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Research Article

Determination of *Candida albicans* proteins concentration by enzyme-linked immunosorbent assay method at subcutaneous introduction in candidiasis therapy

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Abstract

Therapeutic effect of *C. albicans* proteins in concentrations 1, 2, 3, 4 and 5 mg/ml has been examined in white mice. Animals were infected i.p. with suspension of *C. albicans* strain CCM 335–867 in the amount of 20 million cells per 1 ml volume. After 5 days and repeatedly after 14 days mice were injected s.c. in the upper part of right hind paw with proteins of *Candida* cells of volume 0.2 ml. 14 days after each injection, the determination of the animal body protective functions has been carried out by the titer of specific antibodies during immunoassay.

According to the data obtained, it has been found that in the s.c. administration, after the first and second injection with *C. albicans* protein of concentration 1, 2, 3, 4 and 5 mg/ml antibody titers increased 2 times, indicating that there is no stimulation of immune protection.

Keywords

antigen, candidiasis, dose, immunity, therapy, vaccine

Introduction

Candidiasis is an infectious disease caused by yeast-like fungi of the genus *Candida*: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guillierinondii*, *C. krusei* and others (Borshch and Masliak 2011; Diekema et al. 2012; LeibundGut-Landmann et al. 2012). There are mild forms of candidiasis of mucous membranes, skin, and more complex forms – visceral form of candidiasis, more often with a predominant lesion of the gastrointestinal tract and respiratory organs (Saigal et al. 2011; Edwards 2012; Kabir and Ahmad 2013). Complex forms of candidiasis are more commonly found in HIV-infected people. More often the causative agent is not one species of *C. albicans*, but at the same time the following species: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guillierinondii*, *C. krusei*. Yeast-like fungi of the genus *Candida* inhabit the skin and mucous membranes and are part of the normal microflora.

The isolation of cultures from sputum, phlegm mucus, faeces, smears and scrapes from the affected mucous membranes, skin, nails cannot serve as an evidence of

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disseminated (visceral) candidiasis (Golubka 2011; LeibundGut-Landmann et al. 2012). Of diagnostic value are cultures isolated from blood, cerebrospinal fluid, intra-articular fluid and biopsy tissues. In the case of skin and mucous membranes, the diagnostic value has the detection (by microscopy) of a large number of candida in the phlegm, mucus from the throat, as well as the detection of high titers of antibodies in the reaction of complement binding and agglutination reaction with a specific antigen. Positive are considered high titers (1: 160–1: 1600) or increase of antibody titers during the course of the disease (Saigal et al. 2011; Diekema et al. 2012).

The main points in the treatment of candidiasis are the following measures: elimination of factors, contributing to the emergence of candidiasis, the use of B vitamins and ascorbic acid. At skin lesions, treatment is carried out in an open way using ointments with antimycosis action. In the case of mucous membranes lesion (oral, vaginal candidiasis), topical suspension containing nystatin should be used. If the esophageal mucosa is affected, the clinical guidelines recommend the use of ketoconazole 200–400 mg per day (about 50% efficiency), if the symptoms of esophageal involvement do not disappear within 5–10 days, then fluconazole should be used. It is prescribed 100 mg daily for two days (efficiency is about 90%). In these cases, i.v. administration of amphotericin B at 0.3 mg/kg is also possible. With candidiasis of the bladder carry out instillation of amphotericin B solution at 50 µg/ml for 5 days. With desiminated (systemic, visceral) candidiasis, amphotericin B is administered i.v. as 5% solution in the form of drip infusions at the rate of 250 UA/kg body weight (0.3 mg/kg). The course of treatment may take several weeks. An effective drug for the treatment of systemic candidiasis is fluconazole, which is administered internally 200 mg daily for two days. Adverse reactions are noted relatively rarely (2–4%) in the form of nausea, diarrhoea, headache (Rybalkin and Strelnikov 2017; Rybalkin et al. 2017).

It should be noted that in recent years there has been a loss in sensitivity of *Candida* fungi to antifungal agents. This is due to the long-term application of traditional antifungal agents and the increase in the proportion of various pathogenic species of *Candida* fungi that have different sensitivity to traditional antifungal agents (Diekema 2012; Rybalkin and Strelnikov 2017, Rybalkin et al. 2017). In this regard, the search for new methods of candidiasis treatment is necessary.

Vaccines for the prevention and treatment of candidiasis are being actively developed in many countries (Grover et al. 2010; Han and Rhew 2012). *Candida* vaccines cover a wide range of different vaccine variants with different composition ranging from whole inactivated and virulently attenuated fungal cells to subunit vaccines based on a single recombinant protein or peptide fragment or glycoconjugate: inactivated cells of pathogen, attenuated living pathogen cells, *Candida* fungal cell enolases, iC3b receptors of *Candida* fungal cell surface, *Candida* fungal cell mannans, *Candida* fungal cell β-glucans, *Candida* fungal cell heat shock protein Hsp90, SAP protein genes, ALS protein genes, glycoconjugates (mannans and β-glucans) of *Candida* fungal cells.

At this stage of research, it is necessary to select the most promising vaccines for clinical trials from a variety of potential variants of anti-candida vaccines. To do this, all the vaccine options should be analyzed to choose the ones having high reliable efficacy and safety, as well as those that can be standardized in composition and technology and produced with minimal financial costs, minimal use of technological stages and equipment.

It should be borne in mind that some vaccines developed by different researchers are extremely difficult to manufacture, and some of the proposed vaccines are difficult to characterize for the mixture of antigens. It is also necessary to consider the strengths and weaknesses of each specific vaccine. For example, whole inactivated cells are quite complex chemically, which creates serious problems with safety and standardization. Inactivated cells showed weak immune responses as compared to live attenuated fungal cells. Live vaccines are usually one of the best immunogens, but they have the disadvantage of limited use in immunocompromised patients, because even weakened cells can sometimes cause disease in immunocompromised individuals. On the other hand, for subunit vaccines, which may be the best option for production, standardization and safety, there is a greater likelihood of further clinical trials. However, subunit vaccines also have their drawback – weak immunogenicity, that usually requires the addition of an adjuvant to induce long-term protective immunity. Although this is not a problem in experimental animal models, there is a shortage of good adjuvants suitable for use in clinical practice. An alternative to adjuvants is the use of liposomes, virusomes, inert nanoparticles, derived PAMP components and other bioengineered preparations.

It should be noted that no candidiasis vaccine is currently produced or registered in Ukraine. Therefore, the development of such a vaccine is a topical issue in modern medicine and pharmacy.

In previous studies, the method of *C. albicans* cells disintegration has been substantiated, which provides proteins and carbohydrates release. The proposed potential protein-carbohydrate composition of the vaccine provides the widest effect on all determinants of the pathogen, which provides greater efficiency, in contrast to individual antigens (Grover et al. 2010; Han and Rhew 2012; Cassone 2013).

Subsequently, safety studies (allergenicity, reactogenicity, toxicity) have been conducted to confirm the safety of the proposed vaccine (Rybalkin 2014) and its efficacy (Rybalkin 2014; Rybalkin and Strelnikov 2017, Rybalkin et al. 2017).

It is now necessary to check whether the antigens of the *C. albicans* fungal cells have therapeutic effect that is, whether they are able to activate protective mechanisms against candidiasis infection at i.m. and s.c. administration to candidiasis mice, that will further ensure the recovery of diseased animals.

The purpose of this work was to determine the *C. albicans* fungus protein concentration at s.c. introduction in therapy of candidiasis.

Materials and methods

All studies were performed in laminar box maintaining aseptic conditions. *C. albicans* strain CCM 335–867 was pre-cultured in test tubes on Sabouraud agar at 25 ± 2 °C for 48 hours. Then the fungal cells were washed with 10 ml of sterile isotonic 0.9% sodium chloride solution. The resulting suspensions of fungal cells were transferred to Sabouraud agar mattresses incubated at 25 ± 2 °C for 6 days. After this, washed the fungal cells with 25 ml of sterile isotonic 0.9% sodium chloride solution. The microbiological purity of the *C. albicans* fungal cells suspension has been determined visually and by microscopy. Next, centrifugation was carried out at a speed of 3000 rpm for 10 minutes. The obtained precipitate of fungal cells was brought with a sterile isotonic 0.9% sodium chloride solution to (8.5–9) × 10^8 in 1 ml.

Received suspensions of fungal cells in a volume of 10 ml were subjected to sonication for the destruction of fungal cells on the apparatus USUU-21 at a frequency of 22 kHz, intensity 5 W / cm^2 and at a temperature 25 ± 2 °C within 15 min (Rybalkin 2014). Temperature 25 ± 2 °C was constantly monitored for the cell suspensions and maintained by adding cold water to the surrounding tank. Further carried out filtering through the membrane "Vladipore" MFA – MA No. 3, which provides the separation of biological material with the size of 10 kD and its concentration (Rybalkin and Strelnikov 2017, Rybalkin et al. 2017). The filtrate obtained is a mixture of proteins and polysaccharides. In each case, the protein content was determined according to SPU. Considering the fact that *Candida* fungus cell extract contains proteins and polysaccharides that have antigenic properties, according to the requirements of the SPU, the determination of the active substance has been carried out in terms of the substance having more pronounced properties, ie on the protein. Further pre-filtration was performed using filters with a pore diameter of 0.45 µm and sterilization filtration using filters with a pore diameter of 0.22 µm.

Therapeutic effect of *C. albicans* proteins in concentration 1, 2, 3, 4 and 5 mg/ml has been studied in white mice of two months of age, weighing 18–22 g, of 6 animals in the control and experimental groups, which were kept under standard diet in the same conditions. The studies have been conducted at the State Institution "I.I. Mechnikov Institute of Microbiology and Immunology". Before the study, the animals were acclimatized in the experimental room. Animals were infected i.p. with suspension of *C. albicans* fungus strain CCM 335–867 in the amount of 20 million cells per 1 ml volume. After 5 days mice were injected i.m. in the upper part of right hind paw with proteins of *Candida* fungus cells of volume 0.2 ml. After 14 days, the determination of the protective functions of the animal body by titer of specific *C. albicans* antibodies during enzyme immunoassay has been carried out. For this purpose, a set of reagents was used for the enzyme-linked immunosorbent detection of G antibodies to *C. albicans* using the Vector-Best ELISA test system. 14 days after the first injection, repeatedly in the upper part of the left hind paw s.c. injected proteins of *Candida* fungus cells of volume 0.2 ml and after 14 days, carried out the determination of the protective functions of the animal body by titer of specific *C. albicans* antibodies Animals in the control group were administered saline.

Results and discussion

The results of the studies have shown that the antibody titers of healthy animals were in the range 1: 200–1: 500. This can be explained by the possible contact with the fungus of the genus *Candida* during the life of mice or the possible carriage of this type of fungi, since they are part of the normal microflora of animals.

At the s.c. introduction to candidiasis animals after the first injection of *C. albicans* fungal proteins in concentration 1, 2, 3, 4 and 5 mg/ml *C. albicans* antibody titers also doubled compared to the titers in healthy animals. The results of the studies are summarized in Table 1.

After the second s.c. injection of *C. albicans* fungus proteins at 14-day intervals, antibody titers remained at the same level, ie increased 2-fold when using protein concentrations 1, 2, 3, 4 and 5 mg / ml.

Table 1. Therapeutic effect of *C. albicans* fungal cell antigens.

Animals	*C.albicans* protein content, mg / ml	Administration method	*C. albicans* AB titers in ELISA		
			healthy animals	ill after 1st injection	ill after 2nd injection
Mice	1	s / c	1:200	1:400	1:400
Mice	2	s / c	1:300	1:600	1:600
Mice	3	s / c	1:250	1:500	1:500
Mice	4	s / c	1:300	1:600	1:600
Mice	5	s / c	1:500	1:1000	1:1000

Note: $n = 6$

The antibody titer in the control group at s.c. route of administration did not grow. Comparing the results obtained in the experimental and control groups, it is safe to say that s.c. introduction of *C. albicans* fungal cell proteins does not stimulate the production of antibodies responsible for humoral immunity.

All animals that were infected and did not receive antigens died. Animals that were infected and received antigens all survived for a follow-up period of 30 days.

In the future, it is necessary to study the proteins of *C. albicans* at concentrations of 1, 2, 3, 4 and 5 mg / ml at i.m. injection and investigate the growth of *C. albicans* antibody titers after the first and second injections. Next, it will be expedient to compare the results obtained at s.c. and i.m. injection and determine the pathway and concentration of proteins of the fungus *C. albicans.*

Conclusion

According to the data obtained during studies on the treatment of candidiasis, it has been found that in the s.c. route of administration after the first and second injection with *C. albicans* protein in all studied concentrations antibody titers increased only 2 times.

Thus, it can be concluded that these antigens of *C. albicans* do not activate the body's protective reactions.

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