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Immunobiotic lactobacilli reduce viral-associated pulmonary damage through the modulation of inflammation–coagulation interactions

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ABSTRACT

The exacerbated disease due to immune- and coagulative-mediated pulmonary injury during acute respiratory viruses infection results in severe morbidity and mortality. Identifying novel approaches to modulate virus-induced inflammation–coagulation interactions could be important alternatives for treating acute respiratory viruses infections. In this study we investigated the effect of the probiotic strain *Lactobacillus rhamnosus* CRL1505 on lung TLR3-mediated inflammation, and its ability to modulate inflammation–coagulation interaction during respiratory viral infection. Our findings reveal for the first time that a probiotic bacterium is able to influence lung immune-coagulative reaction triggered by TLR3 activation, by modulating the production of proinflammatory and anti-inflammatory cytokines as well as expression of tissue factor and thrombomodulin in the lung. We also demonstrated that the preventive treatment with the probiotic bacteria beneficially modulates the fine tune balance between clearing respiratory viruses (respiratory syncytial virus and influenza virus) and controlling immune-coagulative responses in the lung, allowing normal lung function to be maintained in the face of a viral attack. Our data also pinpoint a crucial role for IL-10 in the immune protection induced by *L. rhamnosus* CRL1505 during respiratory viral infections. These observations might be helpful to propose new preventive or therapeutic approaches to better control virus-inflammatory lung damage using probiotic functional foods.

1. Introduction

Influenza virus (IFV) and respiratory syncytial virus (RSV) are common causes of upper respiratory tract infection and pneumonia. Although several studies have examined the host inflammatory/immune responses to these viruses, some investigations have demonstrated an important role of the hemostatic system in the outcome of viral respiratory infections. Besides inflammatory pathways, respiratory viruses can trigger the coagulation system. They increase the expression of tissue factor (TF), the main initiator of coagulation, in endothelial cells and monocytes inducing a prothrombotic state by concurrent stimulation of coagulation and inhibition of fibrinolysis [1–4]. Although enhanced coagulation may be considered host protective in containing the infection [5], excessive procoagulant activity may result in alveolar fibrin formation and enhancement of inflammation and lung injury. Moreover, much information has accrued demonstrating the close interaction of inflammation, atherosclerosis and thrombosis. Several case–control studies have repeatedly confirmed the common clinical observation that viral respiratory tract infections often shortly precede or accompany acute ischemic strokes or acute myocardial infarctions [6,7].

Abbreviations: APTT, activated partial thromboplastin time; BAL, bronchoalveolar lavage; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFV, influenza virus; IL, interleukin; LAB, lactic acid bacteria; LDH, lactate dehydrogenase; Lr1505, *Lactobacillus rhamnosus* CRL1505; Lr1506, *Lactobacillus rhamnosus* CRL1506; MDCK, Madin–Darby canine kidney; MIP, macrophage inflammatory protein; MOI, multiplicity of infection; MPO, myeloperoxidase; MRS, Man–Rogosa–Sharpe; NAD, nicotinamide adenine dinucleotide; OD, optical density; PAI, plasminogen activator inhibitor; PBS, phosphate buffer saline; Pen/Strep, penicillin–streptomycin; PFU, plaque-forming unit; Poly(I:C), polyinosinic:polycytidylic acid; PRRs, pattern-recognition receptors; PT, prothrombin time; RIG-I, retinoic acid-inducible gene I; RSV, respiratory syncytial virus; TATc, thrombin–antithrombin complexes; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR, Toll-like receptor; TGF, transforming growth factor; TM, thrombomodulin; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor.

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Inflammatory and hemostatic alterations in respiratory viral infections have been associated to double-stranded RNA (dsRNA) intermediates produced during the replication of respiratory viruses such as IFV and RSV, which are recognized by a variety of pattern-recognition receptors (PRRs) in respiratory epithelial, endothelial and immune cells, including Toll-like receptor (TLR)-3 and retinoic acid-inducible gene I (RIG-I). *In vivo* studies using mice have demonstrated that the viral-associated molecular pattern polyinosinic:polycytidylic acid (poly(I:C)), treatment results in TLR3- and CXCR2-dependent neutrophilic pulmonary inflammation, interstitial edema, bronchiolar epithelial hypertrophy, and altered lung function [8,9]. These changes were accompanied by elevated levels of proinflammatory cytokines and type I interferons in broncho-alveolar lavages (BAL) [8] and, increased airway epithelial cell TLR3 protein expression [9]. In addition, studies have reported that poly(I:C) can upregulate TF and downregulate thrombomodulin (TM) expression on endothelial cells. Moreover, *in vivo* application of poly(I:C) induces similar changes in the aortic endothelium of mice and increases D-dimer levels indicating enhanced coagulation and fibrinolysis [10].

Certain probiotic lactic acid bacteria (LAB) strains can exert their beneficial effect on the host through their immunomodulatory activity. These strains, termed immunobiotics [11], have been used for the development of functional foods with the ability to stimulate mucosal immunity. Moreover, studies have demonstrated that some immunobiotic LAB can stimulate the common mucosal immune system to provide protection in other mucosal sites distant from the gut [12]. In this regard, several lines of evidence demonstrated that oral administration of immunobiotics is able to increase resistance against respiratory viral infections. It has been described that several aspects of respiratory antiviral immunity can be beneficially modulated by immunobiotics, including the production of type I interferons, the activity of NK cells, the generation of Th1 responses as well as the production of specific antibodies and the regulation of inflammatory lung injury [11]. We recently initiated a series of studies seeking to establish the capacity of *Lactobacillus rhamnosus* CRL1505 to improve respiratory antiviral immunity. Our research work has demonstrated that mucosal (oral and nasal) administration of the CRL1505 strain is able to beneficially modulate the immune response triggered by TLR3 activation in the respiratory tract and to increase the resistance to RSV challenge [13–15]. Moreover, *L. rhamnosus* CRL1505 administration efficiently reduces inflammatory lung tissue damage produced by poly(I:C) or RSV through its capacity to beneficially modulate proinflammatory/IL-10 and Th1/Th2 balances in the respiratory tract [13–15]. On the other hand, we demonstrated that some immunobiotic strains such as *L. casei* CRL431 or *L. rhamnosus* CRL1505 are able to beneficially modulate the inflammation–coagulation interaction during respiratory infections, indicating that LAB is able to modulate the immune-coagulative response [16–19]. Much research of our group has been done on coagulation activation during severe bacterial infections, and no data on the modulation of coagulation/inflammation interaction by immunobiotics in viral infections are available.

The exacerbated disease due to immune- and coagulative-mediated pulmonary injury during acute respiratory viruses infection results in severe morbidity and mortality. Then, identifying novel approaches to modulate virus-induced inflammation–coagulation interactions could be important alternatives for treating acute respiratory viruses infections. In this sense, studying the effect of orally administered immunobiotics on the immune-coagulative response triggered by respiratory activation of TLR3 would contribute to the knowledge of the mechanism of probiotics' protective effect against respiratory viral infections. Therefore, the aim of the present study was to deepen the understanding of the mechanisms of *L. rhamnosus* CRL1505 immunoregulatory activity by evaluating a) its effects on lung TLR3-mediated inflammation; b) its ability to modulate inflammation–coagulation interaction; c) and its influence on the outcome of respiratory viruses challenges.

2. Materials and methods

2.1. Microorganisms

Lactobacillus rhamnosus CRL1505 (Lr1505) and CRL1506 (Lr1506) were obtained from the CERELA culture collection. The culture were kept freeze-dried and then rehydrated using the following medium: peptone, 15.0 g; tryptone, 10.0 g; meat extract, 5.0 g; and distilled water, 1 l, pH 7. It was cultured for 12 h at 37 °C (final log phase) in Man–Rogosa–Sharpe broth (MRS, Oxoid, Cambridge, UK). The bacteria were harvested through centrifugation at 3000 ×*g* for 10 min and washed 3 times with sterile 0.01 mol/l phosphate buffer saline (PBS), pH 7.2, and resuspended in sterile 10% non-fat milk.

2.2. Animals and feeding procedures

Male 6-week-old BALB/c mice were obtained from the closed colony kept at Tohoku University. They were housed in plastic cages in a controlled atmosphere (22 ± 2 °C temperature, 55 ± 2% humidity) with a 12 h light/dark cycle. *L. rhamnosus* CRL1505 or CRL1506 were administered to different groups of mice for 5 consecutive days at a dose of 10^8 cells/mouse/day in the drinking water, which is the optimal dose with immunoregulatory capacities [20,21]. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care at Tohoku University, Japan.

2.3. Intranasal administration of poly(I:C)

Mice were lightly anesthetized and 100 μl of PBS, containing 250 μg poly(I:C) (equivalent to 10 mg/kg body weight), was administered dropwise, via the nares. Control animals received 100 μl of PBS. Mice received three doses of poly(I:C) or PBS with 24 h rest period between each administration [13,14].

2.4. Lung tissue injury

Forty eight hours after the last poly(I:C) challenge, whole-lung samples from all experimental groups were excised and washed out with PBS. Then, tissues were immersed in 4% (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56 °C. Finally, lungs were cut into 4 μm serial sections and stained with hematoxylin–eosin for light microscopy examination. All slides were coded and evaluated blindly. Albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were also determined in the acellular BAL fluid 48 h post-challenge [13,14]. Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina). LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide (NAD) using the Wiener reagents and procedures (Wiener Lab) [13,14].

2.5. Total and differential leukocyte counts in blood and bronchoalveolar lavages

Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals at 48 h post-challenge and were collected in tubes containing EDTA as an anticoagulant. Total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May-Grünwald Giemsa stain using a light microscope (1000×), and absolute cell numbers were calculated [22]. A portion of the BAL