ORIGINAL PAPER

Subcutaneous administration of live lactobacillus prevents sepsis-induced lung organ failure in rats

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The leading cause of death in intensive care units around the world is the syndrome of exaggerated and prolonged systemic inflammation response (SIRS), which if not controlled will lead to irreversible, often multiple, organ dysfunction and organ failure syndrome (MODS). Host phagocytic cells, predominantly macrophages and neutrophils, and their soluble products, play a central role. Accumulation of neutrophils is often observed, especially in the lungs,[1-3] intestines,[4] liver[5] and kidneys.[6] Neutrophilic infiltration of distant organs,[7] and especially of the lungs,[1] has been a characteristic finding of patients dying from sepsis. However, the mechanisms responsible for the exuberant inflammation and neutrophil infiltration are not yet fully understood. It has recently been demonstrated that this, at least in part, is due to an inhibition of neutrophil apoptosis,[8] and there is a suggested association with activation of nuclear factor — κB (NF-κB), reduced activity of caspases-9 and -3 and maintenance of mitochondrial transmembrane potential.[8]

A composition of four lactic acid bacteria (LAB) and four plant fibres (Synbiotic 2000™) has proven extraordinarily effective in reducing inflammation and rate of infection in patients after extensive operations, including transplantations,[9] severe acute pancreatitis, trauma and in critically ill patients. The LAB for the composition were identified — and to a large extent selected — for their unique ability to transcribe NF-κB.[10] This study was undertaken in order to investigate whether neutrophil infiltration could be inhibited by supplementation with these LAB. However, as many critically ill patients cannot eat and it takes several hours for the LAB to reach the large intestine when orally administered, we wanted to explore the efficacy of subcutaneous administration. Subcutanous administration of live LAB was recently shown to attenuate both murine colitis and murine arthritis.[11]

MATERIALS AND METHODS

The study was conducted at the Experimental Animal Research Laboratory, Faculty of Medicine, Ege University, Izmir, Turkey. Eighty male adult Wistar albino rats weighing 250–300 g were randomly selected and divided into eight groups. All the animals had free access to water and a standard rat chew containing 4.5% plant fibres.

The lactic acid bacteria for the study are ingredients in a synbiotic composition called Synbiotic 2000 FORTE™ (S2000F), which consists of a mixture of four LAB, one from each of the four main genera of *lactobacillus*; 10^{11} of *pediacoccus pentosaceus* 5–33:3, 10^{11} of *leuconostoc mesenteroides* 32–77:1,10^{11} of *lb paracasei* subsp *paracasei* 19 and 10^{11} of *lb plantarum* 2362, i.e. a total of 40 billion LAB per dose, plus a mixture of four well studied bioactive plant fibres: 2.5 g betaglucan, 2.5 g inulin, 2.5 g pectin and 2.5 g resistant starch, in total 10 g of plant fibres per dose. The composition was determined after extensive studies of >350 human and >180 plant strains by microbiologists from Lund University, Sweden, Åsa Ljungh and Torkel Wadström.[10,12] The LAB to be used in the composition were chosen after investigations of a series of microbial functions such as ability to produce bioactive proteins, transcribe NF-κB, produce pro- and anti-inflammatory cytokines, produce antioxidants, and to function and complement each other. The four LAB have partially different functions and demonstrate, in combination, potentiating/synergistic effects. S2000F is produced by Medipharm, Kågeröd, Sweden and Des Moines, Iowa, USA. Further details regarding preparation of the bacterial solutions are set out below.

The animals were randomly divided into eight groups and received treatment as shown in Table 1.

Preparation of bacterial solution

The preparation procedure was performed at the Bacteriology Laboratory of Celal Bayar University, Manisa, Turkey. The four LAB strains were individually prepared. Stock cultures of *Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus mesenteroides* and *Pediococcus pentosaceus* strains were provided by Medipharm, Kågeröd, Sweden. Each bacterial strain was cultured by the use of the BACTEC 9120 system (Becton Dickenson, USA) and subcultures made from the BACTEC bottles onto MRS agar (Oxoid, UK). The plates were incubated overnight under micro-aerophilic conditions at 37^0C. Each bacterial colony was suspended in sterile saline in separate tubes. The bacteria were harvested after centrifugation, washed three times with saline, and re-suspended into saline with a concentration of 10^{11} bacteria per ml. The concentration of the bacteria was first estimated by spectrophotometry (UV 1601, Shimadzu Corporation, Japan) at 620 nm. The number of viable bacteria was directly determined by enumeration of developed colonies after serial dilution (10^{-2}–10^{-10}) in saline, inoculation on MRS agar plates, and incubation at 37^0C for 24 hours. The concentration in the original culture was determined by multiplying the dilution factor of those

Table 1. Administration of LAB or control to eight groups of rats

Group	Administered	Dose	Method	Timing and frequency
Group 1	Composition of *Lactobacillus paracasei, Lactobacillus plantarum,saline, Lactobacillus mesenteroides* and *Pediococcus pentosaceus*	10^{11}	Dissolved in 1 ml administered subcutaneously	One day before CLP
Group 2	Composition of *L. paracasei, L. plantarum, L. mesenteroides* and *Pediococcus pentosaceus*	10^{11}	Dissolved in 1 ml saline administered subcutaneously	Three days before CLP
Group 3	1 ml saline		Administered subcutaneously	One day before CLP
Group 4	1 ml saline		Administered subcutaneously	Three days before CLP
Group 5	Composition of *L. paracasei, L. plantarum, L. mesenteroides* and *Pediococcus pentosaceus*	10^{11}	Dissolved in 1 ml saline, administered subcutaneously	One day before surgery
Group 6	Composition of *L. paracasei, L. plantarum, L. mesenteroides* and *Pediococcus pentosaceus*	10^{11}	Dissolved in 1 ml saline, administered subcutaneously	Daily for three days before surgery
Group 7	Served as a CLP sham group			
Group 8	Served as a negative sham group			

plates. To obtain the bacterial mixture with 10^{11} CFU/ ml, 0.25 ml of bacterial solution was taken from each of four tubes and then mixed into one tube. Stock cultures of bacteria were maintained on MRS agar at 4^0C. When the assay was performed, the bacteria were revived and the procedures were repeated with young cultures.

Surgical technique

All animals were anaesthetised with intra-muscular 75 mg/kg ketamine and the peritoneal cavity opened by a 3-cm midline incision. Lung injury was produced using a modification of the caecal ligation and puncture (CLP) technique described by Chaudry *et al.*[1] The cecum was identified and exteriorised through the incision. The avascular portion of the mesentery was sharply incised and the cecum was ligated just below the ileocecal valve with a 3–0 silk suture, so that intestinal continuity was maintained. Using an 18-gauge needle, the cecum was perforated in two locations on the antimesenteric surface, the colon positioned in the abdomen and the incision closed. All rats were destroyed 24 hours after CLP.

Histological examination

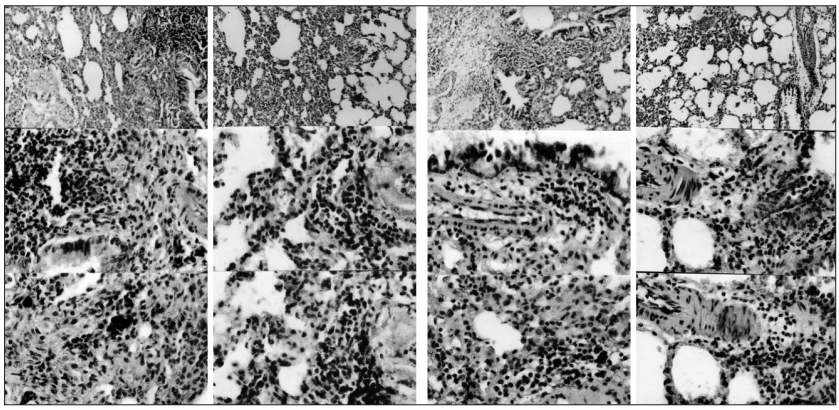
The left lung was excised and fixed in a solution of 10% formalin over 24 hours. Then they were embedded in paraffin using a routine processing protocol. Sections (5 mm thick) were cut and prepared for both histochemical and immunohistochemical techniques. After being deparaffinised in xylene and rehydrated in sections, they were stained with HE and primary antibodies; anti-TNF-a (1/100, Santa Cruz SC-7317), anti IL-1b (1/100, Santa Gruz , SC-1252). Afterwards, sections were incubated with biotinylated IgG (Dako) and then with streptavidin-peroxidase conjugate (Dako). After washing with PBS, sections were incubated with a solution containing 3-amino-9-ethylcarbazole (Dako) for 5 min to visualise immunolabelling and then with Mayer's haematoxylin. Sections were then analysed light-microscopically with an Olympus BX 40 microscope (Olympus, Tokyo, Japan). Control samples were processed in an identical manner, but the primary antibody was omitted. Two observers, blinded to clinical information, evaluated the staining scores independently. Staining intensity was graded as mild (+), moderate (++) and strong (+++), respectively.

The slides were first reviewed at low magnification for an overview to exclude sections containing bronchi, connective tissue, large blood vessels and areas of confluent atelectasis; only regions reflecting the degree and stage of parenchymal injury were evaluated. The areas of the slides not excluded were assessed at magnification (100x). Five power fields (PF) were randomly sampled. The number of neutrophils were noted in each of the five PFs (5PF). The total number of neutrophils was counted in each of the five PFs and expressed as the total number/5PF for each animal.[2] All data were expressed as means ± SEM.

BIOCHEMICAL ANALYSES

Myeloperoxidase (MPO) assay

Lung tissue was washed twice with cold saline solution, snap-frozen using liquid nitrogen to -70°C and stored until biochemical evaluation at -80°C. It was homogenised at pH 6 in ice-cold 50 mM potassium phosphate buffer, containing 0.5% hexade-



1A. 1B. 1C. 1D.

Figure 1A (group-1), Figure 1B (group-2), Figure 1C (group-3), Figure 1D (group-8). *HE (upper) and Immunohistochemical (TNF-a and IL-1ß) staining of lung tissues. Fig 1A CLP+3-day LAB, Fig 1B CLP+1-day LAB, Fig 1C 3-day saline, Fig 1D CLP+sham.*

cyltrimethyl ammonium bromide and centrifuged for 2 min at 5000 rpm. The homogenate was then centrifuged at 5000 x *g* for 60 min at +4°C and the supernatants used for further analysis. The MPO activity was determined using 25 mM 4-aminoantipyrine/2% phenol solution as substrate for MPO-mediated oxidation by 1.7 mM H_2O_2. Changes in absorbance were recorded at 510 nm per minute. One unit of MPO activity is defined as the enzyme activity which degrades 1 µµmol H_2O_2/ min at 25ºC. Data are presented as U/g lung tissue.[3]

MDA assay

Lipid peroxidation in the lung tissue was determined by measuring the level of MDA, the end product of lipid peroxidation. Then 20 mg of the dried-frozen tissue homogenate was dissolved in 1.5 ml of cold saline solution containing 0.001% butylated hydroxytoluene and 0.07% sodium dodecyl sulfate, with a Potter-type glass homogeniser. The saline butylated hydroxytoluene solution had been aerated with nitrogen gas for 10 min before each use. The dissolved homogenate was treated with ethanol/chloroform (3:2) to remove remaining haemoglobin. Malondialdehyde was assayed as described by Shin *et al*.,[4] using a calorimetric reaction with thiobarbituric acid, and expressed as nmol/mg protein. The protein concentration of the homogenate was determined by the method of Lowry *et al*.[5]

DATA ANALYSIS

All values in the text are expressed as mean ±SEM. The results were analysed by one-way-ANOVA analysis of variance. A value of $p < 0.05$ was considered as significant.

RESULTS

Histology and immunohistology

Caecal ligation and puncture caused marked leukocyte infiltration and immunostaining (IL-1ß TNF-α) consistent with acute lung injury (Figure 1C, Table 2) in the control groups (groups 3,4,7). These pathological changes were significantly reduced in the LAB-treated group (Figure 1B, Table 2). The neutrophil infiltration was significantly more reduced by pretreatment for three days compared to only one day of pretreatment ($p < 0.01$).

MPO (myeloperoxidase) and MDA (malondialdehyde)

At 24 hours after induction of CLP, the activity of MPO, an indicator of PMN infiltration, was significantly lower in the LAB-treated groups (Table 1). There was no significant difference in means of MPO between the two LAB-treated groups ($p = 0.25$). The concentration of MDA, an indicator of the degree of lipid peroxidation, was significantly less increased in the LAB-treated groups (Table 1). There was no significant difference in means of MDA between the two LAB-treated groups ($p = 0.9$).

DISCUSSION

Multiple organ dysfunction syndrome (MODS), a progressive deterioration in function of several organ systems, is frequently seen in more extensive — often multiple — trauma, shock, burns and in severe pancreatitis.[13,14] Rapidly progress-

Table 2. Analyses of results of lung injury measurements (Mean ±SEM)

Groups	Group	MPO mU/g protein	MDA nmol/mg protein	IL-1ß	TNF-α
1-day LAB+CLP	1	61,63±8,61 **	0,57±0,22 *	+	+
3-day LAB+CLP	2	45,52±5,59 **	0,55±0,15 *	+	±
1-day saline+CLP	3	107,79±13,86	0,59±0,19	++	++
3-day saline+CLP	4	109,08±14,64	0,62±0,32	++	+++
1-day LAB	5	24,81±2,38	0,67±0,16	+	±
3-day LAB	6	26,68±1,73	0,65±0,13	+	+
Sham+CLP	7	108,42±15,250,9	0±0,11	+++	++
Sham (negative)	8	25,43±2,57	0,59±0,11 *	+	+

** p <0.05 vs group 7, ** p < 0.05 vs groups 3, 4, 7, * p <0.05 vs group 7*
CLP= cecal ligation and puncture; LAB=lactic acid bacteria; MPO = myeloperoxidase; MDA = malonaldehyde

ing cases of MODS are often first detected in dysfunction of the lungs. The most common early (within 24 hours) single organ failure is pulmonary (91%,[15] 81%[16]), followed by impaired renal function (4.5%,[15] 5%[16]) and impaired coagulation (4.5%,[15] 14%[16]). If the early superinflammation in these systems can be prevented or made transient it will significantly contribute to a more favourable outcome.

Central to pulmonary dysfunction is priming of the airway endothelial cells, induced by release of free oxygen radicals, and characterised by alveolar endothelial cell injury, increased capillary permeability and hypoxia. An early event is activation and migration of neutrophils into the lung tissues, causing damage to lung tissue due to release of neutrophil-associated products: reactive oxygen species, proteolytic enzymes and various eicosanoids.

The American College of Chest Physicians and Society of Critical Care Medicine published in 1992 special guidelines for use of innovative therapies in sepsis.[17] Extensive attempts have also been made in recent years to prevent lung dysfunction and subsequent MODS, including improved modalities for early resuscitation, antioxidants,[18,19] antiadhesion molecules,[20] and various anti-inflammatory agents.[21-23] Most of the attempted treatments were performed on experimental animals and the improvement in outcome was most often modest. However, a recently published study carried out among critically ill, mainly trauma, patients reports a 19% reduction in pulmonary morbidity and an impressive 57% lower incidence of MOF in a group of patients receiving supplementation with α-tocopherol and ascorbate.[24] Glutathione is an important antioxidant, which is both synthesised by the body, and supplied from foods, mainly fruits and vegetables. Levels are significantly decreased after surgery as well as in the critically ill, a change associated with impaired lymphocyte and neutrophil function. Glutamine, an important substrate for production of glutathione,[25] has been shown in experimental studies to reduce cytokine release, organ damage and mortality.[26] Supplementation with glutamine, trialled in several controlled clinical studies performed in recent years, has also shown benefits such as reduced mortality, length of stay and morbidity in critically ill patients.[27] A recent experimental study in patients with major burns found, following supplementation with *N*-acetylcysteine, a significant increase in tissue glutathione accompanied by significant reductions in both MDA and MPO.[28] It was also recently reported that preoperative enteral supplementation with glutamine or glutamic acid attenuated intestinal and lung damage in rats following manipulation of intestines during surgery.[29]

Most of the attempts to use antioxidants to control superinflammation has been carried out with traditional vitamins: the many times stronger polyphenol and flavonoid antioxidants have not yet been tried. The effect of intravenous curcumin, a strong natural antioxidant and natural NF-κB, COX-2, LOX and iNOS inhibitor, on reduced damage to the pancreas was studied in two different pancreatitits models: cerulein-induced and ethanol-and-CCK-induced pancreatitis.[30] A significant reduction of histological injuries and tissue neutrophil infiltration was observed, accompanied by reduced activation of NF-κB, I κB degradation, activation of activator protein (AP)-1, and significant reductions in various inflammatory molecules such as IL-6, TNF-α, chemokine KC, iNOS and acidic ribosomal phosphoprotein (ARP). It was specially observed in both models that curcumin significantly stimulated activation of caspase-3.

The focus in recent years has been changing from combating infections to preventing the exaggerated inflammation accompanying severe emergencies. It was recently suggested that 'the challenge in critical illness is less the infection than the exuberant inflammatory response'.[8] Superinflammation precedes and paves the way for the subsequent infection, as demonstrated in a study in liver transplant patients. Patients who, in the late stage of operations, demonstrated a 6 times increase in TNF-α and IL-6 would all in the subsequent days develop signs of septic complications.[31] More recent studies suggest a key role of NF-κB in the pathogenesis of sequelae of inflammation,[32,33] as well as in the associated inhibition of neutrophil apoptosis.[34,35]

Immune suppression, signs of hyperinflammation and suppressed expression of monocyte histocompatibility leukocyte antigen (HLA)-DR, all strongly associated with subsequent development of septic complications and poor outcome, occur early in the disease process.[36-38] Much evidence supports the existence of a therapeutic window of about 24 hours if more profound modulation of the inflammatory cascade is expected. Immuno-supportive treatment should thus be instituted, if not possible before, then from the earliest possible moment after the injury/induction of disease.

The collective genome of microbes with which we live in symbiosis contains about 100 times more genes than our own human genome.[39] The intestinal flora, microbiota, is clearly a large metabolic organ of extensive importance for release and absorption of numerous nutrients and antioxidants, for regeneration and growth of various human cells, especially mucosal cells, for the function of the innate, but also adapted, immune system and for control or reduction of various toxic and mutagenic agents and potentially pathogenic micro-organisms including viruses and fungi. Recent observations also suggest that microbionta plays a key role in the regulation of inflammation and immune response not only in the gut but in the whole body[40] — see further a recent review by Bengmark.[41] In people with Western lifestyle, incidence of such flora is significantly reduced already before trauma,[42] and detoriorates further very quickly after trauma.[43-46] A recent study found no LABs at all in the faeces of ICU patients, but also that the levels of LABs could be returned to normal by supplementing with LAB and plant fibres.[47]

Enteral supplementation is often difficult in the critically ill, which warrants the use of parenteral nutrition. The effects in attenuating both murine colitis and murine arthritis[11] encouraged us to perform the present study. Clearly the impressive effects in prevention of neutrophil accumulation, lung tissue destruction and in inhibition of MDA and MPO are not inferior to what we have observed in other studies after oral supplementation of LAB and plant fibres. However, the animals in this study were pretreated for one and three days before the induction of trauma. Clearly significantly more powerful effects were obtained with longer pretreatment. Most trauma are not foreseeable and it is yet not known if similar effects can also be obtained if instituted early after the induction of trauma.

Neutrophil infiltration of the lungs is strongly associated with severity of disease and outcome, both in acute illnesses such as acute pancreatitis, and in chronic diseases such as cystic fibrosis (CF). Inflammation in CF is caused by persistent bacterial infection in the lung from *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* and is characterised by the persistent infiltration of massive numbers of neutrophils, which leads to lung injury. In addition, neutrophils from CF patients tend spontaneously to generate higher levels of superoxide and to release more elastase than control neutrophils. Reduction or prevention of lung neutrophil infiltration has the potential to trigger a dramatic improvement of disease and outcome both in acute illness and chronic lung disease. For this parenteral administration of specific lactic acid bacteria might offer an effective tool.

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