Pretreatment With Pro- and Synbiotics Reduces Peritonitis-Induced Acute Lung Injury in Rats

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Background: **To study whether enteral pretreatment with a synbiotic composition of lactic acid bacteria and bioactive fibers can reduce peritonitis-induced lung neutrophil infiltration and tissue injury in rats.**

Materials and Methods: **Rats were divided into five groups, and subjected to induction of peritonitis-induced lung injury using a cecal ligation and puncture model (CLP). All animals were pretreated for 3 weeks prior the CLP by daily gavage with either (1) a synbiotic composition (10^{10} CFU of *Pediococcus pentosaceus* 5-33:3, 10^{10} CFU of *Leuconostoc mesenteroides* 77:1, 10^{10} CFU of *L. paracasei* subspecies paracasei, 10^{10} CFU of *L. plantarum* 2362 plus fermentable fibers), (2) fermentable fibers alone, (3) nonfermentable fibers, (4) a probiotic composition (10^{10} CFU of *P. pentosaceus* 5-33:3, 10^{10} CFU of *L. mesenteroides* 77:1, 10^{10} CFU of *L. paracasei* subsp. paracasei, 10^{10} CFU of *L. plantarum* 2,362), or (5) a heat-killed probiotic composition. All animals were killed 24 hours after CLP and lung tissue samples were studied for degree of neutrophil infiltration and levels of tumor necrosis factor (TNF)-α, Interleukin (IL)-1β. In addition the lung wet-to-dry tissue weight ratio, the myeloperoxidase activity, and malondialdehyde content were also assessed.**

Results: **No mortality was encountered in any of the groups. Histologic signs of lung injury (number of neutrophils and TNF-α, IL-1β staining) were observed in all groups except the synbiotic and probiotic treated groups. Myeloperoxidase activity and malondialdehyde content were significantly lower in the two lactobacillus-pretreated groups, with no difference between them. Heavy infiltration of lung tissue with neutrophils was observed only in fiber-treated (302.20 ± 7.92) and placebo-treated (266.90 ± 8.92) animals. This was totally abolished in the synbiotic-treated group (34.40 ± 2.49). Lung edema (wet-to-dry lung weight ratio) was significantly reduced in the synbiotic-treated group (4.92 ± 0.13 vs. 5.07 ± 0.08 and 5.39 ± 0.10, respectively).**

Conclusion: **Three weeks of preoperative enteral administration of a synbiotic composition reduced peritonitis-induced acute lung injury in rats in a CLP model.**

Key Words: **Acute lung injury, Cecal ligation and puncture, Peritonitis, Synbiotics, Probiotics, Prebiotics.**

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Excessive tissue infiltration and accumulation of neutrophils is a common phenomenon in conditions such as shock, sepsis, major trauma, major burns, and severe acute pancreatitis. It can lead to paralytic ileus,[1,2] tissue destruction, and organ failure particularly in the lungs,[3–5] intestines,[6] liver,[7] and kidney.[8] Neutrophilic infiltration of distant organs,[9] especially of the lungs[3] is associated with septic death and has been suggested to be a consequence of "generalized autodestructive inflammation".[9] The extent of infiltration is aggravated by mechanical therapeutic efforts such as handling of the bowels during an operation,[1] and positive pressure ventilation of the lungs.[10] The most common, and often most severe, clinical manifestations of organ failure are seen in the lungs. In severe acute pancreatitis, the organ systems most often involved in early (within 24 hours) organ failure are pulmonary (91%,[11] 81%[12]), renal (4.5%,[11] 5%[12]), and coagulation (4.5%,[11] 14%[12]).

Acute lung injury is characterized by alveolar capillary endothelial cell injury, increased capillary permeability, and subsequent hypoxia, induced by neutrophil-associated inflammatory products. These products include reactive oxygen species, proteolytic enzymes, eucosanoids, and various other mediators of the inflammatory response. Neutrophil-mediated tissue injury to the lung is thought to be initiated by splanchnic hypoperfusion with subsequent endothelial cell injury. This leads to increased expression of intercellular adhesion molecule-1,[13] as well as to the release of serine proteases by the hypoxic pancreas.[14] In addition, mesenteric lymph and transport of toxic lymph within the lymphatic system,[13–15] as well as circulating cytokines,[16] contribute to the damage in the lung.

Significant reduction of inflammation and infection by treatment with a combination of lactic acid bacteria (LAB) and plant fibers (synbiotic treatment) has been documented both experimentally[17,18] and clinically: in severe acute pancreatitis,[19] liver transplantation,[20,21] and extensive operations.[22] We demonstrated in a recent study that 3 days pretreatment with repeated subcutaneous injections of live LAB abolished the lung injury induced by cecal ligation and puncture (CLP).[23] The goal of this study is to investigate whether oral pretreatment with LAB and fibers

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is equally effective in preventing neutrophil-induced lung injury in sepsis.

METHODS

The study was conducted in Ege University Faculty of Medicine, Experimental Animal Research Laboratory. The local ethical committee of the Celal Bayar University Hospital approved the experimental protocol. Fifty male adult Wistar albino rats weighing 250 to 300 g were randomly selected. They were divided into five groups. All the animals had free access to water and a standard rat chew containing 4.5% plant fibers.

The animals were randomly divided into five groups and received the following treatments daily, by gavage, each dissolved in 1 mL of saline, beginning 3 weeks before CLP:

Group-1: 100 mg of Synbiotic 2000 (Medipharm, Kågeröd, Sweden)[24,25] consisting of 10^{10} CFU of *Pediococcus pentoseceus* 5-33:3, 10^{10} CFU of *Leuconostoc mesenteroides* 77:1, 10^{10} CFU of *L. paracasei* subsp. paracasei, and 10^{10} CFU of *L. plantarum* 2,362 + 80 mg of four fermentable fibers (betaglucan, inulin, pectin, and resistant starch). The LAB for the composition were to a large extent selected to be used in the composition for their unique ability to transcribe nuclear factor (NF)-κB.[24]

Group-2: 80 mg of four fermentable fibers (betaglucan, inulin, pectin, and resistant starch).

Group-3: 80 mg of a nonfermentable fiber (crystalline cellulose) administered by gavage during the 3 weeks before the surgery.

Group-4: 100 mg of Probiotic 2000 (Medipharm) 10^{10} CFU of *P. pentosaceus* 5-33:3, 10^{10} CFU of *L. mesenteroides* 77:1, 10^{10} CFU of *L. paracasei* subsp. paracasei, and 10^{10} CFU of *L. plantarum* 2,362.

Group-5: 100 mg of X-ray-killed Probiotic 2000 (Medipharm) 10^{10} CFU of *P. pentosaceus* 5-33:3, 10^{10} CFU of *L. mesenteroides* 77:1, 10^{10} CFU of *L. paracasei* subsp. paracasei, and 10^{10} CFU of *L. plantarum* 2,362.

Surgical Technique

All animals were anesthetized with intramuscular 75 mg/kg ketamine and the peritoneal cavity opened by a 3-cm midline incision. Lung injury was produced using a modification of the CLP technique described by Chaudry et al.[26] The cecum was identified and exteriorized through the incision. An avascular portion of the mesentery was sharply incised and the cecum was ligated with a 3–0 silk suture just below the ileocecal valve, maintaining intestinal continuity. The cecum was perforated with an 18-gauge needle in two locations on the antimesenteric surface, left open, and the abdominal incision was closed. All rats were killed 24 hour after CLP.

Histologic Examination

The left lung was excised and fixed in a solution of 10% formalin for 24 hours and embedded in paraffin for routine histology. Five-millimeter-thick sections were cut and prepared for histochemical and immunohistochemical studies. The sections were stained after deparaffinization in xylene and rehydration with hematoxylin-eosin and primary antibodies: anti-tumor necrosis factor (TNF)-α (1/100, Santa Cruz SC-7317) or anti-Interleukin (IL)-1β (1/100, Santa Cruz, SC-1252). The sections were then incubated with biotinylated immunoglobulin (Ig)G (Dako) followed by streptavidin-peroxidase conjugate (Dako), washed with phosphate buffer solution (PBS), and incubated for 5 minutes with a solution containing 3-amino-9-ethylcarbazole (Dako) aimed to visualize immunolabeling followed by incubation with Mayer's hematoxylin. The sections were studied under light microscopy using an Olympus BX 40 microscope (Olympus, Tokyo, Japan) (Fig. 1). Control samples were processed in an identical manner using only secondary antibodies. Two observers, blinded to clinical information, evaluated the staining independently and graded the sections as mild (+), moderate (++), or strongly (+++) staining for individual antigens.

The slides were reviewed at low magnification to exclude sections containing bronchi, connective tissue, large blood vessels, and areas of confluent atelectasis. Only regions dominated by lung parenchyma were chosen to evaluate stage and degree of parenchymal injury. The chosen areas were assessed under magnification (100×) in the following manner: five power fields (PFs) were randomly sampled; the total number of neutrophils were counted in each of the five PFs and expressed for each animal as the total number/5 PF.[27] All data were expressed as mean ± SEM.

Wet–Dry Weight Ratio

The right lung was excised and the wet-to-dry (W/D) lung weight ratio was evaluated.[28] Representative tissue samples from the right lung were sharply dissected free of nonparenchymal tissue. Samples were placed in a dish and weighed, dried in an oven at 65°C for 24 hours, and weighed again. This was repeated until there was no weight change during a 24-hour period, at which time the samples were determined to be dry. Lung water was expressed as a W/D weight ratio.

Biochemical Analyses

Myeloperoxidase Assay

Lung tissues were washed twice with cold saline solution, and snap frozen in liquid nitrogen and stored at −80°C until biochemical evaluation. Lung tissues were homogenized in ice-cold 50 mmol/L potassium phosphate buffer pH 6, with 0.5% hexadecyltrimethyl ammonium bromide for 2 minutes at 5,000 rpm. The homogenate was then centrifuged at 5,000×g for 60 minutes at 4°C. Myeloperoxidase (MPO) activity in the supernatant was determined using 25 mmol/L 4-aminoantipyrine/2% phenol solution as the substrate for MPO-mediated oxidation by 1.7 mmol/L H_2O_2. Changes in absorbance were recorded at 510 nm. One unit of MPO activity is defined as the enzyme activity which degrades 1 μmol H_2O_2/min at 25°C. Data are presented as U/g lung tissue.[5]

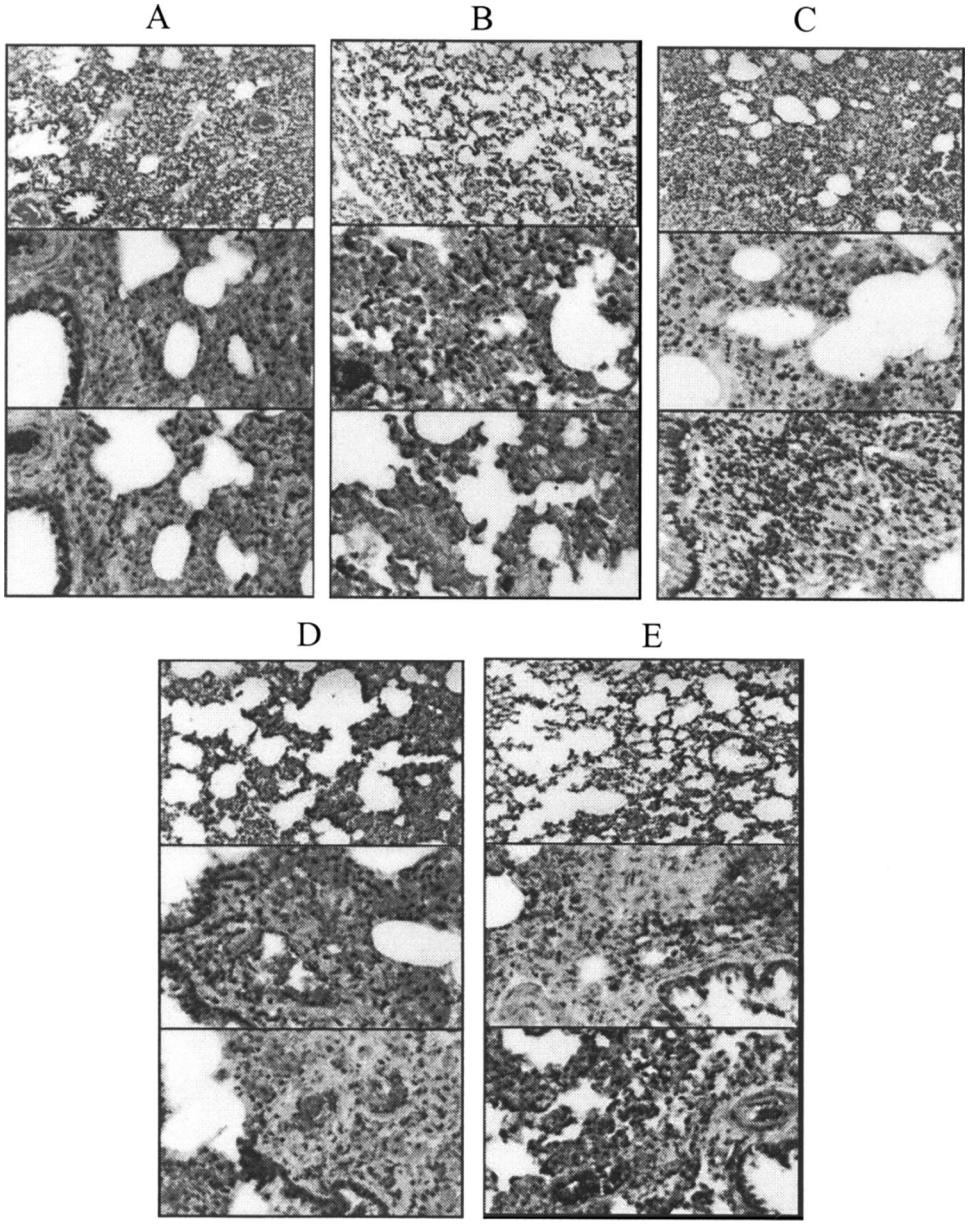

Fig. 1. *Hematoxylin-eosin (*upper*) and immunohistochemical (TNF-α and IL-1β, respectively) (*lower*) staining of lung tissues from group 1 (*A*), group 2 (*B*), group 3 (*C*), group 4 (*D*), and group 5 (*E*).*

Malondialdehyde Assay

Lipid peroxidation in the lung tissue was determined by measuring the level of malondialdehyde (MDA), which is an end product of lipid peroxidation. Twenty milligrams of dried-frozen tissue was homogenized in 1.5 mL of cold saline solution containing 0.001% butylated hydroxytoluene and 0.07% sodium dodecyl sulfate, using a Potter-type glass homogenizer. The saline-butylated hydroxytoluene solution was aerated with nitrogen gas for 10 minutes before each use. The homogenate was treated with ethanol/chloroform (3:2) to remove hemoglobin. MDA was assayed using a calorimetric reaction with thiobarbituric acid. The protein concentration of the homogenate was determined by the method of Lowry et al.[7] The MDA concentration was expressed as nmol/mg protein.

Nitric Oxide Assay

Tissue nitrite (NO_2) and nitrate (NO_3) have been used as an index of nitric oxide (NO) production. Therefore, we measured lung tissue samples for the concentrations of the stable NO oxidative metabolites (NO_2 and NO_3). Quantitation of nitrate and nitrite was based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthlethylenediamine and sulfanilamide.[29] Samples were deproteinized with Somogyi reagent. For total nitrite detection, an aliquot of

the sample was treated with copporized cadmium in glycine buffer at pH 9.7 to reduce nitrate to nitrite. After cleanup, the sample was mixed with fresh reagent and the absorbance was measured in a spectrophotometer to give the total nitrite concentration. A standard curve was established with a set of serial dilutions of sodium nitrite. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as μmol/g wet tissue.

Data Analysis

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

RESULTS

No mortality was encountered in any of the groups 24 hours after CLP.

Histology and Immunohistology

CLP caused marked leukocyte infiltration and immunostaining (IL-1 β, TNF-α) consistent with acute lung injury (Fig. 1B, C, and E and Table 1) in control groups (groups 2, 3, and 5). These pathologic changes were significantly reduced in the LAB-treated groups (groups 1 and 4) (Fig. 1A and D and Table 1).

MPO and MDA

At 24 hours after induction of CLP, the activity of MPO, an indicator of polymorphonuclear (PMN) infiltration, was significantly lower in the LAB-treated groups (Table 1). There was no significant difference in means of MPO between the LAB-treated groups and prebiotic-treated group (groups 1, 2, and 4) (Table 1).

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 and between the probiotic-treated and the X-ray killed probiotic groups (groups 4 and 5, Table 1).

Nitric Oxide

The concentration of NO was significantly less in the LAB-treated groups (Table 1) than in the other groups. There was no significant difference in means of NO between the two LAB-treated groups (Table 1).

Lung W/D Weight Ratio

CLP caused a significant increase in lung water (expressed by W/D weight ratio) in the control groups (groups 3 and 5) compared with synbiotic, probiotic, and prebiotic-treated groups (Table 1).

DISCUSSION

The ability of lactic acid bacteria in combination with plant fibers (synbiotics) to abolish or reduce inflammation and infection are well documented in models such as acetic acid induced colitis,[30] methotrexate-induced enterocolitis,[31] galactosamine-induced acute liver injury,[32–34] and CLP.[7] Bacteremia after CLP was reduced by 50% and endotoxins reduced by 47% after 24 hours in the LAB-pretreated rats, in sharp contrast to gentamycin, which had no effect.[7] Furthermore, intestinal transit was significantly enhanced in the synbiotic-treated animals.

The normal flora has pronounced effects on numerous body functions, including regulation of gastrointestinal motility, various immune functions, and resistance to disease (see further Bengmark et al.[35–37]). However, a significant reduction of the commensal flora occurs in the hospitalization process, because of both disease and pharmaceutical treatment. For example, in experimental pancreatitis, anaerobic bacteria and lactobacilli are significantly reduced already within 6 to 12 hours after induction of pancreatitis both in the distal small bowel and in the colon. These changes are almost instantly followed by significant overgrowth with potentially pathogenic microorganisms such as *Escherichia coli*, and dramatic increases in mucosal barrier permeability (lumen to blood) and in endothelial permeability (blood to tissue).[38,39] This is associated with increased microbial translocation and microbial growth in mesenteric

Table 1 Analysis Results of Lung Injury Measurements (Mean ± SEM)

Group	Wet–Dry Ratio	Neutrophils	MPO	MDA	NO	IL-1β	TNF-α
1	5.32 ± 0.21*	9.00 ± 0.44†	25.62 ± 2.19‡	0.22 ± 1.31§	17.16 ± 2.03¶	+	±
2	6.04 ± 0.36*	31.20 ± 0.98†	56.59 ± 1.73‡	0.48 ± 5.32	47.71 ± 3.20‖	++	+++
3	7.80 ± 0.44	51.10 ± 0.70	145.53 ± 7.53	0.67 ± 2.94	66.22 ± 5.92	+++	+++
4	5.71 ± 0.53*	8.40 ± 0.42†	26.75 ± 2.61‡	0.28 ± 3.55#	18.91 ± 2.24¶	+	+
5	9.37 ± 0.69	52.10 ± 1.44	109.58 ± 16.38	0.43 ± 2.77	71.82 ± 5.96	++	+++

* $p < 0.05$ vs. groups 3 and 5.
† $p < 0.05$ vs. groups 3 and 5.
‡ $p < 0.05$ vs. groups 3 and 5.
§ $p < 0.05$ vs. groups 2, 3, and 5.
¶ $p < 0.05$ vs. groups 2, 3, and 5.
‖ $p < 0.05$ vs. group 5.
# $p < 0.05$ vs. groups 2 and 3.
MPO, myeloperoxidase; MDA, malonaldehyde; NO, nitric oxide.

lymph nodes and pancreatic tissue.[40] A recent study demonstrates a total loss of LAB in most ICU patients, and that it can successfully be replaced by supplementation of synbiotics.[41]

We reported previously that the pretreatment with subcutaneous injection of the four LAB species prevented the CLP-induced acute lung injury.[23] In that study, as in the present study, synbiotic-treated animals have significantly reduced inflammation and tissue destruction compared with controls. The effects obtained in the present study from 3 weeks of daily oral supplementation with LAB and fiber were equal to those observed after 3 days of a parenteral supply of live LAB.

NO is known to be a crucial mediator of the inflammatory response, but its in vivo role as a determinant of lung inflammation and tissue destruction remains unclear.[42,43] However, recent studies suggest a key role in neutrophil migration in sepsis,[42,44] neutrophil sequestration,[43,45] and oxidant action. Reduced levels of NO in the LAB-pretreated animals revealed a possible role of NO in lung inflammation. Further studies are needed to clarify the mode(s) of action of NO in sepsis and prevention of lung destruction and dysfunction.

Inflammation and tissue destruction is an early phenomenon in trauma. Multiple studies support the hypothesis that there is a rather limited therapeutic window to reduce the inflammation and prevent subsequent manifestations such as posttrauma infections, venous thrombosis, and development of serosal adhesions. There are indications that treatment must be initiated either before or within 24 to 48 hours of the injury. Clearly, the best results are obtained from preoperative or immediate synbiotic modulation of inflammation, but significant effects are still observed if instituted within 48 hours after trauma. The best results are observed in elective surgery when probiotic/synbiotic treatment can be initiated before or immediately in connection with trauma. This is exemplified by the experience mentioned in a study of liver transplantation. Sixty-six patients were randomized to either receive Synbiotic 2000 or only the fibers in Synbiotic 2000 in connection with human orthotopic liver transplantation.[21] The treatment started on the day before surgery and continued for 14 days after surgery. During the first postoperative month, only 1 out of 33 patients in the Synbiotic 2000-treated group (3%) showed signs of infection (urinary infection) compared with 17 out of 33 (51%) in the patients supplemented with only the four fibers.[21] The use of antibiotics was in average 0.1 ± 0.1 days in the synbiotic-treated patients and 3.8 ± 0.9 days in the fiber only-treated group.

Similarly, good results are obtained both in a recent, yet unpublished, study where synbiotic treatment was instituted in a study of 113 patients with multiple injuries (Spindler-Vesel et al., personal communication), and in another yet unpublished multicenter study in 65 multiple injury patients (Kotzampassi et al., personal communication). For example, in the last-mentioned study, synbiotic treatment significantly reduced the rate of infections, systemic inflammatory response syndrome (SIRS), severe sepsis, and mortality. The number of days stayed in the ICU and days under mechanical ventilation were also significantly reduced in relation to controls (Kotzampassi K et al., personal communication).

Patients with severe acute pancreatitis often arrive several hours after the onset of disease, such that early treatment is most often impossible. In a recent study, sixty-two patients with severe acute pancreatitis supplemented for 14 days with either Synbiotic 2000 or only the same amounts of fibers, beginning at the latest within 48 hours after onset of disease. Nine of 33 patients (27%) in the Synbiotic 2000-treated group and 15 out of 29 patients (52%) in the fiber only-treated group developed subsequent infections. Eight of 33 (24%) Synbiotic 2000-treated and 14 of 29 (48%) of the fiber-treated patients developed SIRS, multiple-organ failure (MOF), or both ($p < 0.005$) (Olah, personal communication). Although less dramatic than in liver transplantation, the effects of synbiotic treatment were still significant in this group of patients.

One can assume that effects in ICU patients who have already passed the acute phase of trauma and are already in a phase with deep immunoparalysis will be less significant. Early observations in a controlled study with synbiotic treatment in a general ICU population of 270 patients support such an assumption (Knight et al., personal communication).

Numerous attempts have been made in the past to control the posttrauma exaggerated inflammation and subsequent uncontrolled tissue infiltration but most often with limited or no success. Synbiotic treatment is a promising alternative and worth further exploration. It has significant efficacy without side effects and can be provided at limited costs. To pretreat for a period as long as 3 weeks, as done in this experimental study, might not be necessary. It is highly possible that, as was the case with parenteral pretreatment, 3 days or less could be enough. However, in most emergency situations, pretreatment is not possible, regardless of route of administration. In addition, enteral supply is not always possible. Parenteral supply might in such situations offer a better alternative. Future studies are necessary to elucidate that.

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