Effect of recombinant *Lactococcus lactis* producing myelin peptides on neuroimmunological changes in rats with experimental allergic encephalomyelitis

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Abstract

Multiple sclerosis (MS) is a human autoimmune neurodegenerative disease with an unknown etiology. Despite various therapies, there is no effective cure for MS. Since the mechanism of the disease is based on autoreactive T-cell responses directed against myelin antigens, oral tolerance is a promising approach for the MS treatment. Here, the experiments were performed to assess the impact of oral administration of recombinant Lactococcus lactis producing encephalogenic fragments of three myelin proteins: myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein, on neuroimmunological changes in rats with experimental allergic encephalomyelitis (EAE) – an animal model of MS.

Lactococcus lactis whole-cell lysates were administered intragastrically at two doses (10^3 and 10^6 colony forming units) in a twenty-fold feeding regimen to Lewis rats with EAE. Spinal cord slices were subjected to histopathological analysis and morphometric evaluation, and serum levels of cytokines (IL-1b, IL-10, TNF-α and IFN-γ) were measured. Results showed that administration of the L. lactis preparations at the tested doses to rats with EAE, diminished the histopathological changes observed in EAE rats and reduced the levels of serum IL-1b, IL-10 and TNF-α, previously increased by evoking EAE. This suggests that oral delivery of L. lactis producing myelin peptide fragments could be an alternative strategy to induce oral tolerance for the treatment of MS.

Key words: MS, autoimmunity, oral tolerance, Lactococcus lactis, EAE, myelin proteins.

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Introduction

Multiple sclerosis (MS) is an autoimmune disease that targets young people (20-40 years old) and is more common in women than in men. It is most frequently encountered in Northern Europe and North America, affecting approximately 1 in 1000 people. The genesis of MS remains unknown, but some evidence has supported the contributions of genetic and environmental factors or viral infection [35]. Despite its unidentified origin, the mechanism of MS involves humoral and cellular immunity and is based on T-cell autoreactivity directed against myelin antigens. Autoreactive T-lymphocytes migrate through the open blood-brain barrier into the central nervous system (CNS), where they recognize myelin proteins as pathogenic and induce an antigen-specific inflammatory process. Inflammation directed against myelin proteins causes demyelination and further deterioration of axons or even whole neurons, which results in deceleration or disruption of neurotransmission [12]. Tissue injury also affects oligodendrocytes function due to glutamatergic excitotoxicity in the sites of inflammatory lesions [10]. CNS damage provokes progressive physical disability and associated mental impairment that occur in MS patients. Currently, there is no effective therapy for MS. The most common treatment is systemic immunosuppression, which may also weaken the immune system and expose the patient to frequent infections [3,12,42]. However, new therapies targeting specific mechanisms involved in the pathology of MS, such as natalizumab, alemtuzumab or daclizumab may cause severe side effects or result in lower efficiencies than previously expected [7-9]. Thus, there is a need to identify novel therapies that are more effective and safe for patients. Among the most promising approaches developed in recent years that could potentially be applied for the treatment of autoimmune diseases is the induction of oral tolerance [11].

Oral tolerance is a natural state of a decreased immunoresponse to the previously fed antigen. The mechanism induces tolerance toward ingested antigens, which are normally encountered in the body (e.g. commensal bacteria, food). The phenomenon of oral tolerance has been applied for ovalbumin-induced anaphylaxis, contact hypersensitivity or the suppression of antibody production. The same approach has been also proposed for the treatment of autoimmune diseases, and many preclinical trials, including MS therapy, have been conducted [11,37,38]. In most of those trials, myelin basic protein (MPB) or its fragments served as the antigen, and all were shown to reduce the clinical symptoms of induced experimental allergic encephalomyelitis (EAE) [15,26]. However, MBP is not the only antigen that is considered to be immunogenic in MS. Antibodies directed against proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been detected in the cerebrospinal fluid of patients with MS [1,17]. Previously, in our laboratory, experiments with the use of pig spinal cord hydrolysate as the source of broad spectrum of myelin antigens to evoke oral tolerance in rats were performed [18]. In the presented experiment, we decided to use the most encephalogenic fragments of these three proteins, MBP85-97, PLP139-151 and MOG35-55, to induce a state of oral tolerance [2,23].

Since peptide synthesis is expensive and involves many complicated chemical procedures, other techniques for obtaining peptides are desirable. One example is the application of food-grade lactic acid bacteria (LAB) as biofactories for the production of heterologous proteins [28]. Lactic acid bacteria are present in fermented foods, are safe for human and animal consumption, and are extensively developed as live antigen delivery systems. Heterologous expression of therapeutic proteins, such as enzymes, cytokines or antigens, allows the broad use of these bacteria in various applications [5]. For our purposes, we designed recombinant *Lactococcus lactis* strains for use as a mucosal antigen delivery system. As LAB are present naturally in the gastrointestinal tract, we aimed to develop a minimally invasive method to induce oral tolerance that could be further applied for MS treatment.

In our experiments, EAE was applied as an animal model of MS. As EAE is a predominantly used MS model that is based on autoimmunological aspects of the disease, it was appropriate for our investigations. Also Lewis rats used in this study are commonly employed in such experiments, being one of the strains susceptible for EAE [23,39].

Our previous results demonstrated that feeding animals a mixture of *L. lactis* lysates producing MBP85-97, PLP139-151 and MOG35-55, in a twenty-fold but not a four-fold feeding scheme, could diminish the clinical symptoms of EAE [19]. In this study, we examined the influence of feeding *L. lactis* lysates on histo-

Table I. Strains and plasmids

Strain	Genotype	Source
Lactococcus lactis		
IBB360	Natural isolate, characterized as an autolytic strain	IBB PAS collection
Plasmids		
pIL253:PptcB	Ery[R], pIL253 derivative with regulated *ptcB* gene promoter region	[19]
pIL253:PptcB:MOG35-55	Ery[R], pIL253 derivative with *ptcB* gene promoter, expressing MOG35-55 antigen	[19]
pIL253:PptcB:MBP85-97	Ery[R], pIL253 derivative with *ptcB* gene promoter, expressing MBP85-97 antigen	[19]
pIL253:PptcB:PLP139-151	Ery[R], pIL253 derivative with *ptcB* gene promoter, expressing PLP139-151 antigen	[19]

pathological changes in the CNS and serum cytokine levels in rats with EAE.

Material and methods

Animals

Three-month-old female Lewis rats, provided by the Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, weighing approximately 180 g were used in the study. The animals were provided with food and water ad libitum unless otherwise required. Experiments were conducted in accordance with EU Directive 2010/63/EU for animal experiments and based on the consent of the Fourth Local Ethics Committee for Animal Experiments in Warsaw (Act no. 66/2010 of 08.10.2010).

Bacterial strains and plasmids

Recombinant *L. lactis* derivatives of the natural isolate IBB360, and plasmids that were used in this work are listed in Table I. Construction of the pIL253:PptcB recombinant vectors expressing myelin antigens MBP85-97, PLP139-151 and MOG35-55 have been described elsewhere [19].

Preparation of whole-cell lysates of recombinant lactococci

Recombinant lactococci were prepared for intragastric administration as follows. Cells were grown overnight at 30°C in M17 medium [36] supplemented with 0.5% cellobiose and erythromycin (5 µg/ml). Subsequently, cells were harvested (8000 g, 10 min., 4°C) and washed once with 0.9% NaCl. Cells were then suspended in 0.9% NaCl and disrupted 3 times for 1 min using the MiniBeadbeater (BioSpec Products) and 106-µm glass beads (Sigma). Adequate

dilutions of cell lysates were generated that corresponded to doses of 10^3 and 10^6 colony forming units (CFUs). Single doses were frozen in Eppendorf tubes in liquid nitrogen and stored at –20°C until use.

Study design

A total number of 36 animals were randomly divided into 6 groups, consisting of 6 rats each. Animals with evoked EAE were fed intragastrically. Animals were given preparations containing either bacteria with empty vector (pIL253:PptcB) or a mixture of bacteria producing myelin peptides (MOG35-55, MBP85-97 and PLP139-151). Both preparations were administered in two doses. As reference, a group of animals with EAE, not receiving the bacteria preparations was created. Non-treated animals served as a control group. For the time diagram of the experiment see Figure 1.

Animal feeding

Using a gauged pointed needle, animals were fed intragastrically with bacteria preparations once daily for 20 consecutive days. Animals were deprived of food two hours before administration of the bacteria. The administered preparations contained whole-cell *L. lactis* lysates diluted accordingly in 0.5 ml saline to obtain a dose of 10^3 or 10^6 CFU/rat.
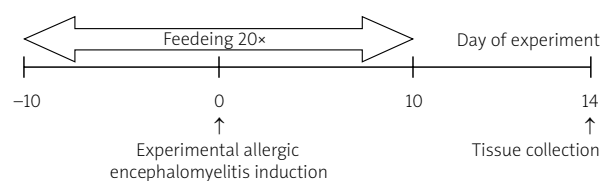

Fig. 1. Time diagram of experiment.

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Experimental allergic encephalomyelitis

Animals were immunized on the 10th day of feeding. The day of immunization was defined as 0 days post-immunization (DPI). EAE was evoked as follows: slightly anesthetized animals (intraperitoneal injection with sodium brietal, Lilly) were injected intradermally into the hind paws with 100 μl (per paw) of the immunization mixture. The mixture contained guinea pig spinal cord homogenate (50% homogenate in PBS) with Freund's adjuvant (1 : 1) and *Mycobacterium tuberculosis* (4 mg/ml). The components were mixed well before application.

Material collection

At 15 DPI, the animals were anesthetized (0.67 ml/kg ketamine + 0.5 ml/kg xylazine *i.p.*, Vetoquinol Biowet) and, after blood collection, perfused transcardially with 4% paraformaldehyde. Lumbar and cervical segments of spinal cords were collected from the spinal canal for histopathological analysis. Serum was obtained from the collected blood.

Histopathological analysis

The collected spinal cord fragments were embedded in paraffin and cut into 8-μm-thick slices. Cresyl violet staining (1% water solution) was performed for cell exposure. A series of labeling experiments were also performed using the sections with the following antibodies: anti-T cells (Anti-CD45R0, DAKO), anti-GFAP (anti-glial fibrillary acidic protein, DAKO) and anti-endogenous albumin (rabbit IgG fraction against rat albumin, MP Biomedicals). The procedure was performed as follows: using dewaxed slices, endogenous peroxidase was blocked with 3% hydrogen peroxide, and the background was blocked with 10% albumin. After incubation with the primary antibodies, the sections were washed and then incubated with a secondary antibody coupled to biotin. After washing, streptavidin coupled to peroxidase was applied followed by a chromogen. The sections were then stained with Mayer's hematoxylin. The chemicals were supplied by DAKO. The sections were analyzed using a light microscope (AH3, Olympus) and images were obtained using a designated camera (3-35AD-4, Olympus).

Morphometric analysis

The morphometric analysis was conducted using both lumbar and cervical spinal cord segment sections stained with Cresyl violet. The area of the whole spinal cord cross-section and the area occupied by the inflammatory infiltrate were measured to determine the ratio, which is presented as the percentage of the slice area occupied by the inflammatory infiltrate.

Measurement of cytokine levels

In serum obtained from collected blood, cytokine levels (IL-1b, IL-10, TNF-α and IFN-γ) were analyzed. Analysis were made using the Bio-Plex Rat Cytokine Assay (Bio-Rad, Poland) according to the protocol supplied by the manufacturer (Bio-PlexTM Cytokine Assay Instruction Manual, Bio-Rad, Poland). Measurements were made using Luminex Bio Plex 200 analyzer, equipped with the software Manager version 5.0. (Bio-Rad, Poland).

Statistical analysis

The results are presented as means ± SD. Statistical assessments were performed using the Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Results

Histopathological studies

Spinal cord cross-sections from control animals showed no signs of inflammation (Fig. 2A and D). Inflammatory infiltrates that were visible in both the gray and white matter of the spinal cord in rats with evoked EAE (Fig. 2B) diminished after oral administration of both doses of *L. lactis* lysates (Fig. 2C). Moreover, there were no visible differences between the intensity of the infiltrates in rats fed preparations containing lysates of bacteria producing myelin peptides and control preparations (data not shown). We also noticed that the infiltrates in animals fed bacterial lysates (both the control and the *L. lactis* producing myelin peptides preparations) had a reduced abundance of inflammatory cells (Fig. 2F) compared with rats with EAE (Fig. 2E), yet they occupied a similar area.

Anti-GFAP labeling revealed active astrocytes in both the gray and white matter of the spinal cord. In untreated rats, a weak reaction was observed, and the astrocytes displayed short tabs and insufficient cytoplasm (Fig. 2G), whereas rats with induced EAE were characterized by active astrocytes with long, branched tabs (Fig. 2H). After oral administration
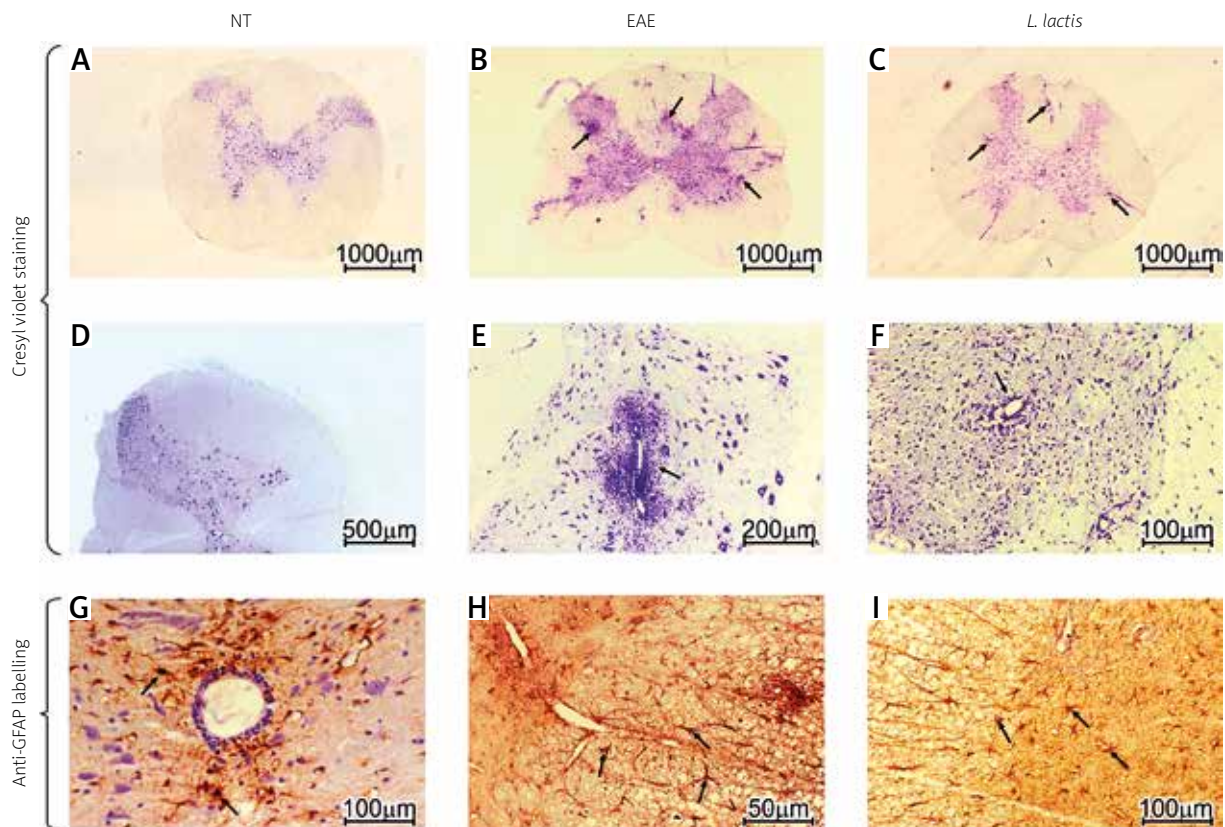
Fig. 2. Spinal cord cross-sections. Cresyl violet staining (**A–F**); inflammatory infiltrates are indicated by arrows. Anti-GFAP labeling (**G–I**); astrocytes are indicated by arrows. Non-treated animals (NT), animals with induced experimental allergic encephalomyelitis (EAE), animals with EAE fed bacteria producing myelin peptides (*Lactococcus lactis*), *n* = 6.

of *L. lactis* lysates, the number of active astrocytes increased in rats with EAE (Fig. 2I).

Anti-endogenous albumin labeling revealed albumin in the perivascular area of rats with induced EAE (Fig. 3A). T cells were observed in the area occupied by inflammatory infiltrates (Fig. 3B).

Morphometric analysis

A morphometric analysis was conducted to assess the area of the spinal cord cross-section occupied by inflammatory infiltrates. No significant differences were observed between groups of fed or non-fed rats with EAE. Animals in the experimental
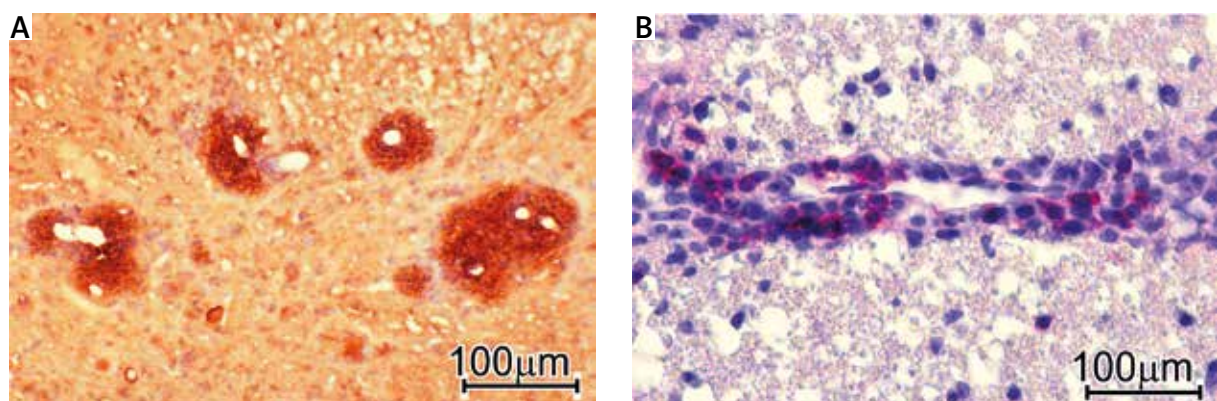

Fig. 3. Spinal cord cross-sections. Anti-endogenous albumin labeling (**A**). Anti-T cell labeling (**B**), *n* = 6.

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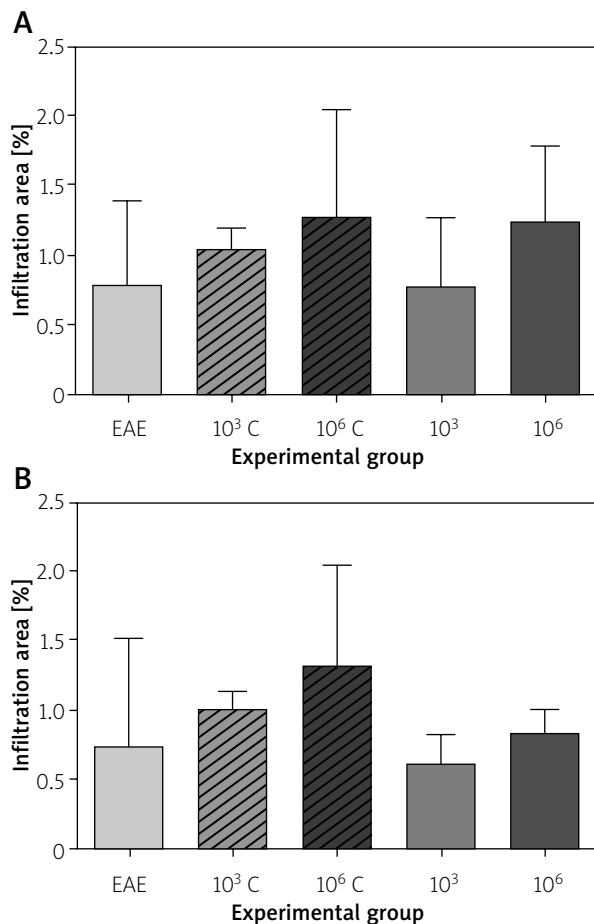
A

B

Fig. 4. Percentage of the section area occupied by inflammatory infiltrates. Cervical segment of spinal cord (**A**). Lumbar segment of spinal cord (**B**), *n* = 6.

groups presented very large individual differences in the infiltration area, resulting in highly dispersive results. Therefore, the changes observed in the area occupied by inflammatory infiltrates can only be described as trends and were similar for both cervical and lumbar spinal cord segments (Fig. 4A and B). In rats that received a lower dose of the lysate (10^3) of myelin peptide-producing bacteria, inflammatory infiltrates occupied a small area of the spinal cord cross-section, while higher dose preparations of both control (10^6 C) and myelin peptide-producing bacteria (10^6) lead to a slight increasing trend in the area occupied by inflammatory infiltrates in comparison to non-fed rats with EAE.

Cytokine levels

The induction of EAE in rats resulted in elevated levels of IL-1b, IL-10, TNF-α and IFN-γ compared

with non-treated animals (Fig. 5A-D). Oral administration of *L. lactis* preparations (control and bacteria producing myelin peptides) at both doses decreased IL-1b, IL-10 and TNF-α to levels that were similar to non-treated animals (Fig. 5A-C). The same effect was observed for rats that received control bacterial preparation. However, in the group that was fed lysates from myelin-producing bacteria, the level of IFN-γ remained elevated and even increased slightly following the administration of preparation at the higher dose (Fig. 5D).

Discussion

Multiple sclerosis is a disease of unknown etiology. As a result, only the symptoms, and not the cause, can be treated. Current MS therapies include mostly non-specific, systemic immunosuppression, which is not free from side effects or can simply be inefficient. Other therapies may be more adequate, but they may also cause severe side effects (e.g. natalizumab) [42]. Our intention in the present study was to exploit the naturally occurring state of oral tolerance for the treatment of MS. As MS is an autoimmune disease in which autoreactive T lymphocytes destroy the myelin sheath, the induction of oral tolerance, which decreases the systemic immunoresponse to previously fed antigen, may be an interesting therapeutic alternative. Gut-associated lymphoid tissue (GALT) produce anti-inflammatory cytokines providing the immunosuppressive environment, helping to maintain the state of tolerance for antigens from food or commensal bacteria. Dendritic cells in the GALT present the gut-derived antigens to naïve T cells in Peyer's patches, what results in differentiation of T cells into regulatory T cells (Tregs) producing IL-10 and TGF-β, or internalization of TCR leading to anergy. Thus, orally administered fragments of myelin peptides induce, depending on the antigen dose, active suppression (inhibiting of Th1-dependent immunoresponse) or anergy/clonal deletion of responsive T lymphocytes (both Th1 and Th2) in the mucosal immune system [4,27,40]. Anergic T cells are not able to migrate from the site of tolerance induction, and remaining in the GALT produce anti-inflammatory cytokines. Tregs induced in the Peyer's patches pass into the bloodstream and spread the antigen-specific tolerance throughout the body. Additionally, apoptosis of autoreactive T cells is induced in the thymus after oral administration of the autoantigen [27,34]. This process results in
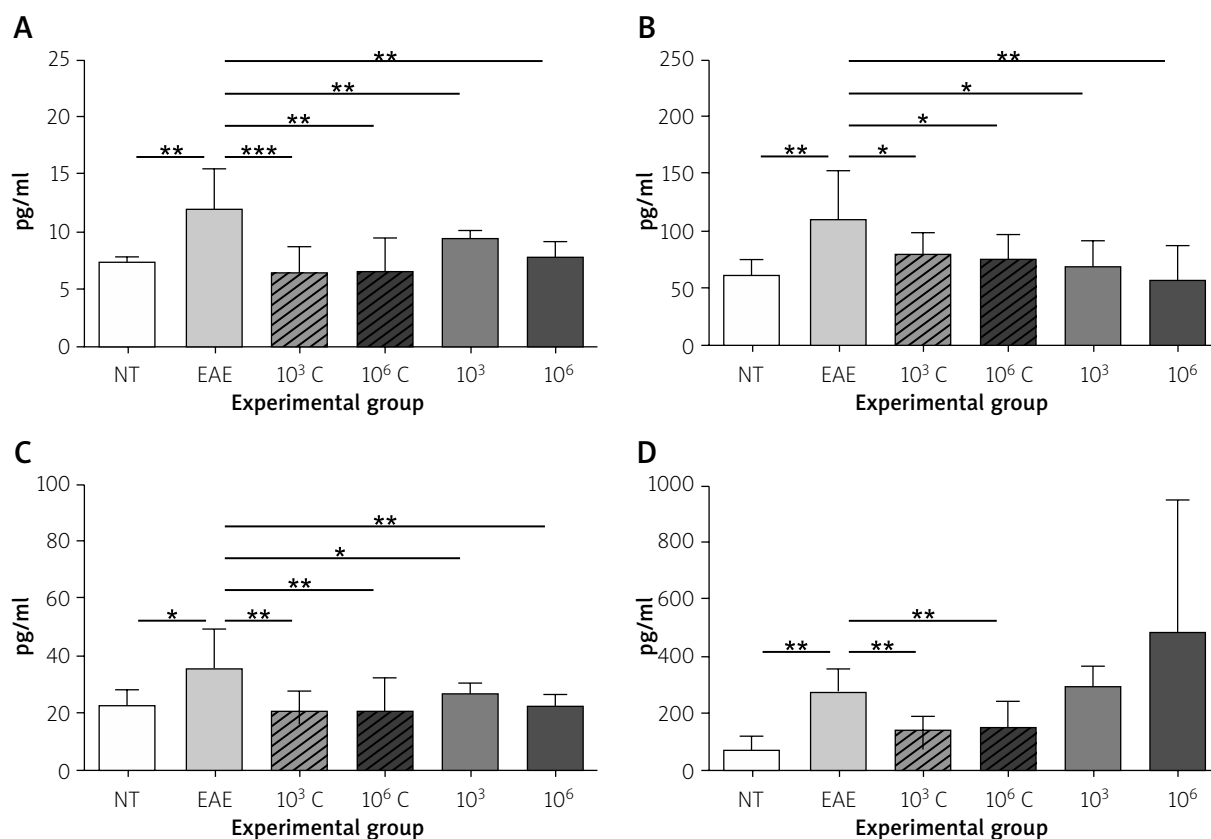
Fig. 5. Serum cytokine levels. Levels of IL-1b (**A**), IL-10 (**B**), TNF-α (**C**), and IFN-γ (**D**), $n = 6$.

a diminished immunoresponse against myelin peptides. Such an approach is anticipated to lead to an antigen-specific, non-systemic, non-specific immunosuppression for the treatment of MS. In addition to oral tolerance, mucosal-associated tolerance (e.g. respiratory tract), generally known as mucosal tolerance, has been considered for many decades as a potential approach for the treatment of illnesses such as uveitis, diabetes, rheumatoid arthritis or allergy [38,41]. Immunological tolerance is an important mechanism that protects the organism from developing an immunoresponse to the exogenous antigens that constantly contact the mucosa, by treating them as self-antigens. Oral tolerance in particular is responsible for maintaining tolerance to dietary antigens and gut microflora. *Lactococcus lactis* bacteria are non-invasive, non-pathogenic and non-colonizing microorganisms with a GRAS (Generally Regarded As Safe) status. There is a large amount of evidence supporting the utility of these bacteria as heterologous protein expression systems. Recombined, leptin-secreting *L. lactis* administered intranasally in mice resulted in a decrease in body mass and food intake

[6]. Additionally, feeding with ovalbumin-secreting *L. lactis* has been reported to induce ovalbumin tolerance in mice. Furthermore, mice that received *L. lactis* alone were able to mount a slight immune response, indicating that this bacterial species itself can enhance the induction of oral tolerance [16,25].

We have previously shown that twenty-fold, but not four-fold, oral administration of a mixture of lysates from *L. lactis* producing MBP85-97, PLP139-151 and MOG35-55 in two doses of 10^3 and 10^6 CFU per dose reduces clinical symptoms and body mass drop in rats with EAE [19]. Here, we describe the effects of bacterial lysates on some neuroimmunological parameters of induced EAE, an animal model of MS. During the progression of EAE, the blood-brain barrier opens. This phenomenon is consistent with the theory regarding the mechanism responsible for the development of MS, in which autoreactive T cells migrate into the CNS and induce an inflammatory response against myelin epitopes [13,24]. Our histopathological analysis of the spinal cord cross-sections demonstrated that evoking EAE resulted in the appearance of perivascular infiltrates in the CNS. Anti-endoge-

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nous albumin labeling revealed its proximity to the vessels, confirming the opening of the blood-brain barrier. T-cell labeling showed that the infiltrates consisted of those immune cells. In our experiments, the inflammatory process observed after evoking EAE in rats was diminished by oral administration of *L. lactis* lysates. The decrease in inflammation intensity was manifested by a reduced number of immune cells rather than by a smaller infiltration surface.

Anti-GFAP labeling was performed to expose active astrocytes. The induction of EAE resulted in an increase in the activity of a greater number of astrocytes, as compared with control rats, and feeding the animals with bacterial lysates further increased the number of these cells. This phenomenon could account for the increased astrocyte proliferation associated with repair processes and glial scar formation in the CNS [26,30].

Serum cytokine levels were measured to determine the peripheral immunological state of the animals [20,31,33]. The results showed that the induction of EAE resulted in an increase in all measured cytokine levels, including both pro- and anti-inflammatory cytokines. Feeding animals with recombinant *L. lactis* lysates decreased IL-1b, IL-10 and TNF-α to the levels detected in non-treated animals, which could imply that animals fed bacterial preparations did not develop an immune reaction. However, this explanation is inconsistent with the histopathological analysis showing infiltration into the CNS. CNS inflammation must be a result of a peripheral process because the immunization mixture was injected peripherally (paw pads). Therefore, it is more likely that our results capture the moment when the inflammatory process was completely cleared in the periphery but still present in the CNS. Differences in the reaction time of the peripheral versus the central immune system may result from the initial induction of antigen tolerance in the gut mucosa, followed by the periphery and then the CNS [29].

Interesting effects have been documented with respect to IFN-γ. After EAE induction, IFN-γ was elevated in comparison to non-treated animals, and feeding with control bacterial preparations resulted in decreased IFN-γ levels. However, when the animals were fed either dose of the bacterial lysate producing myelin peptides, the level of IFN-γ remained elevated. Previous investigations have shown that IFN-γ influences apoptosis of autoreactive T cells by astrocytes [14,32], which may underlie the reduced number of inflammatory cells observed in the infiltrates in rats fed bacterial lysates.

An important observation was made with respect to the effect of oral administration of lysates containing *L. lactis* cells carrying the empty vector (pIL253:PptcB). Results showed that the control preparation may produce a similar but less intense effect in rats as lysates from bacteria producing myelin antigens. It is known that lactococcal cells themselves are able to enhance immunotolerance in mucosa. Our observations are similar to those of Lavasani *et al.* [22] who showed that, due to their immunomodulatory activity and ability to induce regulatory T cells, three probiotic lactic acid bacteria strains (*L. paracasei*, *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313) could be helpful for the treatment of autoimmune diseases.

Difficulties associated with the development of effective MS therapy are linked to the observation that almost every patient presents a unique course of the disease [21]. Similarly, in experimental animals, even inbred animal lines, the clinical symptoms will differ among individual cases. In our trials, EAE was induced using guinea pig spinal cord homogenate as the source of myelin antigens to imitate MS, in which many encephalogenic antigens are believed to play a role. Our previous study has also shown that the three main myelin peptide fragments selected to induce oral tolerance appear to be effective [19].

Experimental results in this work indicate that *L. lactis* is a good antigen delivery system for the induction of oral tolerance in animals with EAE. This notion is confirmed not only by the diminished clinical symptoms of the ongoing disease but also by the decreased inflammatory activity. We suggest that oral administration of lysates from recombinant *L. lactis* cells accelerates the resolution of peripheral inflammation, decreases the intensity of inflammation in the CNS, and boosts the repair process. Considered together, these phenomena may elucidate a perspective for further development of this approach in MS therapy.

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Disclosure

Authors report no conflict of interest.

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