blood macrophage, propagates to the

bloodstream and crosses the blood–brain barrier, ultimately colonizing brain tissue and leading to substantially fatal consequences if it is not cured properly [15-17].

(2) The phase of sexual reproduction also contributes to the existence of genotypic variability of *Cryptococcus* species inside the host, which may lead to improvement of *Cryptococcus* cell's fitness and virulence [18],

(3) Some genes located within the MAT locus are important during mating and during infection [12,19].

Therefore, the understanding of the relationship between the development of *Cryptococcus* and its life cycle is also crucial in terms of studying cryptococcal pathogenicity [13]. Its antigenicity can be detected and measured relatively compared to its number and development condition, and this approach could help to direct the timely initiation of antifungal therapy [20].

This organism can defend predation by various organisms in nature, ranging from protozoans to even metazoans, through several ready-made virulence properties [17] The free living *Cr. neoformans* is able to compete closely with other type of unicellular organisms, *e.g*, amoebas, paramecia, and to some extent this condition is a training ground for them before they established an infection in their host. Phagocytic amoeboid predators such as amoeba have been proposed to select for survival traits in soil microbes, including *Cr. neoformans*, that can also function in animal virulence by defeating phagocytic immune cells, such as macrophages. Several prior studies have shown that incubation of various fungal species with amoeba can enhance their virulence [21]. Inside their host's body, it interacts with some type of cells dedicated to the innate immune response (macrophages or dendritic cells) that naturally exhibiting various propensities of phagocytosis and intracellular killing of substance considered as corpus alienum [22,5,8].

The fungus *Cr. neoformans* grows best under certain host-control conditions, including the availability of serum low glucose level, 5% CO2, and low Fe level, among others, the cells produce a characteristic and prominent capsule composed mostly of sugar (polysaccharides) [23,24]. The size of the capsule depend on certain condition, under harsh low nutrition condition, it might developed thicker capsule [24,25]. Capsule formation is actually part of its coping mechanism [17,26].

Under routine ordinary staining, *e.g,* Gram-stained smears, *Cr. neoformans* appearance may be stain variably or even poorly with; the cell appearance might disturbed by the existence of the massive thick scale gelatinous non-staining capsule. This polysaccharide based capsule actually inhibits the yeast-like cells to absorb color during staining. In Gram staining, it may appear either as granular cells with Gram-positive rounded inclusions impressed upon its cytoplasmic that coloured pale lavender or sometimes seen as Gram-negative lipoid bodies. Under the regular light microscope examination, the India ink stain is widely used for easy but reliable visualization of the capsule in clinical specimen of cerebral spinal fluid [27].There is a 'easy to spot' zone of clearance or clear "halo" around the sphreical cells due to the non-absorbable portion of capsule that prevent particles of ink pigment to enter the capsule portion. This quick and easy method is reliable to identify *Cr. neoformans*. India ink stain is still considered the best methods of direct examination for identification of *Cryptococcus* spp from spinal fluid [24,27]. Unusual morphological forms actually are not common and uniformity of shape is usually made them very easy to recognize. For attempting to identify this yeast in tissue, expert suggest that the option of using mucicarmine stain might help to specifically staining the polysaccharide portion that located in the cell wall of *Cr. neoformans*.

How to make a direct diagnosis without any delay is also a problem. Its predilection, first in the lung and then when it disseminate to the brain, make it more difficult to find. Cryptococcal antigen (CrAg) acquired from the liqour cerebrospinalis is thought to be the best screening for diagnosis of cryptococcal meningitis (CM) [28]. But unfortunately, this option have some limitation because the result might be questionable if the test conducted in HIV-positive patients. In adults living with HIV who have CM symptoms, serum CrAg negativity may rule out CM, while positivity should prompt induction early antifungal therapy if the option of lumbar paracentesis is not available, e.g lack of` resources to perform the procedure. Only in the first episode of CM, cerebrospinal fluid CrAg positivity is considered diagnostic [20,28].

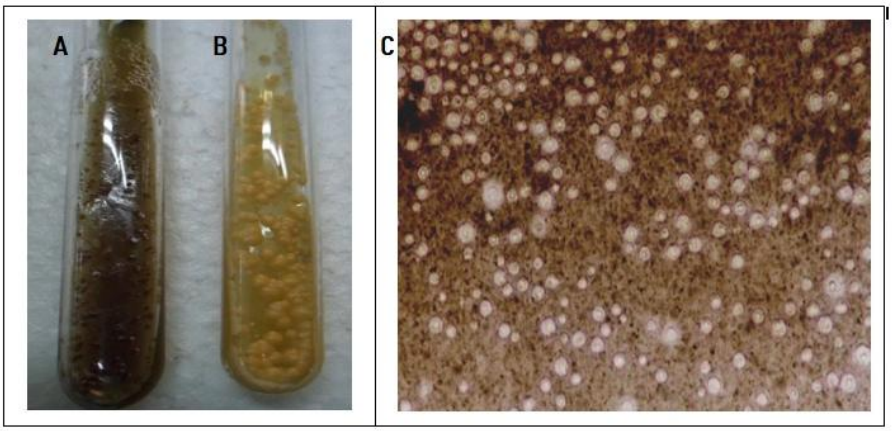

Fig. 1. Macroscopic appearance of *Cryptococcus* spp colony on Bird Seed Agar /BSA medium (A) and on Sabouraud Dextrose Agar/SDA medium (B), while (C) microscopic appearance of *Cryptococcus* spp with India Ink stain (400× magnification). (All pictures taken from specimens belonging to the department of Parasitology, Faculty Of Medicine, Universitas Kristen Indonesia, Jakarta Indonesia)

Adaptation to the host environment (nutrients, pH, and free radicals), mechanism of immune evasion (which include phenotypic variations and the ability to behave as a facultative intracellular pathogen) that makes *Cryptococcus* to survive [8,11,26]. *Cryptococcus neoformans* has two phenotypic characteristics, the capsule and synthesis of melanin, that have a profound effect in the virulence of the yeast, because they both have protective effects for the fungus and at the same time induce host damage as virulence factors [22,15,17]. Finally, the mechanisms that result in dissemination and brain invasion are also of key importance to understand cryptococcal disease, a mechanism facilitated by its facultative intracellular properties and the ability of evasion cellular immune cells [7,9-11].

3. PHAGOCYTOSIS, FACULTATIVE INTRACELLULAR CRYPTOCOCCUS AND EVASION MECHANISM

Phagocytosis is actually a series of receptor-mediated manner that started with activation of the phagocyte cells, chemotaxis, attachment to the foreign particles and finally leads to the internalization of these foreign particles. This action takes place mostly inside the inner portion of phagocytic cells and then those succesfully internalized particles normally will be processed further for being crushed inside a vacuole named phagosome [29]. The size of internalized objects usually ≥ 0.5 μm [29,30]. Recognition by macrophages is a very important key process in generating immune response against invading pathogens; and recognition of pathogens occured through surface receptors present on the macrophage's surface [5].

Cells that have phagocytosis function, *e.g.* macrophages, neutrophils and dendritic cells are recognized by their capability to express a number of receptors on their outer surface that is responsible for phagocytosis; those receptors are meant to recognize, bind, and trigger the internalization of *corpus alienum*, which includes microorganism with obligate intracellular properties, cellular debris and even pieces that come from apoptotic cells [5,29-32]. After phagocytic uptake, ingested particles are processed further; and destroyed using several lytic enzymes, as they progress along certain degradative endocytic steps. This process followed by the formation of the mature phagolysosome inside the phagocytic cells [33]. All of these three different cells: macrophages, dendritic cells, and neutrophils reflect the first line of normal defense against any invading microorganisms, by protecting the host with a unique and specific method for the process of isolating, removing and in the end destroying any harmful object in the host [34,35].

In term of infection, defining the exact mechanisms by which the host immune reaction is able to limit and minimize the impact of infection remains a grey area. Limitless studies on the sophisticated origin of multi-pathway host-pathogen interactions still continues, including for facultative intracellular pathogens. How such

organisms maintain their persistence without being eliminated or killed inside the host's immune cells is to some extent still an enigma [9,29]. These microorganisms have the capability to stay, withstand and even multiply, both intra- and extracellularly, leading to more active and dynamic cross talk with all arms of the host immune system [36]. From this point of view, the act of phagocytosis actually is a two sided sword to the host; it can be considered either an opportunity or hindrance to microorganisms [37].

Viruses, many bacteria, and some protozoa that are obligate intracellular parasites in origin can only multiply inside their host's specific cells [38]. Without being inside a phagocytic cells, these microorganisms will soon be killed by other immune cells, because they will not be able to withstand the harsh condition outside their host's specific cell. Other kind of pathogens, including some type of bacteria and fungi, have the ability to multiply and even survive in both condition, extra- and intracellularly [39]. The choice of microorganism's lifestyle inside their host depends on the availability of specific pathogen's factors that can make them able to survive and/or on conditions that facilitting their existence inside the host [40]

Facultative intracellular yeast, *Cryptococcus* spp, have thrived their built-in capability to avoid phagocytosis by two ways: (1) by blocking adhesion to and/or internalization by phagocytic cells and (2) deactivate some part or some function of the immune cells (will be discuss in following section) [9]. In order to withstand and multiply intracellularly, *Cr. neoformans*, a facultative intracellular pathogen, has built up unique factors that can organize their invasion and even be able to disseminate to a place that is far from its original place. This properties is mainly based on its ability to choose between the intra- and extracellular compartments [22,8]. The need to search for the right genes or factors of the pathogen (or on contrary the host) that might facilitate a certain lifestyle that facilitate their existence and well being should be studied more extensively because gaining those kind of information will benefit us on the development of novel prevention methods (*e.g* vaccines) and treatment strategies for not only to cure cryptococcosis but also, potentially, other intracellular microorganisms.

Eventually, *Cr. neoformans* may rupture phagocytes by lysis or it may escape via vomocytosis, leaving the host cell intact, and then with the aid of monocyte they enter the central nervous system and causing Cryptococcal meningitis [22,5,7-10]. The ability of *Cr. neoformans* to exit cells non-lytically, without causing macrophage damage or death, avoids stimulation of the innate immune response and further allows dissemination of the pathogen to the brain, in order to fulfill their need of inositol [41]. *Cryptococcus neoformans* can also proliferate within immune cells and show remarkable adaptation during infection, including modulation of virulence mechanisms such as polysaccharide capsule expansion [25].

3.1 Capsule Formation

Cryptococcus cell morphology's have a role on its virulence. It is mainly supported by the establishment of a sugar based capsule that cover the whole cell wall. The antigenic componenet of the capsule have the ability to impede the phagocytosis process of the fungus that is supposed to be done by macrophages, dendritic cells , and neutrophils; and further also blocks the internalization of fungal cells by endothelial cells [9,23,24] The first host defense that react against the invasion of *Cr. neoformans* conducted by alveolar macrophages (AM) [5]. The interaction between *Cr. neoformans* and macrophages partly also rely on the dynamic role of the capsule; and this will determine its fate and existence intracellularly. After internalized by macrophages, *Cr. neoformans* may (i) multiply rapidly and result in lysis of the macrophage, (ii) directly be killed by the macrophage or other phagocytic cells, or (iii) manage to live within the macrophage, maintain their number in an equilibrium condition for some time, while waiting for the possibilities of escape and reach the brain without making the host's cell disrupted. Inside the lungs of the host, all three conditions are possible to happen at the same time in one host. Interestingly, more recent studies provide data that the capsule is able to inhibit phagocytosis and also prevent killing by macrophages [9].

Studies have identified some genes and pathways that are involved in capsule production, *e.g* the CAP and CAS gene families and the STE12, UXS1, CHS3, MAN1, AGS1, GPA1, PKA1 and PKR1 genes; all of these genes are responsible for capsule formation or biochemical pathways that are related to capsule formation [42-44]. These genes and pathways that regulate capsule production during infection still remain to be exploited extensively, and this open the horizon for scientist to understand the virulence

of this yeast. Acapsular cryptococci are fragile and easily internalized by neutrophils and macrophages, whereas on contrary, the form of encapsulated yeast are more resistant and difficult for phagocytosis [11,45]. Encapsulated cells are not resistant to phagocytosis but it seems that they still can keep their virulence; this suggests that there is probably other virulence properties of the capsule beside simply preventing phagocytosis [45]. *In vitro*, the dynamic modulation of this phagocytic process by the capsule is determined by (1) capsule size, (2) capsule structure and composition, (3) the presence or absence of serum, and (4) the availability of phagocytic cells.

3.2 Antiphagocytic Protein

Actually, microorganisms have developed several mechanisms to modulate the host immune system to increase their survival and propagation in the host. Their presence in the host is not only revealed by self-produced peptides but also through host-derived chemokines and active complement fragments [46,47] Beside the gene that is responsible for capsule formation, another crucial virulence properties of *Cr. neoformans* is the Antiphagocytic Protein 1 (App1), which prevents the alveolar macrophages from phagocytizing the yeast cell in its surrounding. Beside that, App1 also facilitate melanization [48]. App1 that is located at the outer part of the cell wall inhibits phagocytosis by binding to complement receptors CR2 and CR3 and thus avoids phagocytosis mediated through these receptors [46,49,50].

Furthermore, if these receptors are not available on the macrophage cell surface, the antiphagocytic activity of macrophage is actually missing [50]. App1 has been found in serum of infected patients [49].It is considered to modulate the interaction between *Cr. neoformans* and macrophages. *Cr. neoformans* app1 mutants is actually avirulent in immunocompetent host, because they are efficiently phagocytosed by alveolar macrophages [50].

The role of the gene App1 in the initial stages of cryptococcosis and the differing effects of App1 on its host depends on the immune status/immune level, especially on the availability of cellular immune cells [8,15]. The effort to reveal the mechanisms that arrange its expression as well as how it govern the yeast susceptibility to certain antifungal drugs might help in determining better treatment option.

Treatment of infection caused by *Cryptococcus* spp is crucial, especially if given at the very early stage of infection because it can change the prognosis of the patient [51-53]. Antifungals like amphotericin B and fluconazole or flucytosine to some extent are used widely all over the world to treat cryptococcosis. Antifungal combotherapy are more succesful in treating CM rather than monotheraphy regimen [52]. Usually for already confirmed CM patient, at the inital 1-week amphotericin B regimen given; it consist of amphotericin B (1 mg per kilogram per day administered intravenously) plus either fluconazole (1200 mg per day) or flucytosine (100 mg per kilogram per day) for 7 days, followed on days 8 through 14 by fluconazole (1200 mg per day) [53].

Cryptococcus's virulence factors have been shown to influence the susceptibility to certain antifungal drugs, and that is why more recent studies explore this topic as a more promising approach to succesfully treat the patient [54]. As in the study conducted by Ghaffar *et al*, [3] they examined the relationship between App1 and antifungal drugs. The fact that single amphotericin B in short term actually downregulates App1 expression while exposure to single fluconazole upregulates App1. In addition, App1 was found to increase the susceptibility of the yeast to both amphotericin B and fluconazole, while given in combination. This study is a very good example of how treating virulence factors of *Cryptococcus* spp might be promising.

3.3 Which Cells is Responsible for Phagocytosis?

The specific type of phagocytic cells may determine the outcome and progression of cryptococcosis [5]. In murine models of cryptococcosis study, rat and mouse alveolar macrophages will surely eradicate Cr. neoformans, without the necessity of the presence of serum or gamma interferon (IFN-γ) or other types of cytokines in the reaction [55]. Different stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cr. neoformans* gave an interesting result. Murine resident peritoneal macrophages actually are able to destroy acapsular *Cryptococcus* cells without prior macrophage activation or yeast opsonization, but they can

eradicate encapsulated cells only if IFN-γ is available or in condition if yeast cells are previously opsonized with either fresh serum or an anticapsular antibody [56]. Cryptococcosis in animal model gave us a lot of important information, eventhough when it comes to human cases, it is not immediately readily available as a conclusion [57].

But on contrary to the result on rodent macrophage, human alveolar macrophages have built-in anticryptococcal activity; eventhough its fungicidal capability can be boosted after the addition of fresh serum [58]. But what was interesting,in contrast to murine macrophages, human macrophages have more ability to destroy the acapsular strains compared to the encapsulated strains.

Activation of human alveolar macrophages by adding IFN-γ do not necessarily enhance its anticryptococcal activity but actually has an unwanted deleterious effect because a higher IL-4/IFN-γ ratio contributes to a greater polarization toward the M2 phenotype, which makes macrophages more permissive and tolerant [59-61]. Human blood monocytes or neutrophils, in fresh condition, have the ability to kill*Cr. neoformans*. However, if they are induced to differentiate into macrophages during *in vitro* culturing, their killing activity dissapear, although they can still prevent cryptococcal growth [60,61]. What factor actually make the loss of macrophage killing activity is still unclear, but perhaps may be because of the dissipated myeloperoxidase activity during macrophage differentiation. Although there is some clear differences in the process of yeast's killing by human and murine macrophages that have been noticed, the relative importance of the murine model used as a model of human disease basically is supported by some similarity between the human and mouse pathologies of cryptococcosis.

The complexity of relationship between *Cr. neoformans* and the human arsenal of phagocytic cells is far from clear eventhough there have been many research results obtained. Phagocytic cells are involved in fungal clearance, but their specific functions, separately or in groups, should be investigated more extensively, with consideration given to (1) what is their optimum milleu, (2) their relationship with other arms of the immune response (cellular and humoral), and of course (3) the specific factor(s) produced by the microorganism(s) that interfere with the relationship between this yeast and the phagocytic cells.

4. CONCLUSION

Regarding the course of this host-pathogen relationship and adaptation, there is much to be learned about the possibility of decreasing the hate factor: "its ability to grow and disseminate within the host". Thus, modulation of "the love factor": the expression level of antiphagocytic factors that may play a crucial role in the outcome of the infection or disease progression. These factors may also reflect objectives for novel therapeutic option to cure cryptococcosis.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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DOI: 10.3390/jof6030111

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/65830