Original Article

Ultrastructural Patterns of Interactions between Murine Lung Macrophages and Yeast Cells of *Cryptococcus neoformans* Strains with Different Virulence

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ABSTRACT

This article presents the ultrastructural patterns of interactions between the murine lung macrophages and cells of low- (RKPGY-881, -1165, -1178) and high-virulence (RKPGY-1090, -1095, -1106) strains of *Cryptococcus neoformans* at the seventh post-experimental day. It was found that if macrophages ingest living yeast cells, the latter can: 1) become completely free from polysaccharide capsules, after that their contents undergo lysis, and cell wall debris are extruded from the macrophage (first scenario); 2) become partly free from their capsules, destroy the phagosomal plasma membrane and induce destructive processes inside the macrophage causing their death (second scenario); or 3) not lose their capsules and localize inside macrophage in latent state (third scenario). Macrophages can also ingest senescent and dead *C. neoformans* cells surrounded by capsules that are lost at the ingesting and phagosome stages (fourth scenario). The study revealed the dependence of cell-mediated immunity on the stage of development of ingested *C. neoformans* yeast cells. Here we describe a new mechanism of capsular polysaccharide elimination of *C. neoformans* yeast cells by murine macrophages.

Key words : murine lung, *C. neoformans*, macrophages, virulence, strains, ultrastructure

Introduction

Macrophages play the leading role in the pathogenesis of cryptococcosis¹⁾. However, there are limited data on interactions between *Cryptococcus neoformans* cells and macrophages at the ultrastructural level²⁻⁶⁾. This process was previously investigated by scanning²⁾, transmission³⁻⁵⁾, quick-freezing and deep-etching electron microscopy methods⁶⁾.

Walters et al.²⁾ investigated the peculiarities of surface morphology of phagocytosis between macrophages and *C. neoformans* cells. Kalina et al.³⁾ found out that phagocytic cells in rabbit lung have many pseudopodia penetrating into the polysaccharide capsules of *C. neoformans* cells and demonstrated the disintegration of the first. Papadimitriou et al.⁴⁾ found that macrophages increased in size and came in contact

with fungal cells by means of thin filopodia and cytoplasmic flaps. Nessa et al.⁵⁾ suggested that the sizes of ingested cell capsules were smaller compared to the yeast cells alone and may be due to degradation of some capsular material inside macrophages. Sakaguchi et al.⁶⁾ found that *C. neoformans* cells were ingested by phagocyte in murine brain. They also observed a dense network of actin filaments in pseudopodia and explained their role as one of the main factors in the force generating system in phagocytic process. Thus, only fragmentary data concerning the ultrastructural aspects of interactions between macrophages and *C. neoformans* cells are available in literature.

Previously⁷⁾, we investigated the peculiarities of interactions between murine alveolar macrophages and *C. neoformans* cells of strains with different virulence at the seventh post-experimental day on light microscope. The purpose of the

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Table 1. Properties of *C. neoformans* strains used and interactions with murine macrophages (occurrence of each scenario)

Strains RKPGY	Cell diameter	Thickness of polysaccharide capsule	Serotype	Virulence	Occurrence of each scenario			
					First scenario	Second scenario	Third scenario	Fourth scenario
881	7.10 ± 0.22	4.95 ± 0.30	A	Low	+	-	+	+
1165	6.75 ± 0.64	4.95 ± 0.30	A	Low	+	-	+	+
1178	7.64 ± 0.36	2.40 ± 0.35	D	Low	+	-	+	+
1090	6.93 ± 0.11	2.21 ± 0.13	A	High	+	-	+	+
1095	7.81 ± 0.48	1.72 ± 0.54	A	High	+	+	+	+
1106	7.13 ± 0.21	3.77 ± 0.19	A	High	+	+	-	+

present work is to further study the dynamics of this process in detail using transmission electron microscopy (TEM).

Materials and methods

We studied six strains of *C. neoformans* (Table 1) from the Russian collection of pathogenic fungi (Kashkin Research Institute of Medical Mycology, Russia) isolated from patients. They were cultured on Sabouraud's agar for 3 days at 37°C. For morphometrical investigations, we measured 100 cells of each strain using "crush drop" technique and India ink staining (Table 1). For serological typing, we used PCR-fingerprinting with primer M13 and RFLP (Restriction Fragment Length Polymorphism) of *URA5* with *HhaI* and *Sau96I* restriction enzymes⁸. The virulence of strains (LD₅₀ after intravenous injection varied from 8×10^2 to 1×10^7 cells/mouse) had been previously determined⁹. The strains with LD₅₀ > 10^6 cells/mouse were considered as low-virulence and the strains with LD₅₀ = 1×10^2 – 6×10^4 cells/mouse as high-virulence⁹.

At the time of infection, male mice were 18 to 22 weeks old and had a body weight of 18–20 g. The yeast cell suspensions in 0.5 ml 0.9% NaCl solution were injected intravenously through the lateral tail vein. For light microscopy and TEM, the pieces of lung at the seventh post-experimental day were fixed in 3% glutaraldehyde (in 0.1 M cacodylate buffer) and post-fixed in 1% osmium tetroxide. Then, the samples were dehydrated and embedded in epoxy resin. For light microscopy, the semi-thin sections were cut on Pyramitome 1180 (LKB) and colored with Toluidine blue. We used Alcian blue for histochemical detection of acid polysaccharides as described earlier^{9,10}. The ultrathin sections were cut by Ultratome LKB V and stained with uranyl acetate and lead citrate. The sections were then observed on the JEM 100 SX (JEOL, Tokyo, Japan).

Results

Light microscopy

At the seventh post-experimental day, the majority of

encapsulated yeast cells tended to form large colonized foci in the lung, measuring between 30 and 500 µm (Fig. 1a). A limited number of macrophages were found within the center of colonized yeasts, while at the periphery, more numerous alveolar macrophages surrounded the colonized yeasts. Peripherally located macrophages usually engulfed a few yeast cells, while centrally located macrophages tended to ingest more yeast cells.

Electron microscopy

A series of interactions between alveolar macrophage and *C. neoformans* cell were sequentially illustrated in accordance with the events involving phagocytosis and intracellular fate of the yeast cell (Fig. 1 and 2).

When alveolar macrophages ingest intact yeast cells, the fate of yeast cells are assumed to follow three different pathways partly depending on the virulence of organisms: 1) capsular polysaccharide is absorbed followed by the lytic process that may involve cell wall degradation, where usual phagocytosis and phagolysosome dependent digestion play an essential role throughout the yeast cell killing (first scenario of interaction: phagocytosis and successful intracellular killing); 2) if the yeast cell potentially produces abundant capsular substance, the phagocytosis and killing mechanism does not work successfully, where excessive capsular substance is spilt over into the macrophage cytoplasm (second scenario of interaction: failure of phagocytosis and intracellular killing). In these two scenarios, the nascent and mature yeast cells mainly participate in the host cell interaction. On the other hand, if the electron-dense yeast cells appearing like the desiccated form with intact capsule are contained in the macrophage, they behave as dormant cells (third scenario of interaction). In contrast, if alveolar macrophages engulf the senescent or dead organisms, usually remaining encapsulated, the yeast cells gradually lose their capsule, and the cell wall debris is then extruded from the macrophages (fourth scenario of interaction).

In the first scenario, the interaction of macrophage with encapsulated *C. neoformans* cell began with a tight contact between them (Fig. 1b). Gradually, the macrophage plasma

Fig. 1. Light (a) and transmission electron microscopy (b - o) of macrophages and *C. neoformans* yeast cells in the murine lung at the seventh post-experimental day. a, e – RKPGY-1095; b, c – RKPGY-1165; d, f – RKPGY-1178; g - j – RKPGY-1090; k, l – RKPGY-881; m - o – RKPGY-1106. Explanation for this and other figures: CW – cell wall, DeYC – dead yeast cell; DG – dark granule; DYC – desiccated yeast cell (s); IYC – intact yeast cells, LI – lipid inclusions; M – mitochondrium (a); Mc – microfibrills; Mf – macrophage (s); Mv – microvacuole (s), N – nucleus, Nu – nucleolus, PC – polysaccharide capsule; YC – yeast cell.

membrane obtained the outer contour of polysaccharide capsules and concave shape (Fig. 1b-d). In the zone of contact between the yeast cell capsule and macrophage plasma membrane, the latter formed a well-developed system of extremely thin

(on average 30 nm), long tubules (Fig. 1d-e, arrows), which formed an intensively branched network (Fig. 1f, arrows). The lumen of these tubules was electron-light. The tubules had local, spherical (from 0.4 to 0.6 µm) extensions filled with thin

Fig. 2. Ultrastructure of *C. neoformans* yeast cells and macrophages in the murine lung at the seventh post-experimental day. a, b, d, e – RKPGY-1106; c, i – RKPGY-1095; g, m – RKPGY-881; g, h – RKPGY-1165; k, l – RKPGY-1178.

fibrillar material (Fig. 1g, arrows). The texture of the latter was the same as the yeast cell capsule, localized directly in the lung tissue. The capsular substance was gradually reduced, the outer contour of which appeared irregular in shape (Fig. 1h, i). Thus, transformation from the initial channel-like structures into vacuoles may reflect the phagocytosis activity. The acidic capsular polysaccharide in these vacuoles was more clearly

visualized with Alcian blue stain (Fig. 3).

The fine structure of capsular polysaccharide was greatly variable in its appearance: from an ill-defined amorphous substance (Fig. 1e) to a laminated halo structure (Fig. 1h), which depended on the strain. Such a difference was not observed for the desiccated, senescent, and dead yeast cells. Continued pinocytotic activity of the macrophage's cell

Fig. 3. The Alcian blue histochemical coloring of capsular acid polysaccharides and microvacuoles in cytosol. RKPGY-1095.

membranes may facilitate uptake of capsular substance, leading to reduced capsular thickness (Fig. 1e), and then finally the yeast cells lose their capsules (Fig. 1j). Naked yeast cells devoid of capsular layer were readily exposed to lysosome-mediated hydrolytic enzymes followed by complete degradation (Fig. 1k). The degraded yeast cells showed pale cytoplasm and lysis of nucleus, leading to the disruption of the fine structure. Other organelles, including mitochondria, ribosomes, storage lipid globules, and vacuoles, simultaneously disintegrated. Swollen mitochondria lost their cristae associated with the lysis of their matrix. Plasma membrane was disrupted. Finally, only wrecked cell wall debris was extruded from the macrophage cytoplasm (Fig. 1l). Phagocytosis and killing process (first scenario) occurred independent of the virulence of *C. neoformans* strains.

The first scenario was prevalent and found in all the experiments regardless of strain virulence. Within the macrophage, we often found fusions between the microvacuoles and dark granules (Fig. 1m, n) with variable diameters that made the content of microvacuoles light and homogenous (Fig. 1o).

In murine lungs infected by high-virulence strains (RKPGY-1106 and -1095), we rarely observed intact *C. neoformans* cells inside the macrophages surrounded by the fragmented plasma membrane (Fig. 2a, arrows), which later completely underwent lysis (Fig. 2b). Rare fragmentation or absence of the phagocyte plasma membrane may indicate the occurrence of the second scenario. In this process, the hydrolytic enzymes of ingested yeast cells destroyed the phagocyte plasma membrane leading to destructive processes of macrophages, causing their death. In the phagosomes of macrophages, we sometimes observed more than one yeast cell (Fig. 2c). The lysis of macrophages includes clarification of cytosol, reduction in the number of ribosomes, mitochondrial swelling, clarification of matrix, and reduction of cristae number. At early (Fig. 2d) and subsequent stages (Fig. 2e, f) of ingestion of yeast cells by macrophages, the membrane tubules around the fungal cells were not observed.

As for the third scenario, the fungal cells in latent (dormant) condition were found in murine lungs infected by three low- and two high-virulence (RKPGY-1090, -1095) strains. The internal tubular system was not formed during ingestion of dormant fungal cells (Fig. 2g) and under the phagosome condition (Fig. 2h, i). We never observed “gnawing” of fungal capsules, typical for the first scenario. In macrophages of this type, synthesis and accumulation of storage lipid globules often took place. They were numerous, spherical (from 0.2 to 0.3 μm) and formed aggregations (Fig. 2i). All these characteristics significantly distinguish the third scenario from the first and second. In addition to the ultrastructure of alveolar macrophages, the evidence of their latent existence also confirms the ultrastructural peculiarity of yeast cell content. They were small ($2.9 \times 2.0 \mu\text{m}$), ellipsoidal in form with dense cytosol, masking all the cell components (Fig. 2g, h, i), except lipid droplets.

We often observed that senescent or dead yeast cells ingested by macrophages (fourth scenario) were surrounded by polysaccharide capsules (Fig. 2j). Those cells gradually lost capsules during ingestion (Fig. 2k) and under phagosome condition (Fig. 2l, m), like it was in the first scenario, and formed canals (Fig. 2k, arrows) by the macrophage plasma membrane for capsular polysaccharide “utilization”. Finally, the cell wall debris of undigested fungal cells was extruded from the macrophages.

Table 1 shows that in murine lungs infected by high-virulence strain RKPGY-1095, all the four scenarios occurred. The first and fourth scenarios occurred when all the strains were used. We observed the second scenario only in experiments with two high-virulence strains (RKPGY-1095, -1106). The third scenario was recognized when we used three low- and two high-virulence (RKPGY-1090, -1095) strains. Thus, the second scenario was observed only in high-virulence strains.

Discussion

At the seventh post-experimental day, the interactions between murine macrophages and *C. neoformans* intact cells occurred according to the three scenarios described above, whereas the ingestion of senescent and dead cells occurred only once (fourth scenario).

In the first scenario of interaction, macrophages ingested both growing yeast cells of all six strains and low-virulence mature cells. In the second scenario, macrophages ingested the mature fungal cells of high-virulence strains. Previously, we showed the presence of mitochondrial reticulum, numerous vesicles, and large microbodies in mature yeast cells of these strains^{11, 12}. In the third scenario, macrophages ingested the desiccated yeast cells. We believe that the second scenario was not observed in low-virulence strains because the yeast cells

were not active^{11, 12}. It is possible to expect the appearance of desiccated yeast cells in host tissue infected by low-virulence strains, the presence of which we previously described¹³.

Previously, Harrison and Levitz¹⁴ revealed by light microscopic investigation three types of relationships between *C. neoformans* and macrophages: 1) the fungal cells can be killed by macrophages; 2) fungal cells can grow inside macrophages and cause their lysis; and 3) the fungal cells can exist “in balance” with macrophages (latent state). Our research confirms these data, but we have revealed the fourth type of scenario based on the internal fungal cell ultrastructure.

In all scenarios (excluding the latent condition of fungal cells in macrophages), the macrophage plasma membrane develops the system of thin and long tubules, along which the material of capsular polysaccharide is transported and accumulated in their lumen in the form of local extensions. Later, they are isolated in macrophage cytosol in the form of microvacuoles. We suppose that fusions between microvacuoles and dark granules represent the cellular mechanism of capsular polysaccharide “neutralization” within the macrophage cytoplasm.

The system of macrophage plasma membrane tubules in the phagosome surrounds *C. neoformans* cells in the form of a “corona” and takes part in the process of removing their capsules. It is possible to suppose that: 1) the capsular polysaccharide is transformed into more plastic, flexible form by macrophage enzymes and undergoes hydrolysis; 2) the capsular polysaccharide is transported by macrophage tubules; they develop local extensions that are separated in the cytosol as microvacuoles.

Finally, it would be interesting to see if the mechanisms discovered in this study are also found in other mammalian species including man.

Conflicts of interest

None.

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