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Efficiency of Lyticase (Bacterial Enzyme) in Experimental Candidal Vaginitis in Mice

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Lyticase (a bacterial enzyme) was tested as a new antimycotic drug. Of all objects studied, *Cellulomonas cellulans* AC-870 strain proved to be most productive for this enzyme. A technology for lyticase isolation and purification was proposed. An experimental model of recurrent vaginal candidiasis was created. The model includes combined antibiotic and estradiol therapy. Antimycotic effect of lyticase on the model of recurrent vaginal candidiasis in mice was demonstrated.

Key Words: *relapsing vaginal candidiasis; lyticase; Cellulomonas cellulans*

Candidal vaginitis (CV) is a prevalent gynecological disease. About 75% women have had at least one CV episode. In many cases, CV is a recurrent infection resistant to therapy [1]. Antimycotic drugs are not always effective, because chronic transformation of the infection is sometimes paralleled by the formation of drug resistance. This fact determines the importance of the search for alternative means for cleansing the microecological niche in CV. A possible approach to the solution of the problem is study of the effects of enzymes of bacterial origin modulating specific morphofunctional structures of *C. albicans* yeast-like fungi (YLF) [6].

One of these enzymes is *Micrococcus luteus* lyticase capable of destroying the carbohydrate part of the mannoprotein complex, one of the main structural elements of YLF cell wall [3].

We studied lyticase effect in experiments on mice with induced CV. Our tasks were as follows: isolation and standardization of biological preparation of lyticase; creation of a mouse model of infection adequate to recurrent CV in women; evaluation of the effect of the resultant lyticase preparation on the course of relapsing CV in mice infected with *C. albicans*.

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MATERIALS AND METHODS

Two strains of *Micrococcus luteus* were studied as lyticase producers. One was a variant from the collection of Microbiology Department of University of Peoples' Friendship, the other was strain No. 4698 from Living Culture Museum, L. M. Tarasevich Institute of Standardization and Control. One more producer was *Cellulomonas cellulans* strain AC-870 from the National Collection of Institute of Genetics and Selection of Industrial Microorganisms.

The productivity of these strains was evaluated by comparing activities of filtered *Micrococcus luteus* and *Cellulomonas cellulans* cultures with activity of the commercial specimen (enzyme from Difco) by a previously described method [5]. The values were expressed in activity units (U/ml) in comparison with the internal reference sample (commercial lyticase;

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Sigma), which served as the control. Proteolytic activities of the same filtered cultures were evaluated by reduction of protein concentrations measured by the previously described methods [4].

The target product was purified by gel filtration through Diaflo membranes XM-300, XM-100, and UM-20 (Amicon). The degree of purification was evaluated by specific activity (U/mg protein). Protein concentration was measured as described previously [4]. The safety of the resultant preparation was evaluated by intravenous injections in outbred mice of both genders (18-20 g).

Candidal vaginitis was induced by a clinical strain of *C. albicans* isolated from a woman with manifest infection. Before the experiment, two passages of the strain were carried out intravaginally in intact animals. The strain was then stored at -70°C in buffered saline (pH 7.2) with 15% glycerol. The virulence of YLF strain used in the study was evaluated by the minimum infective dose at intravaginal infection of mice with dysbiosis [2].

The dynamics of mycotic infection was monitored by inoculations of vaginal lavage fluid in Sabouraud solid medium and counting CFU/ml material.

Since mycotic infection develops under conditions of dysbiosis [2], antibiotics suppressing bacterial flora and creating optimal conditions for YLF reproduction were used in the study. The animals received oral doxycycline hydrochloride and intravaginally penicillin (40 mg/kg both) for 5 days before infection. In addition, the infection and provocation of relapse were carried out after induction of the estrous status by estradiol (Mesalin, Intervet) in a dose of 25 mg/kg.

The dynamics of infection was monitored by vaginal contamination and clearance of YLF from the vagina at 2-day intervals. The clearance index was calculated by the following formula:

$$CL=C_0-C_t/C_0\times100\%,$$

where CL is clearance, C_0 is number of CFU after vaginal discharge inoculation after infection of mice at the beginning of the study, and C_t is CFU number after a certain period after the first test.

The results were analyzed by biometric methods using Student's *t* test and Advanced Grapher 2.11 and Excel software.

RESULTS

Comparison of lyticase producer strains showed higher activity of *C. cellulans* strain AC-870. The biological mass of the producer was accumulated in a modified cardio-cerebral broth [5]. The bacterial culture was incubated at 37°C with constant shuttling, the growth dynamics was monitored by optical density. Lyticase accumulation in growth medium increased during the exponential growth of the culture and reached the maximum during the stationary phase, after which it decreased (Table 1). The optimal period for effective culturing was no longer than 65-70 h. The greatest accumulation of the product in filtered culture was observed during this period. Longer culturing led to excessive accumulation of intrinsic endoproteases released during autolysis of the culture and destroying lyticase. This process is confirmed by the results of evaluation of proteolytic activity of the culture fluid during culturing (Table 1).

Culture fluid collected after this period was centrifuged and subjected to sterilizing filtering through Diaflo membranes. Each fraction was concentrated to a volume of 20 ml, after which lyticase activity was measured. No enzyme activity was detected in fractions containing biopolymers with molecular weights >100 and 300 kDa; activity appeared in the interval

TABLE 1. Dynamics of *Cellulomonas cellulans* Strain AC-870 during Lyticase Accumulation and of Proteolytic Activity of Culture Fluid (CF) (*M*±*m*)

Duration of culturing, h	Time course of growth (by changes in optic density – OD), $Unit_{OD}$	Lyticase accumulation in CF during *C. cellulans* culturing, U/ml	Changes in proteolytic activity of CF during *C. cellulans* culturing, U/U reference specimen
12	0±0	0±0	0±0
24	0.028±0.004	0.80±0.15	0±0
36	0.098±0.009	1.50±0.24	0.010±0.004
48	0.300±0.015	2.70±0.45	0.026±0.009
60	0.380±0.012	2.60±0.30	0.090±0.015
72	0.377±0.014	3.20±0.32	0.175±0.034
84	0.371±0.018	2.90±0.49	0.230±0.030
96	0.363±0.015	1.70±0.24	0.267±0.036

of 100-30 kDa. These data are in line with published data indicating that the molecular weight of lyticase is 55 kDa [5]. After washing and concentration of this fraction, specific activity of the final product was brought to 48.3 U/mg protein, which more than 2-fold surpassed specific activity of commercial preparation (20 U/mg protein) taken for the reference in our study.

The preparation was tested for acute toxicity. Outbred mice (n=10) were intravenously injected with 3 ml test preparation. The animals were observed during 10 days. No mortality or clinical changes were detected over this period. Since the preparation in this concentration was not toxic, it was used in further experiments in a sufficiently wide range of doses.

In order to evaluate the therapeutic effect of the resultant preparation, we created a CV model maximally

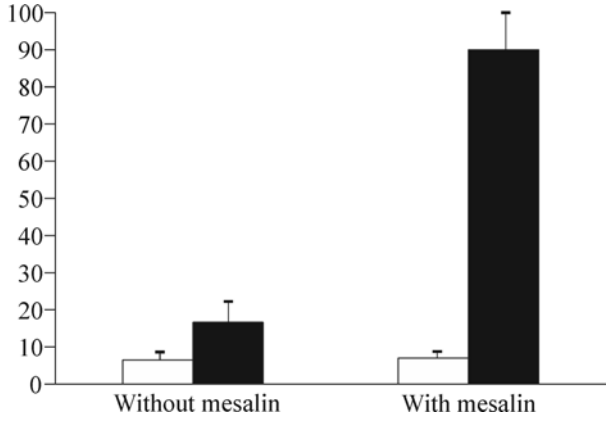

Fig. 1. Efficiency of mesalin in simulation of CV in mice. Light bars: CFU/μl cultured vaginal discharge (contamination); dark bars: percentage of infected animals.

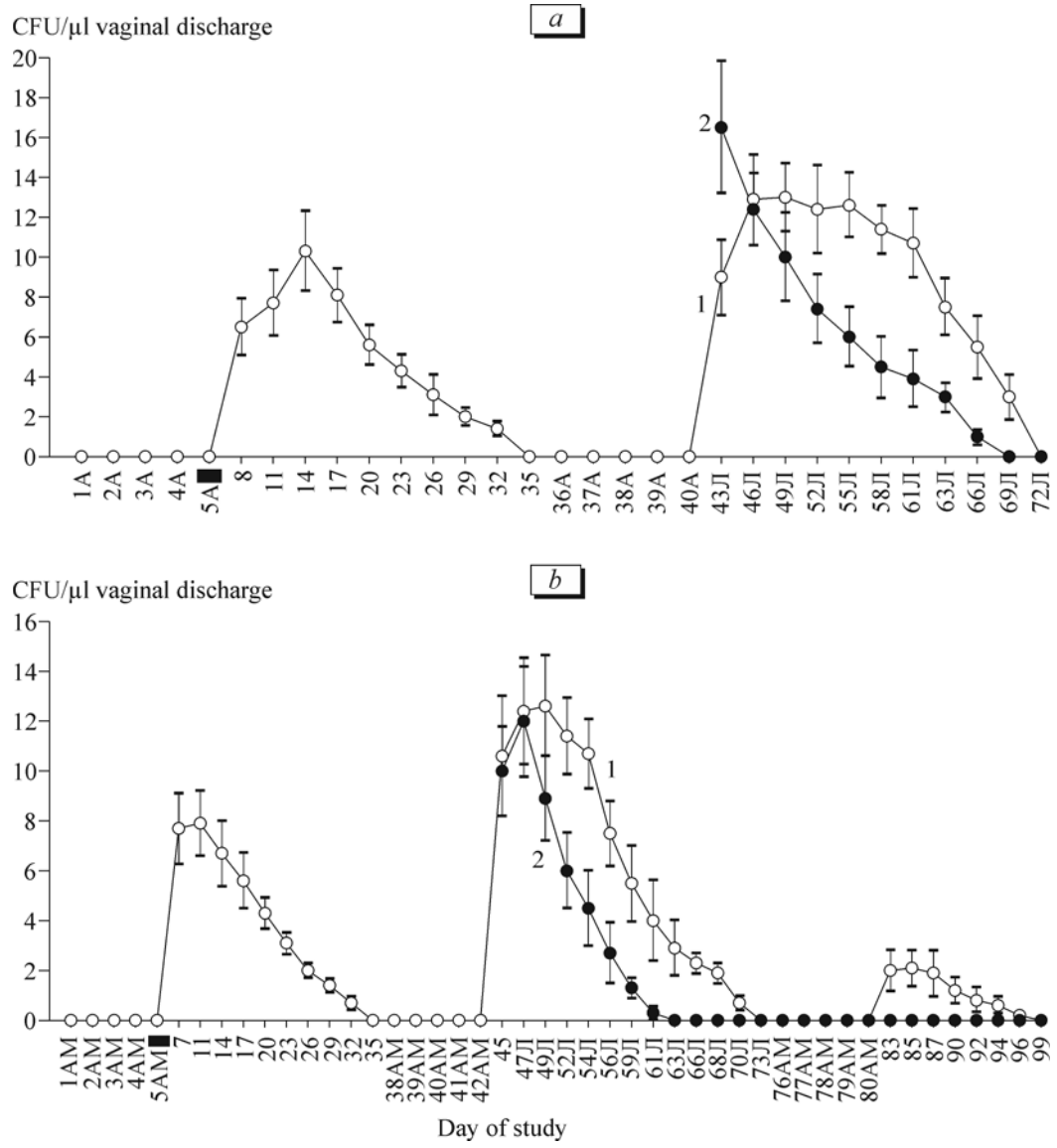

Fig. 2. Changes in contamination of vaginal discharge with *C. albicans* YLF *in vivo*. *a*) 2 U/ml lyticase; *b*) 10 U/ml lyticase . A: antibiotics; L: lyticase, M; mesalin. Dark symbol on the abscissa: a single infection with *C. albicans*. *1*) control; *2*) experiment.

approximated to the mycotic infection in women. We selected a variant of recurrent CV for our model. The first step of our study was evaluation of the minimum infective dose of *C. albicans* strain after passages in mice. The minimum dose was 10^6 CFU/ml. Not more than 20% mice were infected. Increasing the dose to 10^8 CFU/ml resulted in infection of 80% animals. Further increase in the dose did not lead to appreciable changes in infection rate.

We suggested that animal susceptibility and pattern of infection depended on the basal hormonal profiles of experimental animals. It was therefore desirable to synchronize the experimental mice by stimulation of the estrous status. Additional treatment of animals with mesalin increased the percent of infected animals to 90%. After infection, contamination of the vagina increased by days 8-9, after which clearance started and by day 30 it was impossible to isolate YLF from specimens. Infection relapse was induced by repeated antibiotic and estradiol treatment without infection. After antibiotics alone, the incidence of relapses did not exceed 16.6±5.4%, while additional mesalin treatment increased the incidence of relapses to 90.0±9.5% (Fig. 1).

The CV model implied single intravaginal infection of adapted *C. albicans* strain to animals in a dose of 10^8 CFU/ml under conditions of antibiotic-induced dysbiosis and artificially (by mesalin) induced estrus. Two days after elimination of the agent from the vagina and provocation of relapse by repeated antibiotic+hormone treatment of experimental animals, the mice received intravaginal lyticase. The degree of cleansing in experimental and control groups was evaluated by YLF isolation rate. Two series of experiments were carried out with different lyticase doses (2 and 10 U/ml). A significant reduction of vaginal contamination was observed after the very first dose of lyticase (Fig. 2). When lyticase was used in a dose of 10 U/ml, complete cleansing was achieved by 8 days earlier than in experimental series with lyticase dose of 2 U/ml (Fig. 2).

In order to find out whether elimination of the fungus after lyticase treatment was complete or partial, a relapse was repeatedly provoked. CV relapse developed in animals receiving no lyticase. No relapses were recorded in mice treated with the agent.

Hence, the results indicate that lyticase as an enzyme destroying the main structure of the YLF cell wall can be referred to antimycotic agents and included in CV treatment protocols. The cleansing effect of lyticase is experimentally validated and the enzyme can be tried in the complex of means recommended for arresting the relapses of CV caused by *C. albicans* YLF.

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