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STUDY OF INTERFERONOGENOUS ACTIVITY OF THE NEW PROBIOTIC FORMULATION DEL-IMMUNE V®

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Running head: Interferonogenous activity of Del-Immune V®

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Key words: Del-Immune V®, Bifidim, probiotics, peptidoglycan, muramyl peptides, DNA, interferons, tumor necrosis factor, immune system

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Abstract

Context: Prior testing of Del-Immune V® has indicated effectiveness for immune system support; however, mechanisms of action and optimal doses have not yet been researched.

Objective: To study the mechanisms of the immunomodulating effects of Del-Immune V and to investigate its dose-dependent effects on production of immunoregulatory cytokines in vivo and in vitro.

Design, Setting, Participants, Interventions: One hundred forty nondescript laboratory mice with body mass ranging from 14 to 16 grams were divided into 7 test groups. Groups I, II, and III received 0.5 ml of aqueous solution of Del-Immune V by mouth in doses of 5, 50, and 500 μg per mouse respectively for 5 days at 24-hour intervals. Group IV mice received 0.5 ml Bifidim suspension by mouth in a dose of 50 μg/mouse on the same schedule. Group V mice (control group) received 0.15 M NaCl. Group VI and VII mice received a single dose of 50 μg/mouse Del-Immune V (Group VI) or Bifidim (Group VII) on day 1 of the test period. Eight hours after administration, and every 24 hours thereafter for 5 days, several mice from each group were killed by cervical dislocation; blood serum, peritoneal exudate macrophages, and splenocytes were obtained from each group of mice for testing.

Main Outcome Measures: Interferonogenous activity of cultured splenocytes and serum levels of interferon.

Results: Groups I-IV showed a marked increase of IFN levels in blood serum after administration of Del-Immune V or Bifidim; the optimal daily dose was found to be 50

μg/mouse. The highest serum IFN level was reported 24 hours after administration. The control group remained unchanged. Maintenance of elevated circulating IFN was possible only through repeated administration.

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INTRODUCTION

Among a large number of presently known therapeutic products that utilize lactobacilli, cell wall peptidoglycan is enjoying growing popularity as an immunomodulator that contains, among other things, fragments of DNA and cell peptidoglycan of the strain *Lactobacillus rhamnosus* V. 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.[1-6] 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 its dose-dependent effects on production of immunoregulatory cytokines in vivo and in vitro.

The last 5 years have been marked by increasingly active study of the mechanisms of the immunobiological effects of probiotics and bacterial medications.[7-16] 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.(1-4) The adjuvant effect of BCG and the immunomodulating activity of formulations containing 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 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).[17-26] 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).[9-12]

These properties of peptidoglycans indicate a basis for creating immunomodulating formulations for clinical use. Lactobacilli in the 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.[27] 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.[11,15] 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.[16] 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.[27-29]

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 14-16g. 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 mice received 0.5 ml of aqueous solution of Del-Immune V orally in doses of 5, 50, and 500 µg/mouse respectively for 5 days at 24-hour intervals. Group IV mice were administered 0.5 ml Bifidim suspension (control probiotic medication) orally in a 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 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 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,[30] peritoneal exudate macrophages (PEM),[31] and spleen,[32] from which splenocytes were harvested[33] 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, 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). Levels of cytokine production (IFN and TNF) were determined 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.[30] 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.[34]

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(15). The significance of the results was analyzed by Student-Fisher t-test. Differences of $P < .05$ were considered to be significant.[35]

RESULTS

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 a marked increase in IFN levels in blood serum (see 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 $\log_2$ U/ml ($P > .05$). After repeated administrations, levels reached 5.5±0.7 $\log_2$ U/ml ($P > .05$), 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.

Insert Figure 1 about here.

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 (see Table 1).

Insert Table 1 about here.

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. Maintenance of circulating IFN levels was possible only through repeated administration.

A 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 a 2-fold increase of interferon response in comparison with intact animal cells (see 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-γ production by immunocompetent cells as a response to adequate in vitro stimulation, and spontaneous IFN production.

Insert Figure 2 about here.

After administration of certain IFN inductors, capacity for enhanced production of IFN-α and IFN-γ was seen in splenocytes 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 control levels on day 5. In the Bifidim group, it was possible to see 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 an 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 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 an optimal dose for Del-Immune V is more likely to be close to 50 μg/ml.

Insert Figure 3 about here.

The highest IFN levels in supernatants were reported on day 1 of cell culturing with experimental formulations. However, 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 a temperature of 60^0 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.[36]

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.[37-42]

IFN-γ also participates in the immune response of macrophage cells, inducing production of TNF and IL-1[43] and modulating their functions.[44] Therefore, the level of TNF, a pleiotropic cytokine produced by primed monocytes and macrophages, lymphocytes, and NKC, was assessed in murine serum (see Figure 4).[45-57] 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 a dose of 50 μg/ml, serum TNF was 0.6 ng/ml ($P < .05$) and 0.8 ng/ml ($P < .05$), 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.

Insert Figure 4 about here.

Del-Immune V administered in a dose of 5 μg/mouse resulted in an insignificant increase in circulating TNF concentration to 0.4 ng/ml ($P > .05$), in comparison with 0.3 ng/ml in the control group. It should be noted that oral administration of Del-Immune V in 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.[48-54]

A 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.

Insert Figure 5 about here.

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 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.

DISCUSSION

Derivatives of microbial origin, including lipopolysaccharides (LPS), MDP, and CpG DNA, are identified by immunocompetent cells with TLR receptors.[27,55,28] Thus, LPS *E. coli* stimulates mainly monocytes and macrophages.[7] The LPS receptor is a histocompatibility antigen characterized by a proteinic nature and marked as CD 14. It can be found on monocytes, macrophages, neutrophils, lymphocytes, and bowel epithelial cells. Fixation of microbial derivatives with receptors results in a 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, which are 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.[56] 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.[57] 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.[58] These cytokines synergistically stimulate macrophage cells that, in turn, intensify killing activity. Intercellular cooperation of epithelial and immunocompetent cells in response to cytokine and chemokine molecules is effected via MHC (major histocompatibility complex) class I and II molecules,[45] which are receptors for IFN-γ, IL-1, TNF-α, TGF-β, IL-2, IL-4, IL-7 and IL-10.[55,59]

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.[60-64]

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.[65] 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.[56]

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.[67]

In this study, the new probiotic formulation Del-Immune V, in a dose of 50 μg/mouse, was shown to 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 secure Del-Immune V a worthy place among modern immunomodulating medications.

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