



Lactoferrin Regulates the Immune Responses in Post-Surgical Patients

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Abstract. The effect of oral administration of lactoferrin (LF) was studied to determine if it could modify post-surgical immune response. The action of LF was evaluated in 18 LF-treated patients vs 28 placebo counterparts. Patients (women and men, mean age 50 years) were given daily oral doses (20 mg each) of LF for 5 consecutive days prior to thyroid surgery. The following immune response parameters were determined in blood samples taken from the patients day before, day after, and 5–7 days following surgery: cell morphology, the proliferative response of peripheral blood mononuclear cells to phytohemagglutinin, and the spontaneous and lipopolysaccharide (LPS)-induced production of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6). As a consequence of the thyroid surgery, the total leukocyte count increased on the post-operative day by about 50% in all patients and the percentage of lymphocytes fell by 26 and 35% in the control vs LF-treated group. The content of neutrophils, on the other hand, elevated on day 1 post-operation by 51 and 68%, respectively. The percent of neutrophil precursors was markedly higher in LF-treated patients, particularly on the day before and the day after surgery (4.1 and 4.8 vs 2.5 and 3.7%, respectively). The post-surgical values were, however, comparable in both groups for neutrophils. The proliferative response of lymphocytes showed a slight decrease in the control group and an increase in the LF-treated patients on day 5 post-operation (20% over control group). LPS-induced TNF- α production was higher in LF-treated patients both one day before and one day following surgery (28 and 24%, respectively). LPS-induced IL-6 production was comparable in both placebo and LF-treated patients before surgery, however, on day 1 and 5 following surgery, the production of IL-6 was higher in LF-treated patients by 65 and 27%, respectively. Taken together, the data presented in this study revealed an increased immune responsiveness in all patients treated with LF and subjected to thyroid surgery. This suggests that treatment with LF could constitute an effective protective measure against post-surgical complications.

Key words: lactoferrin; clinical insult; immunoregulation; prevention.

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Introduction

Clinical interventions alter the natural defenses. In addition, invasive procedures that breach the external barrier of the skin provide added portals of the entry for opportunistic and pathogenic organisms. Therefore, despite tremendous advances made in both the understanding and management of critical illness and injury over the last decades, mortality remains high and, in some cases, virtually unchanged from the time of their initial description.

The problem is predominantly generated by post-surgical complications leading to various forms of systemic inflammation, shock and organ failures^{3, 26, 28, 30}. The severity of such complications often increases when patients become immunocompromised^{7, 11, 18, 34}.

Surgical procedures cause changes in several immune parameters, particularly early after surgery^{13, 16, 23, 29, 31, 36}. The extent of these changes depends on the severity of clinical insult and relates mostly to T cell reactivity, as measured by their proliferation rate and ability to produce cytokines^{22, 30, 31, 36}.

All the hitherto investigated strategies aimed at diminution of post-surgical complications have proved, however, unsuccessful¹. Moreover, intervention in the cascade of mediators of inflammation and in the process of hemodynamic disturbances appears to be usually too late to stop the generalized state of hypo- or hyperreactivity^{1, 3, 27}. Therefore, much more attention has been paid recently to evaluating the immune status of surgical patients, so that adequate preventive measures can be taken¹⁷. The *in vitro* production of cytokines and the proliferative response of peripheral blood lymphocytes can be measured in all patients undergoing surgery, who may then be treated adequately. Immunomodulation, in particular a non-invasive, orally administered regimen with natural immunomodulators, seems to be the most appropriate clinical approach to alter the immune responses of patients undergoing surgery.

Numerous studies have been conducted on the potential use of lactoferrin (LF), a natural glycoprotein, to modulate the immune systems of humans and other animals^{2, 8, 25, 26, 45, 46, 48, 49}. LF is an iron-binding, 80 kDa protein found in the exocrine secretions of mammals and secondary granules of neutrophils^{8, 25}. Its serum levels increase rapidly upon infection as a consequence of its release from neutrophils by a sequential action of several inflammatory cytokines^{4, 28}. Receptors for LF can be found on many cell types including activated T and B cells, monocytes/macrophages, platelets and neoplastic cells²⁵. Of particular relevance to this clinical trial may be the existence of specific LF recep-

tors on intestinal brush border cells¹⁸ involved in the active transfer of the protein into adjacent lymphoid tissue and the circulation¹⁰. LF was found to be an important regulator and inducer of several cytokines^{9, 37, 45, 48, 49}. We also demonstrated that LF promoted maturation of T and B cells in mice^{43, 44}. The protein was also protective with regard to gut microflora³³ and gut-wall integrity²¹, in experimental sepsis³⁹ and endotoxemia^{26, 41}. Our study in mice revealed that pretreatment with LF intravenously or *per os* modulated post-surgical immune response by reducing serum levels of interleukin (IL)-6 and, to a lesser degree, tumor necrosis factor (TNF)- α ⁴⁹. Investigations were also conducted on the immune status of cardiac surgery^{36, 37} trauma and septic patients² where LF exhibited regulatory and enhancing effects on lymphocyte proliferation and production of TNF- α and IL-6 by mononuclear blood cell cultures. These studies were extended to volunteers taking bovine lactoferrin (BLF) orally^{48, 49}. In these trials we showed an increased turnover of neutrophils, regulatory effects on the magnitude of mitogen-induced lymphocyte proliferation, and preferential regulation of spontaneous but not lipopolysaccharide (LPS)-induced, cytokine production by blood cell cultures. In this report we describe the effectiveness of orally administered LF on post-surgical immunological status in patients undergoing thyroid surgery. Moreover, reported in this article are results demonstrating the mechanism by which LF protects against post-surgical complications.

Materials and Methods

Patients: treatment with lactoferrin and clinical evaluation. The trial was conducted on 46 patients scheduled for elected thyroid surgery in the years of 1997–1999 at the First Clinic of General and Endocrine Surgery and the Second Clinic of Surgery, Wrocław Medical Academy. The study was approved by the local Ethical Committee and each patient was informed of the purpose of the investigation and had given prior personal consent to participate in the trial.

The patients were randomly selected in a double-blind, placebo-controlled trial. The patients took daily oral 20 mg doses of BLF in capsules for 5 days prior to surgery. Placebo capsules contained lactose only. Heparinized blood samples were taken on the pre-operative day, one day after and 5–7 days following operation and transported immediately to the Institute of Immunology and Experimental Therapy (Wrocław, Poland) for immunological testing.

In both groups of patients, males constituted 33.3% of the total number. The average age of patients in the control group was 48 and 50 years for men and women, respectively. In the LF-treated group, the respective average ages were 47 and 48 years.

Histopathological studies of the surgically removed tissue revealed that the indication for the surgery in 62.5% of the control patients was multinodular goiter of differential size, mostly of endemic type with symptoms of trachea compression. In the LF-treated group, such patients represented 55.5% of the total number. In both groups a subtotal, subcapsular resection of both thyroid lobes was performed.

In the control group, 29.5% of patients exhibited hyperfunction of the thyroid gland, confirmed by elevated serum concentrations of thyroid hormones (fT_3 , fT_4) and lowered content of thyroid-stimulating hormone. These patients had been treated for a prolonged period with thyreostatics until clinical euthyrosis was attained. Analogously, in the LF-treated group hyperfunction of the thyroid gland was present in 38% of patients, and all patients exhibited symptoms of Grave-Basedov's disease. The state of clinical euthyrosis was attained by treatment with Methizol or Methyliouracyl. In this category of patients, a subtotal resection of both thyroid lobes, including the isthmus, was performed. In addition, 2 patients in the control group and 1 patient in the LF-treated group had a thyroid carcinoma. In the one case where the cancer was advanced a total resection of the gland was performed, while in the other cases only the invaded lobe was removed with subtotal resection of the remaining lobe.

The patients were in generally good condition and in euthyrosis. Blood transfusion and steroid treatment were not applied. The patients were routinely treated with a single dose of antibiotic 30 min before surgery. Wound healing was without complications.

Bovine lactoferrin was provided by FerroDynamics, Inc. (Houston, TX 77042, USA). It was essentially LPS-free, as determined by Amebocyte Limulus assay. The BLF was admixed in capsules with lactose. Placebo capsules contained lactose only.

Isolation of cells. Venous blood was taken into heparinized syringes on admission to the ITU, and 1, 2 and 5 days later. Blood was diluted twice with PBS and centrifuged on Ficoll-uropoline gradient (density 1.077 g/ml) at 2400 rpm, 25 min at 4°C. Cells from the interphase were washed 3 times with Hanks' medium. The cell fraction consisted of 80% lymphocytes and 20% monocytes. Cells were resuspended in a culture medium (RPMI 1649 with addition of glutamine, sodium pyruvate, 2-ME and antibiotics). For the pro-

liferation assay, 10% FCS (final concentration) was added and, for cytokine induction, 2% human AB serum.

Preparation of blood cell cultures. Heparinized blood was diluted with RPMI-1640 culture medium to achieve a concentration of 10^6 cells/ml. The cells were distributed in 2 ml aliquots to 24-well culture plates and cultured overnight in a cell culture incubator. The supernatants were harvested and used for cytokine determination.

Determination of IL-6 activity. The assay was performed according to VAN SNICK et al.³⁴. Briefly, 7TD1 indicator cells were washed 3 times with Hanks' medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics to a density of 2×10^4 cells/ml. Then, the cells were distributed in 100 μ l aliquots into 96-well, flat-bottom plates containing 100 μ l serially diluted plasma of supernatant in triplicate. After 72 h of culture, the proliferation of 7TD1 cells was determined using the MTT colorimetric method¹⁴. The results of IL-6 activity are presented in pg/ml, a concentration value of IL-6 which corresponds to the activity of IL-6 expressed in U/ml³⁴. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

Determination of TNF- α activity. For the determination of TNF- α activity, the indicator clone WEHI 164.13 was used¹². The cells were washed 3 times with Hanks' solution and resuspended in RPMI 1640 supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2×10^5 /ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4 /well). Serially diluted plasma or supernatant samples were prepared on separate plates and transferred to microtiter plates containing WEHI 164.13 indicator cells. The medium contained, in addition, 1 μ g/ml of actinomycin D to increase the sensitivity of the assay. After an overnight incubation, cell survival was determined using the MTT colorimetric assay¹⁴. The results of TNF- α activity are presented in pg/ml where 10 pg of TNF- α correspond to 1 U of activity when tested a recombinant human TNF- α (kindly provided by Prof. W. Stec, Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Łódź, Poland). One unit of TNF- α was calculated as the inverse dilution of a given plasma sample where a 50% survival of WEHI 164.13 cells took place.

Proliferation test. The cells, resuspended in a culture medium at a concentration of 10^6 /ml, were distributed into 96-well, flat-bottom culture plates in 100 μ l aliquots. PHA (5 μ g/ml) was added to induce cell proliferation. After a 3-day incubation the proliferative response was determined using the colorimetric MTT method¹⁴.

Table 1. Blood picture of the patients in the perioperative period

Cell types	Patients					
	control			LF-treated		
	Days before and after operation					
	-1	1	5	-1	1	5
	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$	$\bar{X} \pm SE$
Leukocytes/mm ³	5728±337.8	8655±499.2	6821±516.6	5992±482.9	8772±546.9	7157±367.2
Lymphocytes(%)	33.9±1.6	25.1±1.4	30.4±1.4	33.2±2.4	21.7±1.9	30.2±1.7
Neutrophils (%)	60±1.4	69±1.2	63±1.3	59±2.1	71±2.3	62±1.6
Neutrophil precursors (%)	2.5±0.3	3.7±0.4	3.4±0.5	4.1±0.8	4.8±0.9	3.9±0.7

Colorimetric determination of cell proliferation/death. The assay was performed according to HANSEN et al.¹⁴. Briefly, MTT solution, 5 mg/ml in 0.9% NaCl, was added at a volume of 25 µl/well and incubated for 2–4 h. Then, 100 µl of a lysing buffer was added (20% SDS, 50% DMF, pH 4.7). After an overnight incubation at 37°C the optical density (OD) was measured using ELISA reader Dynatech 5000, at the wavelength of 550 nm and reference wavelength of 630 nm.

Morphology of blood cells. A drop of heparinized blood was applied onto a microscopic slide and a smear was made. After drying, the preparation was treated with May-Grünwald reagent and then with Giemsa reagent. The cells (up to 200) were counted (differentiated) at a magnification of 800× in an immersion oil. The results are presented as a percentage of the following cell types in the preparation: immature forms (bands), neutrophils and lymphocytes.

Statistics. The results were evaluated using Student's *t*-test. The results are presented as mean values of the parameters studied $\pm$ standard error (SE). The differences were regarded as significant when $p < 0.05$.

Results

Changes in blood cell count and cell morphology

The total numbers of leukocytes in the peripheral blood and the percentages of lymphocytes, neutrophils and neutrophil precursors in the patients subjected to surgery are presented in Table 1. In response to surgery, the leukocyte counts increased on the next day by 51 and 68% in the control group and LF-treated group, respectively (statistically significant elevation: $p < 0.001$), showing a tendency to drop on day 5. The counts of lymphocytes were lower on day 1 post-surgery by 26 and 35%, respectively (statistically signifi-

cant decline: $p < 0.01$) and returned to the initial level on day 5. The percentage of neutrophils, on the other hand, increased after operation, that increase being stronger in the LF-pretreated group. The increase of the neutrophil's percentage on day 1 post-operation was significant in both groups of patients ($p < 0.001$). The increase in the neutrophil count was also transient. More characteristic changes related to the percentage of neutrophil precursors between the studied groups. The percentage of these precursors was higher in the LF-pretreated group before surgery, 4.1 vs 2.5% (a statistically significant difference: $p < 0.01$). However, the subsequent rise in the level of these cells (day 1 post-surgery) in LF-treated patients was not significant. In the control, placebo-treated patients, the rise in the level of neutrophil precursors was statistically significant with: $p < 0.02$. All other relations in the levels of the respective cell types between both groups were not relevant.

The proliferative response of PBMC to PHA

The ability of peripheral blood mononuclear cells (PBMC) from the patients to respond by proliferation to phytohemagglutinin (PHA) is presented in Fig. 1. The proliferative response of lymphocytes was similar in both groups of patients before surgery and on day 1 post-operation, with a little tendency to decline. However, the proliferation was higher in the LF-pretreated patients by about 20% on day 5 post-operation, and this was statistically significant ($p < 0.05$). Moreover, the proliferation of lymphocytes in the control group had a steady tendency to decrease.

The production of TNF- α by peripheral blood cell cultures

The ability of blood cells to produce TNF- α upon LPS stimulation is presented in Fig. 2. The results show

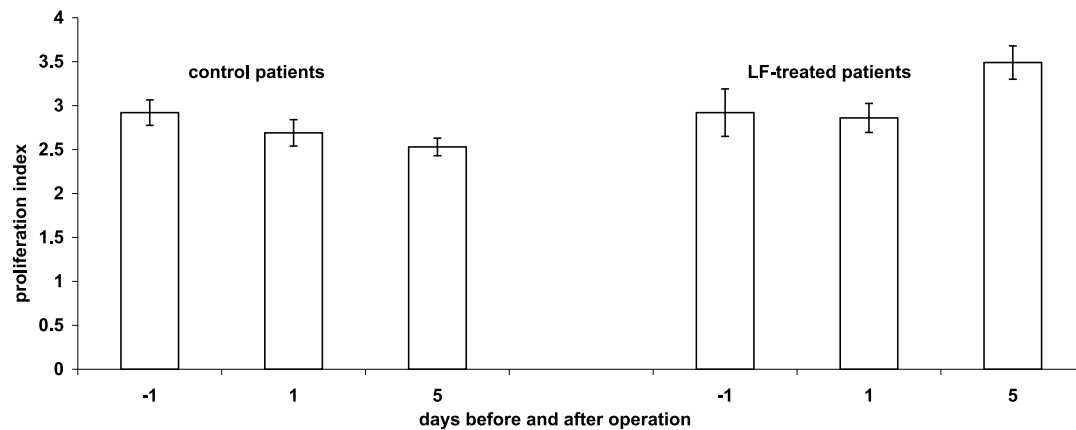


Fig. 1. The proliferative response of PBMC to PHA in the perioperative period. The results are shown as the mean values of proliferation indices $\pm$ SE. The increase of proliferation index on day 5 in the LF-treated group in comparison with the control group is statistically significant ($p < 0.05$)

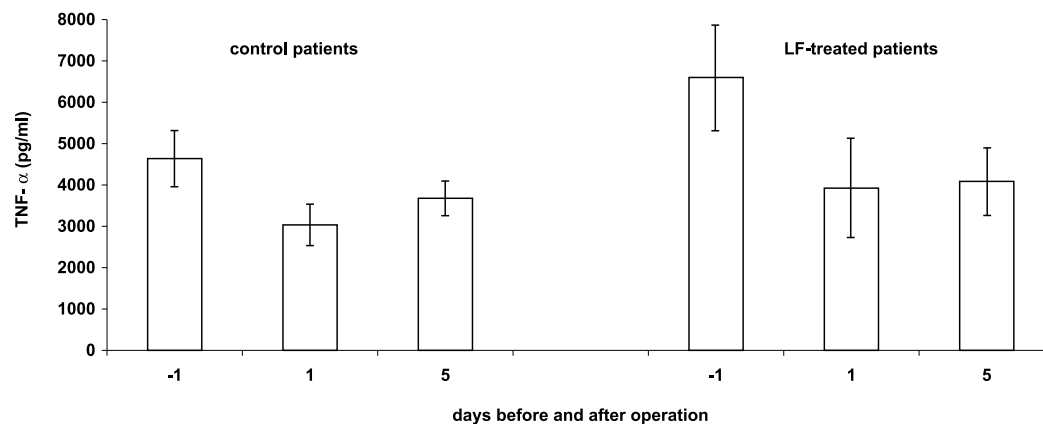


Fig. 2. The production of LPS-inducible TNF- α by peripheral blood cultures. The results are shown as the mean values $\pm$ SE. The differences within the groups and between the groups on respective days are not significant

that the initial (pre-operative) ability of cells to produce TNF- α was higher by 28% in LF-pretreated patients. In both groups of patients the production of TNF- α fell by day 1 post-operation, however, cells from LF-pretreated patients still produced more (by 24%) TNF- α than their control counterparts. On day 5 following surgery, the production of this cytokine was comparable in both groups. To gain a better insight into the effects of LF pretreatment on TNF- α production, we divided the patients into categories, i.e. those producing low (below 4000 pg/ml) or high (above 4000 pg/ml) levels of TNF- α . TNF- α production is under genetic control in humans²⁴, so that a low and high production of that cytokine can be distinguished. Fig. 3 shows that, in the control group of patients, the surgery did not affect production of TNF- α in the low-responder category. The effect of surgery on the high-reactive ca-

tegory was small (a transient, insignificant drop). In contrast, in the LF-pretreated group, the surgery deeply affected this cytokine's production in both categories of patients and was regulatory. This regulation was reflected by a sharp decrease of TNF- α production in the high-reactive patients and an enhancement (although not statistically significant) in the low-reactive group, so that the production of TNF- α was comparable on day 5 in both categories. It also appeared that the surgery significantly decreased spontaneous TNF- α production in both groups, low production was not much affected (not shown).

Production of IL-6 by peripheral blood cell cultures

These results, shown in Fig. 4 revealed that LPS-induced IL-6 production, although similar in both groups of patients before surgery (control: 1187 pg/ml, LF-

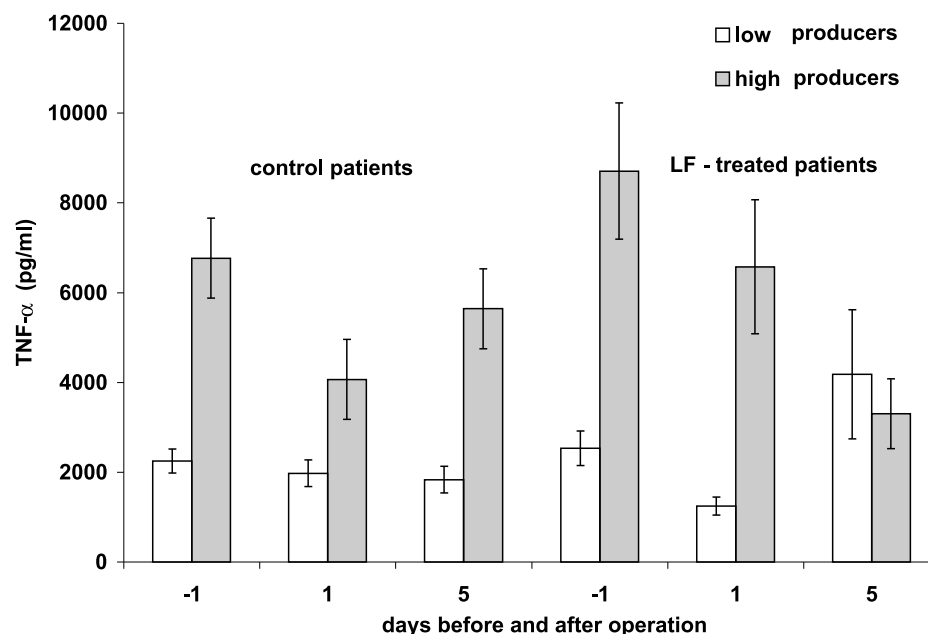


Fig. 3. The production of LPS-inducible TNF- α by peripheral blood cell cultures of low and high cytokine producers. The results are presented as the mean values $\pm$ SE. The differences between high vs low responses on respective days within control and LF-treated group are statistically significant except of day 5 in the LF-treated group (<0.001 , <0.05 , <0.001) and ($p<0.01$, <0.01 , NS). The difference between day -1 and day 1 values in the high responder control group is significant ($p<0.05$), but not between day -1 and 5. In the respective high-responding LF-treated group, the decrease on day 1 was NS, but significant when comparing day -1 and 5 ($p<0.001$). The changes in the low-responding subgroups of control patients were not significant and in the analogous subgroup of the LF-treated patients the decrease on day 1 was significant ($p<0.02$) and the rise between day -1 and 5 not significant. The comparison of high responders between control and LF-treated patients on respective days revealed no statistical difference. In the low-responding category, the response of LF-treated patients was significantly higher than that of control patients ($p<0.05$) on day 5

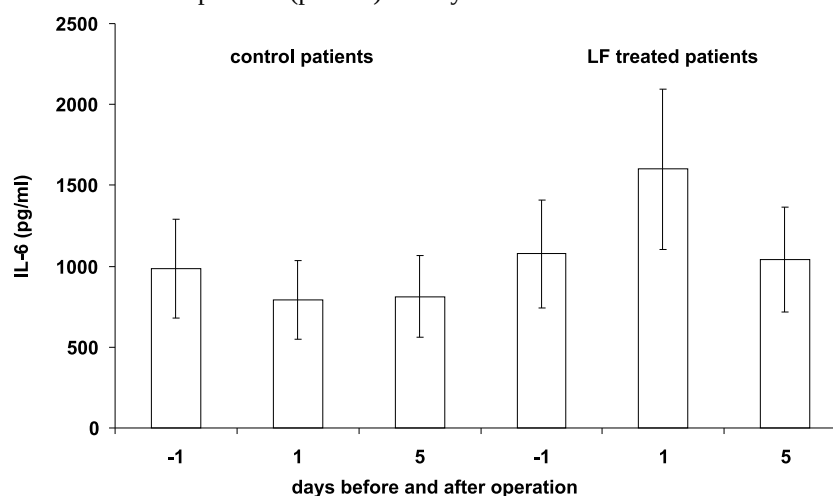


Fig. 4. The production of LPS-inducible IL-6 by peripheral blood cell cultures. The results are presented as the mean values $\pm$ SE. The differences are not statistically significant

-treated: 1297 pg/ml), was slightly lowered after surgery in control patients, but stimulated on day 1 post-operation in LF-pretreated patients. This upstimulatory effect was marked (about 2-fold), although not statisti-

cally significant. Upon division of patients to high and low IL-6 producers, no change in the kinetics of the production of this cytokine following surgery was noted (not shown).

Discussion

In this paper we described results of our first clinical trial aimed at an evaluation of the medical benefits of LF given orally to patients before surgery. In particular, we were interested in finding out whether LF would regulate the immune response to surgery during the early post-operative period. As expected, surgery alone and LF pretreatment elicited some characteristic changes in the blood picture. The lymphocyte count transiently dropped in both groups of patients, a phenomenon described earlier³⁰. The total increase of the white cell count observed on day 1 post-operation could be attributed to the increase of the neutrophil level (Table 1) and to the output of neutrophil precursors from the bone marrow. This increased turnover of neutrophils is characteristic of clinical insult^{3, 31} or infection^{5, 28, 38} and is caused by the sequential action of proinflammatory mediators²⁸. Such a phenomenon is associated with the preventive action of LF on the immune system. First, released LF contributes to the significant drop of free iron ions, which are a potential cause of free-radical generation and a stimulus for bacterial growth²⁵. Second, release of LF, and, consequently, GM-CSF, leads to a rapid production of neutrophil precursors, thus expanding the pool of the most efficient phagocytic cells²⁸. Although the level of neutrophil precursors rose in control patients (from 2.5 to 3.7%) after surgery, the level of these cells was already much higher before operation in the LF-treated patients. This supports our earlier data on healthy volunteers, where orally administered LF significantly enhanced the output of neutrophil precursors into circulation in a dose-dependent manner^{47, 48}. The similar phenomenon observed in our clinical study suggests that pre-operative treatment of patients with LF may be advantageous i.e. the patients have already an increased pool of these cells, so that the effect of surgery is less violent.

Of particular interest is the upregulation of the proliferative response of lymphocytes to PHA in the LF-pretreated group of patients. The proliferative response of lymphocytes to mitogens is decreased after surgery, however, the most evident decrease occurs 1 day after surgery^{30, 36}. In our study, that decrease was slight, probably because the extent of surgery was minor, not involving manipulation within the peritoneal cavity. However, even in patients subjected to cardiac surgery³⁶, the proliferative response of PBMC to PHA after surgery only decreased in patients showing normal or high pre-operative proliferation indices; low-reactive patients were even stimulated. Nevertheless, the fact of upregulation of the T cell function in LF-pretreated pa-

tients on day 5 after surgery may be regarded as beneficial.

The ability of blood cell cultures to produce TNF- α was decreased following surgery^{30, 35}, as expected. Our previous studies showed that the production of LPS-induced TNF- α in blood cell cultures of septic and trauma patients was also inhibited^{2, 36}. Other studies on patients subjected to major surgery¹⁶, showing that production of TNF- α by T cells but not by monocytes, was inhibited, indicated that T cell, but not monocyte, function was impaired despite MHC class II expression inhibition on monocytes. In our study we did not investigate separately the ability of lymphocytes and monocytes to produce TNF- α , so that the measured TNF- α could derive from both these sources. Our *in vitro* experiments showed that the reduced ability of blood cells from septic and trauma patients could be partially reversed by LF². The preclinical trials on volunteers showed that administration of oral LF regulated rather than enhanced LPS-induced TNF- α production⁴⁸. The fact of an increased production of TNF- α by cells from LF-pretreated patients suggests that it could be caused by an upregulation of TNF- α production in low-responding patients. Nevertheless, the elevated ability to produce TNF- α upon LPS challenge in LF-pretreated patients on the day before and the day after operation may be regarded as an advantageous phenomenon since TNF- α plays an important role in the defence against pathogens^{6, 15}. Of particular interest was the regulatory effect of LF pretreatment on LPS-induced TNF- α production when low- and high-reactive categories of patients in both groups were analyzed. Although high responders in the control group were subjected to regulation by surgery (a drop in TNF- α production on day 1 post-operation), there was no change in TNF- α production in low responders. The ability of high responders to make TNF- α remained high on day 5 which may be a beneficial feature, whereas the low production of TNF- α by cell cultures of the low-reactive category may not be a desirable phenomenon. On the other hand, low TNF- α responses in LF-treated patients were upregulated on day 5. The high preoperative TNF- α values in LF-pretreated patients were inhibited early by surgery, but the 5 day production was still in the optimal range. The pre-operational treatment of patients with LF also resulted in an increased ability of cells to produce IL-6. That effect, although not statistically significant, indicated an enhanced resistance of LF-treated patients to potential infection.

The immunoregulatory mechanisms of LF actions may have a different basis. Orally administered BLF is likely to bind LPS in the gut lumen. Such LF-LPS

complexes were shown to be very efficient in the protection of mice against endotoxic shock in mice (our unpublished data). Receptors for LF are abundant on brush border intestinal cells, and there is evidence that some LF crosses the gut barrier as an intact molecule³². As a result of oral treatment of mice, both local (GALT-associated) and generalized immune responses are elicited¹⁰. We recently showed that LF given orally to mice exhibited adjuvant properties to several antigens⁴⁵. Mice pretreated with LF and subjected to surgery demonstrated a diminished release of surgery-elicited cytokines, particularly TNF- α ⁴⁹. This phenomenon could be due to a property of LF to induce a proinflammatory IL-10 and to increase the IL-10 to TNF- α ratio in endotoxemic mice⁴¹. Studies are underway to establish the immune status of surgical mice, pretreated with LF, more thoroughly.

From the data presented in this study it is clear that LF enhances the immune reactivity of surgical patients in general and that it is regulatory with regard to selected immune parameters when patients were analyzed according to their initial immune status. This is a first clinical attempt of LF application in surgical patients. Further efforts will be aimed at the pre-operative application of LF in patients at higher risk, e.g. those selected for abdominal surgery. The application of LF in major abdominal surgery may have a more evident advantage when taking into account the protective effects of LF on the gut structure²¹ and its ability to bind LPS⁴⁰ and combat pathogenic bacterial strains³³. In addition, BLF pretreatment may be of great advantage in some categories of patients particularly susceptible to post-operative complications, such as obstructive jaundice^{11, 35}, alcoholic liver disease⁷, and diabetes¹⁹. BLF was shown to be protective in experimental pancreatitis in rats²⁰. The ability of BLF to bind LPS and to restore physiological intestinal flora may be relevant in the diminution of postsurgical shock in obstructive jaundice and cirrhosis.

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