Effects of Gram-Positive Microorganisms and Their Products on *In Vivo* Survival of Hemopoietic Clonogenic Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 4, pp. 441-445, April, 2008
Original article submitted December 24, 2007

The effects of gram-positive bacterial strains (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) and their subcellular components on the survival of hemopoietic clonogenic cells were evaluated by the formation of endogenous splenic colonies. The effects of these preparations on NO production were studied by the spin-trap paramagnetic resonance spectroscopy. Bacterial preparations from gram-positive bacteria stimulated survival of hemopoietic clonogenic cells, but did not induce NO production in contrast to *E. coli* LPS.

Key Words: *hemopoietic clonogenic cell survival; nitrogen oxide; gram-positive bacteria; lipoteichoic acid; peptidoglycan*

LPS, a component of gram-negative bacterial wall, is one of the most potent and best studied stimulants of hemopoietic clonogenic cells (HCC; multi-, oligo-, and unipotent precursors of differentiated blood cells) [15]. In high concentrations, this toxic agent plays the key role in the pathogenesis of sepsis, while normally LPS is constantly released by enteric bacteria through the portal vein, is exposed to immunocytes, and causes no pathological consequences in normal subjects [15]. The radioprotective effect of LPS on bone marrow and intestinal epithelial stem cells is caused by activation of the NFκB transcription factor, TNFR1 and proinflammatory cytokine expression, and prostaglandin synthesis [13].

The discovery of TLR1 and TLR4 receptors involved in the recognition of pathogen-associated molecular invariants [15] in hemopoietic stem cells showed that LPS directly (without cellular and biochemical mediators) regulates metabolism, recovery, and differentiation of HCC. Presumably, membrane components of gram-positive bacteria can also be factors of HCC survival.

We studied the possibility of regulating HCC survival by gram-positive microorganisms (and their components) from endogenous enteric microflora.

MATERIALS AND METHODS

Experiments were carried out on outbred (initial genotype Swiss strain) albino 5-month-old mice (males and females, 28-32 g) and male (CBA× C57Bl/6)F1 mice (20-22 g) kept in cages in a room protected from noise at natural light and 22-24°C on standard diets with free access to food and water.

The animals were exposed to a single dose of 6.5 Gy on a Luch γ-device with ^{60}Co source at a dose power of 0.3 Gy/min.

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0007-4888/08/1454-0460

The following reagents were used: sodium diethyldithiocarbamate trihydrate (DETC), iron sulfate heptahydrate, sodium citrate trisubstituted dihydrate (Baum-Lux); LPS (*E. coli* 0111:B4), lipoteichoic acid (LTA, *Staphylococcus aureus*), peptidoglycan (PG; *S. aureus*), *Bordetella pertussis* toxin (PT, Sigma-Aldrich-Rus).

Lactobacteria and preparations from them were prepared at N. F. Gamaleya Institute. *Lactobacillus acidophilus* strains NK1, K3, 100ASh and *Lactobacillus rhamnosus* strains V300 and 57 were used in experiments as living cell suspension (20×10^{11} CFU/ml) and as subcellular components. Subcellular components were obtained by a single cycle of freezing/defrosting of bacterial suspension and ultrasonic treatment at 0-4°C, after which the suspension was centrifuged at 23,000 rpm, the precipitate was separated and diluted in Ca^{2+},Mg^{2+}-free Dulbecco phosphate buffer (0.05 M, pH 7.2) to the needed concentration.

The following preparations were made: Cyto-1 (supernatant fraction from a mixture of strains NK1, V300, 57 strains in 1:1:1 proportion), Mem-1 (destroyed cell precipitate), Cyto-2 (supernatant fraction from a mixture of *Lactobacillus acidophilus* strains NK1, K3, 100ASh in 1:1:1 proportion), and Mem-2 (destroyed cell precipitate).

Eight days after irradiation, experimental animals were sacrificed by ether overdose, the spleens were removed and fixed, and colonies formed by hemopoietic CFU which survived by this term (CFU-S-8) on their surface were counted [4]. Each group consisted of 12 mice, experiments were repeated 2-3 times.

NO-inhibitory activity of synthesized substances was studied on outbred male albino mice (original genotype: Swiss strain). Four hours before sacrifice by ether overdose, the animals were injected with LPS (intraperitoneally, 1.5 mg/kg in 0.5 ml saline per mouse). Three hours after LPS injection the animals were injected with the test preparations and after 1 h liver samples were removed. NO production was evaluated by spin-trap EPR spectroscopy by a method modified by A. F. Vanin [1,5].

Statistical differences in the means and survival curves were evaluated by the analysis of dispersions and Newman—Keuls and Dunnett tests [2].

RESULTS

The effects of biopreparations on splenic weight and number of CFU-S-8 in mice irradiated in a dose of 6.5 Gy are presented in Table 1. A pronounced effect of LPS and *L. rhamnosus* (strain 57) cells on splenic weight was noted, which increased by 65% in mice treated with these preparations in comparison with the control animals (34 mg). In other groups, this parameter virtually did not change in comparison with the control.

The effect of bacterial preparations on the number of CFU-S-8 was more pronounced. The LTA+PGN mixture of membrane components of pathogenic gram-positive (GP) bacteria induced minimum increment in CFU-S-8. The effect of PT exotoxin was the same: 3 CFU-S-8 *vs.* 2.2 CFU-S-8 in the control. Bacterial cells more actively stimulated the growth of CFU-S-8. Their number in the spleens of animals treated with these preparations increased 3-4-fold. The counts of CFU-S-8 were the same after injections of NK1 and V300, but the increase in the spleen weight after V300 injection attested to an increase in the size of endogenous colonies and hence, in the count of HCC under the effect of V300 cell preparation.

The effects of subcellular preparations from a mixture of GP of two bacterial species are summed up in Table 2. All the preparations caused a significant (2.5-3-fold) increase in CFU-S-8 number in

TABLE 1. Effects of Living *Lactobacillus* Preparations on Survival of HCC, Evaluated by Production of Endogenous Splenic Colonies by CFU-S-8

Preparation, dose	Treatment	Spleen weight, mg	Mean number of colonies formed by CFU-S-8 per spleen
Control	6.5 Gy	34±6	2.2±1.9
LPS (1.5 mg/kg)	LPS+1 day	58±14$^+$	15±4$^+$
LTA (3 mg/kg)+PG (10 mg/kg)	(LTA+PG)+1 day+6.5 Gy	33±5	3.0±1.3$^+$
PT (200 ng)*	PT+2 days+6.5 Gy	37±11	3.0±1.1$^+$
NK1 (1.2×10^9 CFU)*	NK1+1 day+6.5 Gy	39±6	6.9±2.1$^+$
V300 (1.2×10^9 CFU)*	V300+1 day+6.5 Gy	37±5	9.0±2.6$^+$
57 (1.2×10^9 CFU)*	57+1 day+6.5 Gy	54±8$^+$	9.0±2.6$^+$

Note. Here and in Table 2: *dose per mouse. $^+p<0.05$ compared to the control.

TABLE 2. Effects of Subcellular Preparations of *Lactobacillus* on HCC Survival, Evaluated by Formation of Splenic Colonies by CFU-S-8

Preparation, dose	Treatment	Spleen weight, mg	Mean number of colonies formed by CFU-S-8 per spleen
Control	6.5 Gy	33±7	1.6±1.5
LPS (1.5 mg/kg)	LPS+1 day+6.5 Gy	52±14+	11.4±5.7+
Mem-1 (4×10^9 CFU)*	Mem-1+1 day+6.5 Gy	36±4	4.0±2.9+
Cyto-1 (1.2×10^9 CFU)*	Cyto-1+1 day+6.5 Gy	37±5	5.3±3.7+
Mem-2 (4.3×10^9 CFU)*	Mem-2+1 day+6.5 Gy	37±4	4.0±2.5+
Cyto-2 (4.3×10^9 CFU)*	Cyto-2+1 day+6.5 Gy	34±6	4.0±2.9+

Note. Mem: membrane fraction; Cyto: cytoplasmatic fraction.

comparison with the control. It is noteworthy that Cyto-1 was used in an almost 3-fold lesser dose than other preparations and its effect was more pronounced. However, the increase in the number of CFU-S-8 in these groups was significantly lower ($p<0.05$) in comparison with that in LPS-treated group. The spleen weight also increased significantly in this group.

The effects of some of the bacterial preparations on NO production in mice were studied (Fig. 1). Our findings and published data suggest that many bacterial preparations, mainly from pathogenic and GP microorganisms, are characterized by proinflammatory activity, which manifests, among other things, in NO induction in mammalian phagocytic cells over 3-6 h after injection of the preparation. All the studied preparations were injected 4 h before collection of liver samples for analysis. Animal treatment with LPS in a dose of 1.5 mg/kg caused a significant (almost 13-fold) increase in NO content in the liver in comparison with the basal level. After LPS treatment in a dose of 0.15 mg/kg, the content of NO in the liver was only 25% lower than after higher dose. The production of NO

TABLE 3. Bacterial Preparations Stimulating the Survival of Hemopoietic Stem Cells *In Vivo*

Preparation	Source/composition	Biological activity	Notes	References
IRS-19	Mixture of 4 GP and 4 GN bacterial lysates	Leukocytes↑ IL-6, IFN-γ↑ CFU-S-8↑	Effect observed after preparation injection before and after lethal γ-irradiation	[10]
Muramyl-tripeptide	Derivative of polysaccharide, a component of GP bacterial membrane	Reticulocytes↑ Lymphocytes↑ BM cell composition↑	Superadditive effect with indomethacin	[14]
Lactobacillus casei	Heat-inactivated	Animal survival↑ CFU-S-8↑	Effect after preparation injection between 48 h before and 9 h after irradiation	[17]
Broncho-Vaxom	Mixture of 4 GP and 4 GN bacterial lysates	CFU-S-8↑	Superadditive effect with indomethacin	[11]
OK-432	*Streptococcus haemolyticus* (GP) preparation	Animal survival↑	Effect is observed after preparation injection following a lethal γ-irradiation and stimulated by repeated injections	[13]
Bificol	Lyophilized *Bifidum* and *E. coli* bacteria (10:1)	CFU-S-8↑ Animal survival↑	Preparations contain endotoxin	[3]

Note. BM: bone marrow; GN: gram-negative.

in the liver of animals treated with LPS in a dose of 1.5 mg/kg was taken for 100%. None of the preparations induced significant extra production of NO in comparison with the basal level (9% of NO content in liver samples from LPS-treated mice) in animals which received the spin trap alone (Fig. 1, group 2). On the other hand, combined treatment with LPS and Mem-2 fraction (mixture of *L. acidophilus* strains) caused a significant (almost 1.6 times) increase in NO production in comparison with that after LPS alone.

Published data indicate that apathogenic GP bacteria-based preparations, including preparations on the base of endogenous enteric bacteria, seem to be promising candidates for prevention and therapy, including stem cell therapy, due to promotion of cell survival (Table 3) [7]. This hypothesis is supported by experimental data indicating some important features in the mechanisms of the effects of GP bacteria and their components on the immune system, for example, on inflammatory reaction induction [15].

Our data indicate that stimulation of HCC survival by GP bacteria or their components is realized by mechanisms differing from LPS-induced reaction, which is usually associated with activation of NFκB transcription factor, synthesis of inducible NO-synthase, and many-fold increase of NO production [15].

According to tentative quantitative evaluation of the doses of bacterial preparations used in our study, if the content of LPS molecules in *E. coli* cells is 3.5×10^6 molecules [15] and their mean molecular weight is about 15,000 Da, the dose of 1.5 mg/kg corresponds to about 1.7×10^{11} cell/kg. It is almost 3-fold more than the *Lactobacillus* dose used in our study. However, our data indicate that even a 3-fold lower dose of LPS causes approximately the same stimulation of NO production and HCC survival [6].

A minor effect of *Bordetella pertussis* exotoxin on HCC survival was detected. Though this toxin also induced the proinflammatory cytokines, the mechanism of HCC activation remained not clear [12]. Our data indicate that additional synthesis of NO can be hardly an important component of this process.

Hence, apathogenic strains of GP bacteria or their components stimulate the survival of hemopoietic stem cells, the mechanism of their action *in vivo* differing from the reaction induced by gram-negative *E. coli* endotoxin.

The authors are grateful to I. M. Fedosov (EPR spectroscopy), N. G. Kucherenko, A. I. Trishkina, and L. V. Stein (preparation of samples).

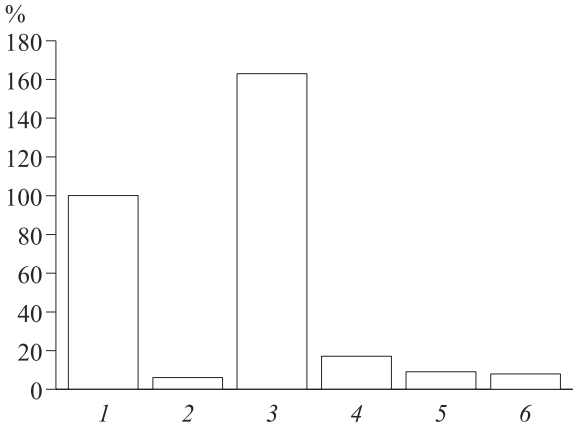

Fig. 1. Effects of bacterial preparations on NO production in mouse liver. *1*) LPS; *2*) spin trap; *3*) LPS+Mem-2; *4*) Mem-2; *5*) Mem-1; *6*) NK-1. Ordinate: NO content in liver samples from animals treated with bacterial preparations in percent of that in animals treated with LPS.

The study was supported by the state budget, Russian Foundation for Basic Research (project 06-04-08193ofi_a), Russian foundation for Basic Research and the Government of the Kaluga region (project 07-04-97607r_ofi).

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