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Beneficial immunomodulatory activity of *Lactobacillus casei* in malnourished mice pneumonia: effect on inflammation and coagulation

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Abstract

Objective: The effect of *Lactobacillus casei* CRL 431 immunomodulatory activity on inflammation and coagulation during pneumococcal pneumonia was investigated in malnourished mice.

Methods: Weaned mice were malnourished after they consumed a protein-free diet for 21 d. Malnourished mice were treated for 7 d with a balanced conventional diet (BCD) with *L. casei* supplementation (BCD+Lc) or without it. The malnourished control group received only a protein-free diet whereas well-nourished control (WNC) mice consumed BCD ad libitum. Mice were challenged by the intranasal route with pneumococci at the end of each dietary treatment. Lung injury, leukocyte recruitment, cytokine production, coagulation tests, and fibrin(ogen) deposition in lungs were evaluated.

Results: Malnourished control mice showed impaired leukocyte recruitment and cytokine production, and more severe lung injuries when compared with WNC mice. Coagulation tests were significantly impaired in malnourished control group versus WNC group. Repletion with BCD or BCD+Lc improved these parameters, but only BCD+Lc mice achieved the values of WNC mice. In addition, the interleukin-10 level was higher in the BCD+Lc group than in the WNC group.

Conclusion: Repletion with supplemental *L. casei* accelerated recovery of the defense mechanisms against pneumococci by inducing different cytokine profiles. These cytokines would be involved in the improvement of the immune response and in the induction of a more efficient regulation of the inflammatory process, limiting the injury caused by infection.

Keywords: *Lactobacillus casei*; Malnourished mice; Inflammation; Coagulation system; Pneumococcal pneumonia

Life-threatening complications from bacterial infections are a major and growing clinical problem, aggravated by the emergence and spread of antibiotic resistance in bacterial pathogens and by an increase in the number of immunocompromised patients [1].

During bacterial infection, the host responds to invading microbes with a number of different defense mechanisms. Blood coagulation and inflammation are universal responses to infection and there is a cross-talk between them that can either amplify or dampen their respective functions. Loss of appropriate interactions between these systems contributes to tissue damage in infectious diseases [2].

Bacterial pneumonia is a leading cause of morbidity and mortality and *Streptococcus pneumoniae* remains the most common pathogen responsible for community-acquired pneumonia in both developed and developing countries [3]. Pneumococcal pneumonia is characterized by an intense inflammatory reaction that is known to be directly induced by pneumococcal cell wall components and pneumolysin [4]. In addition, there is growing evidence that aspects of the immune response greatly contribute in pneumococcal pathogenesis: although immunosuppressed individuals die as a consequence of poor host response, immunocompetent hosts face overwhelming inflammatory reactions that contribute to tissue injury, shock, and death [5].

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Modern therapeutic approaches to infectious diseases focus on the modulation of the host response, especially in immunocompromised individuals [6]. In this sense, the use of probiotics to beneficially modulate the immune system has greatly increased in recent years. Probiotic lactic acid bacteria has several immunomodulatory effects, documented in different studies by various research groups, which include improvement of the immune response [7–10] and antiinflammatory properties [11–13].

In a previous study we demonstrated in a pneumonia-sepsis model [7] that malnourished mice are more susceptible to *S. pneumoniae* infection. In addition, we reported that repletion of malnourished mice with a balanced conventional diet (BCD) with supplemental *Lactobacillus casei* CRL 431 (BCD+Lc) significantly accelerates the recovery of the defense mechanisms against pneumococcal respiratory infection.

The effect of protein malnutrition on the interaction between inflammation-coagulation has not been studied extensively. Moreover, there are no reports concerning the potential effect of probiotic lactic acid bacteria in this situation. In this work we undertook a series of experiments to investigate the effect of *L. casei* immunomodulatory activity on inflammation and coagulation in malnourished mice challenged with a dose of *S. pneumoniae* that induces pneumonia without sepsis.

Materials and methods

Microorganisms

L. casei CRL 431 was obtained from our culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). The culture was 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 8 h at 37°C (final log phase) in Man-Rogosa-Sharpe broth (Oxoid, Wesel, Germany). The bacteria were harvested by centrifugation at 3000 × g for 10 min and washed three times with sterile 0.01 mol/L phosphate-buffered saline (PBS), pH 7.2. Capsulated pneumococcus (serotype 14) was isolated from the respiratory tract of a patient from the Department of Clinical Bacteriology of the Niño Jesús Children's Hospital in San Miguel de Tucumán, Argentina. Pneumococci serotypification was performed in Administración Nacional de Laboratorios e Institutos de Salud, Buenos Aires, Argentina.

Animal model

Male 6-wk-old Swiss albino mice were obtained from the closed colony kept at our bioterio. They were housed in plastic cages at room temperature. Mice were housed individually during the experiments, and the assays for each parameter studied were performed in 5 to 6 mice per group for each time point. Weaned mice were malnourished after they consumed a protein-free diet [7] for 21 d. At the end of this period, mice that weighed 45% to 50% less than well-nourished mice were selected for experiments. Well-nourished control (WNC) mice consumed ad libitum a BCD [7].

Malnourished mice were separated into two groups for repletion treatment. One group of malnourished mice was fed BCD for 7 consecutive days. Because administration of *L. casei* for 2 d is the optimal dose to provide protection against *S. pneumoniae* in malnourished mice [7], the second group of mice received 7 d of BCD+Lc with *L. casei* supplementation (10^9 colony-forming unit/mouse/d) on Days 6 and 7. The malnourished control (MNC) group received only the protein-free diet whereas WNC mice consumed the BCD ad libitum. Our ethical committee for animal care approved the experiments.

Experimental infection

S. pneumoniae was first grown on blood agar for 18 h; freshly grown colonies were suspended in Todd Hewitt broth (Oxoid) and incubated at 37°C overnight. The pathogen was harvested through centrifugation at 3000 × g for 10 min at 4°C and then washed three times with sterile PBS. Cell density was adjusted to 4×10^4 colony-forming unit/L. The size of the inoculum was confirmed by serial dilutions and quantitative subcultures on blood agar. Challenge with *S. pneumoniae* was performed on the day after the end of each dietary treatment (Day 8). Mice were infected by dropping 25 μL of the inoculum containing 10^3 log-phase colony-forming unit of *S. pneumoniae* in PBS into each nostril and allowing it to be inhaled. To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min. WNC and MNC mice were infected in the same way. Mice were sacrificed on Day 0 (before infection) and at 12 h and 1, 2, 3, 5, 10, and 15 d postinfection. The infecting dose was selected on the basis of its inability to produce translocation to blood, as determined from the results of the bacterial cell counts performed in the blood of mice with severe pneumonia (unpublished data). During the 15 d postinfection all groups were fed only with BCD, with the exception of MNC group, which received protein-free diet.

Determination of leukocytes in bronchoalveolar lavages and biochemical assay of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) samples were obtained according to the technique previously described [7]; briefly, the trachea was exposed and intubated with a catheter and two sequential BALs were performed in each mouse by injecting 0.5 mL of sterile PBS. A portion of the fluid was used to determine the total number of leukocytes using a hemocytometer. The remaining sample of fluid was centrifuged for 10 min at 900 × g, the pellet was used to make smears, and differential cell counts were performed by

counting 200 cells stained with May Grünwald-Giemsa. The supernatant fluid was frozen at −70°C for subsequent biochemical analyses. Albumin content, a measure to quantify increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid. Albumin content was determined colorimetrically on the basis of albumin binding to bromocresol green using an albumin diagnostic kit (Wiener Laboratory, Buenos Aires, Argentina). LDH activity, expressed as U/L of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide using reagents and procedures (Wiener Laboratory).

Myeloperoxidase activity in lung homogenates

Neutrophil infiltration in lung tissue was evaluated by the quantification of myeloperoxidase (MPO) activity as follows. Lungs were cleared of blood with 3 mL of PBS by intracardiac puncture. Lungs were removed and homogenized in 3 mL of 50 mmol/L acetate buffer, pH 5.4 (MPO assay buffer). Homogenates were frozen at −70°C for 15 min, thawed, sonicated for 60 s, and centrifuged at 3600 × g for 15 min at 4°C. After the cellular lysate was equilibrated to room temperature, MPO was evaluated by adding 200 μL of an appropriate dilution of the lysate to 20 mmol/L 3,3',5,5'-tetramethylbenzidine in dimethylphormamide and 30 μL of 2.7 mmol/L of hydrogen peroxide in MPO assay buffer. The reaction mix was incubated for 3 min at 37°C and stopped by addition of ice-cold 200 mmol/L sodium acetate buffer (pH 3). Absorbance was read at 655 nm against a standard curve made with commercial MPO (Catalog No. M-6908, Sigma-Aldrich Co, St Louis, MO, USA). The results were expressed as specific activity of MPO (MPO U/mg of total proteins in lung homogenate). Total proteins concentration was determined [14] in the cellular lysates by the method of Bradford [15].

Histophatology

At prechosen intervals, whole-lung samples from control and infected mice were excised and washed out with PBS 0.01 mol/L, pH 7.2. Then, tissues were immersed in 4% (vol/vol) 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 hematoxilin-eosin for light microscopy examination. All slides were coded and evaluated blindly.

Immunofluorescence test for immunoglobulin A+ cells in bronchus-associated lymphoid tissue

To determine the number of immunoglobulin (Ig)A+ cells in bronchus-associated lymphoid tissue (BALT), immunofluorescence assays were performed on histologic sections. Mice were sacrificed on Day 0 and on Days 1, 5, 10, and 15 postinfection and lungs were aseptically removed and processed following Sainte-Marie's technique [13]. The slices were incubated with α-chain monospecific antimouse antibody conjugated with fluorescein isothiocyanate (Sigma-Aldrich Co). Cells were counted with a fluorescent microscope using ×100 magnification. The results were expressed as the number of positive fluorescent cells per 10 fields. They represent the mean of three histologic slices for each animal (6 mice/group for each time point).

Cytokine concentrations in BAL

Acellullar BAL fluid was obtained as described above. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6, and IL-10 concentrations in BAL were measured with a commercially available enzyme-linked immunosorbent assay kits following the manufacturer's recommendations (R and D Systems, Minneapolis, MN, USA).

Blood collection and coagulation tests

Blood samples were obtained by cardiac puncture and collected in a 3.2% solution of trisodium citrate at a ratio of 9:1. Correction of the anticoagulant volume for hematocrit values was made before sample collection. Blood was collected in EDTA for platelet counting. Platelet-poor plasma was prepared by centrifugation at 2000 × g for 15 min; then it was removed and transferred to a plastic container. The plasma was recentrifuged at 2000 × g for a further 10-min period. Routine coagulation test, prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were performed manually on fresh plasma samples. PT was determined by a one-step method (Thromboplastin-S, Biopool International, Buenos Aires, Argentina). The APTT was determined by a one-step method (STA APTT, Diagnostica Stago, Asnières, France). Fibrinogen was measured by the method of Clauss (FIBRI PREST 2, Diagnostica Stago).

Immunohistochemical analysis

For fibrin(ogen) immunostaining, lung sections were deparaffinized and endogenous peroxidase activity was quenched with a solution of methanol/0.03% H_2O_2 to inhibit the activity of the endogenous peroxidase in the lungs (Merck, Buenos Aires, Argentina). The sections were incubated in 10% normal sheep serum and then exposed to sheep antimouse fibrinogen (purified IgG, Cedarlane, Hornby, Ontario, Canada). After washes, slides were incubated with donkey antisheep IgG peroxidase conjugate (Sigma-Aldrich Co). Peroxidase activity was detected with a 3,3'-diaminobenzidine peroxidase substrate solution (0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.015% H_2O_2 in 0.01 mol/L PBS, to detect the activity of the peroxidase conjugated to the IgG, pH 7.2, Sigma-Aldrich

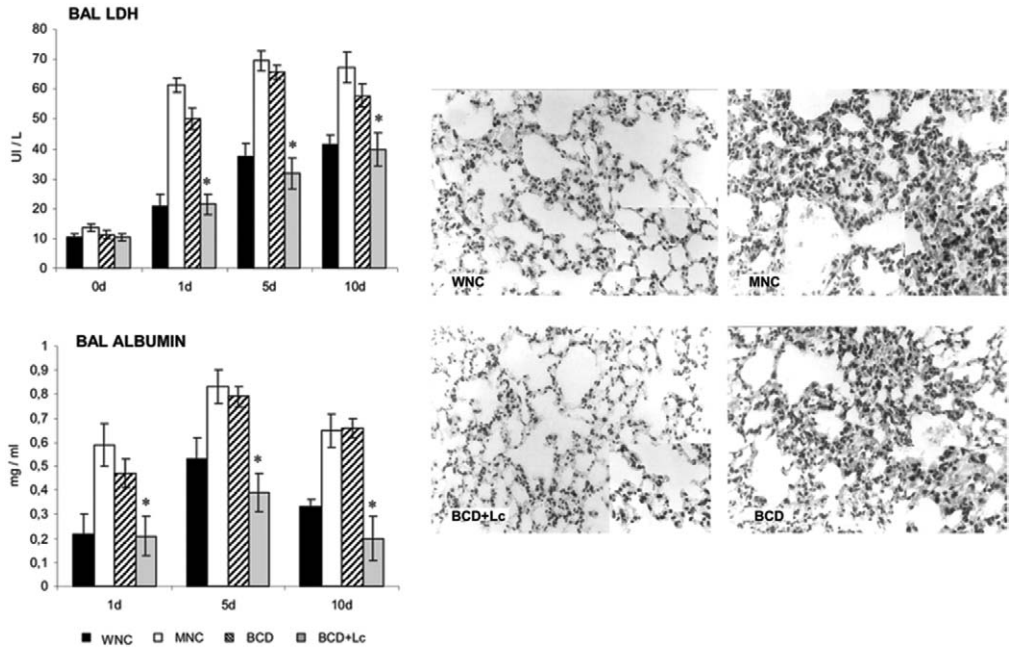

Fig. 1. Lung injury evaluated by assessing albumin content and lactate dehydrogenase (LDH) activity in bronchoalveolar lavages (BAL) and by histologic analysis of lung tissue. Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Photographs show lung parenchyma on Day 10 postinfection. (Original magnification ×200.) Results are expressed as means ± SD. *Different from MNC mice and no differences with WNC group ($P < 0.05$).

Co), after which a light counterstain with hematoxylin was performed.

Statistical analysis

Experiments were performed in triplicate and results were expressed as the mean ± SD. A two-way analysis of variance test was used. Tukey's test (for pair-wise comparisons of the means of the different groups) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

Results

Lung tissue injury

Albumin content and LDH activity, measured in the acellular BAL fluid, were used as indices of lung injury. Challenge with *S. pneumoniae* caused increases in both BAL albumin content and LDH activity in the MNC and WNC groups (Fig. 1), but these parameters were significantly higher in MNC mice than in WNC mice. In the WNC group there was a descending trend in albumin concentration on Day 10, suggesting that lungs were recovering from the inflammatory injury. Values of LDH activity and albumin concentration in mice replete with BCD+Lc were similar to the WNC mice whereas BCD mice showed values similar to those in the MNC group (Fig. 1).

The effects of pneumococci on the lung structure were assessed by studying histologic changes. The histopathologic study revealed significant lung injury in the MNC mice with a marked reduction in the alveolar spaces, an intense inflammatory response, fibrosis, and hemorrhage (Fig. 1). The histologic changes found in the lungs of the WNC mice were always less severe than those in the lungs of the MNC group (Fig. 1). When *L. casei* was added to the repletion diet (BCD+Lc group), the injury caused by the pathogen to the lung tissue decreased significantly, the histologic characteristics of this group being similar to those of the WNC group during the whole period assayed. Mice treated with BCD showed histologic signs intermediate to those of WNC and MNC mice (Fig. 1).

In addition to its direct cytotoxicity, *S. pneumoniae* can produce further lung tissue injury through its ability to induce inflammation. Thus, inflammatory cells and proinflammatory and antiinflammatory mediators in lungs were studied.

Inflammatory cells in lung

Alveolar macrophages were the predominant resident leukocytes in the alveolar spaces of non-infected mice, as we have previously described [7]. WNC mice showed an important recruitment of neutrophils and macrophage migration into the alveoli, resulting in high leukocyte counts in BAL during the infection (Fig. 2). MNC mice showed a significant impairment in the recruitment of neutrophils and

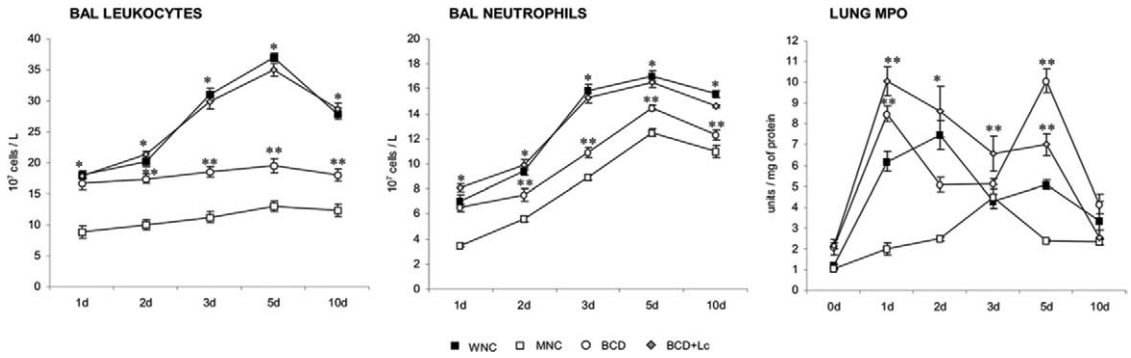

Fig. 2. Leukocyte and neutrophil count in bronchoalveolar lavage (BAL) and myeloperoxidase (MPO) activity in lung homogenates. Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as means ± SD. *Different from MNC mice and no differences with WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

macrophages. However, BCD+Lc treatment was enough to normalize the response to the infection, with values of neutrophils and macrophages similar to those in WNC mice. The mice treated with BCD had lower values than the WNC mice (Fig. 2).

Neutrophil recruitment into lung tissues was quantified by the intracellular enzymatic marker MPO (Fig. 2). MPO activity in the MNC group was significantly lower than in the WNC group throughout the assayed period. Repletion with BCD+Lc induced a significant increase in MPO activity with respect to the WNC group on Days 1, 2, and 3 postinfection; from then on there was a decrease, normal values being reached on Day 10 postinfection (Fig. 2). In the BCD group, MPO activity exhibited a different behavior from that found in the other experimental groups, with peaks on Days 1 and 5 postinfection.

Cytokines in lung

The levels of TNF-α and IL-1β in BAL before the infection were similar in all groups (Fig. 3). This finding indicates that malnutrition does not alter basal levels of BAL TNF-α or IL-1β. After the challenge with *S. pneumoniae*, both cytokines increased significantly, reaching a peak at 12 h postinfection. Afterward, TNF-α and IL-1β decreased gradually until they returned to basal levels. Dif-

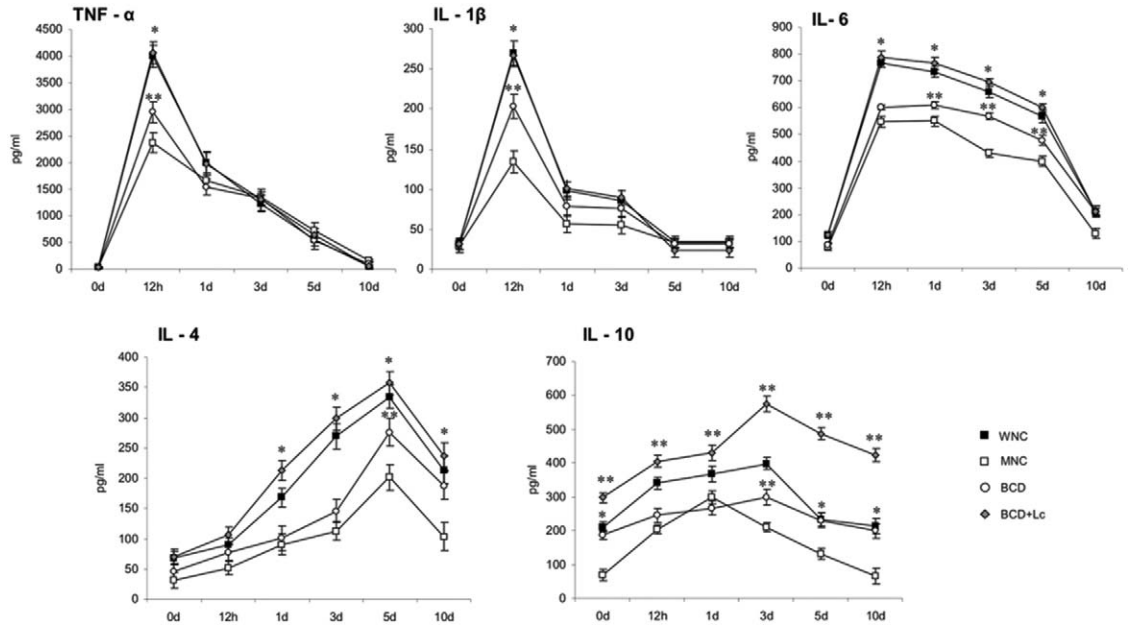

Fig. 3. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6, and IL-10 concentrations in bronchoalveolar lavages. Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as means ± SD. *Different from MNC mice and no differences with WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

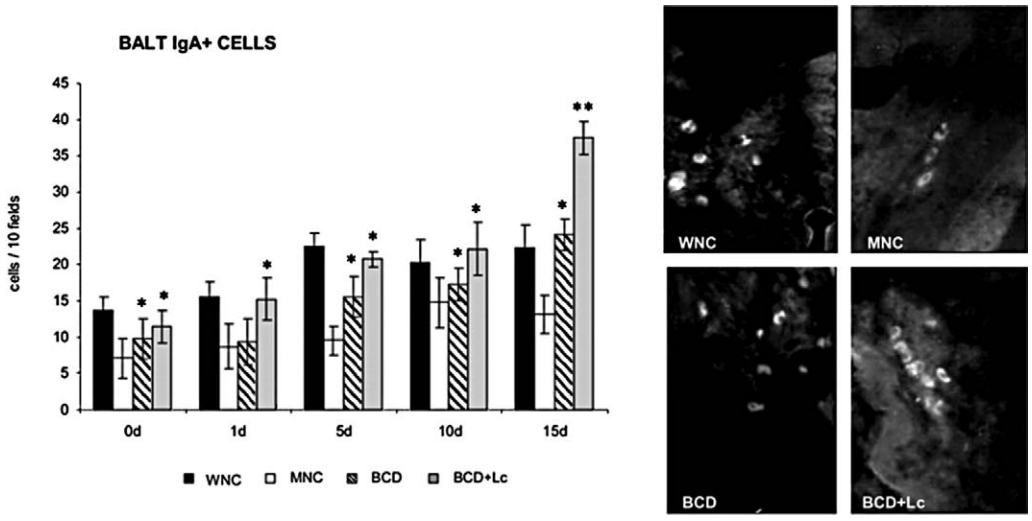

Fig. 4. Number of immunoglobulin (Ig)A+ cells in bronchus-associated lymphoid tissue (BALT). Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Photographs show BALT IgA+ cells on d 15 postinfection. (Original magnification ×200.) Results are expressed as means ± SD. *Different from MNC mice and no differences with WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

ferences between the groups were found only at the earlier stages of infection. Concentration of BAL TNF-α and IL-1β were significantly lower in the MNC group than in the WNC group. Mice fed the BCD treatment showed higher values than MNC mice but they did not reach the levels of the WNC group. However, mice repleted with BCD+Lc showed similar values of TNF-α and IL-1β to those in the WNC mice.

No differences between the groups were found in basal levels of IL-6 (Fig. 3). The infection induced an increase in the concentrations of this cytokine, which remained high until Day 5, in all experimental groups. BAL IL-6 was significantly lower in MNC than in WNC mice during the whole postinfection period. Both repletion treatments enhanced IL-6 production, but only mice fed BCD+Lc showed similar levels to the WNC group.

Malnourished control mice showed basal levels of IL-10 and IL-4 significantly lower than those of the WNC mice (Fig. 3). Repletion with BCD normalized the basal levels of both cytokines whereas the BCD+Lc mice showed normal IL-4 values but IL-10 levels were significantly higher than the WNC mice. The infection induced a progressive increase in IL-4 values in all experimental groups up to Day 5 postinfection; however, the IL-4 levels in the WNC mice were significantly higher than those in the MNC mice. Only repletion with BCD+Lc was capable of normalizing IL-4 response to infection.

After challenge with the pathogen, IL-10 levels increased in all the groups, WNC mice showing values significantly higher than the ones in the MNC group. Animals repleted with BCD showed a behavior similar to the WNC mice during the initial stages of the infection, reaching WNC values on Day 5 postinfection. In the BCD+Lc group, IL-10 in BAL showed higher values than those found in the WNC group throughout the assayed period.

The results obtained showed that the addition of *L. casei* to the repletion diet normalized the innate immune response to the infection and, at the same time, increased IL-10 production, which would contribute to limiting injury caused by the inflammatory process. In addition, the animals repleted with BCD+Lc showed normal values of IL4, a fact that would imply the induction of a more effective humoral immune response. This is why we evaluated this response in lung by determining the number of IgA+ cells in BALT.

IgA+ cells in BALT

Malnutrition decreased IgA+ cells in BALT, which were significantly increased by both repletion treatments (Fig. 4). Repletion with BCD+Lc not only normalized this parameter but also induced a significant increase in the number of IgA+ cells on Day 15 postinfection compared with WNC group. The BCD group showed a behavior similar to that of the BCD+Lc group; however, the increase in IgA+ cells was significantly lower on Day 15 postinfection. These results agree with the antipneumococcal IgA levels in BAL previously reported [7].

Activation of coagulation system

Malnutrition induced a significant decrease ($P < 0.001$) in the percentage of prothrombin activity and in APTT and fibrinogen values (Fig. 5). All these parameters reached normal values with both repletion diets. The number of platelets decreased significantly in the MNC compared with

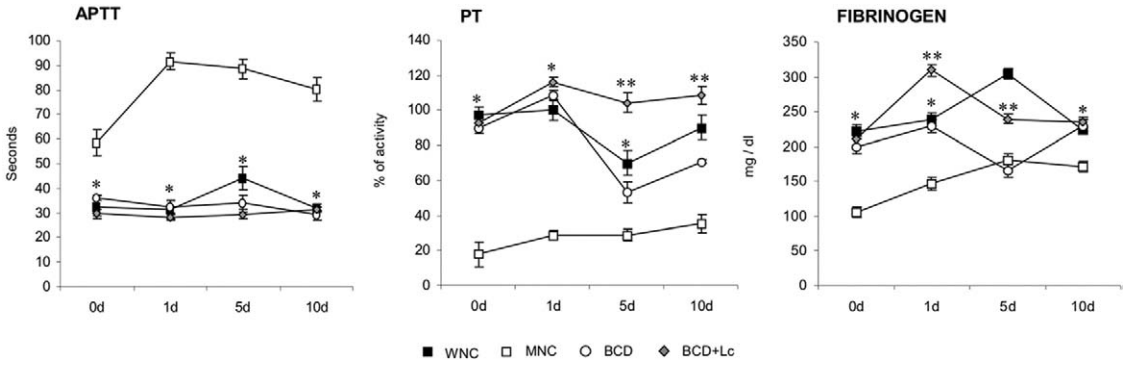

Fig. 5. Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration. Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (BCD+Lc). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Results are expressed as means ± SD. *Different from MNC mice and no differences with WNC group ($P < 0.05$). **Different from MNC and WNC groups ($P < 0.05$).

the WNC mice (WNC, $10.71 \pm 0.65 \times 10^5$ cells/μL; MNC, $8.96 \pm 0.55 \times 10^5$). Both repletion diets caused a significant increase in platelet counts, but only the BCD+Lc treatment normalized this parameter ($11.14 \pm 0.68 \times 10^5$ cells/μL).

The APTT values tripled after challenge on Day 1 postinfection in the MNC mice. These values were normalized with both repletion diets with no significant differences with respect to WNC mice throughout the postinfection period studied.

After challenge with the pathogen, WNC mice showed a significant decrease in prothrombin activity only on Day 5 postinfection. Mice repleted with the BCD treatment showed no differences with the WNC group, whereas repletion with BCD+Lc caused prothrombin activity to remain normal during the whole assayed period.

The infection caused an increase in fibrinogen concentration in WNC mice, maximum value being reached on Day 5 postinfection. On Days 1 and 5 postinfection, fibrinogen values were higher in the BCD+Lc group than in the BCD group ($P < 0.05$); on Day 10 they were similar, approximately 50% higher than those in the MNC group.

When animals were infected, platelet counts were high in all groups, with no significant differences between them (data not shown).

Fibrin(ogen) deposition in lungs

After challenge, the MNC group showed fibrinogen deposits of moderate intensity at the pleural level and low intensity in some areas of the parenchyma. The reaction became stronger and more extensive on Day 5 postinfection, involving the whole pleura, some bronchi, and interalveolar walls. On Day 10 postinfection the whole parenchyma was compromised (diffuse pattern) (Fig. 6). In the WNC group, the reaction was limited to the pleura on the first days postinfection, reaching its maximum intensity on Day 10 postinfection (Fig. 6). At the level of the parenchyma, deposits were slightly positive, a focal pattern being observed. Animals repleted with BCD showed a similar behavior to WNC mice whereas repletion with BCD+Lc caused the reaction to become limited to the pleural zone without altering the lung parenchyma (Fig. 6).

Discussion

Malnutrition is common in developing countries and is associated with impaired cellular and humoral immunity to microorganisms. Much of the morbidity and mortality that afflicts malnourished people is caused by infections. In previous investigations we found that in our experimental model, malnourished mice were more susceptible to pneumococcal infection than well-nourished mice and pathogen colonization of lung was significantly greater in the MNC

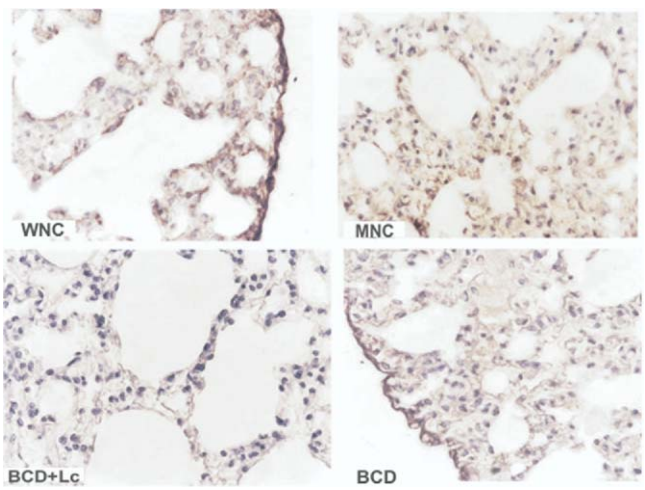

Fig. 6. Immunohistochemical analysis for fibrin(ogen) deposition in lungs. Malnourished mice replete with balanced conventional diet (BCD) or BCD with supplemental *Lactobacillus casei* (*BCD+Lc*). Malnourished control (MNC) and well-nourished control (WNC) mice were used. Photographs show lung sections on Day 10 postinfection. (Original magnification ×400.)

group [7]. In addition, we found that the supplementation of a repletion diet with *L. casei* accelerates the recovery of the innate immune response and improves the specific immune mechanisms against an *S. pneumoniae* respiratory infection in malnourished mice [7]. In the current article we studied the influence of *L. casei* administration to malnourished mice on the inflammation induced by an *S. pneumoniae* infection, including studies of alterations of the coagulation process.

The magnitude and duration of the inflammatory process in lungs are considered to be important determinants of bacterial clearance; however, this process can also determine the severity of, and mortality from, pneumonia [4,5,16]. Although a vigorous response by phagocytic cells at the early stages of infection is assumed to protect the host from bacterial infection, increasing evidence also suggests that persistent high levels of inflammatory cells and cytokines play a prominent role in acute and chronic lung injury [17].

Effective host defense against lung bacterial infection is primarily dependent on rapid bacterial clearance mediated by macrophages and neutrophils. The sequential recruitment of leukocytes toward sites of infection is the most fundamental process of innate immunity [18]. When we determined the MPO activity in lung as a measure of neutrophil and monocyte recruitment, we observed that pneumococcal pneumonia induced an important recruitment of inflammatory cells into lungs, with significant differences in the MPO activity and in the kinetics of neutrophil recruitment between the different groups. Sustained neutrophil recruitment was observed early after infection in WNC mice with a peak on Day 2, whereas in MNC mice there was a delay in this process and MPO activity was significantly diminished with respect to the WNC group. When malnourished mice were fed BCD+Lc, MPO activity was significantly higher than in the WNC group, especially on Day 1 postinfection, which is probably related to the more effective pathogen clearance. Repletion with BCD increased this parameter, but with different kinetics from the WNC mice, with a second peak on Day 5, probably because the inflammatory process (albumin, LDH) observed in lung was similar to the MNC group.

It has been reported that dietary restriction impairs neutrophil migration into local inflammatory sites by reducing adhesion molecule expression and cytokine chemokine production [19]. In addition, Hidemura et al.[20] have reported that oral administration of a probiotic *Bifidobacterium longum* enhanced neutrophil recruitment into local inflammatory sites in a diet-restricted murine peritonitis model by increasing local cytokine production. The main physiologic function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes [21]. It has been suggested that early and sustained TNF-α/IL-1β production takes place in *S. pneumoniae* pneumonia [22].

In the current study we observed a significant decrease in BAL TNF-α and IL-1β levels in the MNC group. When malnourished mice were repleted with the BCD+Lc diet, these cytokines reached similar concentrations to those found in the WNC group. In addition, we observed normalization in the recruitment of neutrophils into lung tissues. Repletion with BCD increased TNF-α and IL-1β in BAL, but it was not enough to achieve the values of WNC mice. Consequently, *L. casei* administration accelerated the normalization of the cytokines involved in the innate immune response. When we analyzed plasma levels of fibrinogen, we observed similar values in BCD+Lc and WNC mice, but the peaks were on Days 1 and 5, respectively, which could indicate an earlier inflammatory response in the former group.

In the current work we found that MNC mice showed an important increase in LDH and albumin in BAL with respect to the WNC group during the pneumococcal respiratory infection. These results and the histopathologic studies evidenced a higher lung injury in MNC than in WNC mice. Repletion with BCD was not enough to avoid the tissue injury induced by pneumococcal infection. However, the addition of *L. casei* to the BCD induced an important decrease in albumin and LDH in BAL and the results of the histopathologic examination were similar to those of WNC mice.

Interleukin-10 is an inhibitor of activated macrophages and is, thus, involved in the homeostatic control of innate immune reactions [21]. The MNC group showed significantly lower basal levels of IL-10 than WNC mice. Repletion with *L. casei* was able to increase IL-10 to significantly higher levels than those found in the WNC group. After infection, IL-10 increased in all experimental groups. However, the group repleted with BCD+Lc showed significantly higher levels than WNC group. This result is probably related to the regulation of the lung injury despite the increase in inflammatory cytokine and neutrophil recruitment observed in this group.

Antigen-presenting cells process the pathogens into antigenic fragments and acquire the capacity to activate surrounding T-helper cells, which in turn stimulate B cells to proliferate and mature into polymeric IgA-producing cells under the influence of cytokines that include IL-4, IL-5, IL-6, IL-10, and transforming growth factor-β [23]. In this work we observed an increased number of IgA+ cells in the BALT of the BCD+Lc group when compared with WNC group. These findings are probably related to the increase in IL-6, IL-4, and IL-10 in that group.

To study whether malnutrition affects coagulation in mice, we determined PT (extrinsic pathway) and APTT (intrinsic pathway) in plasma in the different experimental groups. MNC animals showed an impaired coagulation mechanism when compared with the WNC group. Malnutrition induced a prolonged APTT and a decreased activity of PT. BCD+Lc and BCD were able to normalize these parameters.

Blood coagulation and the inflammatory process are in-

terrelated as part of the innate host defense mechanisms [24]. To determine whether pneumococcal pneumonia affects coagulation in the systemic compartment of our experimental model, we measured PT and APTT after infection with pneumococci. After infection APTT was affected only in malnourished mice whereas there was no alteration of this parameter in the other groups.

Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 stimulate one of the most potent prothrombotic agents, the tissue factor [25], which stimulates the extrinsic coagulation cascade resulting in thrombin production and ultimately formation of a fibrin-rich thrombus. An indication of the importance of the tissue factor in the inflammatory process was obtained in an animal model of sepsis in which tissue factor–dependent procoagulant activity correlated with levels of TNF-α [26]. In the current work we found that WNC mice and the group fed BCD showed a significant decrease in PT activity on Day 5 postinfection. However, in the animals fed BCD+Lc PT was not altered; this fact is probably related to the lower tissue injury observed in this group and its moderate inflammatory response.

Platelets can also mediate inflammatory reactions through the production of cytokines and chemokines such as IL-1 and IL-8 [27,28]. The MNC group showed a significant decrease in platelet numbers with respect to WNC group and repletion with BCD+Lc was enough to normalize those values, which could contribute to the normalization of the inflammatory response in this group.

It has been established that the pneumococcal cell wall activates procoagulant activity at the surface of endothelial cells during pulmonary inflammation [29] that leads to fibrin deposition in lung tissues [30]. Exuberant coagulation relative to anticoagulation and fibrinolysis in the lung and the systemic circulation are important in the pathophysiology of acute lung injury. In the early stages of acute lung injury, fibrin is deposited in the alveoli, thus, increasing the inflammatory response [31]. In the current study, to evaluate the local activation of coagulation, we also examined lung tissue deposition of fibrin(ogen). Antifibrin(ogen) immunostaining showed fibrin(ogen) deposition in lung in the different infected groups.

In the animals fed BCD+Lc there was a lower fibrin(ogen) deposition than in the MNC group, without alterations of the lung parenchyma. In a previous work we demonstrated that *L. casei* administration induces macrophage activation [8]. Macrophages appear to play a pivotal role in the transition between wound inflammation and repair [32] and, as fibrin(ogen) can modulate macrophage activity, it can thereby influence the rate of this transition.

It has been demonstrated that the antiinflammatory cytokines IL-4 and IL-10 inhibit IL-1β and lipopolysaccharide-induced tissue factor activity in monocytes [33] and that pretreatment of animals with IL-10 limits the size of the thrombi formed in response to stasis-induced venous thrombosis [34]. In our experimental model, *L. casei* was able to induce an increase in IL-10 in BAL, which would limit fibrin(ogen) deposition in lung during the pneumococcal infection. In addition, in mucosal tissues the IgA is likely to contribute to the control of complement-dependent inflammation resulting in alterations of the mucosal barrier through its antiinflammatory potential [35]. Consequently, the antiinflammatory mechanisms elicited by *L. casei* administration could be responsible for the lower tissue injury observed in this group with respect to the other ones.

According to the results of the current work, the addition of *L. casei* to a repletion diet fed to malnourished mice would accelerate the recovery of the defense mechanisms against pneumococci by inducing different cytokine profiles. These cytokines would be involved in the improvement of the innate and specific immune response and in the induction of a more efficient regulation of the inflammatory response, thus, limiting the injury caused by the infection.

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