THE SYNERGISTIC EFFECT OF LACTIC ACID BACTERIA AND ALKYLGLYCEROLS ON HUMORAL IMMUNITY IN MICE

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Summary: Investigations on immune suppression and reconstitution of immune functions dependent on the presence of physiological microflora allow us to conclude that symbiotic microorganisms such as Lactobacillus sp. are essential for adequate activity of the defense system in humans. In addition to their beneficial influence on the intestinal microbial balance, these microorganisms exert a variety of immunomodulatory effects on the host immune system. On the other hand, immunostimulatory animal-derived substances rich in alkylglycerols have been shown to enhance lactic acid bacteria proliferation. Therefore, the aim of the present study was to evaluate the effects on murine humoral response of the combined administration of lyophilized combination of three lactic acid bacteria: L. acidophilus, L. bulgaricus and Bifidobacterium bifidum together with alkylglycerol-rich shark liver oil. The lactic acid bacteria mixture induced markedly stronger enhancement of the humoral response than alkylglycerols did. A significant synergistic stimulatory effect of lactic acid bacteria and alkylglycerols was observed in both treatment schedules: post- as well as in preimmunization with sheep red blood cells. However, their concomitant administration exerted stronger immunomodulatory effect than did the alternative route of treatment.

Introduction

A number of studies carried out in recent years allow us to conclude that lactic acid bacteria, which are primary elements of intestinal flora, possess a significant stimulatory effect on humoral as well as cellular immune response (1). Lactic acid bacteria mixtures have traditionally been regarded as food supplements with the potential to reestablish the balance of gut microflora. However, recent data indicate that symbiotic intestinal microflora is essential for physiological maturation as well as for the adequate activity of the defense system in humans and animals. Antibiotic decontamination of the Balb/c mouse gastrointestinal tract resulted in a lack of generation of immunopriming microbial substances, which led to immunosuppression (2). Lactobacillus sp., Propionibacterium sp. and Bacteroides sp. have been shown to act on basic immune functions liberating biologically active products such as low molecular weight peptides (MW < 6.5 kD) or enzymes (reductases, oxidases) (3). In lactic

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acid bacteria fed laboratory animals the number of B lymphocytes in Peyer's patches as well as the proliferative and antibacterial activity of T lymphocytes increased, thus strengthening resistance to infection (4-6). Likewise, Perdigon observed higher levels of immunoglobulins in murine intestinal fluid following oral administration of _L. casei_ and _L. acidophilus_ (7). Other studies proved that _L. casei_ significantly up-regulated the dynamics of inflammatory response increasing macrophages tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) production as well as IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ) gene expression in human lymphocytes (8). Apart from the significant stimulatory effect on the host's immune response, lactic acid bacteria therapy has been suggested to possess other important clinical activities. Its application in the prophylactics and management of allergic disorders as a Th1-type immune reaction trigger and IgE production suppressor has been widely discussed (9). Similarly, the role of lactic acid bacteria in the prevention of neoplastic diseases due to their antitumorigenic and antimutagenic effects is currently being evaluated (7, 10).

In the intestinal ecosystem a number of microorganisms, both symbiotic and pathogenic, coexist in a subtle balance. Therefore, the beneficial effects of lactic acid bacteria mixtures delivered as a food supplement or medication greatly depend on their ability to reach the large intestine in viable and intact form, as well as on their capacity to establish themselves in the large intestine and become active. Consequently, data proving that alkylglycerols of animal origin administered concomitantly with lactic acid bacteria increase their proliferative potential are of special interest (11).

Alkylglycerols or glycerol ethers occur in higher amounts in the lipids of animal organs: bone marrow, spleen, liver and plasma. The most important source of alkylglycerols is the liver of certain elasmobranch fish i.e., shark or ray. Numerous biological effects have been attributed to the alkylglycerols, the most important of which are a significant stimulatory effect on hemopoiesis and on humoral and cellular immunity (12, 13). Alkylglycerols were shown to enhance mice macrophage metabolic activity and TNF-α production (14, 15). Our previous studies have proven that in mice fed shark liver extract the number of circulating granulocytes and their metabolic activity estimated in the chemiluminescence assay were significantly increased (16). We also observed that shark liver extract inhibited the cutaneous angiogenesis induced in Balb/c mice by syngeneic sarcoma L-1, human urinary bladder and human kidney tumor cells (16). These results correspond with those of other reports showing significant inhibition of endothelial cell migration toward basic fibroblast growth factor (bFGF) in the presence of alkylglycerols (17). It has been suggested that the antiangiogenic effects and proapoptotic properties of alkylglycerols might be responsible for their beneficial influence in tumor treatment, as reported by several clinical studies (18-20).

Because both preparations, which are rich in alkylglycerol shark liver oil and lactic acid bacteria lyophilized mixture, possess immunomodulatory properties and possibly, as suggested in Brohult's study (11), might interact in vivo, we designed our study to evaluate the effects exerted by the combined administration of these preparations on the murine immune system. Animals were fed substances rich in alkylglycerols, Greenland shark liver extract (containing 20% alkylglycerols, mainly selachyl, chimyl and batyl alcohol, together with their methyl-derivatives) and a lyophilized combination of three lactic acid bacteria: _L. acidophilus, L. bulgaricus_ and _B. bifidum_.

**Materials and methods**

_Anomals_. Experiments were performed in 2-month-old inbred male Balb/c mice from our own breeding colonies (breeding material was obtained from the
Effect of lactic acid bacteria and alkylglycerols on murine humoral response

Oncology Center, Warsaw, Poland) distributed in six groups that orally received: i) filtered water for 7 days; ii) shark liver oil (Ecomer batch no. 991201, Exposan AB, Sweden) 5 μl/mouse/day for 7 days; iii) shark liver oil 10 μl/day for 7 days; iv) lyophilized lactic acid bacteria (L. acidophilus, L. bulgaricus, B. bifidus) mixture (Trilac batch no. 543099032, Pharmacia and Upjohn Allergon AB, Sweden) 32 x 10⁶ cells/mouse/day for 7 days; v) shark liver oil and lyophilized lactic acid bacteria in doses as above for 7 days; vi) lyophilized lactic acid bacteria for 7 days, followed by shark liver oil for 7 days at the same doses as above.

The mice were fed for 7 days before or after immunization with sheep red blood cells (SRBC).

At the end of the experiment the mice were subjected to narcosis by means of chloral hydrate, and peripheral blood was collected from the peri-orbital plexus. Afterwards, serum was isolated and frozen at −80 °C for further evaluation.

Hemagglutination assay: Examined mice sera were inactivated (56 °C, 30 min), titrated and incubated for 60 min at room temperature. Afterwards, 0.5% SRBC was added and incubated as above, then centrifuged (10 min, 150 x g). Hemagglutination was evaluated in an optical microscope as a last dilution with at least three cell conglomerates present in at least three consecutive fields.

Statistical analyses. The results are presented as activation indices (K) i.e., mean number of SRBC conglomerates following preincubation with treated mice sera per mean number of conglomerates formed in the presence of sera from control animals. The statistical significance of each treatment group compared with control animals was tested at the level of p = 0.05 using Student’s two-tailed t-test.

**Results**

Pre-immunization feeding schedule. All examined feeding schedules except for shark liver oil at the lower dose (5 μg) resulted in a significant increase of SRBC conglomerates in the hemagglutination assay that mirrored the elevation of antibodies levels in mice sera (Table 1).

However, concomitant administration of lactic acid bacteria and shark liver oil induced a more marked increase in the activation index than other feeding schedules. The effects of shark liver oil and lactic acid bacteria given subsequently were similar to those of the group administered shark liver oil only

<table>
<thead>
<tr>
<th>Substances</th>
<th>Number of mice</th>
<th>K (mean ± SE)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (water)</td>
<td>38</td>
<td>1.00 ± 0.044</td>
<td>—</td>
</tr>
<tr>
<td>2. Shark liver oil 5 μl</td>
<td>12</td>
<td>1.13 ± 0.075</td>
<td>NS</td>
</tr>
<tr>
<td>3. Shark liver oil 10 μl</td>
<td>10</td>
<td>1.15 ± 0.063</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>4. Lactic acid bacteria 20 μl</td>
<td>21</td>
<td>1.21 ± 0.048</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>5. Shark liver oil 5 μl plus lactic acid bacteria 20 μl</td>
<td>12</td>
<td>1.59 ± 0.089</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>(from groups 2, 3 and 4 p &lt; 0.001)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Shark liver oil 10 μl plus lactic acid bacteria 20 μl</td>
<td>12</td>
<td>1.36 ± 0.067</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>(from groups 2 and 3 p &lt; 0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Lactic acid bacteria 20 μl, followed by shark liver oil 5 μl</td>
<td>12</td>
<td>1.15 ± 0.031</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>(from group 5 p &lt; 0.001, from group 6 p &lt; 0.01)</td>
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*Mice were fed for 7 days, immunized with sheep red blood cells and were sacrificed after another 7 days (K = activation index).
and were even less expressed than in the group administered lactic acid bacteria only.

Postimmunization feeding schedule. Up-regulation of antibody production in mice fed after immunization with SRBC was observed in groups given lactic acid bacteria or shark liver oil and lactic acid bacteria concomitantly, but was not observed in the shark liver oil only groups (Table II). However, unlike in the preimmunization fed groups, the effects of lactic acid bacteria and shark liver oil concomitant administration were similar to those exerted by lactic acid bacteria alone.

The immunostimulatory effect of lactic acid bacteria and shark liver oil (10 μg) fed concomitantly, as well as lactic acid bacteria mixture alone, was significantly stronger when administered before immunization with SRBC rather than afterwards (Fig. 1).

Discussion

The increasing number of studies on immunomodulatory and antineoplastic properties revealed by natural substances of plant or animal origin reflects the growing interest in their application in modern medicine. However, the mechanisms of their immunomodulatory activity are often incompletely known and their interactions are rarely examined.

Both preparations examined in the present study (lyophilized lactic acid bacteria and shark liver oil containing alkylglycerols of animal origin) possess proven immunostimulatory activity. However, data from the literature on their synergistic interaction are extremely scarce even though Brohult postulated this possibility some years ago (12).

Therefore, the aim of this study was to evaluate, in an animal model of humoral immunity, the effects of combined administration of two preparations containing lyophilized lactic acid bacteria or alkylgly-

Table II The effect of lactic acid bacteria and alkylglycerols in shark liver oil on antibody production

<table>
<thead>
<tr>
<th>Substances</th>
<th>Number of mice</th>
<th>K (mean ± SE)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (water)</td>
<td>12</td>
<td>1 ± 0.16</td>
<td>—</td>
</tr>
<tr>
<td>2. Shark liver oil 5 μl</td>
<td>9</td>
<td>0.9 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>3. Shark liver oil 10 μl</td>
<td>17</td>
<td>1.23 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>4. Lactic acid bacteria 20 μl</td>
<td>15</td>
<td>1.71 ± 0.04</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(from group 2 p &lt; 0.001, from group 3 p &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>5. Shark liver oil 5 μl and lactic acid bacteria 20 μl</td>
<td>8</td>
<td>1.61 ± 0.08</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(from group 2 p &lt; 0.001, from group 3 p &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>6. Shark liver oil 10 μl and lactic acid bacteria 20 μl</td>
<td>16</td>
<td>1.93 ± 0.11</td>
<td>(from group 2 p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(from groups 2 and 3 p &lt; 0.001)</td>
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</table>

*Mice were immunized with sheep red blood cells and were fed for 7 days afterward. K = activation index.
cerols of animal origin. For this purpose, a simple laboratory model was chosen as a screening test to reveal clinically relevant in vivo effects. Examined substances were administered before or after immunization, as it respectively happened, while introducing prophylactic or therapeutic treatment.

As a result, humoral response in mice following lactic acid bacteria mixture feeding was significantly enhanced. This result is in agreement with other reports revealing adjuvant effect of certain lactic acid bacteria on antigen-specific antibody production. It was proven that L. acidophilus and B. bifidobacterium strains (present in the lactic acid bacteria mixture examined in our study) augment local immune response enhancing IgA production in the digestive tract as well as stimulating the systemic humoral response to the influenza virus (21, 22). Experimental evidence suggests that the lactic acid bacteria-driven enhancement of B cell proliferative response plays a key role in these processes (6).

As expected, lactic acid bacteria-driven humoral response up-regulation was observed in both experimental settings both before and after immunization. However, the stimulation was distinctly stronger when lactic acid bacteria were administered after SRBC. This confirms the distinct role of B cell activation in their stimulatory effect on antibody production, although other mechanisms, such as TNF-α and IL-6 production enhancement should not be overlooked (6, 23).

In contrast, alkylglycerols revealed a direct immunomodulatory effect only when administered before immunization. In agreement with our results, Ngwena observed a significant adjuvant effect on the humoral response solely in mice fed alkylglycerols before immunization (13). Mice immunized 3 days later did not produce increased amounts of antibodies. It was also proven that supplementation of glycerol esters in the diets of lactating rats resulted in elevated immunoglobulin levels in milk and subsequently stimulated greater IgG and IgM production in the pups (24). Since alkylglycerols were shown to up-regulate macrophage ability to present antigens to B lymphocytes (13), the observed effect might be due to the priming of the defense system and the increase in its potential to develop a specific immune reaction to antigens.

This immunostimulatory effect of alkylglycerols in shark liver oil and lactic acid bacteria on humoral response was even more pronounced when outcomes of their simultaneous administration were examined. Combined schedules delivered a much stronger stimulatory effect on antibody production than did separate treatment. Moreover, the consecutive route of administration (shark liver oil for 7 days followed by lactic acid bacteria for 7 days) showed results similar to those of separate treatment but were significantly weaker than the concomitant feeding protocol (shark liver oil and lactic acid bacteria for 7 days). These data clearly prove that alkylglycerols in shark liver oil and lactic acid bacteria when administered at the same time act synergistically, probably due to the complementary ways of modulating antibody production. Enhancement of B cell proliferative activity by lactic acid bacteria as well as alkylglycerol-driven up-regulation of the ability of macrophages to present antigens seems to be the key mechanism. However, certain positive interactions between both preparations are also likely to play a role.

The main known site of alkylglycerol action at the cellular level is the cell membrane. Their accumulation changes lipid composition, altering membrane physicochemical structure, fluidity, shape and permeability by membrane pore formation (25). Moreover, it has been proven that alkylglycerols inhibit protein kinase C, thus indirectly blocking important intracellular signal transduction pathways, as well as down-regulating other membrane enzymes, such as phospholipase C, phosphatidylinositol-3-kinase and others (25-28). This increases cellular susceptibility to some external stimuli such as anti-influenza drugs as well as to certain prodifferentiation impulses. It was
postulated that in addition to the immunomodulatory effect, changes produced by alkylglycerols in the structural and functional balance of the microorganisms' cellular membrane are responsible for the significant antibacterial effects. This kind of activity was proven in vitro against pathogens: Corynebacterium hofmannii, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans and Staphylococcus pyogenes strains (28). On the other hand, a significant stimulatory effect of alkylglycerols on the proliferative activity of symbiotic bacteria was shown (25). However, the mechanisms underlying this phenomena are still poorly understood. Apart from the direct influence on lactic acid bacteria strain proliferative activity, the negative effect of alkylglycerols on the intestinal pathogens might indirectly support lactic acid bacteria in finding a suitable biological niche in a complicated intestinal ecosystem.

Finally, our study provides some conclusions of possible, although preliminary clinical value. The results of preimmunization treatment, which clinically corresponds to prophylactic therapy, were most impressive after combined administration of shark liver oil and lactic acid bacteria. Interestingly, no significant benefit was observed in this model when the shark liver oil dose was increased.

In contrast, the postimmunization-feeding scheme, resembling therapeutic treatment, was most effective when using the combination of lactic acid bacteria and higher shark liver oil dose. This combination administered as therapy exerted a significantly stronger immunomodulatory effect than in the prophylactic schedule (preimmunization). In contrast, the effect of the lower postimmunization shark liver oil dose added to lactic acid bacteria was no greater on humoral response than that of lactic acid bacteria alone. Therefore, it is quite possible that in these two experimental settings shark liver oil alkylglycerols administered pre- or postimmunization act through different mechanisms. Hypothetically, effects on the pathogenic microflora might play a marked role in prophylactic activity while significantly enhancing antigen presentation by macrophages in the therapeutic, postimmunization period. The present study is the first to confirm the synergistic effects exerted on humoral response by the combined administration of lyophilized lactic acid bacteria and alkylglycerols from shark liver extract. Moreover, the significant advantage offered by the concomitant treatment scheme, in comparison with the consecutive one, seems to be of vital importance and is of great interest from the theoretical and clinical point of view. Since the choice of a simple experimental method as well as the animal model does not permit implementation of our results directly into the clinical situation, further studies on the interaction between lactic acid bacteria and shark liver oil alkylglycerols should be performed.

References

Effect of lactic acid bacteria and alkylglycerols on murine humoral response


