CLINICAL STUDY

Comparative study of disintegrated cells influence of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* on human and mouse immune mechanisms

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Abstract

The study presents comparison of immunomodulatory effects of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* disintegrated cells on selected immune mechanisms of human and mouse leukocytes. We measured their phagocytic activity, phagocytic index and microbicidal activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* cells as well as peroxidase and lysozyme activities of human and mouse leukocytes. Our results revealed predominantly inhibitory effect of disintegrated microorganisms on nonspecific immune functions of human leukocytes, but mainly stimulatory effect on mouse leukocytes monitored immune functions. *(Tab. 7, Ref. 7.)*
Key words: immunomodulatory activity, phagocytic activity, microbicidal activity.

Ongoing problems associated with pathogenic microorganisms antibiotic resistance, as well as occurrence of immunodeficiency, allergic, autoimmune and cancer diseases lead to search of new prophylactic and therapeutical approaches. One of the possible and auspicious ways of treatment is the modulation of immune response. It was found, that many components and products from studied and commonly used microorganisms belong to the most effective substances with immunomodulatory activity. In the last 25 years many advances have been made towards understanding the interactions between microorganisms and their hosts. It has been shown, that the substances, which promote immunity of the host, can be effective in prevention and treatment of bacterial, viral and allergic diseases. They may be used also in cases where antibiotic treatment failed and they also successfully help to deal with severe post surgery infections. The ailments from immunodeficiency are also common in geriatrics and lead to post surgery complications.

Effects of immunomodulatory substances on different parts or components of the immune system are various. While some cells or even the whole system can be stimulated by the same substance, the others are suppressed. Fridman (1991) suggested the similarity between immune system (which he called "mobile brain") and central nervous system as well as in their interaction. The examples of such interaction are the development of fever or the cooperation with the endocrine system in inflammatory or other defense mechanisms. Impairment of function one of these systems may result in defects of others and consequently proceed to disease. Resistance of each individual against diseases is particular and conditioned by his health, age, sex, genetic factors and environment. Therefore both the immunomodulatory and antibiotic therapy should be approached individually on the base of previous examinations. Moreover, up-to-date research and the resulting immunomodulatory therapy are focused on the selective suppression or stimulation of particular immune system cells.

This paper is aimed on analysis of immunomodulatory activities of selected microorganisms. Their effects were tested in vitro on leukocytes and in vivo on immune system of inbred mice, predominantly with respect on phagocytic, microbicidal and metabolic activity of phagocyte cells. The effects were determined as phagocytic activity, phagocytic index, bactericidal activity on *Staphylococcus aureus*, colicidal activity on *Escherichia coli* and candidacidal activity on *Candida albicans*, as well as lysozyme and peroxidase activities. The results enabled not only to compare the activities of tested substances, but also revealed some differences in immune mechanisms of human and mouse cells.

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Material and methods

Animals

Inbred male black mice C57BL/6 (Velaz, Prague, Czech Republic) with average weight 18.0±2.0 g were kept in standard conditions with food and water intake ad libitum.

Microorganisms

It was used the collected strains of *Candida albicans* SC 1539, *Escherichia coli* ATCC 11229, *Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* MAU 2958, *Streptococcus faecalis* (Dpt. of Cell and Molecular Biology of Drugs, Faculty of Pharmacy CU, Bratislava, Slovakia).

Preparation of samples

The freeze-dried strains of *Staphylococcus aureus* (S), *Escherichia coli* (E) and *Candida albicans* (C) were added to 2 ml of saline in amount of 0.05 g and suspension were destroyed in ice (18 kHz on SONIPREP 150 MSE) during the period after 5 minutes (3-times). Next the volume of mixture was completed up to 5 ml with saline and the ultrasonication was repeated. The suspensions were cultivated on agar media as a control of sample sterility.

Culture media

The blood agar, Endo agar and Sabouraud agar were used for cultivation of microorganisms (Imuna, Sarisske Michalany, Slovakia). The blood agar was prepared by adding of 10 % of defibrine sheep's blood to the melted and cooled (50 °C) competent components.

Human leukocytes

Human leukocytes (HL) from healthy volunteers (OHT — Derer's Hospital, Bratislava, Slovakia) were added to RPMI 1640 medium with 0.5 % growth proteins GPBOS (Institute for Serum and Vaccine Production, Prague, Czech Republic) according to producer and adjusted to concentration of $2x10^6$ cells per ml.

Application of samples and isolation of mouse leukocytes

The homogenates S, E, C and control saline were added to suspension of human leukocytes or they were injected intraperitoneally (i.p.) in mice with 50 μg/g substances in 100 μl of sterile physiological saline during 5 days at 24 h intervals. Each experiment was administrated to five parallel samples. The saline was added in the same volume alike as in experiments with homogenates.

Human leukocytes were cultivated for 48 h at 37 °C RPMI 1640 with 0.5 % growth proteins in 5 % CO_2 atmosphere in aseptic environment. The mice were killed at seventh day (after two days without adding of homogenates) by interruption the spinal cord. Peritoneal cells from the mice peritoneal cavity were pooled with 5 ml of saline. The liquid with leukocytes was taken out from the mice. Leukocytes were then microscopically counted and adjusted to $2x10^6$ cells/ml with adding of RPMI 1640 with 0.5 % growth proteins alike in HL.

Phagocytosis determinations

A suspension of human and mouse cells ($2x10^6$/ml) were incubated for 60 min at 37 °C with 50 μl of killed *Streptococcus faecalis* ($5x10^8$/ml) (by heating in an autoclave). Particles were then placed on a microscope slide. The slides were dried at laboratory temperature and stained according to Wright (1 ml of colour was put on the slide and it was affected on for 1 min; then it was added 1 ml of water (pH 7.0) and the slide was mixed for 3.5 min; the slides were dried at laboratory temperature). Phagocytic activity was calculated as percentage of the phagocytic cells from the total amount of 100. The index of phagocytosis was calculated as the number of particles ingested by one phagocyte.

Bactericidal activity

The bactericidal activity of human and mouse cells was determined on *Staphylococcus aureus* or *Escherichia coli*. A 24 h culture of *Staphylococcus aureus* on blood agar or *Escherichia coli* on Endo agar were adjusted to optical density 0.35 at 540 nm. In next the suspensions were resuspended to concentration $1:10^5$ cells/ml. 100 μl of ultrasonically disintegrated (18 kHz, 10 s) human or mouse cells and 75 μl of *Staphylococcus aureus* or 50 μl of the *Escherichia coli* suspensions were incubated for 60 min at 37 °C. The surviving microbes were calculated as a colony number after cultivation on a solid media.

Candidacidal activity

The candidacidal activity has been tested by using *Candida albicans* SC 1539. A 48 h culture of *C. albicans* on Sabouraud agar was used for preparing of sample. The suspension of microorganism was adjusted to absorbance A_{540}=0.35 and resuspended to concentration $1:10^3$ cells of *C. albicans* in 1 ml. Aliquots (100 μl) of ultrasonically disintegrated human or mouse cells (10 kHz, 10 s) and the microbial suspension were incubated at 37 °C for 60 min. The surviving microbes were calculated as colony number after 48 h cultivation (at 25 °C) on Sabouraud agar and they were recalculated as the number of cells per 1 ml.

Peroxidase activity

The ultrasonically disintegrated leukocytes (18 kHz, 10 s) were centrifuged 10 min at 39 200 ms^{-2}. The supernatant (150 μl) was added to 50 μl of the peroxidase substrate (29.4 g trisodium citrate, 1000 ml distilled water, 1.0 ml 30 % hydrogen peroxide, 5 mg 1,2-phenylenediamine, pH 5.0). The reaction was stopped after 20 min by adding H_2SO_4 (4 mol/l). The changes in absorbance were measured spectrophotometrically at 490 nm.

Lysozyme activity

The ultrasonically disintegrated leukocytes (18 kHz, 10 s) were centrifuged 10 min at 39 200 ms^{-2}. The amount 150 μl of the supernatant was mixed with 50 μl of the *Micrococcus luteus* suspension (A_{410}=0.8) in the phosphate buffer pH 6.2. Turbidity changes were measured in interval 0 and 25 minutes spectrophotometrically at 410 nm in comparison to the control suspension.

Results

The aim of these experiments was to evaluate the effects of ultrasonically disintegrated freeze-dried cells of *S. aureus* (S), *E. coli* (E) and *C. albicans* (C) on immune system of human leukocytes (in vitro) and mouse leukocytes (in vivo).

The results of phagocytic activity of human and mouse macrophages after the activation by S, E or C homogenates are shown in Table 1. The homogenates have not affected human macrophages, but the phagocytic activity of mouse macrophages significantly increased.

Tab. 1. Phagocytic activity of human (H) and mouse (M) macrophages.

	Group (n=5)	Arithmetic mean %	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	84.0	10.2	1.0	
	S	69.5	7.7	0.8	NS
	E	80.0	7.7	0.9	NS
	C	84.0	8.0	1.0	NS
M	K	51.4	6.7	1.0	
	S	83.2	9.6	1.6	0.001
	E	93.8	3.0	1.8	0.001
	C	79.2	11.7	1.5	0.01

Tab. 2. Phagocytic index of human (H) and mouse (M) macrophages.

	Group (n=5)	Number of engulfed particules	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	1.3	0.4	1.0	
	S	1.7	0.2	1.3	NS
	E	2.9	0.9	2.2	0.05
	C	2.3	0.4	1.7	0.01
M	K	2.6	0.2	1.0	
	S	6.2	0.4	2.3	0.001
	E	8.3	1.3	3.2	0.001
	C	6.0	1.1	2.3	0.001

Tab. 3. Bactericidal activity of human (H) and mouse (M) leukocytes against *Staphylococcus aureus.*

	Group (n=5)	Number of living cells x 10^5	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	71.2	11.6	1.0	
	S	73.8	12.7	1.1	NS
	E	63.4	7.2	0.9	NS
	C	79.2	7.4	1.1	NS
M	K	52.3	5.5	1.0	
	S	81.7	8.9	1.5	0.01
	E	85.4	23.1	1.6	NS
	C	86.7	7.8	1.6	0.01

Tab. 4. Bactericidal activity of human (H) and mouse (M) leukocytes against *Escherichia coli.*

	Group (n=5)	Number of living cells x 10^5	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	84.0	4.7	1.0	
	S	83.8	0.7	0.9	NS
	E	94.6	8.6	1.1	NS
	C	69.6	9.0	0.8	0.05
M	K	16.0	7.2	1.0	
	S	27.6	14.5	1.7	NS
	E	18.0	7.1	1.1	NS
	C	24.8	12.1	1.5	NS

Tab. 5. Microbicidal activity of human (H) and mouse (M) leukocytes against *Candida albicans.*

	Group (n=5)	Number of living cells x 10^3	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	10.0	1.4	1.0	
	S	6.7	3.4	0.7	NS
	E	5.3	4.7	0.5	NS
	C	4.7	2.0	0.5	0.05
M	K	478.8	235.3	1.0	
	S	29.3	31.7	0.06	0.05
	E	23.3	22.7	0.05	0.05
	C	18.6	15.1	0.04	0.01

Tab. 6. Peroxidase activity of human (H) and mouse (M) leukocytes.

	Group (n=5)	Absorbance (490 nm)	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	0.27	0.01	1.0	
	S	0.25	0.01	0.9	0.05
	E	0.26	0.01	0.9	0.01
	C	0.26	0.01	0.9	0.05
M	K	0.03	0.01	1.0	
	S	0.4	0.08	11.7	0.001
	E	0.3	0.04	8.5	0.001
	C	0.5	0.03	17.4	0.001

In the case of human macrophages, except for the S homogenate, all other samples significantly increased the values of phagocytic index (Tab. 2).

In the group of human leukocytes we found that the bactericidal activity (Tab. 3) against *S. aureus* was not affected by any homogenate. On the contrary, treatment of mouse leukocytes by the homogenates S and C significantly increased their capability to kill staphylococci. This effect has not been revealed when *E. coli* was used as test organism (Tab. 4). Only C homogenate, when acting on human leukocytes, was able to enhance their capability to kill the E. coli bacteria. In the group of mouse leukocytes treated by all homogenates we determined that the candidacidal activity was statistically significantly increased. In the case of tested human leukocytes, only C homogenates revealed the similar effectiveness (Tab. 5). The most pronounced differences between human and mouse leukocytes after treatment by the microbial homogenates are in their metabolic activity. While human leukocytes were affected, in the case of mouse leukocytes both peroxidase and lysozyme activities were significantly stimulated after activation by disintegrated microbial cells (Tab. 6 and 7).

Discussion

From the large number of compounds with immunomodulatory activity many have the microbial origin and many of them are commercially available.

Mostly they are lysates of gram-positive and gram-negative bacteria or isolated glucans from fungi. Although their activities have been studied, their effects on human and other mammalian immune mechanisms are still not fully understood.

Zembala et al. (1994) found out differences between the activity of BCG and endotoxin at human and mouse macrophages. Ferencik et al. (1986, 1988) tested immunomodulatory activity of particular glucans and of their soluble chemically modified derivatives on human and guinea pig leukocytes.

In this paper we would like to demonstrate the effect of homogenates prepared from three different microorganisms. The nonspecific defensive mechanisms of the immune system are the first line of defence during the protection against pathogens. Initiating these mechanisms could form also the best conditions for starting the next activities of immune system. The homogenates were gained from the cells of *Staphylococcus aureus* as a gram-positive bacterium, *Escherichia coli* as a gram-negative bacterium and *Candida albicans* as a yeast. The bacteria and yeast were isolated from exponential phase of growth at the agar media. Freeze-dried microorganisms were destroyed by sonication and the homogenates were absent of any living cells.

We tried to get the same and standard quantity of the leukocytes as well as the amount of disintegrated microorganisms despite the different conditions of cultivation the human (in vitro) and mouse cells (in vivo). The phagocytic activity of mouse leukocytes was activated with homogenates S, E and C. Meanwhile, the activity on human macrophages has not been affected in this way.

Bukovsky et al. (1998) demonstrated, that the phagocytic activity on human leukocytes was stimulated by the outer membrane from *E. coli*. Notable is, that control group of human leukocytes had a high number of phagocyting macrophages (84 %) in comparison with control group of mouse macrophages (51 %). It means, that human leukocytes could be activated by donor of leukocytes.

The phagocytic index was determined as the number of ingested particles of *Streptococcus faecalis* by one phagocyte. Contrary to the phagocytic activity, the number of ingested particles was increased in all groups in comparison with the sample of physiological control. Only in the group of human macrophages was significantly reduced by the treatment with homogenate S.

Baronikova et al. (1999) observed, that phagocytic index of human mononuclear cells was increased by outer membrane from *E. coli*. Bukovsky et al. (1994) demonstrated an increase in phagocytic activity and index of peritoneal mouse macrophages after application (i.p.) of zymozan (from *Saccharomyces cerevisiae*). The bactericidal activity of mouse leukocytes against *S. aureus* was decreased by activation by all three homogenates. Contrary to this, the activity of human leukocytes against *E. coli* was not affected at the same conditions.

The candidacidal activity of human and mouse leukocytes was tested by using *C. albicans* homogenate and it was not influenced on the level of statistical significance after treatment with homogenates S and E. The activity of human leukocytes after treatment with homogenate C was increased and the activity of mouse leukocytes were stimulated by all homogenates. The results were compared with the control group of leukocytes.

Baronikova et al. (1999) and Bukovsky et al. (1998) found stimulation of candidacidal activity of human leukocytes activated with outer membrane from *E. coli.* Also the peroxidase and lysozyme activity of human and mouse leukocytes were tested.

Tab. 7. Lysozyme activity of human (H) and mouse (M) leukocytes.

	Group (n=5)	Difference in absorbation (405 nm) between 0 and 25 min	Standard deviation (±SD)	Activity (k=1)	Statistical significance p<
H	K	0.27	0.01	1.0	
	S	0.25	0.01	0.9	0.05
	E	0.26	0.01	0.9	0.01
	C	0.26	0.01	0.9	0.05
M	K	0.02	0.01	1.0	
	S	0.2	0.05	11.7	0.001
	E	0.2	0.03	11.4	0.001
	C	0.2	0.03	11.2	0.001

The activity of human leukocytes was decreased and mouse leukocytes were stimulated by tested samples.

Enhancement of lysozyme activity of peritoneal mouse macrophages was shown also by Bukovsky et al. (1994) after i.p. application of zymozan.

Despite the extensive research and practical use of immunomodulators the mechanism of various extracts and substances are still not satisfactory explained. The results of our work suggest, that the function and activities, though on the first sight similar, could be realized in different ways. Consequently, it can be evidence of reality, that after treatment with the same homogenate at the same concentration the bactericidal and fungicidal activity of leukocytes may be different. These differences are not only between the human and mouse mechanisms, but also in single groups of human and mouse leukocytes. Mechanisms of immunomodulatory activity could be affected by environmental factors as well as by differences between human and other mammalian organisms.

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