Wound Healing and Angiogenic Properties of Supernatants from *Lactobacillus* Cultures

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Extracts or supernatants from cultures of *Lactobacilli* are used for their medicinal effects, including wound healing and immune system stimulating activity. We have studied the *in vivo* and *in vitro* effects of supernatants from bacterial cultures of two strains of *Lactobacillus* (LS) on tissue repair and angiogenesis. Subcutaneous injection of LS into rodent ears led to proliferation of blood vessels that also exhibited strong immunostaining for *Flk-1* receptor. Some inflammatory cells were scattered among the blood vessels. The continuous influx of polymorphonuclear leukocytes (PMNs) and macrophages into transcutaneous wounds in mice treated with LS resulted in prolonged inflammatory phase of wound healing and delayed wound closure, including reepithelialization. Subcutaneous injection of Matrigel impregnated with LS into the abdominal wall led to rapid and transient influx of PMNs in the vicinity of the gel. LS stimulated the proliferation of murine macrophage J774.A1 cell line and porcine lymphocytes but not that of murine fibroblast AKR-2B cells. LS also induced production of TNF-α by J774.A1 cells and by porcine kidney epithelial LLC-PK1 cells. LS did not appear to have an effect on collagen production. In conclusion, our study demonstrates the potential of LS to function as a stimulator of the inflammatory stage of tissue repair, TNF-α production, and of angiogenesis. Exp Biol Med 228:1329–1337, 2003

Key words: supernatant from *Lactobacillus* cultures; angiogenesis; acute inflammatory response; TNF-α production; lymphocyte blastogenesis

Inflammation followed by tissue repair is a complex physiological process aimed at restoration of normal function after infection or wounding (1, 2). Advances in growth factor discovery, biochemistry of extracellular matrix, and immunology have enhanced our knowledge of inflammation and wound healing, including angiogenesis. While inflammation stimulates the production of angiogenic growth factors, angiogenesis is an event separate and independent from inflammation (3, 4). Angiogenesis occurs during later stages of tissue repair as a process essential to restoration of tissue damaged by injury and inflammation (3, 5–7). Though many growth factors possess angiogenic properties and are commercially available in recombinant form, their widespread clinical use is hindered by their prohibitive costs (8–12). In addition, their half-life in the blood stream is likely to be short and unpredictable due to the presence of a variety of binding proteins. Therefore the identification and characterization of alternative compounds actively promoting wound healing constitutes an important part of development of new therapeutic strategies.

Products and extracts from *Lactobacillus* have been used in food fermentation, and for their alleged wound healing and antiviral properties for many years in the Far East. *Lactobacillus* extracts are believed to boost energy and to be effective remedies for allergies, common cold, lactose intolerance, and to be active in reducing the risk of colon cancer (13, 14) and reducing cholesterol levels (15–17). We have studied the effects of supernatant collected from the culture of two strains of *Lactobacillus* (which we called LS) on several aspects of cell proliferation and wound healing. Our previous studies have found that the application of LS to chorioallantoic membrane of 8-day-old chicken embryos leads to thickening of the membrane due to proliferation of blood vessels, inflammatory cell infiltrates, and deposition of proteoglycans (Li *et al.*, manuscript in preparation). Here we report that subcutaneous injection of LS into rodent ears resulted in angiogenesis and in influx of polymorphonuclear leukocytes (PMNs). The administration of the supernatant to murine transcutaneous wounds led to an increased acute inflammatory response. LS also induced TNF-α production in murine macrophage J774.A1 cells and in porcine kidney LLC-PK1 cells, and it also activated porcine lymphocyte blastogenesis. As such, LS appears to have strong angiogenic and proinflammatory properties.

Materials and Methods

Materials. Two strains of *Lactobacillus acidophilus* (ATCC strains 4356 and 43121) were plated (1:1) in MRS

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broth (pH 5.5, Difco Laboratories, Detroit, MI) and co-cultured at 37°C for 24 hrs under microaerophilic conditions. The MRS culture medium contains polysorbate, acetate, magnesium, and manganese, which are known to act as special growth factors for *Lactobacilli*, as well as a rich nutrient base. Overnight bacterial cultures contained 2.5×10^8 colony-forming units. After centrifugation (10,000 × g for 15 min, 4°C), the supernatants were filtered through a 0.2-μm filter to remove remaining bacteria and debris and lyophilized before storage at −20°C. At the time of the experiments, the lyophilized *Lactobacillus* supernatant (LS) was reconstituted with deionized water, filtered with Puradiscs (0.22-μm pore size, Whatman Inc., Ann Arbor, MI) and termed as LS. Because of its acidity (pH was 2.35 ± 0.15) LS had to be first neutralized for some (but not all) applications. The presence of lipopolysaccharide (LPS) in LS was excluded with a diagnostic kit from Cambrex Corporation (East Rutherford, NJ). The kit is based on the *Limulus* amebocyte lysate test, which is specific for LPS diglucosamine backbone (18). Supernatants from several different bacteria (e.g., *Enterococcus*, *Bacillus mesentericus*, *Clostridium butyricum*, *Bifidobacterium bifidum*) were used as controls in the *in vivo* angiogenesis assay described below.

The use of all animals was approved by the Animal Use Committee at The University of Georgia. Swiss NIH mice and Sprague Dawley rats were purchased from Harlan (Indianapolis, IN). Gilts used for peripheral blood collection were healthy pigs (Landrace × Yorkshire × Hampshire or Landrace × Yorkshire × Durac) raised indoors at The University of Georgia swine center. Matrigel®, growth factor reduced, was from Becton Dickinson Biosciences (Bedford, MA). Mouse monoclonal antibody directed against *Flk-1* receptor (A-3) and polyclonal goat anti-type I collagen antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Detection systems for use in immunohistochemistry and for Western blotting were from Santa Cruz Biotechnology and Vector Laboratories (Burlingame, CA), respectively.

Angiogenesis Assay. Two- to three-month-old female Swiss NIH mice and Sprague Dawley rats were first anesthetized with an intraperitoneal injection of mixture of xylazine (5 mg/kg) and ketamine (75 mg/kg). Ear lobes were shaved and disinfected with 70% isopropanol before receiving a subcutaneous (sc) injection of LS or of supernatants from control bacteria over the posterior aspect of the left ear lobe cartilage of mouse (40 μl) or rat (100 μl). Before administration, LS was neutralized with sodium hydroxide solution. The contralateral right ear lobes served as a control and received an equivalent volume of phosphate-buffered saline (PBS). After euthanasia 1, 4, and 7 days later, ear lobes were excised and fixed in 10% formalin.

Wound Healing in Mice. Transcutaneous punch biopsies, 6 mm in diameter, were performed on the backs of anesthetized (as above) 2- to 3-month old female Swiss NIH mice (two biopsies per mouse) as we described previously (19, 20). LS suspended in 2% methylcellulose (40 μl of LS in 50 μl 2% methylcellulose in PBS) was applied to the wound at the time of biopsy. The contralateral wound in the same animal served as a control, receiving PBS in 2% methylcellulose. Several mice received 2% methylcellulose in 0.04 N HCl in control wounds. Mice were euthanized on days 1, 3, 5, 7, 11, and 12, biopsy sites were excised, fixed in 10% formalin, and processed for routine histology. Two experiments with 3 to 6 wounds per data point per experiment were performed.

Chemotactic Effect of *Lactobacillus In Vivo*. To evaluate angiogenic and chemotactic effects of LS in mice, LS was delivered suspended in growth-factor-reduced Matrigel into 2- to 3-month-old NIH Swiss mice according to the method described by Carter and Halper (19). Upon sc injection of 0.4 ml Matrigel mixed with 40 μl LS (left side) or PBS (right side) near the abdominal midline, the gel solidified and persisted for several days. Mice (3 per data point per experiment) were sacrificed at specified times. Recovered gels with attached adjacent tissues were fixed in 10% formalin and processed for histology.

Histological Evaluation. Excised tissues were fixed in 10% formalin and embedded in paraffin; 5 μ-thin sections were cut and stained with hematoxylin/eosin for light microscopy. Selected sections were also stained with Masson's trichrome for collagen and with Gomori's silver impregnation method for reticular fibers to document collagen fiber formation.

Immunohistochemistry. After deparaffinization and rehydration of 5 μ-thin tissue sections, antigen unmasking was performed by microwave heating 2 × 5 min in PBS. After quenching the activity of endogenous peroxidase with 0.1% hydrogen peroxide/water for 30 min at room temperature, nonspecific binding sites were blocked with 1.5% normal goat serum in PBS for 1 hr at room temperature. After a 5-min wash in PBS, the primary mouse monoclonal antibody to *Flk-1* receptor (1:200 in PBS) was applied to tissue sections overnight in a humidified chamber at 4°C. Next morning, the slides were washed with PBS (3 × 5 min) and incubated with secondary biotinylated anti-mouse antibody (1:200 dilution). The presence of antibody–antigen complexes was visualized using a Santa Cruz kit utilizing the ABC method. DAB was used as the chromogen. The sections were counterstained with hematoxylin.

Cell Proliferation Assays. Mouse embryonal kidney fibroblastic AKR-2B cell line was used in a procedure described previously (21). AKR-2B cells were plated at a density of 5×10^3 cells/ml in 12-well cell culture plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After 24-hr incubation at 37°C in atmosphere with 5% CO_2 the medium was discarded and replaced with DMEM with 10% FBS or with DMEM with 1% FBS. Aliquots of 5, 10, and 20 μl/ml of LS diluted 1:2 in PBS were added to replicate wells. Cells were trypsinized and counted after 3 (DMEM with 10% FBS) and 5 (DMEM with 1% FBS) days of incubation,

respectively. Mouse macrophage J774.A1 cell cultures were maintained in DMEM/Ham's F12 (1:1) supplemented with 5% FBS. In cell proliferation assays, 5000 J774.A1 cells were plated in 50 μl/well medium in 96-well flat-bottom microtiter plates. After 6 hrs, cells were treated with serial dilution of LS in DMEM, together with 1 μCi/well ^{3}H-thymidine (methyl-^{3}H-thymidine, specific activity 6.7 Ci/nmol; NEN Life Sciences Products, Inc., Boston, MA) in 8 replicates, and incubated for 96 hrs. Cultures were harvested onto fiberglass filter discs with an automated cell harvester (Skatron, Sterling, VA). Filter discs were placed in scintillation vials containing 3 ml scintillation cocktail, and the ^{3}H-thymidine incorporated into DNA was determined in a Beckman liquid scintillation counter. Each experiment was performed three times.

Western Blotting. For type I collagen determination by Western blotting, AKR-2B cells were grown in DMEM supplemented with 10% FBS in 25 cm^2 flasks until 60% to 70% confluent when LS was added. After 2 days of incubation, the medium was discarded. Cell monolayers were rinsed with PBS and lysed with a lysis buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The buffer contained 0.5 mg/ml gelatin and was kept on cells for 15 min (22). The extract was centrifugated at 12,000 *g* for 15 min. Aliquots of the supernatant were suspended in SDS-PAGE sample buffer. After separation on a 6% SDS-polyacrylamide gel, samples were transferred to a nitrocellulose membrane in methanol-glycine buffer using standard methods. Type I collagen was identified using a goat polyclonal antibody from Santa Cruz. Biotinylated secondary anti-goat antibody, Vectastain® *Elite* ABC kit, and DAB substrate kit (all from Vector Laboratories) were used to visualize antibody–antigen complexes.

Analysis of TNF-α mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Murine macrophage J774.A1 cells were plated at a density of 3000 cells/cm^2 in six-well plates and were allowed to attach and grow in DMEM with 10% FBS for 3 days before treatment. Serial dilutions of LS in 50 μl DMEM were added to each well. The control wells received equal volume of DMEM. After 6 hrs of incubation total RNA was isolated from J774.A1 cells using TRI® reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer protocol. Total RNA (2.5 μl) was transcribed to cDNA using oligo(dT)$_{12\text{-}18}$ and superscript II primers (Life Technologies, Grand Island, NY) at 42°C for 50 min. TNF-α (2 μl aliquots) and β-actin (1 μl aliquots) cDNAs were amplified by PCR using Taq DNA polymerase (BD Biosciences Clontech, Palo Alto, CA) and 0.2 μM of each primer (for sequences see below) in 1X PCR buffer containing 2 mM $MgCl_2$. The thermoamplification program consisted of an initial denaturation (5 min at 95°C), followed by 32 cycles (for TNF-α) or 34 cycles (for β-actin) of 30 sec denaturation (94°C), 30 sec annealing (50°C), and 1 min elongation (72°C), with a final extension period of 1 min at 72°C. In preliminary experiments, an exponential increase in DNA was observed up to 40 cycles for both products. The sense and antisense primers were 5′-GTT CTA TGG CCC AGA CCC TCA CA-3′ and 5′-TCC CAG GTA TAT GGG TTC ATA CC-3′ for murine TNF-α, and 5′-ATG GAT GAC GAT ATC GCA-3′ and 5′-ATG AGG TAG TCT GTC AGG T-3′ for murine β-actin, respectively (chosen by Primer3 program; Whitehead Institute, Cambridge, MA).

Porcine kidney LLC-PK1 cells (CRL1392, ATCC, Rockville, MD) maintained in DMEM/Ham's F12 (1:1) supplemented with 5% FBS were plated at density of 6000 cells/well in 24-well plates. The cells were allowed to grow for 72 hrs before the addition of 1 μl aliquots of dilutions of LS ranging from 1:400 to 1:10,000 for 3 hrs. Cells were harvested and RNA extracted as described for J774.A1 cells. Total RNA (2.5 μl) was transcribed to cDNA using oligo(dT)$_{12\text{-}18}$ and superscript II primers (Life Technologies, Grand Island, NY) at 42°C for 50 min. Porcine TNF-α (2 μl aliquots) and GAPDH (1 μl aliquots) cDNAs were amplified by PCR using cycling conditions described previously. The sense and antisense primers were 5′-AAT GGC AGA GTG GGT ATG-3′ and 5′-CTT GAT GGC AGA GAG GAG-3′ for TNF-α, and 5′-TCC CTG CTT CTA CTG GTG CT-3′, and 5′-TGA GCT TGA CAA AGT GGT CG -3′ for GAPDH, respectively (chosen by Primer3 program).

Polymerase chain reaction products were separated on 2% agarose gel containing ethidium bromide, detected by UV light, and quantified using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Density of β-actin and GAPDH cDNA bands in the same gels was used for normalization of murine and porcine TNF-α cDNA, respectively. The experiments were performed three times.

Bioassay for TNF-α. J774.A1 cells were cultured in 12-well plates and were treated with serial dilutions of LS for 6 hrs when 95% confluent. Medium was collected for determination of biological activity of TNF-α. LPS (1 ng) was used as a positive control, because its application leads to more than 10^6-fold increase in TNF-α secretion. Each dilution or sample was tested in triplicate and experiments were performed three times. Cytolytic activity of TNF-α present in the medium was quantified in 96-well microtiter plate bioassays using WEHI 164 clone-13 murine fibrosarcoma cells, as described by Morris *et al.* (23). One unit of TNF-α activity was defined as the dilution of test sample leading to lysis of 50% of WEHI fibrosarcoma cells. The activity of TNF-α in samples was compared with the activity of increasing concentrations of recombinant human TNF-α concomitantly run with each assay.

Lymphocyte Blastogenesis. Peripheral blood was obtained from the jugular vein of healthy female pigs (n = 9) between 9 and 14 months of age, collected in heparin (10,000 IU)-coated 35-ml syringes, and mixed with equal volumes of RPMI-1640 medium containing 100 IU penicillin and 100 μg streptomycin/ml and 10% FBS. Mono-

nuclear cells were separated by gradient centrifugation (600 × *g* for 25 min) on Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) (24), collected from the interface, and resuspended in RPMI-1640 medium with 1% antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml, and fungizone 0.25 μg/ml) and 10% FBS. Cells were washed three times and reconstituted in RPMI-1640 medium containing 10% FBS at a concentration of 2×10^6 cells/ml. Cell viability as determined by the trypan blue exclusion method was always more than 95%. The lymphocyte mitogenesis test was performed as described (25). Porcine lymphocytes, 1×10^6/50 μl medium, were placed into wells containing varying concentrations, but equal volume (50 μl) of LS, along with pokeweed mitogen (PWM, 1/100 dilution of stock solution prepared as indicated by manufacturer, GIBCO BRL, Grand Island, NY), phytohemagglutinin (1:1000 and 1:2000 dilutions of stock solution prepared as recommended by the manufacturer, Difco Laboratories), or concanavalin A (5 and 10 μg/ml, Sigma). Cultures were performed in 96-well flat-bottom microtiter plates and incubated at 39°C with 5% CO_2 in air for 72 hrs. Eighteen hours before the termination of cultures, cells were pulsed with ^{3}H-thymidine (1 μCi/well). All cultures were harvested onto fiberglass filter paper discs. Filter discs were placed in scintillation vials containing 3 ml scintillation cocktail, and the ^{3}H-thymidine incorporated into DNA were determined in a Beckman liquid scintillation counter. Each experiment was performed three times in eight replicates.

Statistical Analysis. Inflammatory cells (i.e., PMNs, macrophages, lymphocytes, and plasma cells) in transcutaneous wounds and in tissues surrounding Matrigel were counted in 10 randomly chosen high-power fields per wound. The mean cell counts were subjected to analysis of variance to determine significant effects of duration of wounding, treatment, and their interaction. Since an unequal number of mice comprised day and treatment groups, significant differences relied on the least-square error determinations using the general linear model algorithm of SAS (SAS Institute Inc., Cary, NC). The effect of LS treatment on lymphocyte blastogenesis and on expression of TNF-α mRNA by J774.A1 cells data were first analyzed by the least-square analysis of variance using the General Linear Models procedure of SAS. Effects of dilutions were further studied using Tukey and Student-Newman-Keuls tests.

Results

In Vivo Effects

Angiogenic Effect. Subcutaneous injection of LS into the posterior aspect of mouse and rat ear lobes led to blood vessel formation and influx of mostly PMNs into the injection site 1 day after administration. Only ears that received LS showed the proliferation of newly formed blood vessels accompanied by a few inflammatory cells in the injection site (Fig. 1). Using immunohistochemistry, the presence of *Flk-1*, vascular endothelial growth factor (VEGF)-Receptor, was noted in rat endothelial cells already on day 4 (Fig. 1D). On day 7, fewer inflammatory cells were present; however, the blood vessels were much larger, with open lumina and lined with active, plump endothelial cells (Fig. 1C). Only a few blood vessels and no inflammatory cells were noted in control ears during the entire experiment (Fig. 1A). None of the supernatants derived from the other four bacterial cultures listed in Materials and Methods had any effect on ear histology 4 days after injection.

Wound-Healing Effects. The first phase of normally proceeding wound healing is marked by the influx of inflammatory cellular infiltrate consisting of PMNs and macrophages under a fibrin plug. Both the control wounds and wounds treated with LS showed good influx of inflammatory cells, which consisted first mostly of PMNs. As expected, the number of PMNs peaked in the control wounds during the first 3 days, only to be replaced by macrophages and lymphocytes (Fig. 2). At day 5 and later, control wounds showed gradual decrease in inflammatory cellular infiltrate forming a less-dense band below the fibrin plug, and newly formed granulation tissue was observed to start filling the underlying wound bed. Control wounds underwent steady reepithelialization and their surface area was at least 80% closed by day 7. The LS-treated wounds ex-

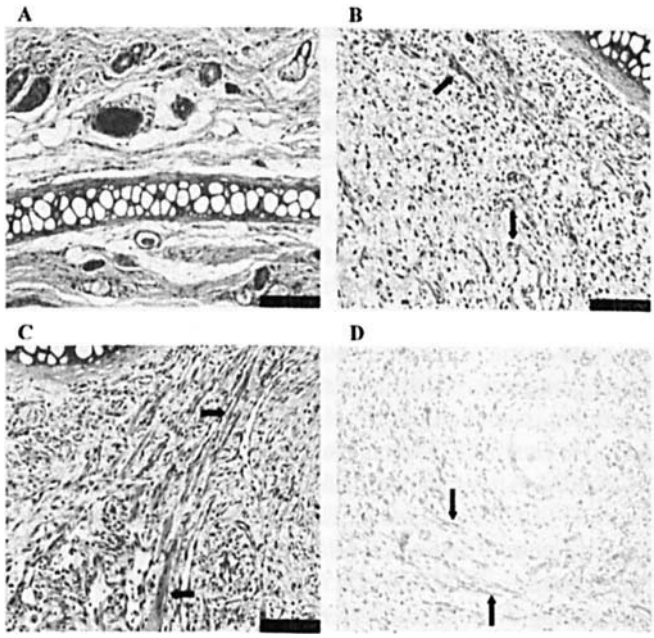


Figure 1. Angiogenic effect of LS *in vivo*. Rat ears were examined microscopically after sc injection of LS or PBS. (A) Only a few capillaries were scattered among normal skin structures and next to ear cartilage in control ears. (B) Numerous small blood (←) and few inflammatory cells filled sc tissue of an ear with 4 days of sc injection of LS. (C) Seven days after sc injection of LS the blood vessels were much larger, with open lumina and were lined with large active endothelial cells (←). (D) The newly appearing blood vessels exhibited intense immunostaining for *Flk-1* protein, a receptor for VEGF (←). LS-treated mouse ear lobes exhibited very similar changes.

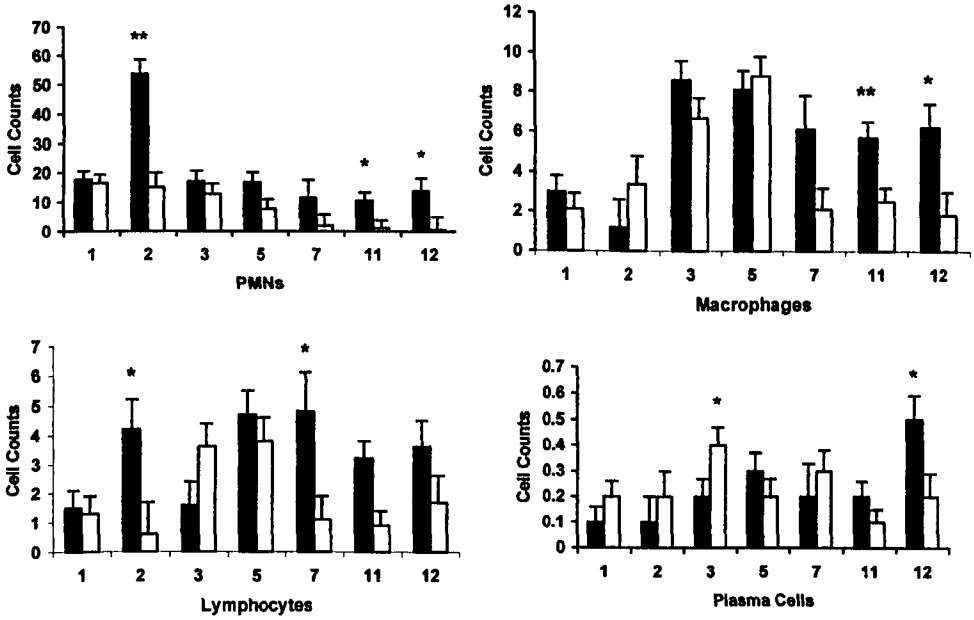


Figure 2. Effect of LS on inflammatory cell infiltrate in murine transcutaneous biopsies. LS suspended in methylcellulose was added to punch biopsies performed as described in Materials and Methods. PBS or HCL in methylcellulose were added to control wounds. Mice were euthanized on days 1, 2, 3, 5, 7, 11, and 12 post wounding; wounds were excised and processed for histology. The number of PMNs, macrophages, lymphocytes, and plasma cells was determined in 10 high-power microscopic fields and statistically evaluated with analysis of variance. Least-square standard errors are indicated. *Significance was taken as $P < 0.05$; **$P < 0.01$. Empty bars: control wounds; filled bars: LS-treated wounds.

hibited a significant influx of PMNs (3- to 4-fold increase) and lymphocytes (2-fold increase) on day 2 post-wounding (Fig. 2). These wounds also remained wide open and showed continuing influx of inflammatory cells. The number of lymphocytes was significantly increased in treated wounds on day 7. The presence of large numbers of PMNs and macrophages persisted in the wound bed, and remained significantly increased with only a little granulation tissue formation on days 11 and 12 (Fig. 2). At day 11 and 12 after wounding, the LS-treated wounds showed no gross or microscopic changes from days 3 and 5: large band of inflammatory cells overlying wound bed mostly void of granulation tissue, including blood vessels, and newly laid collagen and virtually no reepithelialization present. In contrast, control wounds were largely closed and covered with newly formed epidermis, and young connective tissue containing collagen and very few inflammatory cells was filling the wound beds 11 and 12 days after wounding.

In general, only a few plasma cells were scattered in control and treated wounds. There was a small but significant increase in the population of plasma cells on day 3 in the control group (Fig. 1). A similar small but significant increase occurred in the LS group but not until day 12 (Fig. 2).

Chemotactic Effect of *Lactobacillus*. *Lactobacillus* (40 μl/mouse) suspended in Matrigel or Matrigel alone was administered sc into the abdominal wall of Swiss NIH mice as described (18). The mice were sacrificed, gels recovered 1 and 3 days later, formalin fixed, and processed for histology. Matrigel alone elicited only a limited tissue response, consisting primarily of a few macrophages surrounding the gel. One day after application LS prompted statistically significant influx ($P < 0.01$) of PMNs into the space around the gel and into the underlying abdominal muscle. There was a 10-fold increase in the number of PMNs in the treatment group on day 1, which returned to control values by day 3 (Fig. 3A). There was no significant treatment or day effect for lymphocytes, macrophages, or

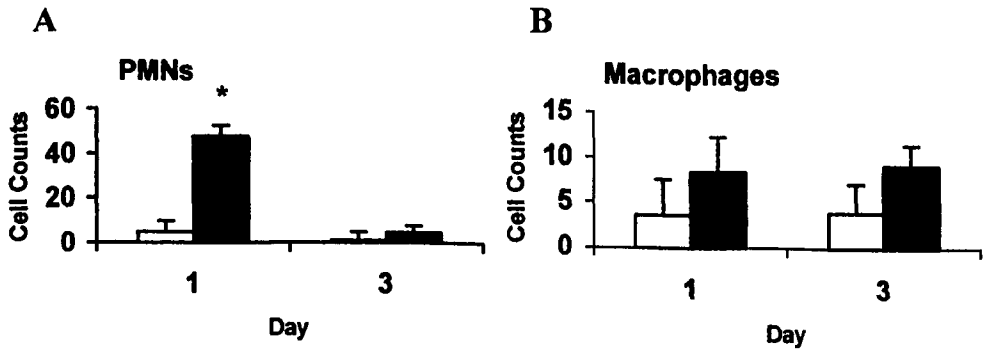


Figure 3. Chemotactic effect of LS. Mice received Matrigel impregnated either with LS or with PBS in an sc injection into the abdominal wall. Mice were euthanized on days 1 and 3 post injection; gels with adjacent tissue were excised and processed for histology. The number of (A) PMNs and (B) macrophages was determined in 10 high-power microscopic fields and statistically evaluated with analysis of variance. Least-square standard errors are indicated. *Significance was taken as $P < 0.05$. There were very few lymphocytes and plasma cells present. Empty bars: control wounds; filled bars: LS-treated wounds.

plasma cells, though there was a trend showing an increase in macrophages on days 1 and 3 (Fig. 3B). Both lymphocytes and plasma cells were present in very small numbers.

In Vitro Experiments

Effect on Cell Proliferation. The addition of LS to AKR-2B cells led to mild inhibition of cell proliferation of AKR-2B cells grown in DMEM supplemented either with 10% or 1% FBS. However, this effect was inconsistent (i.e., it was not apparent in all assays). The maximum achieved was 50% inhibition of cell proliferation with 10 μl of LS/ml medium. Western blotting revealed that LS had no effect on type I collagen production by AKR-2B cells. The production of other collagen types was not evaluated. In contrast, LS stimulated the proliferation of murine macrophage J774.A1 cells (Fig. 4). LS in serial dilutions was added to J774.A1 when 60% to 70% confluent, and ^{3}H-thymidine incorporation was measured for 3 days. A marked stimulation of ^{3}H-thymidine incorporation was observed between 1:100,000 and 1:1,000,000 dilutions of LS (Fig. 4). The activity of LS dropped off rapidly with higher dilutions.

***Lactobacillus* Stimulates the Expression and Secretion of TNF-α.** After 6 hrs of exposure of J774.A1 cells to LS, total RNA was extracted and RT-PCR used to determine the levels of TNF-α mRNA. LPS (1 ng/ml) was used as a positive control, because it is the most potent inducer of TNF-α expression known. As indicated in Figures 5 and 6, the induction of TNF-α mRNA and protein by LS was dose dependent, though LS was a significantly less potent inducer than LPS (data not shown). The increase in TNF-α mRNA expression was matched by LS-induced increase of TNF-α secretion by J774.A1 cells as determined in bioassay measuring the cytolytic effect of media conditioned by J774.A1 in the presence or absence of LS (Fig. 5) though it represented only a 2-fold increase in the amount of secreted TNF-α units. In comparison, LPS-induced stimulation of TNF-α was much higher, up to 1000 times more (or up to 10^6 units, data not shown). Pretreatment of neutralized LS with trypsin (2.5 mg/ml) for 60 min or with heat (56°C for 30 min) did not abolish the effect of LS on TNF-α synthesis.

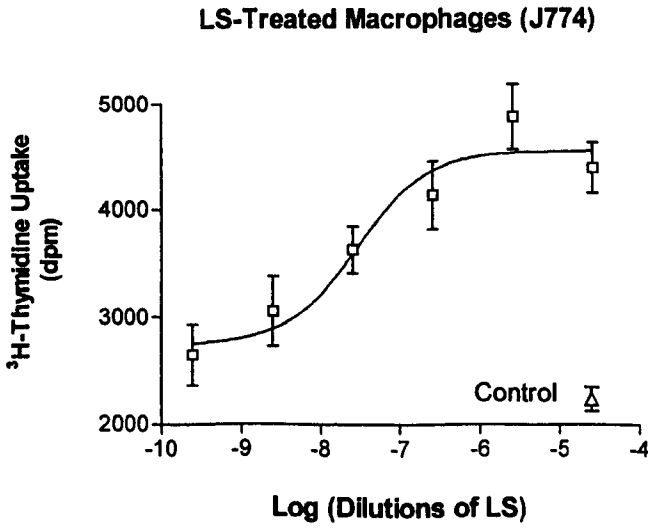


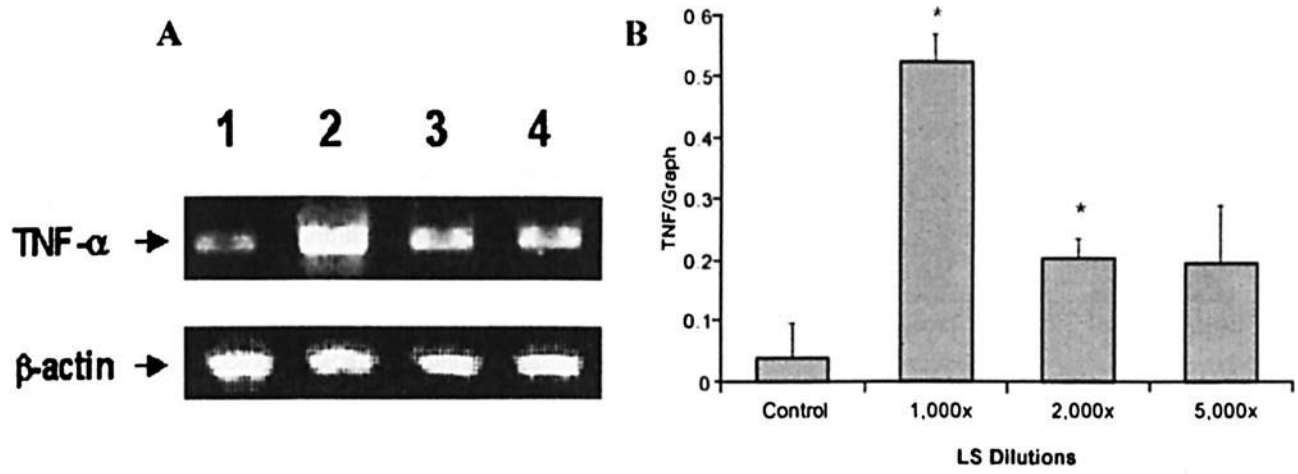
Figure 4. LS stimulates proliferation of macrophage J774 cells. J774 cells (5000 per well) were plated in 50 μl/well medium in 96-well flat-bottom microtiter plates. After 6 hrs cells were treated with serial dilution of LS in DMEM, together with 1 μCi/well ^{3}H-thymidine, and incubated for 96 hrs. All cultures were harvested onto fiberglass filter discs. Filter discs were then placed in scintillation vials containing scintillation cocktail, and the ^{3}H-thymidine incorporated into DNA was determined in a liquid scintillation counter. Each experiment was performed three times in 8 replicates.

The effect of LS treatment on the expression of tumor necrosis factor α (TNF-α) by porcine epithelial kidney cells (LLC-PK1) is summarized in Figure 6. LLC-PK1 cells were seeded at density of 6000 cell/well in 24-well plates. The cells were allowed to grow for 3 days (72 hrs) before 1 μl of LS was added at final dilutions from 1:400 to 1:10,000 for 3 hrs. Cells were then harvested and TNF-α mRNA was quantified by RT-PCR. The addition of LS led to significant increase of TNF-α mRNA. Paradoxically, this increase occurred only at higher dilutions (1:4000 and higher).

***Lactobacillus* Stimulates Lymphocyte Blastogenesis.** Lymphocytes isolated from porcine peripheral blood were exposed to LS in the presence or absence of pokeweed mitogen. ^{3}H-thymidine incorporation was used as an indicator of DNA proliferation. The addition of LS to porcine lymphocytes led to moderate stimulation of lymphocyte proliferation in the presence of PWM (Fig. 7). LS alone or in combination with phytohemagglutinin or concanavalin A, two potent T cell mitogens, did not enhance lymphocyte blastogenesis (data not shown).

Discussion

Our results indicate that supernatants from *Lactobacillus* cultures potentiate acute inflammation. We demonstrated that LS acts as a very potent chemoattractant or a regulator movement of PMNs, and to, lesser extent, of macrophages. Moreover, LS applied to skin wounds in mice extended the inflammatory stage of wound repair by at least 1 week. These effects of LS likely occur through chemoattraction of inflammatory cells and induction of TNF-α production. We showed that LS induces TNF-α gene expression by macrophage J774.A1 and epithelial LLC-PK1 cell lines. The increase in TNF-α mRNA level correlated with the presence of cytolytic activity in media conditioned by J774.A1. This is in agreement with other studies that reported activation of macrophages (26) and increased TNF-α expression by murine macrophages after exposure to *Lactobacillus* (27, 28). The absence of LPS (as determined by the *Limulus* amebocyte lysate test) in the LS preparation indicates that another as yet unidentified compound(s) is responsible for TNF-α induction. In addition, LPS-induced TNF-α production never reached the plateau stage, even when it stimulated the production of more than 10^6 units TNF-α. In contrast LS-induced TNF-α production reached the plateau stage at 25 units, which was achieved with LS preparation diluted 1:1000. Because trypsin treatment did



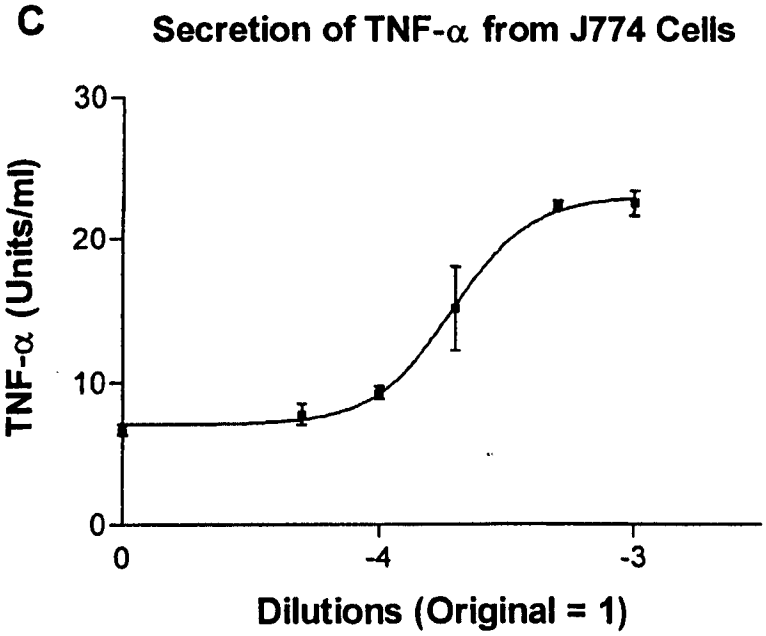


Figure 5. Effect of LS on TNF-α gene expression in murine macrophage cells (J774.A1). J774.A1 cells were plated at density 3000 cells/cm^2 in six-well plates and allowed to attach and grow for 3 days before treatment. Serial dilutions of LS in 50 µl DMEM were added to each well. The control wells received equal volume of medium. After 6 hrs of incubation total RNA was extracted and transcribed to cDNA using oligo(dT)$_{12-18}$ and superscript II primers (Life technologies, Grand Island, NY). TNF-α and β-actin cDNAs were amplified by PCR as described in Materials and Methods. PCR products were separated on 2% agarose gel containing (A) ethidium bromide and (B) quantified using densitometry. Density of β-actin cDNA bands in the same gel was used for normalization. *Significant increase at $P < 0.05$ compared with control. The experiments were performed three times. (A) Lane 1: control; Lane 2: LPS (200 ng) as positive control; Lane 3: LS (1000x dilutions); Lane 4: LS (2000x dilutions). The levels of actin mRNA are shown in the lower left panel as a control. (C) Effect of LS on TNF-α secretion in J774.A1 cells. J774.A1 cells were cultured in 12-well plates and were treated with serial dilutions of LS for 6 hrs when 95% confluent. Medium was then collected for determination of cytolytic activity of TNF-α using WEHI 164 clone-13 murine fibrosarcoma cells grown in 96-well microtiter plates. One unit of TNF-α activity was defined as the dilution of test sample leading to lysis of 50% of WEHI fibrosarcoma cells. Each dilution or sample was tested in triplicates and experiments were performed three times.

not abolish LS activity and because the protein content of LS was very low, in ng/ml (data not shown) it is unlikely that protein or peptide present in LS was responsible for the observed biological activities.

It is noteworthy that the LS-induced stimulation of TNF-α expression by macrophages is not due to an LS-induced increase in macrophage proliferation. First of all, the effect on TNF-α secretion occurred only 6 hrs after the addition of LS, whereas 4 days of incubation of cells with LS was needed to stimulate cell proliferation. Second, the differential effect of LS on macrophages is dose dependent. The dilution of LS required to induce 50% maximum TNF-α production (log 6×10^{-4}) was much higher than that required to stimulate 50% maximum cell proliferation (10^{-6}).

The observation that LS led to moderate stimulation of lymphocyte proliferation in the presence of PWM suggests that LS enhances immune responses, specifically lymphocyte proliferation after exposure of the host to an antigen (pathogen). Phytohemagglutinin and concanalin A act as porcine T-cell specific mitogens, whereas PWM is a mitogen for both T- and B-cells in the pig (25). It thus appears that LS enhances lymphocyte blastogenesis through a B-cell mediated mechanism. Aattour *et al.* (29) have also observed stimulation of lymphocyte proliferation (and interferon γ production) by orally administered *Lactobacillus* in rats.

Multiple, sometimes contradictory effects of *Lactobacillus* bacteria on immune functions have been described. This likely reflects differences among various strains of *Lactobacillus*. For example, *L. acidophilus* has been described to induce TNF-α production in murine macrophages (26, 27), whereas *L. casei* and *bulgaricus* downregulated the level of TNF-α in intestinal mucosal explants from patients with Crohn disease (30). Similarly, while *L. acidophilus* is known to increase interferon γ (29), treatment with *L. bulgaricus* leads to decreased production of interferon γ (31).

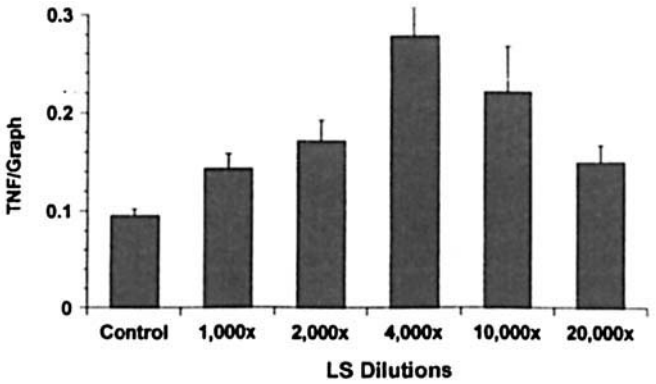


Figure 6. Effect of LS on TNF-α gene expression in porcine LLC-PK1 kidney cells. LLC-PK1 cells were plated at density of 960 cells/well in a 96-well plate, and grown for 72 hrs before the addition of 1μl aliquots of dilutions of LS ranging from 1:400 to 1:10,000 for 3 hrs. Cells were then harvested and RNA extracted as described for J774 cells. Total RNA (2.5 μl) was transcribed to cDNA using oligo(dT)$_{12-18}$ and superscript II primers. TNF-α and GAPDH cDNAs were amplified by PCR as described in Materials and Methods. PCR products were separated on 2% agarose gel containing ethidium bromide and quantified using densitometry. Density of GAPDH cDNA bands in the same gel was used for normalization. The experiments were performed three times. The graph is a densitometric representation of the agarose gel containing PCR products.

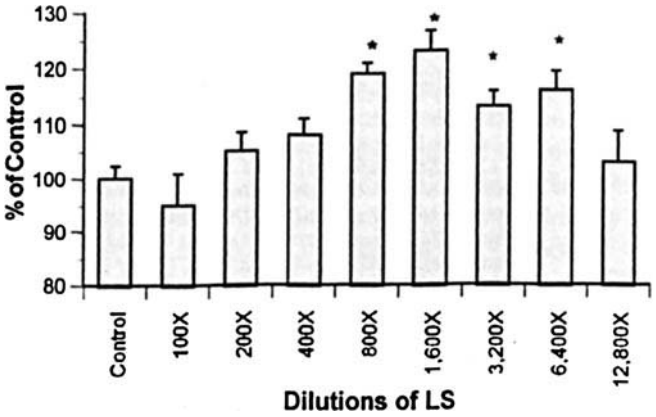


Figure 7. Lymphocyte blastogenesis. Mononuclear cells were separated from peripheral porcine blood by gradient centrifugation on Histopaque 1077 as described in Materials and Methods. When porcine lymphocytes are pre-stimulated with PWM, LS in serial dilutions shows a concentration-dependent enhancement of lymphocyte proliferation. *Significant increase at $P < 0.05$ compared with control group (with PWM only). Each experiment was performed three times in 8 replicates.

The inhibition of secretion of IL-4, IL-5, and IL-8 is a property of many strains of *Lactobacilli* (32, 33).

It is likely that the multiple compounds present in various *Lactobacillus* extracts are responsible for the pleiotropic effects exhibited by such preparations. Exopolysaccharides such as kefiran produced by *Lactobacilli* might be responsible for stimulation of TNF-α by macrophages and other cells (27, 34). A polysaccharide fraction secreted by a strain of *L. bulgaricus* is thought to be responsible for decrease in production of interferon γ (31).

Because *Lactobacillus* extracts and preparations, including LS used in this study, are very acidic, Carbo *et al.* (35) suggested that acetic acid is responsible for the antifungal effect of these bacteria. We did not observe an effect of low pH when we used PBS acidified with HCl in many of our control experiments. We did not use acetic acid because our previous experience indicated mammalian cytotoxicity (Halper, unpublished data). The small volume of acidic solution in one dose administered to cells in culture was quickly neutralized judging from the rapid color changes of the culture medium. It is likely that during *in vivo* administration, the extract would be quickly neutralized as well.

We are the first group to report angiogenic effects of a *Lactobacillus* preparation. LS promoted the formation and growth of capillaries in rodent ear lobes. It is interesting to note that we did not observe angiogenesis in the skin wounds. We attribute the dissimilarity in response to be due to difference in milieu where LS exerts its activity and to the fact that inflammation and angiogenesis occur as separate processes during tissue repair. The disruption of epithelial surface and blood vessels during a punch biopsy leads to tissue destruction, and chemoattraction and activation of inflammatory cells such as PMNs and macrophages, accompanied by increase in TNF-α production rapidly follows such an event. In contrast, sc injection would cause minimal tissue damage, and thus only minimal or no activation of the inflammatory phase of wound healing. Rather, endothelial cells would constitute the primary target. The presence of *Flk-1* protein in blood vessels in ear lobes treated with LS certainly raises the possibility of an indirect effect of LS on endothelial cells.

In conclusion, our data suggest that LS promotes pro-inflammatory processes, including chemoattraction of PMNs and macrophages and angiogenesis in addition to previously described stimulation of production of TNF-α and other cytokines, such as interleukins and interferons. Detailed biochemical characterization is necessary to determine whether the diverse activities of LS are induced by one or multiple compounds present in LS preparations. Because our preliminary data indicate almost no protein presence in LS, we hypothesize that other likely LMW compounds (e.g., carbohydrates) are active components of LS.

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