Multiple disguises for the same party: the concepts of morphogenesis and phenotypic variations in Cryptococcus neoformans†

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INTRODUCTION

Adaptation of pathogenic fungi to the host environment is key to understanding the diseases caused by these microorganisms. During infection, adaptive responses are triggered to evade the immune response and survive in the host. Most of these responses are regulated by signaling pathways, which induce adaptation to the host nutritional environment, pH and osmotic pressure, and also provide resistance to free radicals and antimicrobial molecules. In addition, pathogenic fungi frequently change their cellular morphology. For example, it is well known how different yeasts induce pseudohyphae and hyphae during infection. These cellular forms have been well characterized in Cryptococcus neoformans, which can be divided in three classes. Two of them represent a common feature among fungi, the encapsulated pathogenic yeast Cryptococcus neoformans is found during infection as blastoconidia. However, this fungus exhibits striking variations in cellular structure and size, which have important consequences during infection. This review will summarize the main aspects related with phenotypic and morphological variations in C. neoformans, which can be divided in three classes. Two of them are related to changes in the capsule, while the third one involves changes in the whole cell. The three morphological and phenotypic variations in C. neoformans can be classified as: (1) changes in capsule structure, (2) changes in capsule size, and (3) changes in the total size of the cell, which can be achieved by the formation of cryptococcal giant/titan cells or microforms. These changes have profound consequences on the interaction with the host, involving survival, phagocytosis escape and immune evasion and dissemination. This article will summarize the main features of these changes, and highlight their importance during the interaction with the host and how they contribute to the development of the disease.

Keywords: Cryptococcus neoformans, morphogenesis, capsule enlargement, giant cells, micro-cells, antigenic variations

Although morphological transitions (such as hyphae and pseudohyphae formation) are a common feature among fungi, the encapsulated pathogenic yeast Cryptococcus neoformans is found during infection as blastoconidia. However, this fungus exhibits striking variations in cellular structure and size, which have important consequences during infection. This review will summarize the main aspects related with phenotypic and morphological variations in C. neoformans, which can be divided in three classes. Two of them are related to changes in the capsule, while the third one involves changes in the whole cell. The three morphological and phenotypic variations in C. neoformans can be classified as: (1) changes in capsule structure, (2) changes in capsule size, and (3) changes in the total size of the cell, which can be achieved by the formation of cryptococcal giant/titan cells or microforms. These changes have profound consequences on the interaction with the host, involving survival, phagocytosis escape and immune evasion and dissemination. This article will summarize the main features of these changes, and highlight their importance during the interaction with the host and how they contribute to the development of the disease.

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1 I dedicate this article to my two sons, Javier and David, for filling up my days with joy and happiness.
Cryptococcus neoformans has traditionally been considered a yeast that does not exhibit filamentous growth or dimorphism, except during the mating process (for reviews, see Heitman et al., 2011; Kozubowski and Heitman, 2011). Although some pseudohyphal forms have occasionally been described during infection (Neilson et al., 1978; Williamson et al., 1996; Gazzoni et al., 2010), this is a rare phenomenon, and it is believed that C. neoformans does not undergo morphological changes in the host. While this concept is true, many articles demonstrate that C. neoformans displays a complex morphogenetic program which results in the appearance of multiple phenotypic forms. In fact, the final result of these variations is the production of multiple types of yeast cells that may differ in their recognition by the immune system.

In this review, I discuss the concepts of phenotypic and morphologic variations in C. neoformans with the purpose of illustrating the complexity of different forms that this pathogen exhibits in vivo and how they contribute to the development of the disease. These changes are of three types: changes in capsule structure, changes in capsule size, and changes in total cell size. Some of these different variations are illustrated in Figure 1. I describe the main characteristics of these variations, and also highlight their importance in infection.

**CHANGES IN CAPSULE STRUCTURE**

The capsule of C. neoformans is mainly composed of polysaccharide. The major component is glucuronoxylomannan (GXM), which comprises around 90–95% of the total mass of the capsule (Cherniak et al., 1980). The other component has been classically known as galactoxylomannan (GalXM; Cherniak et al., 1982), which accounts for 5–10% of the capsular polysaccharide. Both components have high molecular weights, around 10^6 Da for GXM and 10^5 Da for GalXM (Cherniak et al., 1982; McFadden et al., 2006b). The structure of these polysaccharides has been largely studied and revised (see reviews in Doering, 2006; Bose et al., 2003; Janbon, 2004; Zaragoza et al., 2009; Janbon and Doering, 2011). Briefly, GXM is composed of a chain of mannose residues, with substitutions of xylose and glucuronic acid. GalXM was previously believed to be composed of a chain of galactose with substitutions of xylose and mannose. Recently, it was found that it also contains residues of glucuronic acid (Heiss et al., 2009), so the name of glucuronoxylomannan-galactan (GXM-Gal) has been suggested for this polysaccharide. GXM is highly 6-O-acetylated (Cherniak et al., 1988a; Cherniak and Sundstrom, 1994; Janbon et al., 2001; McFadden et al., 2006b). In addition to polysaccharides, the capsule consists of a small proportion of mannoproteins (MPs; Levitz and Specht, 2006). While GXM localizes throughout the whole capsule, GXM-Gal and MPs seem to localize to regions close to the cell wall (De Jesus et al., 2010). More recently, other components have been suggested to be present in the capsule too, such as sialic acid and chitin-like structures (Rodrigues et al., 2008; Gahrs et al., 2009), but their role in capsule architecture remains unknown.

The steps involved in capsule synthesis and fibers assembly are poorly understood. A few capsular genes necessary to produce the capsule (known as CAP) have been described (Chang and Kwon-Chung, 1994, 1998, 1999; Chang et al., 1995), but their exact biochemical function is not fully characterized. More recently, more genes have been identified by massive analysis of a C. neoformans mutant library (Liu et al., 2008), but the interplay of these, and how they regulate capsule synthesis remains to be elucidated. Furthermore, it has been shown that secretion of vesicles loaded with capsular polysaccharide is an important process in capsule synthesis (Rodrigues et al., 2007).

Although the main components of the capsule are characterized, how the polysaccharides are spatially organized is still unknown. The polysaccharide is organized in fibers that can self-associate through non-covalent interactions (Pierini and Doering, 2001; McFadden et al., 2006b). In the case of GXM, seven different basic structures have been described (Cherniak et al., 1998; Nimrichter et al., 2007), but how these structures are organized, and repeated is still unknown. Recent findings indicate that the capsular fibers are highly branched (Cordero et al., 2011). A clear conclusion from the basic analysis of the capsule is that multiple structures, repetitions, and ramifications are possible in the same GXM or GXM-Gal molecule (see reviews in McFadden et al., 2006a; Rodrigues et al., 2011; and in Rodrigues et al. in this same issue).

As a consequence, C. neoformans has classically been classified in five different serotypes (A, B, C, D, and AD hybrid), based on the different antigenic properties of the capsular polysaccharide. The use of structural and analytical techniques, such as NMR or mass spectrometry has demonstrated that the capsular variation occurs at inter- and intra-strain levels (Cherniak et al., 1988a,b; McFadden et al., 2006b). The use of monoclonal antibodies that bind to the capsule has been extremely useful to understand the variability and complexity of this structure (Dromer et al., 1987; Eckert and Kozel, 1987; Todaro-Luck et al., 1989; van de Moer et al., 1990; Casadevall et al., 1992, 1994; Pirofski et al., 1995). Most of these mAbs have different affinities and specificities for different epitopes in the capsule, which indicates that the capsule
has a complex and heterogeneous structure. Moreover, the epitope distribution for some of these Abs is not homogeneous through the capsule (Maxson et al., 2007b).

The use of mAbs has been of particular interest to show, not only the variability of the capsule between strains, but also how this structure changes in the same strain depending on the environment. McFadden et al. (2007) demonstrated that the binding of two different antibodies to the capsule changed when the fungal strain was grown in different media. Consistent with these findings, the capsule exhibits a high heterogeneity in the expression of epitopes, which depends on cell age, growth conditions, and serotype (Garcia-Hermoso et al., 2004; Gates-Hollingsworth and Kozel, 2009). The relationship between cell age and capsule structure is supported by the fact that the capsule of the buds is structurally different from the capsule of the mother cells (Pierini and Doering, 2001; Zaragoza et al., 2006; Gates-Hollingsworth and Kozel, 2009). Taken together, these findings clearly illustrate how the capsule is a dynamic structure that undergoes important rearrangements.

Changes of the capsule structure also occur in vivo, which suggest that these variations have consequences on the interaction with the host. A seminal article by Charlier et al. (2005) elegantly demonstrates how the capsule undergoes significant rearrangements during infection. The authors use two mAbs which present different in vitro serotype specificity (E1 mAb, which binds to serotype A, and CRND-8, which binds to serotype D). When animals are challenged with a serotype A strain, the fungus bound E1 mAb at early times of infection. After several days of infection, the binding specificity changed, and the fungal cells preferentially bound the CRND-8 mAb. This evolution of the capsule structure seems to be organ specific (Garcia-Hermoso et al., 2004). Moreover, there is evidence that similar capsular rearrangements occur during human cryptococcal meningitis (Cherniak et al., 1995), where structural changes in the capsule of isolates from patients with recurrent cryptococcosis have been described. The molecular basis underlying the variability in capsule structures are not yet known.

The capsule can also significantly change the polysaccharide density. Many reports indicate that the density of the capsule varies according to its spatial distribution, being denser at the inner locations close to the cell wall (Pierini and Doering, 2001; Gates et al., 2004; Zaragoza et al., 2006; Maxson et al., 2007b). In addition, the density can significantly increase with cellular age and independently of capsule size (Maxson et al., 2007b). This change is associated with a higher resistance of the capsule to factors that induce its separation from the cell, such as γ-radiation, which suggests that increase in density is associated with a higher crosslinking of the polysaccharide fibers (Maxson et al., 2007b). Increase in polysaccharide density also occurs during infection (Gates et al., 2004).

**IMPORTANCE OF CAPSULE STRUCTURE CHANGES DURING THE INTERACTION WITH THE HOST**

The fact that capsule structure can change depending on the environmental conditions has profound consequences for the interaction with the host, since this structure is the first barrier recognized by the immune cells. An example of this situation is provided by the opsonic effect of some antibodies that do not bind to Fc receptors in the macrophages, such as IgM or Fab fragments, and that in consequence, are not opsonic by themselves. In these cases, the binding of the antibody produces a structural change in the capsule that exposes polysaccharide epitopes that can bind to CD18 and induce phagocytosis through the complement receptors (Netski and Kozel, 2002; Taborda and Casadevall, 2002). Conversely, changes in capsule structure alter the recognition of Abs. For example, changes in the acetylation can dramatically influence the binding of Abs to the capsule (Todaro-Luck et al., 1989; Kozel et al., 2003). Capsular differences between serotypes also influence the binding pattern of the same Ab (which can be annular or punctuate, depending on the serotype), and this correlates with opsonic efficiency and protection during infection (Cleare and Casadevall, 1998; Mukherjee et al., 1998). These findings suggest that if a specific adaptive response elicits certain Abs during infection, the ability to change the capsular structure will yield fungal cells which will not be recognized by those antibodies.

Changes in capsular structures occur during the cross of the brain–blood barrier, suggesting that capsular variations are required for dissemination and organ colonization (Garcia-Hermoso et al., 2004; Charlier et al., 2005).

In addition to Ab recognition, capsule structure affects the deposition of other molecules of the immune system. For example, the capsule is a potent inducer of the alternative pathway of complement activation, and it has been shown that differences in capsular structure influence the rate of complement deposition on the capsule. While all the serotypes accumulated the same amount of C3 molecules, the kinetics of the process was much faster when strains from serotypes A and D were used compared to strains from serotypes B and C (Young and Kozel, 1993).

The change in capsule density is another factor that determines some aspects of the interaction with the host. In particular, capsule density influences the penetration of different molecules of the immune system, such as complement or Abs, in the capsule, and affects the biological activity of these molecules (Zaragoza et al., 2003b; Gates and Kozel, 2006; Zaragoza and Casadevall, 2006). More recently, it has been demonstrated that a high degree of branching and viscosity of the capsule enhances some properties of the polysaccharide that are important during the interaction with the host, such as complement activation and phagocytosis avoidance, and the ability to act as a protective agent against free radicals (Cordero et al., 2011).

Another phenomenon that highlights the importance of capsule structure during infection is phenotypic switching. In C. neoformans, two different types of colony morphologies, smooth and mucoid, have been described (Goldman et al., 1998) that present differences in capsule viscosity (McFadden et al., 2007). These phenotypic variations are involved in virulence, since mucoid isolates are hypervirulent (Goldman et al., 1998; Guerrero et al., 2010).

Changes in capsular structure can influence not only the interaction between the yeast cells and the immune system, but can also affect the diagnosis of the infection. The most widely diagnostic tool used for cryptococcosis is based on serological tests which use reactive sera against the capsular polysaccharide which circulates in the serum. However, this circulating polysaccharide can change its structure over time, resulting in lack of reactivity, and false negative diagnoses (McFadden et al., 2004).
A striking feature of the capsule is that it can undergo significant changes in its size according to the environmental conditions. This finding was first described in the 1950s (Littman, 1958), and in this work, a medium which produced capsule growth was described. More factors that induce capsule enlargement have been described, such as CO₂, iron limitation, mammalian serum, low nutrient concentration at moderate basic pH and mannitol (Anna, 1979; Granger et al., 1985; Vartivarian et al., 1993; Zaragoza et al., 2003a; Zaragoza and Casadevall, 2004; Guimaraes et al., 2008). More importantly, while the capsule of C. neoformans is small in the environment and in regular laboratory conditions, it significantly increases in size after a few hours post-infection (Feldmesser et al., 2001).

Capsule enlargement represents a significant change for the cell. In cells with large capsule, this structure accounts for approximately 95% of the total volume of the cell (Maxson et al., 2007a). Capsule enlargement is achieved by addition of new polysaccharide to the old capsule (Maxson et al., 2007b; Frases et al., 2009). An estimation of the mass of the capsule has been made, and it has been found that in media where the capsule enlarges, the total mass of the polysaccharide increases by a 20% in only a few hours. (Maxson et al., 2007a). These changes indicate that capsule enlargement is a dramatic change for the cell, and suggest that it is a high energy cost process. Structural studies have shown that the new polysaccharide fibers are different from the ones that are previously attached to the cell (Frases et al., 2008).

Capsule growth is a regulated process, and does not occur indefinitely. After enlargement, the capsule size reaches a limit, which is directly proportional to the size of the cell body, delimited by the cell wall (Zaragoza et al., 2006).

**CHANGES IN THE TOTAL SIZE OF THE CELL**
Changes in the total cell size of C. neoformans occur during infection. Although C. neoformans is mainly found as rounded yeast cells in the host, the size of the blastoconidia found in the tissues (in particular, lungs) undergoes enormous variations. The regular cell size of C. neoformans cells *in vitro* ranges from 5 to 7 μm. During infection, sizes from 1 to 100 μm are found (see Figure 1 and Crucikshank et al., 1973; Feldmesser et al., 2001). Hence, C. neoformans can produce both microforms and macro cells in the host. These variations have occasionally been described in the literature, but it has not been until recently that these types of cells have raised the interest of the scientific community. These phenomena are extremely interesting and suggest multiple consequences during the interaction with the host.

The best characterized phenomenon is the ability to form cells of a tremendous size in the lungs of infected mice. This transition is observed after several days of infection, so it could be considered as a “late” morphological response of the pathogen. Recently, two independent articles have characterized these cells in detail (Okagaki et al., 2010; Zaragoza et al., 2010). These articles defined them as “giant” or “titan” cells. So I will refer to them as fungal or cryptococcal “giant/titan cells.” Using different approaches, these two groups show that fungal giant/titan cells are reproducibly found during infection, but their proportion is highly variable. During infection in murine models, where disseminated disease is accompanied by a strong inflammatory response, the proportion of fungal giant/titan cells is very low, around 5–10% of the total fungal burden. Okagaki et al. (2010) find that this proportion significantly increases when co-infections with MATα and MATα mating types are performed, indicating that the pheromone signaling pathway plays an important role in the development of giant/titan cells. On the other hand, the proportion of cryptococcal giant/titan cells also increases when the fungal burden in the lungs is low and the mice develop chronic asymptomatic infection (Zaragoza et al., 2010). In addition, cAMP signaling pathway is required for cryptococcal giant/titan cell formation (Zaragoza et al., 2010). In agreement, other elements involved in the cAMP signaling pathway have been expressed in macrophages during infection.
recently described to affect giant/titan cell formation, such as Ste3a and Gpr5 (G-protein coupled receptors), and the transcription factor Rim101 (Okagaki et al., 2011). Moreover, analysis of a collection of gene deletion mutants have identified other proteins involved in cellular enlargement, like G1 cyclins, Rho-GTPases, and GTPases-activating proteins, suggesting that titan formation requires the interplay of different pathways in the cell (Okagaki et al., 2011). Ras1 and MAPK signaling pathway do not seem to participate in giant/titan cell induction (Zaragoza et al., 2010; Okagaki et al., 2011). Fungal giant/titan cells are polyploid (Okagaki et al., 2010; Zaragoza et al., 2010), suggesting that the formation of these cells is achieved by endoreduplication, a well known process that yields cells of enormous size. In addition to an increase in the DNA content, fungal giant/titan cells present other phenotypic differences compared to regular cells. The capsule of titan cells has a higher polysaccharide density and presents different antigenic properties compared to cells grown in vitro (Zaragoza et al., 2010). In addition, the cell wall is significantly thicker, reaching a width of 2–3 μm (Zaragoza et al., 2010). Titan cells present peculiar intracellular features, such as the presence of a fragmented vacuole. Cryptococcal giant/titan cell formation is achieved not only by an increase in the capsule size, but also in the cell body size, which in some cases could reach a diameter of 100 μm (Okagaki et al., 2010).

Besides the formation of titan cells, C. neoformans can also form micro-cells, of a size lower than 1 μm (Feldmesser et al., 2001). These cells have not been studied in detail, but they are a common feature of the C. neoformans infection. The fact that C. neoformans can exhibit giant/titan and micro-cells during infection suggests that this pathogen has developed a complex morphogenetic program that allows adaptation to the host environment.

IMPORTANT OF CHANGES IN TOTAL CELL SIZE DURING THE INTERACTION WITH THE HOST

The presence of cryptococcal giant/titan cells during infection represents a multilevel problem for the immune system. These cells present a higher resistance to oxidative agents (Okagaki et al., 2010; Zaragoza et al., 2010). In addition, titan cells avoid phagocytosis, although macrophages seem to recognize and bind to these fungal cellular forms (see Figure 2 and Okagaki et al., 2010; Zaragoza et al., 2010). These two features confirm that cryptococcal giant/titan cells can evade the host immune system. However, the role of these cells in the pathogenesis of the yeast is still unclear. Co-infection with MATa and MATα cells produced an increase in the proportion of fungal giant/titan cells in the lung, and this correlated with decreased dissemination of the fungus to the brain (Okagaki et al., 2010), suggesting that their large size impairs their ability to exit the lungs and cross biological barriers. During chronic infection, where a low number of yeasts are found in the lungs and there is no inflammation, the proportion of fungal giant cells was around 70–90% (Zaragoza et al., 2010). These observations suggest that, in fact, fungal giant/titan cells are not involved in the development of a disseminated disease. Instead, they provide fungal resistant forms that can persist in the host for long time periods. However, recent findings demonstrate that fungal giant/titan cells are virulent in the non conventional host Galleria mellonella (Garcia-Rodas et al., 2011), suggesting that this type of cells can also contribute to the development of the disease in certain situations, most probably by producing a progeny of cells of regular size.

Cryptococcal giant/titan cells have been also described in other model hosts. Recently, it has been found that incubation of C. neoformans in ameba extracts results in capsule enlargement (Chrisman et al., 2011), and occasionally, also in the appearance of titan cells (Chrisman et al., 2011). Furthermore, unpublished results from our group indicate that during infection in the non-conventional host Galleria mellonella, C. neoformans also forms giant/titan cells (Garcia-Rodas et al., 2011). These findings indicate that gigantism is a general feature elicited by C. neoformans that occurs during infection in different types of hosts, which highlights the idea that this morphological transition plays an important role in fungal survival and persistence in the host.

Cryptococcus neoformans can also produce cells of a smaller size, known as micro-cells. Although their role in virulence has not been described, their presence during infection raises challenging questions. For example, it is tempting to hypothesize that due to their reduced size, these cells could have a particular ability to disseminate and cross biological barriers, such as endothelia and the brain–blood barrier, and in consequence, contribute to the development of cryptococcal meningitis.

FINAL REMARKS ABOUT THE ROLE OF MORPHOLOGICAL AND PHENOTYPIC VARIATIONS IN DIFFERENT PHASES OF THE INFECTION AND DISEASE CAUSED BY CRYPTOCOCCUS NEOFORMANS

As it has been described in this review, C. neoformans can produce a large number of phenotypic forms by inducing changes in the structure and/or size of the capsule, and in the total size of the cell. These changes have profound effects during the interaction with the host. They contribute to immune evasion, adaptation to the host environment, dissemination, and long term survival.
during infection. These phenotypic and morphological variations constitute mechanisms that contribute to the cryptococcal infection, in addition to the well established role of the cryptococcal exopolysaccharide as a virulence factor (see review in Zaragoza et al., 2009). The molecular mechanisms involved in the regulation of these changes, and their contribution to the cryptococcal pathogenesis remain unknown, but it is reasonable to hypothesize that they participate in different states of the cryptococcal disease. After the first contact of the yeast with the host, capsule enlargement (an “early” response of the fungus) contributes to phagocytosis escape and intracellular survival, which allows the yeast to evade the first line of defense elicited by the host. In immunocompetent hosts, the infection is controlled, but the fungal cells are not completely cleared, and it is believed that *C. neoformans* can develop a latent state (Dromer et al., 2011). In these conditions, the production of fungal giant/titan cells (a “late” fungal response) could be a key factor to ensure long term survival. During the most typical clinical manifestation, which is dissemination and meningitis, morphological changes also contribute to the disease. First, the ability to produce capsular rearrangements contributes to the avoidance of recognition by immune cells and antibodies. In addition, capsular rearrangements seem to contribute to yeast dissemination and cross of biological barriers. The capacity to produce micro-cells could also facilitate the dissemination of the fungus and the consequent invasion of the brain. Finally, the large number of different yeast forms that could be found during infection could also play a role in another well known disease caused by *C. neoformans*, which is the immune reconstitution inflammatory syndrome (IRIS) also known as immune restoration disease (IRD). This is a disease developed by some HIV patients who recover their immune system after the initiation of the HAART. In these conditions, if an infection is encountered by the immune system, an exaggerated inflammatory response is elicited, which develops an acute disease. The pathogens which are associated with this disorder are *Mycobacterium tuberculosis* and *C. neoformans* (French, 2009), most probably because their incidence is particularly significant among HIV patients. In the case of *C. neoformans*, I hypothesize that in situations of immune recovery, the presence of multiple variants of cryptococccal cells, with different capsule structures and cell sizes may elicit multiple immune responses simultaneously, which can contribute to the appearance of the IRIS.

**FUTURE PERSPECTIVES**

In conclusion, although *C. neoformans* has not been classically considered a fungus able to undergo morphological changes, it can in fact elicit multiple cellular types which contribute to the survival of the yeast *in vivo*. Although some effects of cryptococcal morphogenesis have been described, many important questions still need to be addressed. These transitions have been described *in vitro* and in murine models, but their presence during human infection has not been fully demonstrated. Clinical and histopathological studies are needed to confirm the occurrence of capsular variations and the formation of micro- and giant/titan cells in humans. In addition, the cellular mechanisms that regulate the variations in structure and size of the capsule and the formation of fungal giant/titan and micro-cells are unknown, and future studies are required to unveil these molecular processes. For this purpose, it is necessary to better characterize the genes involved in capsule synthesis, and in particular, the interplay between them. The finding that vesicle secretion is involved in capsule synthesis suggests that exocytosis processes might be involved in capsular rearrangements, and that induction or inhibition of extracellular transport could result in variations of capsule structure. Concerning the changes in total cell size, the formation of cryptococcal giant/titan and micro-cells implies that *C. neoformans* can induce alterations in their cell cycle, to undergo endoreduplication (to produce gigantic cells), or to reduce the length of the cell cycle (to produce micro-cells). The idea that cell cycle regulation depends on host factors is a challenging hypothesis that deserves special interest in future studies. To conclude, the full characterization of morphological changes in *C. neoformans*, and in particular, the elucidation of the molecular mechanisms involved in these processes will also contribute to the design of new therapeutic strategies.

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