Damaging Effects of Lyticase on *Candida albicans* and Changes in the Response of Rat Alveolar Macrophages to the Contact with Yeast-Like Fungi

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Changes in the cell wall of yeast-like fungi *Candida albicans* caused by bacterial enzyme lyticase were studied under an electron microscope. The results were compared with the findings on phagocytosis of lyticase-treated *Candida albicans* by rat alveolar macrophages. It was shown that destruction of the mannan and fibrous outer layers of the cell wall of yeast-like fungi treated with the enzyme led to the release of intracellular organelles and their content. Lyticase treatment reduced the resistance of *Candida albicans* to intracellular digestion by macrophages contributing to completion of phagocytosis.

Key Words: *lyticase; yeast-like fungi; Candida albicans; phagocytosis; rat alveolar macrophages*

Candidiasis is a prevalent mycotic infection. It is often observed in women taking oral contraceptives, in individuals receiving steroid hormones or long-term courses of antibiotic therapy, as well as in patients with diabetes mellitus and congenital or acquired immunodeficiency, and in tumor patients receiving radiation or chemotherapy. According to WHO data, yeast-like fungi (YLF) *Candida* are present in the vaginal microflora of about one-third of healthy women and *Candida* vaginitis constitutes up to 20-30% of urogenital tract infections [1,3]. Treatment with antimycotic drugs can lead to the formation of drug resistance and chronization of the process. This necessitates the search for new alternative treatments for *Candida*. The impact on the viability of YLF by enzymatic destruction of cell structures could become the new trend. From this standpoint, bacterial enzyme lyticase attracts special interest, but possible application of this enzyme as a biological product for therapeutic purposes has not yet been addressed.

Lyticase, an enzyme produced by *Micrococcus luteus,* is used for a long time as a reagent destroying YLF cell wall [5]. Cell treated with lyticase are transformed into a spheroplast sensitive to osmotic pressure fluctuations and its viability is thus limited.

In our previous work we isolated biological preparation of lyticase and studied its effect *in vitro* on YLF *C. albicans* by their viability, adhesion to the vaginal epithelium, morphogenesis, and phagocytosis by macrophages [2]. The next stage of the research was electron microscopic study of *C. albicans* compared with the findings on the phagocytosis of intact and lyticase-treated YLF by rat alveolar macrophages (AM).

MATERIALS AND METHODS

We used lyticase obtained by culturing *Cellulomonas cellulans* and YLF (*C. albicans*) clinical isolate from a woman with symptomatic infection. Electron microscopic studies were performed under a JEM-100 C transmission electron microscope. Preparations

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were obtained as follows: 24-h *C. albicans* culture was treated with 2 and 10 U lyticase (experiment) or buffered saline (control) for 1 h at 37°C on a shaker. YLF cells were separated from the culture medium by centrifugation at 1000 rpm, washed three times with buffered saline, and fixed with 4% paraformaldehyde.

Phagocytitic function of AM was studied using the method described by J. M. Bakker *et al.* [4] with our modifications. AM were isolated from albino outbred male rats (mean body weight 180-200 g). To this end, the rats were narcotized with ether, the chest was opened, and the lungs with trachea and heart were removed. The trachea was previously ligated with a silk thread. The lungs were washed with medium 199 (without antibiotics) containing heparin (5 U/ml) and 1% DMEM/F12 (pH 7.0-7.2). Washout fluid was transferred in centrifuge tubes, placed on ice, and then centrifuged at 2000 rpm for 15 min. The supernatant was removed and 1.0 ml medium 199 containing 20% sterile bovine serum was added to the precipitate. The count of macrophages in 1 ml was counted in a Goryaev chamber. The suspension of AM was diluted with medium 199 containing 20% bovine serum to a concentration of 500,000 cell/ml; 1 ml *C. albicans* suspension containing 25×10^6 cells was added to 1 ml suspension containing 500,000 AM (optimal load 50 YLF per 1 AM). The mixture of AM and YLF in a volume of 1 ml was transferred into penicillin vials with obliquely positioned coverslips, tightly closed with rubber stoppers, and placed at a slant in an incubator at 37°C (culture medium completely covered the coverslips).

For evaluation of phagocytic activity of AM, the suspension was incubated in a thermostat for 60 minutes; digestive capacity was evaluated after thorough washout from extracellularly located YLF and additional incubation for 1 hour and 30 minutes (total 150 min) in new bottles with fresh medium 199 containing 20% bovine serum.

After the incubation, coverslips with attached AM were removed with forcepts, gently washed in pure medium 199, fixed for 15 min in methanol, dried, and stained after Romanovsky–Giemsa for 30 minutes. Then the glasses were thoroughly washed with distilled water, air-dried, and dehydrated successively in acetone, acetone–xylene (1:1) mixture, and xylene (1 min each).

Then, coverslips were mounted on glass slides with polystyrene (10 slides in control and experimental groups). On each slide, 100 cells were counted and

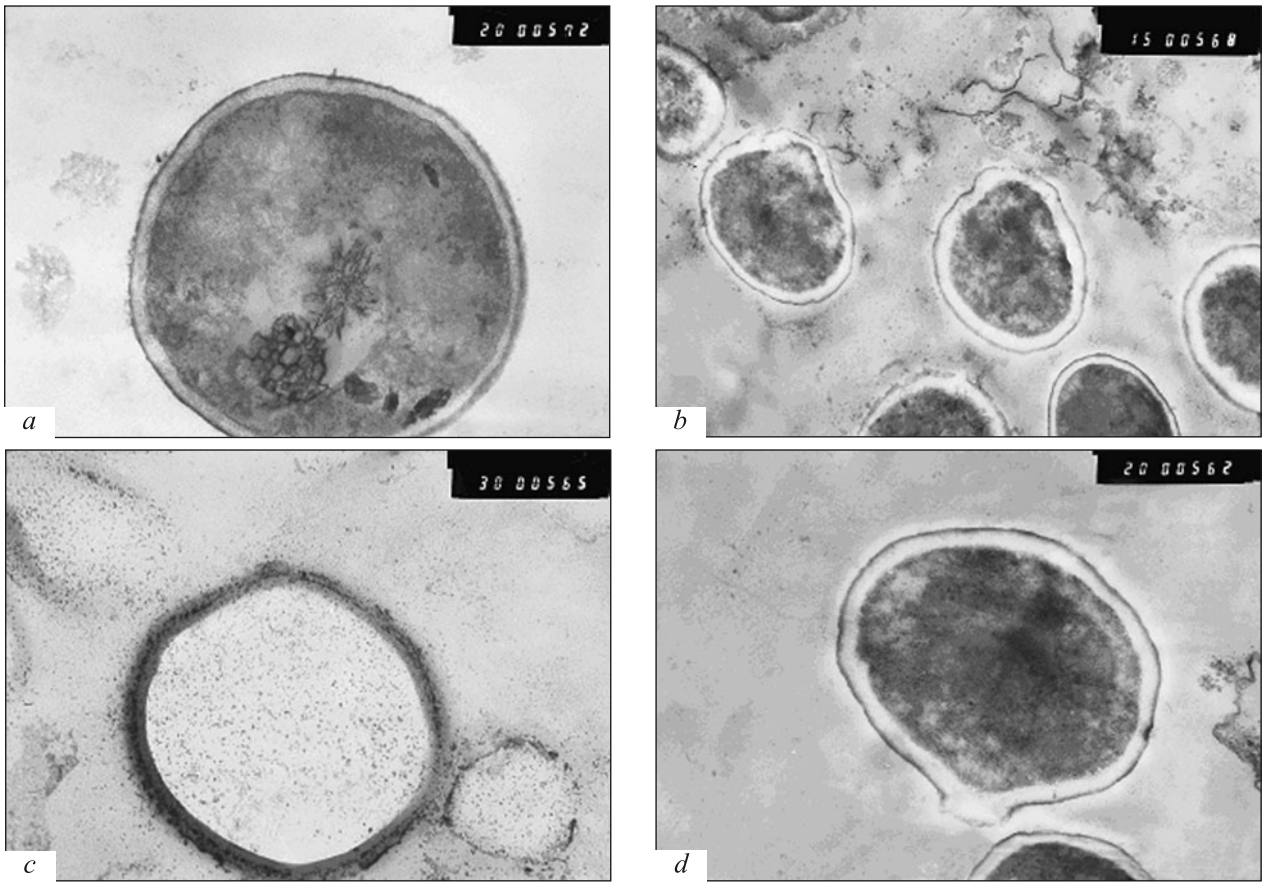

Fig. 1. Electron microscopic study of the effect of lyticase on YLF, ×15,000. *a*) intact YLF; *b-d*) treated with lyticase.

phagocyte number, phagocytic index, total number of YLF per active AM, index of their digestive capacity, and phagocytosis completion index were determined. The experiment was repeated three times.

The data were analyzed using Student's *t*-test and Advanced Grapher Version 2.11 and Microsoft Office Excel software.

RESULTS

Electron microscopic examination revealed significant differences in cell structures of intact and lyticase-treated YLF (10 U). The cell walls of *C. albicans* in control slides had clearly distinguishable chitin, glucan, and mannan layers. Outer fibrous layer presented by well-defined villous structures presumably related to adhesins ensuring adherence of YLF to AM was also detected (Fig. 1, *a*).

After treatment of YLF with the enzyme, mannoprotein layer becomes noticeably thinner than in the control due to cleavage of mannan bonds (Fig. 1, *b, d*), but glucan and chitin layers remained little changed. Ruptures of the entire cell wall of YLF were sometimes seen (Fig. 1, *d*) surrounded by a clear zone; fibrillar layer is absent in these sites, although it is clearly seen in the control (Fig. 1, *a*). Destruction of mannoprotein complex causes degradation of the cell skeleton and death of YLF due to lysis of organelles and detritus release into extracellular medium (Fig. 1, *c*). Damage to the mannan layer is accompanied by separation of the fibrous layer. The outer fibrillar layer is not only a target for the enzyme, but also a functionally active structure participating in adhesion.

It is obvious that lyticase alters the structure and properties of YLF cell wall. We can hypothesize that the resistance of YLF cells to environmental factors and their virulence will decrease even in the absence of coarse alterations and loss of viability. To prove this hypothesis, we studied phagocytosis of lyticase-treated YLF by AM.

Table 1 presents the results of comparative analysis of phagocytosis of intact and lyticase-treated (2 U) YLF by AM.

The data (Table 1) suggest that phagocytic index and phagocytic number after 1-h incubation did not differ significantly in experimental and control samples. However, during subsequent incubation (150 min) phagocytic index and phagocytic number were significantly higher in samples with intact YLF than in samples with lyticase-treated YLF. Moreover, comparison of phagocytic index and phagocytic number at different terms of the experiment showed that digestion of the majority of lyticase-treated microorganisms by AM was completed by the end of incubation, while in the control phagocytosis was still at the capture stage. Index of digestive capacity and index of phagocytosis completion describing the process of digestion, the main indicators of AM function, most clearly demonstrate the antimycotic effect of lyticase (Table 1), because in experimental samples they were significantly (by several times) higher than in the control.

The increase in the index of digestive capacity in experimental samples suggests that the mean capacity of each macrophage to complete phagocytosis increased 3.5 times, and total functional activity of AM resulted in the successful digestion and destruction of absorbed YLF (phagocytosis completion index in the experiment), which 24.6-fold reduced their intracellular content compared to YLF not treated with the enzyme (phagocytosis completion index in the control). The negative values of these parameters in control samples suggest that AM failed to perform phagocytosis and YLF could proliferate and survive for a long time in the target cells. YLF persistence in macrophages is a mechanism of asymptomatic *Candida* infection and recurrent candidiasis.

Thus, treatment with lyticase even in low doses (2 units) significantly reduces the resistance of YLF to intracellular digestion by AM, thus promoting complete phagocytosis of *C. albicans*.

TABLE 1. Parameters of Phagocytosis of Intact and Lyticase-Treated *C. albicans* by AM (*M*±*m*)

YLF	Time of incubation, min	Phagocytic index, %	Phagocyte number	Index of digestive capacity, %	Phagocytosis completion index, %
Treated with lyticase	60	91.0±1.0	8.2±0.9	28.6±2.3*	64.7±0.4*
	150	46.0±0.4*	5.9±0.9*		
Intact	60	88.7±0.8	8.6±1.0	-8.0±0.7	-2.6±0.4
	150	83.7±0.7	9.4±1.1		

Note. *$p<0.05$ compared to that after 60 min.

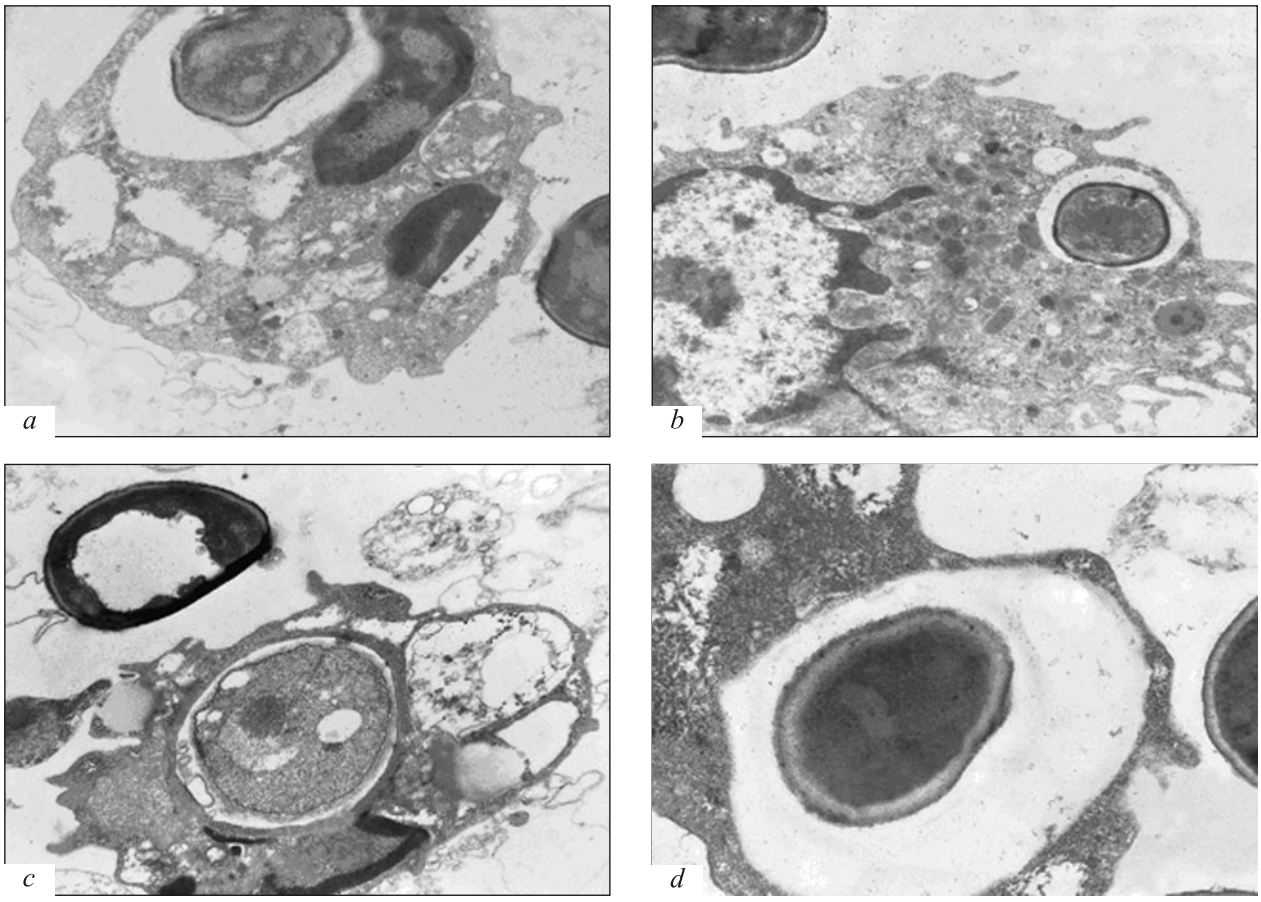

Fig. 2. Phagocytosis of *C. albicans*, ×10,000. *a*, *b)* intact YLF (60- and 150-min incubation, respectively); *c, d)* treated with lyticase (60- and 150-min incubation, respectively).

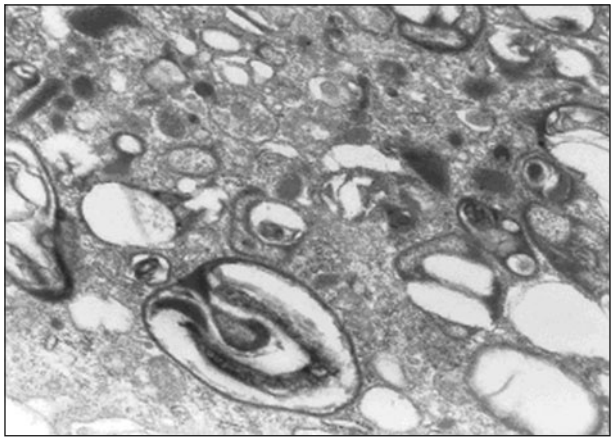

Fig. 3. Residual bodies in the form of myelin figures formed after intracellular hydrolysis of *C. albicans*. Lyticase-treated YLF after 150-min incubation, ×10,000.

The results of electron microscopic examination of AM in the two groups compared with the phagocytic reaction of AM to intact and lyticase-treated YLF (Fig. 1-3) provide a key to the understanding of the mechanism of lyticase impact on YLF *C. albicans*. It can be assumed that these findings will concern the permeability of the cell wall of *C. albicans* for modern drugs. Lyticase as an enzyme that destroys the basic structure of YLF cell wall can be numbered among the antimycotic drugs and included in *Candida* treatment protocol.

The damaging effect of lyticase on *C. albicans* was experimentally substantiated, which is a prerequisite for the development and testing of drug complex designed to treat the recurrent *Candida* vaginitis.

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REFERENCES

1. A. S. Ankirskaya, V. V. Muravyeva, S. A. Fursova, and T. G. Mironova, *Vestn. Ross. Assots. Akush. Ginekol*., No. 1, 106-109 (2000).
2. N. P. Sachivkina, E. G. Kravtsov, E. A. Vasilyeva, and M. V. Dalin, *Byull. Eksp. Biol. Med.,* **148**, No. 8, 180-183 (2009).
3. *Management of Opportunistic Infections and General Symptoms of HIV/AIDS. Clinical Protocol for the WHO European Region* (2007), pp. 68-71.
4. *J. M. Bakker, E. Broug-Holub, H. Kroes, et al.*, *Immunology*, **94**, No. 3, 304-309 (1998).
5. J. H. Scott and R. Schekman, *J. Bacteriol*., **142**, No. 2, 414-423 (1980).