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# Elevated Activity Levels of Serum Antimicrobial Peptides in Mice as Response to Immunization with Yeast Antigens

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## Abstract

Except immunoglobulins and components of complement system, the important part of congenital humoral immunity are antimicrobial (poly) peptides (AMP). Main antimicrobial mechanism of AMP action is the destruction of cytoplasmic membrane. In host organism number of AMP act simultaneously, therefore method for the measuring of overall AMP activity would be helpful. The method was recently applied and modified for estimation of AMP activity in serum: aliquots of low-molecular fraction of serum added to test-culture of *Candida albicans*, and the part of killed cells, i.e. total activity of AMP, calculated by spectroscopy. Aim of present study is the estimation of AMP activity in mice sera after immunization with cells of clinical yeasts compare to the immunoglobulins G.

Yeasts strains-*Candida albicans*, *Cryptococcus neoformans*, *Rhodotorula mucilaginosa*, *Trichosporon cutaneum*, *Geotrichum candidum*, and *Saccharomyces cerevisiae*-were cultivated in liquid defined media up to the end of exponential growth phase. Obtained yeasts cells used for immunization of mice, but yeast antigens-for the estimation of IgG-antibodies levels in mice sera by ELISA method. AMP activity were measured as described above.

Immunization of mice with yeasts cells was accompanied not only with increase of specific IgG levels, but with elevation of total activity of AMP: for example specific IgG in sera of mice immunized by C. albicans cells was 16-fold higher than in non-immunized (control), and AMP activity-1,5 fold higher than in control. Correlation between specific humoral response at the immunoglobulins level and humoral response at the AMP-activity level (Pirson`s coefficient $r=0,735$; $p \leq 0,05$) as a result of immunization of mice by fungal cells was demonstrated for the first time. The method may be used for study of humoral immunity associated with action of AMP in various animal and human models.

## Introduction

The clinically important yeasts are progressively increased group of fungi due to following reasons-appearance of new identification methods and elevation of number of immunosuppressed individuals. Nowadays among 1500 of known yeasts specia more than 80 specia include in this group [1], but most of them are teleomorphs (sexual stages) of the following 7 genera: *Candida*, *Malassezia*, *Rhodotorula*, *Cryptococcus*, *Trichosporon*, *Geotrichum* and *Saccharomyces*. Recently we reported about cultivation of 7 specia of these genera and isolation of specific yeast antigens which are appropriate for the immunodiagnostics of yeast mycoses by the estimation of specific immunoglobulins level [2]. In addition to immunoglobulins and complement system, the important part of congenital humoral immunity are antimicrobial peptides (AMP) [3]. The AMP produced by neutrophils, macrophages, monocytes and epithelial cells [4]. In human blood different classes of AMP–lactoferrin, calprotectin, lysozyme, secretory leucoprotease inhibitor, cathelicidins, defensins and lipocalin-are circulated, however the number of AMP act in other loci is more miscellaneous. Main result of AMP action on microbial cell is the destruction of cytoplasmic membrane. At present every class of AMP investigated in connection with different pathology, but results of the studies are rather discrepant [5-7]. Methods of quantitative estimation of individual AMP are known [8,9], but number of AMP act simultaneously, therefore an adequate method for the measuring of overall AMP activity would be helpful. Such method was recently applied for estimation of AMP activity of vaginal fluid [10,11], based on the action of aliquots of the substrate on test-culture of *Candida albicans* and calculation of killed cells percent. Later we have modified the method with adapt it for the serum by use of two techniques-removal of high-molecular (≥ 100 kDa) protein fraction (i.e. removal of complement system proteins) and estimation the result by spectroscopy [12,13]. Use this approach we measured the total AMP activity in sera of patients with bronchopulmonary candidiasis and showed that the activity was elevated (like the specific serum immunoglobulins) in patients compare to control group [13]. The aim of present study is the estimation of AMP activity levels in mice before and after

immunization with cells of clinical yeasts compare to the corresponding immunoglobulins G.

## Methods

**Yeasts strains** used for all experiments were selected from laboratory collection-*Candida albicans* № 927, *Cryptococcus neoformans* № 3465, *Rhodotorula mucilaginosa* № 132, *Trichosporon cutaneum* № 18, *Geotrichum candidum* № 1206; and from Russian Collection, Pushchino-*Saccharomyces cerevisiae* Y-375.

**Cultivation** of non-lipophilic yeasts was carried out in liquid defined medium at 30°C and speed of rotation 150 per min in 500 ml flasks contained 100 ml (g/l): glucose-20, asparagine-15, $(NH_4)_2SO_4$-1.4; $MgSO_4{}^*7\ H_2O$-0.5; NaCl-0.1; $CaCl_2$-0.02; 10 ml of 1.2 004D phosphate buffer pH 5.5; trace elements, antibiotic [14].

**Yeasts cells and immunization of mice** Yeasts cells were harvested from the end of exponential phase [2], centrifuged, suspended in 0.02% sodium mertiolate at different concentrations, exposed 2 h at room temperature for cells killing and freezed at-25°C up to immunization. 10 white outbred mice were used for each experiment with one yeast strain (in total-60 mice), and 10 naive mice were used as a control. Within each experiment 5 immunization cycles were carried out: step № 1-each mouse was intraperitonealy injected by 0.5 ml of yeast suspension with concentration 250 μg protein/ml; step № 2-one week later-500 μg protein/ml; step № 3-one week later-1000 μg protein/ml; step № 4-one week later-2000 μg protein/ml; step № 5-one week later-again 2000 μg protein/ml; one week later the total mice antisera were collected followed by pool according to yeasts strains and controls.

**Yeasts antigens** Antigen preparations of other yeast strains were obtained from supernatants (cell-free culture liquids) of late exponential cultures by the following procedure: filtration through bacterial filters with pore 0.22 μ («TRP», Germany) lyophilization, solvation in minimal volume of distilled water, 5 cycles of freezing-thawing with removal of high-molecular polysaccharide fraction, filtration through membrane of 100 kDa («Amicon ultra», Germany).

**Specific immunoglobulin G (IgG) levels** were estimated by enzyme-linked immunosorbent assay-ELISA-method [15]. Yeasts antigens-100 μl (10 μg protein/ml in carbonate-bicarbonat buffer pH 9.6)-inoculated in 96-well micro titer plates ("Gr0065iner Bio-One", Germany), followed by incubation at 37°C during 1h and at 10°C during 16 h. Then plates were washed 3 times with phosphate buffer solution (PBS) and 100 μl of mice sera was inoculated in corresponding wells (sera dilution 1:400). Pool of non-immunized mice sera were used as controls in same dilutions. Background wells were inoculated with all components except serum. Plates were incubated again at 37°C during 1 hour with following washing 3 times with PBS, after that anti-IgG mouse conjugate ("Sigma", USA) was added (dilution 1:100) and plates incubated at 37°C during 1 h. After 3 washing procedures with PBS the reaction with tetramethylbenzidine (TMB) was held (100 μl in each well). After 15 min reaction was stopped by inoculating of 50 μl 2 M HCl in each well. IgG level was expressed as optical density units (OD) measured in each well by spectrophotometer ("EFOS 9305", Russia) at wave length of 450 nm. Such experiments were carried out three times.

**Estimation of total AMP activity** was carried out by the following procedure: 50 μl of test-culture *C. albicans* № 927 (final concentration of cells about $10^8$ CFU/ml) was combined with 350 μl of serum filtered through membrane filter («Amicon ultra», Germany) with pores diameter 100 kDa. Control specimen contained 350 μl of normal saline solution instead of the serum. The mixture was incubated on shaker at 32°C during 2 h, after that centrifuged during 5 min at 16000 g, supernatant removed and 500 μl of the dye 2 mM bromocresol purple 10 ml in 1.25 M phosphate buffer pH 4.6 was add and incubated again during 45 min. After centrifugation 50 μl of the supernatant was dissolved in 2.5 ml of the same buffer and the optical density was measured at wave length 440 nm use spectrophotometer ("Genesys 10S UV-Vis", USA). These experiments were carried out three times.

The total AMP activity was calculated:

$A_{total}=(N_{control}-N_{exp})^*100/N_{control}$,

$A_{total}$-total AMP activity of serum, %;

$N_{control}$-optical density of control supernatant,

$N_{exp}$-optical density of experimental supernatant;

$A_{specific}=A_{total}/P$,

$A_{specific}$-specific AMP activity of serum, relative units;

P-serum protein concentration, mg/ml

Serum protein concentration was determined by method Lowry [16].

Statistical evaluation of obtained results was carried out by parametric and nonparametric methods of «MS Excel», «Statistica for Windows».

## Results

For the quantitative evaluation of IgG-antibodies to yeasts antigens in mice sera the ELISA method was used. Immunization of mice with suspensions of yeast cells lead to generation of specific antibodies. Table 1 demonstrates the specificity of obtained antigen preparations-maximal levels of specific IgG (OD 450 nm) correspond to reaction of each antigen with the homological serum. Control pool of sera contained minimal levels of the antibodies. Significance of differences between levels of IgG-antibodies to yeast antigens in homologous serum and this indexes in non-homologous sera were calculated by Mann-Whitney U test ($p \leq 0,05$).

In addition antigens from *C. albicans* and *G. candidum* except the reaction with homologous sera reacted with heterologous sera of mice immunized with these yeasts. This fact may be associated with high concentration of cross-reacted proteins in


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the antigenic preparations which caused with affiliation of these specia to the same taxon-*Ascomycota*.

**Table 1:** Levels of specific immunoglobulin G to yeasts antigens in sera of mice immunized with yeast cells (OD 450 nm) (M ± m).

| ELISA test used yeast antigens | Sera of mice immunized with yeast cells of following specia: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Candida albicans* № 927 | *Rhodotorula mucilaginosa* № 132 | *Cryptococcus neoformans* № 3465 | *Trichosporon cutaneum* № 18 | *Geotrichum candidum* № 1206 | *Saccharomyces cerevisiae* Y-375 | Control (sera of non- imminized mice) |
| *Candida albicans* № 927 | 2,452 ± 0,34 | 0,348 ± 0,04 | 0,131 ± 0,02 | 0,073 ± 0,01 | 1,066 ± 0,25 | 0,306 ± 0,03 | 0,151 ± 0,02 |
| *Rhodotorula mucilaginosa* № 132 | 0,196 ± 0,046 | 1,275 ± 0,31 | 0,06 ± 0,01 | 0,183 ± 0,02 | 0,034 ± 0,01 | 0,071 ± 0,01 | 0,098 ± 0,01 |
| *Cryptococcus neoformans* № 3465 | 0,144 ± 0,02 | 0,07 ± 0,01 | 0,276 ± 0,03 | 0,140 ± 0,02 | 0,154 ± 0,02 | 0,065 ± 0,01 | 0,191 ± 0,02 |
| *Trichosporon cutaneum* № 18 | 0,547 ± 0,05 | 0,238 ± 0,03 | 0,169 ± 0,02 | 2,496 ± 0,33 | 0,096 ± 0,02 | 0,239 ± 0,04 | 0,167 ± 0,02 |
| *Geotrichum candidum* № 1206 | 0,967 ± 0,10 | 0,053 ± 0,01 | 0 | 0,216 ± 0,02 | 1,293 ± 0,30 | 0,027 ± 0,01 | 0,006 ± 0,01 |
| *Saccharomyces cerevisiae* Y-375 | 0,113 ± 0,03 | 0,095 ± 0,01 | 0,019 ± 0,01 | 0 | 0,080 ± 0,01 | 0,333 ± 0,04 | 0,053 ± 0,01 |

Method of estimation of AMP activity based on the property of these compounds to destroy the cytoplasmic membrane. As a result of such destruction the dye-bromocrezol purple-insert into damaged test-culture cells and its concentration in the incubation medium decrease. The control specimen of test-culture, in which majority of cells (70-80%) are alive, absorb less amount of dye than the experimental specimen, so that optical density in control supernatant always higher than in experimental one.

The first task of the study was to estimate of AMP activity of sera of mice immunized with cells of different yeasts. Data concerned the immunization of mice with yeasts cells are demonstrated in Table 2. Each value was the result obtained for pool of sera of 10 mice. Values of total AMP activity of sera in immunized mice were obviously higher than in non-immunized mice (significance of differences calculated by Mann–Whitney U test, $p \leq 0,05$). Maximal values of the activity took place into sera of mice immunized with cells of *Geotrichum candidum*, but minimal-of *Saccharomyces cerevisiae*. Since the protein concentration of serum is not constant value, this parameter for different sera pools was estimated. The ratio of total AMP activity to the corresponded protein concentration of the serum pool was designate as specific activity. Specific AMP activities of immunized mice sera were relatively higher than such index of control pool ($p \leq 0,05$).

Comparison of total AMP activity values with levels of antibodies formed as a result of reaction of corresponded antigen with the homologous serum (bold type numerals in table 2) showed high positive correlation-Pirson`s coefficient $r_1$=0.735; although total AMP activity values almost not correlated with antibody levels in sera of mice immunized with cells of corresponded yeasts specia to antigen of *C. albicans* (italic type numerals in table 2)-$r_2$=0.314. Comparison of specific AMP activity with the same indexes showed the same tendency -$r_1$=0.524, and $r_2$= -0,118.

**Table 2:** Activity of serum antimicrobial peptides (AMP) in mice immunized with cells of clinically important yeasts (M ± m).

| **Genera/specia and strain numbers of yeasts used for immunization of mice** | **Total AMP activity of serum ($A_{total}$) , %** | **Serum protein concentration (P), mg/ml** | **Specific AMP activity of serum ($A_{specific}$), relative units** |
|---|---|---|---|
| *Candida albicans* № 927 | 6,48 ± 0,40 | 2,49 ± 0,25 | 2,61 ± 0,16 |
| *Rhodotorula mucilaginosa* № 132 | 6,29 ± 0,44 | 1,93 ± 0,22 | 3,27 ± 0,19 |
| *Cryptococcus neoformans* № 3465 | 5,33 ± 0,30 | 1,51 ± 0,18 | 3,51 ± 0,21 |
| *Trichosporon cutaneum* № 18 | 8,04 ± 0,50 | 1,45 ± 0,19 | 5,51 ± 0,31 |
| *Geotrichum candidum* № 1206 | 8,23 ± 0,51 | 1,67 ± 0,20 | 4,92 ± 0,29 |
| *Saccharomyces cerevisiae* Y-375 | 4,46 ± 0,27 | 1,80 ± 0,19 | 2,50 ± 0,15 |
| Control * | 4,38 ± 0,25 | 2,05 ± 0,22 | 2,15 ± 0,11 |

## Discussion

Recently we have studied the yeasts growth phases in liquid defined media for further determination of specific antigens localization [2]. The end of exponential growth phase was chosen for the antigen obtaining because it is known that exactly this period of microbial culture growth characterized with specific antigens in addition to relatively high biomass [17]. Western blot analyses indicated that most specific antigens of non-lipophilic yeasts were consisted in cell-free supernatant of culture liquids [2]. These results we used during realization of present study, and high specificity of obtained yeasts antigen preparations was confirmed by ELISA tests: the highest levels of specific mouse IgG were corresponded to homologous sera. Increase of immunoglobulins as response to immunization is well known fact-the estimation of specificity of antigens based on this phenomenon. However, the important factors of antimicrobial humoral immunity in addition to immunoglobulins and complement system are AMP [3,4]. Recently total activity of AMP was studied in different human loci (secrets)-vaginal secret [10,11], hair [11], serum [13] and water solved fraction of skin secret [18]. This activity was shown to decrease in different secrets as response to inflammatory processes, but serum AMP activity was increased in patients with bronchopulmonary mycosis. Nevertheless, since the present time almost no data concerned the reaction of AMP activity or quantities of individual AMPs in response to immunization. The only publication about this question consecrated the increase of defensin production and activity after immunization of house fly pupae by different opportunistic bacteria and fungus [19]. Present study is the first research concerned mammal model of immunization effect on AMP activity. The fact of correlation between specific humoral response at the immunoglobulins level and humoral response at the AMP-activity level as a result of immunization is demonstrated for the first time and need further investigation. Now we can only ascertain the fact of coordinated reaction of humoral components in response to injection of mice by fungal cells.

Interestingly that AMP activity was estimated use *C. albicans* as the test-culture, therefore it is reasonable to propose highest activity in mice immunized with cells of this species, but it is not so. Based on the data one can conclude that unlike the immunoglobulins, AMP-response is not specific. This fact may be explain as follows: small size of AMP molecules-3.5-90 kDa-and linear structure is rather primitive compare to large-150-500 kDa-and complex structure of immunoglobulins.

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## References

1. de Hoog GS, Guarro J (2011) Atlas of Clinical Fungi.
2. Arzumanyan V, Shmeleva O, Serdiyk O, Michailova N (2013) Physiological Parameters of Clinical Yeasts Growth and Isolation of Specific Antigens. Biochemistry and Physiology: Open Access 2: 116-120.
3. Zasloff M (2002) Antimicrobial Peptides in Health and Disease. N Engl J Med 347: 1199-1200.
4. Bahar A, Ren D (2013) Antimicrobial Peptides. Pharmaceuticals 6: 1543-1575.
5. Carothers DG, Graham SM, Jia HP (2001) Production of betadefensin antimicrobial peptides by maxillary sinus mucosa. J Rhinol 15: 175-179.
6. Schaller-Bals S, Schulze A, Bals R (2002) Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. Respir Crit Care Med. 165: 992-995.
7. Gallo RL, Nizet V (2003) Endogenous Production of Antimicrobial Peptides in Innate Immunity and Human Disease. Curr Allergy Asthma Rep 3: 402-409.
8. Paulsen F, Pufe T, Conradi L, Varoga D, Tsokos M, et al. (2002) Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes. J Pathol 198: 369-377.
9. Boesch AW, Zhao Y, Landman AS, Rodriguez GM, Fahey JV, et al. (2013) A Multiplexed Assay to Detect Antimicrobial Peptides in Biological Fluids and Cell Secretions. J Immunol Methods 397: 71-76.
10. Valore EV, Park C, Igreti SL, Ganz TJ (2002) Antimicrobial components of vaginal fluid. Obstet Gynecol 187: 561-568.
11. Arzumanian V, Malbakhova E, Vartanova N (2012) The New About Congenital Antimicrobial Defense of Some Epithelial Tissues-Vaginal Mucosa and Hair. A Search for Antibacterial Agents. InTech 125-146.
12. Arzumanian VG, Malbakhova ET, Foshina EP, Artemeva TA, Butovchenko LM, et al. (2015) Method for determining antimicrobial peptide activity as marker of tissue immunity state of various epithelial tissues. Patent RF № 2015113069/15.
13. Arzumanian VG, Shmeleva OA, Burova SA, Yastrebova NE (2016) Activity of antimicrobial peptides in serum at bronchopulmonary candidiasis. Advances of medical mycology 16: 9-11.
14. Yarrow D (1998) Methods for the isolation, maintenance and identification of yeasts. In: Kurtsman CP, Fell JW editors. Yeasts, A Taxonomic Study. Elsevier 77-100.
15. Johnstone A, Thorpe R (1987) Immunochemistry in practice. Blackwell Scientific Publication 306.
16. Lowry O, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265-275.
17. Basnakyan I (2010) Stress in bacteria: utility and injury for man. Moscow: Meditsina 160.
18. Arzumanian VG, Masyukova SA, Sergeev AY, Grebenyuk DV, Ilina IV, et al. (2017) Secretory antimicrobial peptides activity, immunoglobulins and skin microflora in acne patients. International Journal of Immunopathology In press.
19. Dang X, Wang YS, Huang YD, Yu XQ, Zhang WQ (2010) Purification and characterization of an antimicrobial peptid, insect defensin, from immunized house fly (Diptera:Muscidae). J Med Entomol 47: 1141-1145.


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