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EFFECTS OF DRY ENZYMATIC LYSATE DEL-IMMUNE V® ON CYTOKINE PRODUCTION IN THE EXPERIMENTAL MODELS

Prior testing of Del-Immune V® has indicated effectiveness for immune system support; however, mechanisms of action and optimal doses have not been researched yet. It is shown that the drug Del-Immune V®, a dry enzyme lysate of the strain Lactobacillus rhamnosus V, containing muramyl peptides and DNA fragments in the range of doses of 5–500 mg/mouse (optimum 50 mg/mouse) is an active inducer of IFN, it affects the production factor of tumor necrosis (TNF) in systems in vivo and in vitro. Its inducing activity is comparable with the data obtained while using complex probiotic Bifidim, but the live cells of bifidobacteria, being a part of the drug more efficiently stimulated production of TNF in vitro in comparison with Del-ImmuneV®. The levels of cytokine production under the influence of drugs in the in vivo correlated with the production of cytokines in the system in vitro. The highest serum IFN level was reported 24 hours after the drugs administration. The control group remained unchanged. Maintenance of elevated circulating IFN was possible only through repeated administration.

Key words: Del-Immune V®, Lactobacillus rhamnosus V, *probiotic Bifidim, interferon, tumor necrosis factor, immune system.*

Among a large number of presently known therapeutic products utilizing lactobacilli, cell wall peptidoglycan is enjoying growing popularity as an immunomodulator which contains, among the other things, the fragments of DNA and cell peptidoglycan of the lactic acid bacteria *Lactobacillus rhamnosus* V(DV Strain). Del-Immune V® (manufactured by Pure Research Products, LLC, Boulder, Colorado) was registered by the US Food and

Drug Administration in 2002 as a food supplement for immediate immune system support.

The biochemical structure of Del-Immune V® and preliminary experimental and clinical data indicate that Del-Immune V® may be highly effective in infectious diseases of viral (flu, hepatitis C), bacterial (bronchitis), and fungal etiology, allergies of all severity levels, asthma, chronic fatigue, and fibromyalgia [10, 24]. The mechanism of such a wide scope of biological activity of the formulation is still unclear. The goal of our research, therefore, was to study the mechanisms of the immunomodulating effects of Del-Immune V® and to describe their dose-dependent effects on production of immunoregulatory cytokines in vivo and in vitro.

Last 5 years and previous investigations have been marked by increasingly active study of the mechanisms of the immunobiological effects of probiotics and bacterial medications [8, 9, 12, 25, 26]. As a result, bacterial medications such as liastenum (blasten), deodan, licopid, prodigiosanum, salmosanum, sodium nucleinate, MC (molecular composition — yeast DNA and Tilorone), biostim, BCG, rumurtide, ribomunyl, and lactolin are being used, in both trials and clinical practice, for different pathologies [10, 17, 23, 24]. The adjuvant effect of BCG and the immunomodulating activity of formulations containing the derivatives of lactobacilli, such as liastenum (blasten, *Lactobacillus delbrueckii)* and deodan (*Lactobacillus bulgaricus*), have been associated with peptidoglycans and their structural components, muramyl dipeptides (MDP). The most active analog of MDP, MurNac-L-Ala-D-Glu-NH_2, has demonstrated the adjuvant and pleiotropic effects and is capable of inducing a number of cytokines: IL-1, tumor necrosis factor (TNF-α), IL-2, IL-6, IL-8, IL-12, and interferon gamma (IFN-γ) [9, 12].

These cytokines in turn stimulate nonspecific cytotoxicity of normal and effector lymphocytes and natural killer cells (NK), and coordinate the body's immune response, depending on the nature of the aggressive agent and the T-helper differentiation (Th1 or Th2) [2, 9, 12, 26].

These properties of peptidoglycans indicate the basis for creating immunomodulating formulations for clinical use. Lactobacilli generally recognized as safe (GRAS) group are good sources of peptidoglycans. Toll-like receptors TLR4 and TLR2 for MDP and peptidoglycans have been identified on the surface of lymphocytes and macrophages [25]. The fragments of probiotic bacterial DNA are interesting because of their capacity to stimulate production of cytotoxic lymphocytes and NKC, activate the complement system, heighten cytostatic and cytotoxic activity of macrophages, and regulate production of immunoregulatory cytokines [25].

Owing to the TTTCGTTT DNA pattern of the strain, *Lactobacillus rhamnosus GG* was found to be a factor preconditioning immunobiological activity of the probiotic producer [8]. Thus, CpG DNA are identified with the help of TLR9 and TLR10 expressed in the intercellular (endosomes) cell compartments. CpG DNA identification with TLR9 and TLR10 results in activation of neutrophils and cytokine production [6, 15, 25].

Materials and methods

The study examined the dose-dependent effect of Del-Immune V® on production of immunoregulatory cytokines in nondescript mice with body mass of 14—16 g. One hundred forty animals were selected on the basis of the analogue principle, and were divided into 7 groups of 20. The animals were fed balanced rodent food and water ad libitum. Group I, II, and III of mice received 0.5 ml of aqueous solution of Del-Immune V orally in doses of 5, 50, 100 and 500 μg/mouse respectively for 5 days at 24-hour intervals. Group IV of mice were administered 0.5 ml Bifidim suspension (control probiotic medication) orally in the dose of 50 μg/mouse on the same schedule. The Bifidim was a dry mass of antagonistic bifidus bacteria immobilized on enterosorbent in combination with ascorbic acid (Intervetmed Ltd., Kiev, Ukraine). Group V of mice were administered 0.15 M NaCl. Group VI and VII mice were used to study the interferonogenous activity of a single administration of 50 μg/mouse of Del-Immune V® (Group VI) or Bifidim (Group VII). Cytokine production by IFN and TNF was examined in intact and treated mice in 8 hours after initial administration and then every 24 hours for the next 5 days. For this purpose, several mice from each group were killed by cervical dislocation; blood serum, [13] peritoneal exudate macrophages (PEM) [13] and spleen [16], from which splenocytes were harvested [16] from each group of mice for testing.

The optimal dose of Del-Immune V® was also tested via in vitro induction of immunoregulatory cytokines in splenocytes and PEM ($1x10^7$ cell/ml) of treated and intact mice by culturing cells with the formulation in final concentrations of 5, 50,100 and 500 μg/ml. Interferonogenous activity of the tested formulations was assessed in comparison with Bifidim 50 μg/ml and standard inductors (IFN-α; Newcastle Disease Virus, NDV—10 TCD_{50}/cell; IFN-γ; phytohemagglutinin, PHA—20 μg/ml; *Difco*; TNF, LPS *E. Coli* 0111—4 μg/ml—*Sigma* USA). The levels of cytokine production (IFN and TNF) were determined in 6, 24, and 48 hours after incubation of the cell with the formulations.

Biological activity of TNF was assessed by cytotoxicity in the passaged culture of murine fibroblasts L-929 [13]. The result was recorded on a multiscanner (Dynatech, Switzerland) with a wavelength of 540 nm. The cytotoxicity index was calculated using the formula CI = K-O/Kx100%, where K and O represent optical density values for the cell in the culture medium (RPMI 1640 with 10% FCS). The calibration curve based on standard recombinant TNF formulation *Sigma* was used for standardization of the cytotoxicity index [7].

IFN levels in cell cultures and serum were measured using standard microtitration in the passaged cell culture L-929 against 100 TCD 50 indicator virus (vesicular stomatitis virus, Indiana VSV) with constant CO_2 level [13]. The significance of the results was analyzed by Student-Fisher t-test. Differences of $P < 0.05$ were considered to be significant [11].

Results and discussion

Daily oral administration of Del-Immune V® or Bifidim to Groups I-III in the course of 5 days in doses of 5, 50, or 500 μg/mouse resulted in marked increase in IFN levels in blood serum (Figure 1). The optimal interferonogenous dose was found to be 50 μg/mouse (Group II). After 24 hours of observation, circulating IFN levels in Group II reached 4.5±0.5 $\log_2$ U/ml. After repeated administrations, levels reached 5.5±0.7 $\log_2$ U/ml, in comparison with 2.0±0.7 $\log_2$ U/ml in the control group (Group V). Further administration of Del-Immune V® in a dose of 50 μg/mouse on day 3 allowed for maintenance of the 5.5±0.5 $\log_2$ U/ml level. Administration of the formulation on days 4 and 5 resulted in nonsignificant decreases in circulating IFN levels. When Del-Immune V® was administered in doses of 5 and 500 μg/mouse (Groups II and III), findings were similar, although maximum interferon levels were not as high.

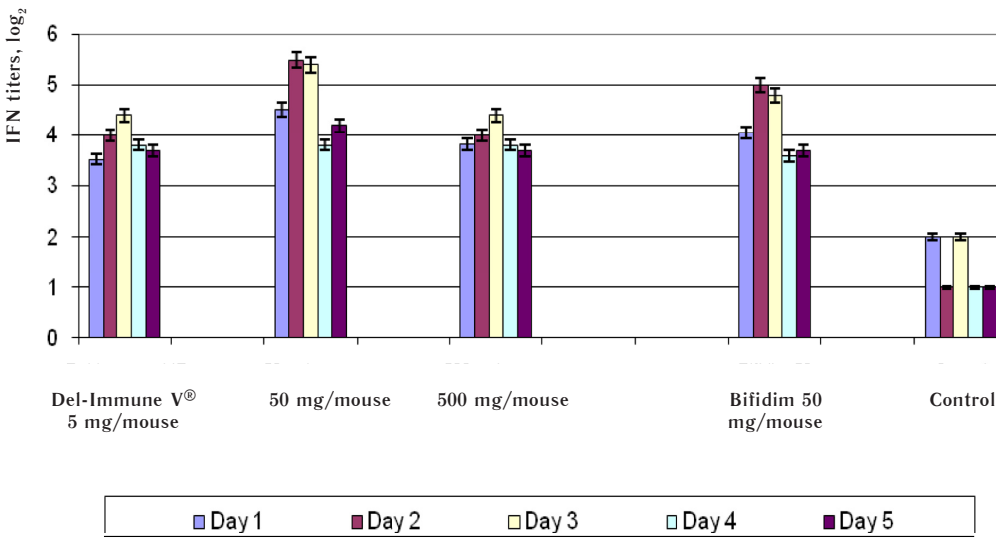

Fig. 1. Interferon activity of Del-Immune V and Bifidim *in vivo*

One-time oral administration of Del-Immune V® or Bifidim to mice in a dose of 50 μg/ml resulted in increased circulating IFN level 8 hours after administration. The highest serum IFN level was reported 24 hours after administration, while levels in control group animals remained unchanged (Table).

Forty-eight hours after administration of Del-Immune V®, serum IFN levels in all active groups remained reliably enhanced in comparison with the control group, but IFN was later eliminated from the body. The maintenance of circulating IFN levels was possible only through repeated administration.

Table

Murine Serum IFN Titers after One-Time Administration of Del-Immune V or Bifidim

Studied formulations; doses of 50 μg/ml	Serum IFN titers, $\log_2$		
	8 h	24 h	48 h
Del-Immune V	4.00 ±0,03*	4.40 ±0,03*	3.41±0,03*
Bifidim	3.60 ±0,01*	4.00 ±0,03*	2.70 ±0,03
Control	2.00 ±0,02	2.00 ±0,03	2.00 ±0,03

* $p \leqslant 0,05$

The comparative analysis of interferonogenous activity induced by formulations made on the basis of living bifidus bacteria cells (Bifidim) or structural components of *Lactobacillus rhamnosus V* (Del-Immune V®) was performed by testing the interferon-synthesis activity of leukocytes. Splenocytes of the mice receiving experimental formulations were cultured with NDV and TNF inductors, resulting in 2-fold increase of interferon response in comparison with intact animal cells (Figure 2), indicating that the experimental formulations positively affected immune response status. Interferon status was determined by assessing circulating IFN titers (serum IFN), IFN-α and IFN-α production by immunocompetent cells as a response to adequate in vitro stimulation, and spontaneous IFN production.

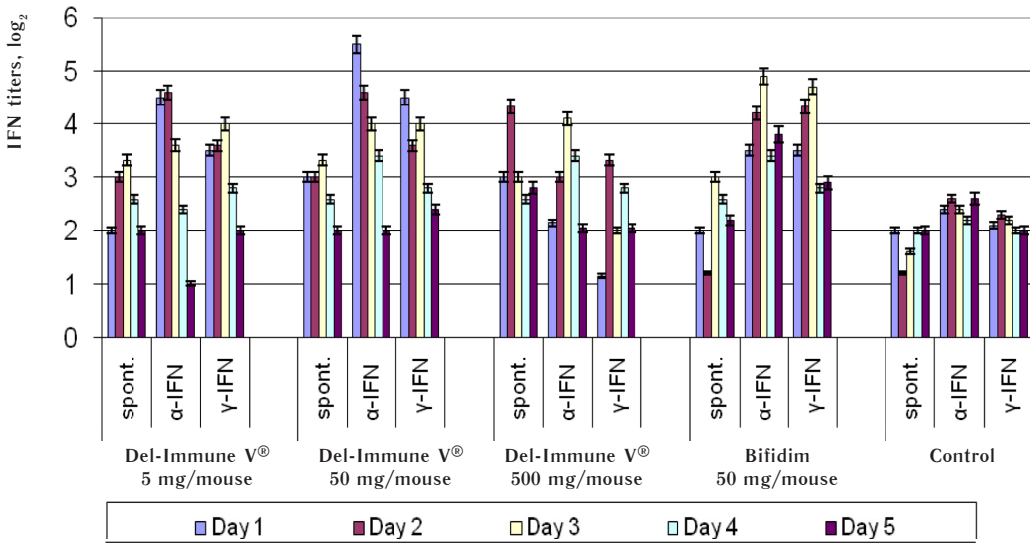

Fig. 2. Interferon activity in splenocytes cultured with NDV and PHA inductors following administration of Del-Immune V or Bifidim

After administration of certain IFN inductors, capacity for enhanced production of IFN-α and IFN-γ was seen in splenocytes in 24, 48, and 72 hours after administration of the experimental formulations. One of the contraindications for IFN inductor use is development of hyporeactivity—inhibition of IFN production after repeated administration of the formulation. Refractoriness of animals was determined by assessing INF-α- and -γ levels in response to adequate stimulation. Decreases in IFN-α and IFN-γ production were reported on day 4 after the initial administration and reached the control levels on day 5. In the Bifidim group, it was possible to observe restoration of the interferon-producing capacity of immunocytes on day 5, when activation of the interferon-synthesis capacity of splenocytes was noted. These findings indicate that administration to mice of optimal doses of the probiotic formulations Del-Immune V® and Bifidim on the appropriate schedule stimulates IFN production and increases efficacy of other interferonogenous inductors.

Preincubation of PEM cultures of experimental and intact animal cells with Del-Immune V® and Bifidim resulted in cytokine synthesis stimulation, as measured by IFN titers (Figure 3) and TNF concentrations (Figure 5). Adding Del-Immune V® or Bifidim in the doses of 5, 50, or 100 μg/ml to PEM cultures of experimental and intact mice resulted in IFN synthesis (Figure 3). It should be noted that the interferon activity of supernatants depended on the concentration of experimental formulations added to PEM. Thus, when the concentration was 5 μg/ml, IFN production was much lower than when it was 50 or 100 μg/ml, although it was still almost 6 times higher than the control level. At the same time, concentrations of 50 and 100 μg/ml resulted in an accumulation of stimulated IFN titers with similar values, indicating that the optimal dose for Del-Immune V® is more likely to be close to 50 μg/ml.

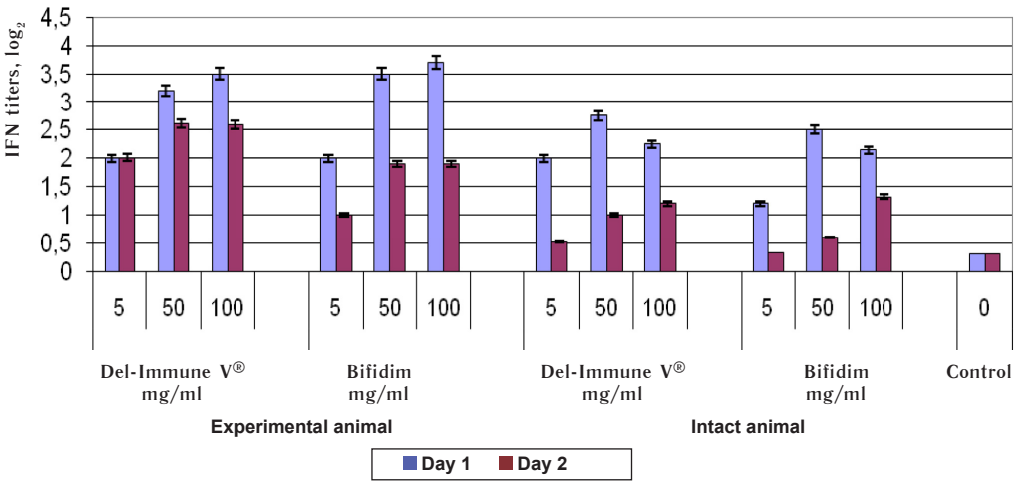

Fig. 3. Interferonogenous impact of Del-Immune V and Bifidim on peritoneal macrophages; comparison of cultures of intact and experimental animal cells

The highest IFN levels in supernatants were reported on day 1 of cell culturing with experimental formulations. However, the levels of IFN in the control group remained lower than in the experimental groups on both days. Heating serum samples of the animals receiving Del-Immune V® or Bifidim for 30 minutes at the temperature of 60 °C decreased their capacity to inhibit reproduction of vesicular stomatitis virus in cell culture L_{929}. The physical and chemical properties of the IFN produced were characteristic of IFN-α/β- and -γ [13].

IFN-γ is produced by sensitized T-lymphocytes $CD4^+$ and $CD8^+$ and NK cells. IFN-γ demonstrates a wide range of immunotropic effects, provides for Th1 differentiation of T-helpers, and stimulates expression on membranes of HLA-DR antigens; without these functions, identification of bacterial antigens or further activation of T-lymphocytes (including T-helpers stimulating maturation of NK-cells as well as some subpopulations of B-lymphocytes) is impossible [13].

IFN-γ also participates in the immune response of macrophage cells, inducing production of TNF and IL-1[13] and modulating their functions. Therefore, the level of TNF, a pleiotropic cytokine produced by primed monocytes and macrophages, lymphocytes, and NKC, was assessed in murine serum (Figure 4) [14, 21, 22, 27].

Oral administration of Del-Immune V® or Bifidim in doses of 5, 50, or 500 μg/mouse resulted in endogenous TNF production. After administration of Del-Immune V® or Bifidim in the dose of 50 μg/ml, serum TNF was 0.6 ng/ml ($P < 0.05$) and 0.8 ng/ml ($P < 0.05$), respectively, while in the control group it did not exceed 0.3 ng/ml. Maximum production of this cytokine was reported 8 hours after administration of these formulations.

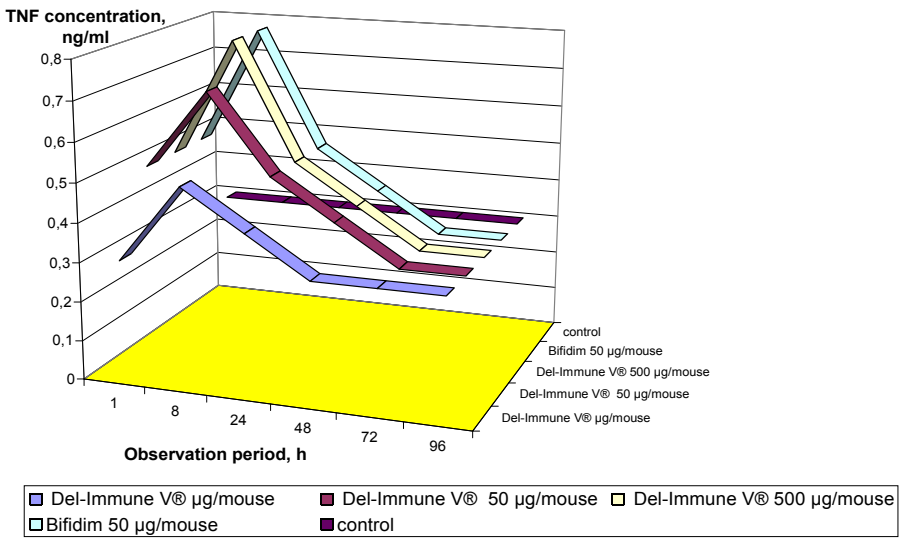

Fig. 4. Murine serum TNF dynamics after administration of Del-Immune V and Bifidim

Del-Immune V® administered in the dose of 5 μg/mouse resulted in insignificant increase in circulating TNF concentration to 0.4 ng/ml ($P > 0.05$), in comparison with 0.3 ng/ml in the control group. It should be noted that oral administration of Del-Immune V® in the doses of 50 and 500 μg/ml resulted in practically equal circulating TNF indices (0.6 ng/ml and 0.7 ng/ml, respectively). This TNF production in vivo calls for further studies since TNF mobilizes leukocytes, terminates inflammatory processes, and plays an important role in the effector and regulatory networks of body immune response. Enhanced TNF production leads to activation of neutrophils, macrophages, and lymphocytes, thus strengthening anti-infection immunity [14, 27].

TNF-induced cascade of induction signals results in gradual production of IL-1 and IL-2, activation of T-lymphocytes, and generation of anti-tumor effector cells—lymphokine-activated killers lysing different tumor target cells. TNF intensifies the proliferative response in mixed culture lymphocytes and tumor cells, and demonstrates adjuvant activity for T- and B-lymphocytes. It should be noted that circulating TNF was quickly eliminated from the body.

In vitro trials showed that adding Del-Immune V® or Bifidim in concentrations of 5, 50, or 100 μg/ml to macrophages of experimental and intact mice resulted in TNF production peaking 8 hours after adding these formulations (Figure 5). TNF production potential of PEM was dose-dependent. The optimal in vitro concentration of Del-Immune V® and Bifidim was 50 μg/ml.

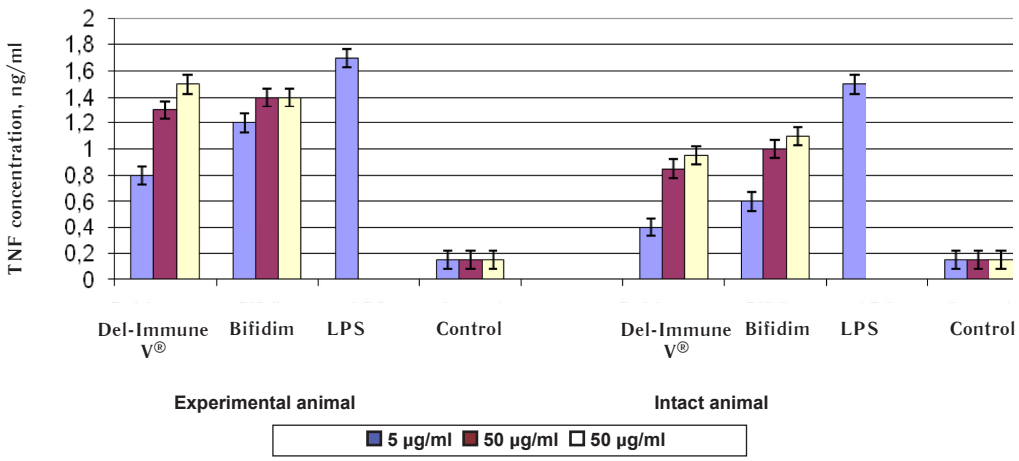

Fig. 5. TNF production 8 hours after adding Del-Immune V, Bifidim, or LPS to peritoneal macrophages of experimental or intact mice

TNF production by the macrophages of the experimental mice after administration of a specific LPS inductor, Del-Immune V®, or Bifidim was

more intensive than by PEM of the intact mice. Both Del-Immune V® and Bifidim induced a higher immune response in macrophage cells of experimental mice, resulting in enhanced production of IFN and TNF. Cell-mediated immune regulation and stimulation of effector function by macrophages are the indicators of the immunomodulating activity of the above formulations. The dose-dependent responses of mice to these immunomodulators should be tested in human subjects to determine whether similar effects will be found.

Derivatives of microbial origin, including lipopolysaccharides (LPS), MDP, and CpG DNA, are identified by immunocompetent cells with TLR receptors [6, 25].

Thus, LPS *E. coli* stimulates mainly monocytes and macrophages [23]. The receptor for LPS is the antigen CD14, which is protein in the family and it first interacts with microbial components aheading of TLR activation.

It can be found on monocytes, macrophages, neutrophils, lymphocytes, and bowel epithelial cells. Fixation of microbial derivatives with receptors results in signal change in the given biological system, which stimulates the synthesis and release of different immunity mediators or cytokines. It should be noted that gram-positive bacteria, including lactobacilli, activate the major class II histocompatibility complex, which induces IFN-γ and IL-12 necessary for Th1 differentiation of T-helpers. Gram-negative bacteria and LPS (a major component of the cell wall of gram-negative bacteria; lipopolysaccharides are endotoxins and important antigens) induce monocytic production of IL-10, inhibiting cytotoxicity activation of IFN-γ and secretion by T- and NK-cells [5]. Since clinical applications of LPS and gram-negative bacteria are limited because of high toxicity, finding selective immunomodulators is one of the main conditions for improving the efficacy of immunostimulating therapy.

In this study, Del-Immune V® stimulated the functional activity of monocyte-macrophagal murine cells. However, higher dosages did not always result in higher efficacy. The success of immune active therapy can be enhanced not only by new medications but also by their rational use.

The living cells of Bifidim stimulated in vitro TNF production more intensively than Del-Immune V®. Cytokine production in vitro induced by Del-Immune V® and Bifidim was compared with cytokine production *in vivo* [3]. Induction of pro-inflammatory cytokines IFN and TNF by Del-Immune V® and Bifidim in vitro suggests that these formulations stimulated a nonspecific immune response in vivo. On the basis of these results documenting the potential of oral Del-Immune V® and Bifidim to stimulate synthesis of IFN-α/β- and -γ as well as TNF, it should also be noted that IFN-γ can induce expression of TNF-α receptors on macrophages [4]. These cytokines synergistically stimulate macrophage cells that, in turn, intensify killing activity. IFN-gamma increases the expression of class II MHC proteins on professional antigen presenting cells, and so

promotes antigen presentation to helper T cells as well. It also enhances the expression of important signaling receptors — Toll-like receptors, important for the development of protection against viral infections and hypersensitivity, as some of the ligands of these receptors regulate the function of adhesion molecules such as CD11b and L-selectin [25]. The synergistic activity of cytokine (IFN and TNF) production induced by Del-Immune V® and Bifidim helps to demonstrate some therapeutic effects of these formulations. The comparative study of Del-Immune V® and Bifidim demonstrated that both formulations had a stimulating effect on cytokine secretion activity of the splenocytes and macrophages necessary for production of IFN and TNF. Bifidim contains living cells of bifidus bacteria, while active substances of Del-Immune V® are MP (muramyl peptides) and nucleoproteids of the probiotic strain *Lactobacillus rhamnosus V*. Del-Immune V® demonstrated higher interferonogenous activity in vivo and in vitro than Bifidim (Figures 1 and 3). However, in vitro, Bifidim stimulated higher levels of TNF in comparison with Del-Immune V® (Figure 5).

The choice of probiotic formulation (live probiotics cells or structural derivatives of probiotic cells) depends on a large number of factors, including potential, mechanism, mode of administration and desired immune response. The mechanisms of action of this group of formulations are most likely multifactorial and include a number of signals, cell types, and receptors. One characteristic of probiotic activity is selective effects on the immune system of the macro-organism, whereby only those parts of the natural immune response that require correction are altered [1, 27].

Probiotics demonstrate a variety of influences on immunological processes, depending on the type and strain of the bacteria. For example, bacteria *L. fermentum* and *L. plantarum* stimulate B-cell proliferation, while *L. acidophilus* mainly causes induction of T-cell immune response [19]. Incubation of different strains of lactobacilli with human peripheral blood mononuclears showed that *L. brevis, L. reuteri, L. lactis, L. casei* and *L. plantarum* stimulate, to varying degrees, production of IL-1, IL-12, TNF-α, and IFN-γ.[66] Similar findings show that *L. plantarum, L. rhamnosus* and *L. paracasei ssp. paracasei*, when cultured with peripheral blood mononuclears, intensify secretion of IL-12 [18].

Certain structural components of lactobacilli, including peptidoglycans and DNA fragments, can also influence the secretion activity of human monocytes in vitro through intensified production of IL-1, IL- 6 and TNF-α; in vivo they can activate synthesis of E2 prostaglandin and activate the system of complement and maturation of T-cell precursors [20].

In this study there were shown that the dry enzymatic lysate powder of a special lactic acid bacteria *Lactobacillus rhamnosus* (DV Strain) Del-Immune V® in the dose of 50 μg/mouse could actively induce IFN and moderately stimulate the production of tumor necrosis factor, showing significant promise as an immunomodulating preparation. Its natural

origin, interferonogenous activity, safety, usability, and the possibility of oral administration allow us to consider Del-Immune V ® as modern immunomodulating medications.

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ВЛИЯНИЕ СУХОГО ФЕРМЕНТАТИВНОГО ЛИЗАТА DEL-IMMUNE V® НА ПРОДУКЦИЮ ЦИТОКИНОВ В ЕКСПЕРИМЕНТАЛЬНЫХ МОДЕЛЯХ

Реферат

Del-Immune V®, как препарат сопровождения, проявил высокую эффективность для поддержки иммунной системы, однако, механизмы действия и оптимальные дозы еще не исследованы. Показано, что препарат Del-Immune V®, сухой ферментативный лизат штамма *Lactobacillus rhamnosus V,* содержащий мурамил пептиды и фрагменты ДНК, в диапазоне доз 5—500 мг/мышь (оптимум 50 мкг/мышь) является активным индуктором ИФН, влияет на продукцию фактора некроза опухоли (ФНО) в системах *in vivo* и *in vitro*. Его индуцирующая активность сопоставима с показателями, полученными при использовании комплексного пробиотика Бифидим, однако живые клетки бифидобактерий, что входят в состав препарата, более интенсивно стимулировали продукцию ФНО *in vitro* сравнительно с Del-Immune V®. Уровни продукции цитокинов под влиянием препаратов в системе *in vivo* коррелировали с продукцией цитокинов в системе *in vitro*. Наиболее высокие уровни ИФН после приема препаратов выявлены на 1 сутки эксперимента. В контрольной группе уровни ИФН оставались неизменными. Обеспечение повышенного уровня циркулирующих ИФН было возможно только повторным введением препаратов.

Ключевые слова: Del-Immune V®, *Lactobacillus rhamnosus V*, пробиотик Бифидим, интерферон, фактор некроза опухоли, иммунная система.

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ВПЛИВ СУХОГО ФЕРМЕНТАТИВНОГО ЛІЗАТУ DEL-IMMUNE V® НА ПРОДУКЦІЮ ЦИТОКИНІВ В ЕКСПЕРИМЕНТАЛЬНИХ МОДЕЛЯХ

Реферат

Del-Immune V®, як препарат супроводу, виявив високу ефективність для підтримки імунної системи, однак, механізми дії та оптимальні дози ще не досліджені. Показано, що препарат Del-Immune V®, сухий ферментативний лізат штаму *Lactobacillus rhamnosus V*, що містить мураміл пептиди і фрагменти ДНК, в діапазоні доз 5—500 мг/мишу (оптимум 50 мкг/мишу) є активним індуктором ІФН, впливає на продукцію фактора некрозу пухлини (ФНП) в системах *in vivo* та *in vitro*. Його індукуюча активність близька до показників, одержаних при використанні комплексного пробіотику Біфідим, проте живі клітини біфідобактерій, що входять до складу препарату, більш інтенсивно стимулювали продукцію ФНП *in vitro* порівняно з Del-Immune V®. Рівні продукції цитокінів під впливом препаратів в системі *in vivo* корелювали з продукцією цитокінів в системі *in vitro*. Найбільш високі рівні ІФН після прийому препаратів виявлені на 1 добу експерименту. У контрольній групі рівні ІФН залишалися незмінними. Забезпечення підвищеного рівня циркулюючих ІФН було можливо тільки через повторне введення препаратів.

Ключові слова: Del-Immune V®, *Lactobacillus rhamnosus V*, пробіотик Біфідим, інтерферон, фактор некрозу пухлини, імунна система.