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Nonlipopolysaccharide Component(s) of *Lactobacillus acidophilus* Stimulate(s) the Production of Interleukin-1α and Tumor Necrosis Factor-α by Murine Macrophages

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Abstract: Previous studies in our laboratory suggested that Lactobacillus acidophilus *strain DDS-1 (LA1) has a suppressive effect on chemically induced tumors in experimental animals. In an effort to understand the possible mechanisms underlying this effect, we investigated the ability of LA1 to induce the production of interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), which have potent cytocidal and cytostatic effects on tumor cells. The mouse macrophage cell line RAW264.7 was incubated with live or heat-killed cells of four strains of* L. acidophilus *or* Bifidobacterium bifidum. Escherichia coli *was used as a source of lipopolysaccharide that is known to induce the above cytokines. The amount of the cytokines present in the culture fluid was quantitated by an enzyme-linked immunosorbent assay. LA1 induced the production of higher levels of IL-1α and TNF-α than other lactobacilli and bifidobacteria. Stimulation of the production of the cytokines was not due to the lipopolysaccharide (LPS) component, since LPS at concentrations equivalent to, or 100-fold greater than, that of LA1 induced only negligible amounts of IL-1α and TNF-α. These results reveal that non-LPS component(s) of LA1 stimulate(s) the production of IL-1α and TNF-α by macrophages, indicating that this organism stimulates the production of immunologic factors.*

Introduction

The beneficial effects of probiotic bacteria have been well documented in the literature. These effects were primarily attributed to the favorable alteration in gastrointestinal microecology, prophylaxis against some types of intestinal infections, and increased tolerance to lactose-containing foods (1). Our previous studies on the beneficial effects of *Lactobacillus acidophilus* strain DDS1 (LA1) indicated that this organism produces B-complex vitamins (2), enzymes that help digest proteins and fats (3), and the natural antibiotic Acidophilin, which inhibits the growth of several undesirable microorganisms (4). It also reduces occurrence of diarrhea (5) and prevents the formation of potentially carcinogenic secondary bile acids (6).

Regular consumption of fermented milks containing lactic acid bacteria has been implicated in lower incidence of colon cancer in several epidemiologic studies (1,7). This observation has prompted the investigations on the tumor-preventing role of lactobacilli. A recent study in our laboratory found that rats given LA1 orally had lower incidence of colon tumor when challenged with a chemical carcinogen (8). Although detailed information is not available on the antitumor activity of lactic acid bacteria, it has been suggested that this activity may be macrophage dependent (9). Macrophages are known to play a key role in the immune response to tumors (10). Antitumor activity of macrophages may involve direct contact with the tumor cells and the secretion of a number of cytokines that are directly or indirectly involved in antitumor activity. Interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) secreted by macrophages exhibit cytostatic and cytocidal effects on several tumor cell lines *in vitro* (11,12). They also costimulate the activation of T-helper cells, which in turn secrete other cytokines, especially IL-2, which induces the proliferation and activity of cytotoxic T lymphocytes that are specific for the tumor cells (13,14). IL-1 and IL-2 also enhance the activity of natural killer cells, which kill the tumor cells in a nonspecific manner (13).

Lactic acid bacteria have been suggested to stimulate macrophages (15). In addition to the enhanced phagocytosis and production of enzymes, stimulation of macrophages enhanced the production of cytokines. In a recent study by Sekine and co-workers (9), intraperitoneal injection of a cell wall preparation of *Bifidobacterium infantis* induced the expression of mRNA for several cytokines in the peritoneal exudate cells of BALB/c mice. Lactobacilli have been shown to induce interferon production in mice (16). The immunomodulatory effect of lactic acid bacteria, including bifidobacteria, was found to be dependent on the inherent properties of each strain rather than on the common characteristics of the bacterial species (17). The correlation between regular con-

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sumption of fermented milk and the lowered incidence of colon cancer, our observation of the prophylactic effects of LA1 on chemically induced tumors in rats, and the importance of macrophages in antitumor immune response prompted us to further investigate the specific effects of LA1 on macrophages. In this study we examined the ability of this bacterium to stimulate murine macrophages to produce IL-1α and TNF-α, the key cytokines in antitumor immune responses. Because mature macrophages produce IL-1α and TNF-α (18), we investigated the production of these cytokines in an *in vitro* macrophage system.

Materials and Methods

Macrophages

The murine macrophage cell line RAW264.7 of BALB/c origin, developed by Raschke and colleagues (19), was obtained from the American Type Culture Collection (Rockville, MD). The macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.2% gentamycin. Macrophage viability was determined by the trypan blue dye exclusion method. This cell line does not constitutively produce IL-1α or TNF-α.

Bacterial Cultures and Lipopolysaccharide

LA1 and *B. bifidum* were obtained from our laboratory culture collection, and *L. acidophilus* strains NRRL 0734, NRRL 6934, and NRRL B4527 were obtained from the National Center for Agricultural Utility Research (Peoria, IL). *Escherichia coli* ATCC 25922 was obtained from American Type Culture Collection. The lactobacillus and bifidobacteria cultures were cultivated in MRS broth (Difco Laboratories, Detroit, MI); the *E. coli* was cultivated in nutrient broth (BBL, Cockeysville, MD). The cultures were harvested after 12 hours, centrifuged at 5,000 *g* for 10 minutes, washed, and resuspended in phosphate-buffered saline (PBS). The counts [colony-forming units (cfu)] of bacterial culture suspensions were determined before the cultures were heat killed at 72°C for 10 minutes. The viability of the heat-killed cultures was checked and found to be zero. Lipopolysaccharide (LPS) from *Salmonella typhosa*, containing ≥500 endotoxin units (EU)/μg, was obtained from Sigma Chemical (St. Louis, MO). LPS dissolved in PBS was used as a positive control for the production of IL-1α and TNF-α by the macrophage cell line. Endotoxin units (as a measure of the LPS content) of the bacterial suspensions were determined by the Limulus amoebocyte lysate assay (20).

IL-1α and TNF-α Assay

The macrophages cultured in tissue culture dishes at 37°C in a humidified incubator with 7.5% CO_2 atmosphere were harvested by scraping with a cell scraper and washed once with PBS. The macrophages were then added to the wells of a 24-well culture plate (5×10^5 cells/well) and incubated until they became 80% confluent on the surface of the wells. The medium was then aspirated, and the samples (media, bacterial broth, bacterial cells, or LPS) mixed with DMEM supplemented with 10% fetal bovine serum were added to the cells and incubated for 24 hours. At the end of the incubation period, the culture supernatant fluid was aspirated, centrifuged at 7,000 *g* for five minutes at 4°C, aliquoted, and stored at −20°C until further use. The amounts of IL-1α and TNF-α present in the culture fluids were quantitated by an enzyme-linked immunosorbent assay (ELISA) using the respective ELISA kits from Endogen (Cambridge, MA) according to the manufacturer's instructions.

Results

Live and Heat-Killed Cells of LA1

Preliminary experiments performed to compare the ability of live and heat-killed LA1 to induce the production of IL-1α showed that live cells stimulated the production of twice the amount of IL-1α compared with the heat-killed cells. Concomitantly, a 4.5-fold increase in the number of live cells was observed at the end of the incubation period. Stimulation of higher levels of IL-1α production by live LA1 cells was very likely due to the increased cell count during the incubation. Because heat-killed LA1 cells also induced production of IL-1α comparable to that of live cells (when the increase in cell numbers was considered), further experiments were conducted with heat-killed cells of bacterial cultures. IL-1α and TNF-α production by the macrophages was not observed with the media and bacterial broths that were used as negative controls (data not shown).

Effect of Various Concentrations of LA1 on Production of IL-1α and TNF-α by Macrophages

To compare the relative amounts of IL-1α and TNF-α produced by the macrophages in response to other test organisms, production of these cytokines by the macrophages in response to various concentrations of LA1 was first determined. The dose-response curve (Figures 1 and 2) showed that $\log_{10}$ 6.6 of the cfu equivalent per milliliter of culture fluid induced maximum production of IL-1α, whereas $\log_{10}$ 5.0 cfu/ml of LA1 induced maximum production of TNF-α.

Cytokine Production Induced by Other Bacteria

Heat-killed cultures of different strains of *L. acidophilus*, *B. bifidum*, and *E. coli* were tested for their ability to induce the production of IL-1α and TNF-α by the macrophages. Although other lactobacilli and bifidobacteria cultures also induced the production of various amounts of these cytokines by the macrophages, LA1 induced the highest amount

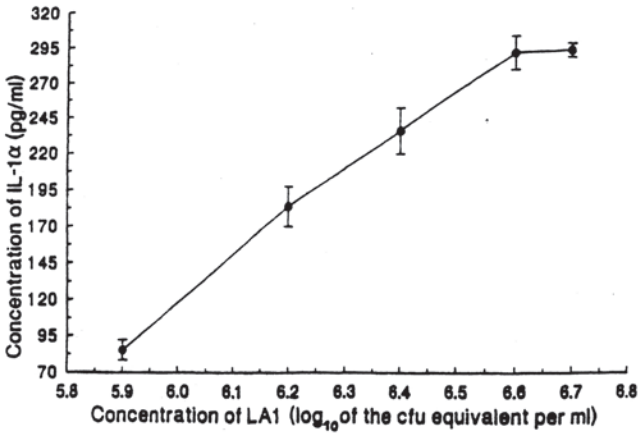

Figure 1. Effect of concentration of heat-killed *L. acidophilus* strain DDS1 (LA1) cells on production of interleukin-1α (IL-1α) by macrophages. Amount of IL-1α produced by macrophages in response to various concentrations of heat-killed LA1 was quantitated by enzyme-linked immunosorbent assay. Concentration of LA1 is expressed as $\log_{10}$ of colony-forming unit (cfu) equivalent per ml. Values are means of triplicates; vertical bars, SD.

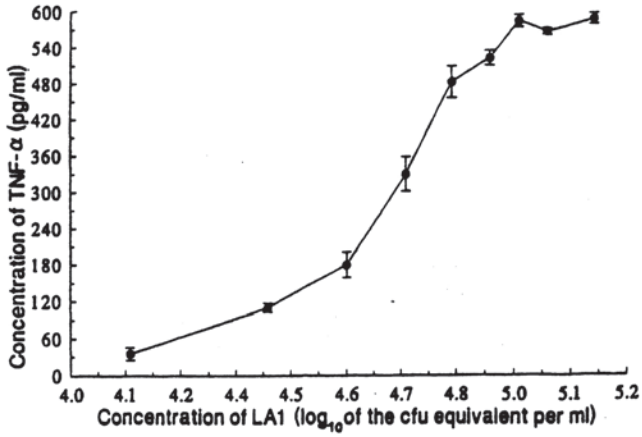

Figure 2. Effect of concentration of heat-killed LA1 cells on production of tumor necrosis factor-α (TNF-α) by macrophages. Amount of TNF-α produced by macrophages in response to various concentrations of heat-killed LA1 was quantitated by enzyme-linked immunosorbent assay. Concentration of LA1 is expressed as $\log_{10}$ of cfu equivalent per ml. Values are means of triplicates; vertical bars, SD.

of IL-1α (315 pg/ml; Figure 3) and TNF-α (588 pg/ml; Figure 4) among the lactic acid bacteria.

Role of LPS in Stimulation of IL-1α and TNF-α Production by Macrophages

As expected, *E. coli*, which has very high amounts of LPS, induced the highest amount of IL-1α (Figure 3) and TNF-α (Figure 4). To rule out the possibility that the cytokine-inducing ability of LA1 may be due to its LPS component, we determined the endotoxin concentration of LA1 and compared it with that of *E. coli*. The Limulus amoebocyte lysate assay revealed that LA1 contained negligible amounts of endotoxin (0.0176 U/ml) compared with *E. coli* (310 EU/ml).

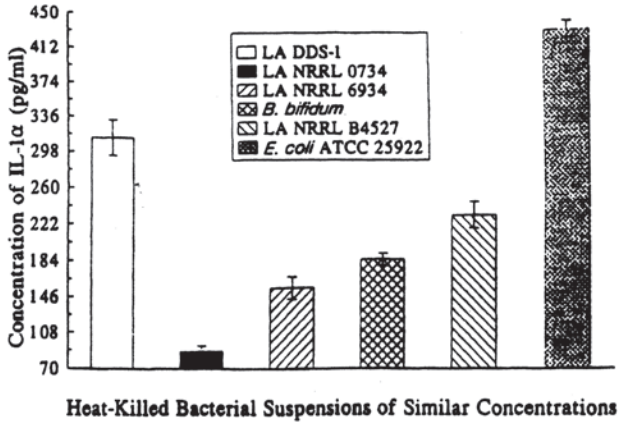

Figure 3. Production of IL-1α by LA1 (LA DDS-1), *L. acidophilus* strains NRRL 0734, NRRL 6934, and NRRL B4537 (LA NRRL 0734, LA NRRL 6934, and LA NRRL B4537), *Bifidobacterium bifidum*, and *Escherichia coli* ATCC 25922. Amount of IL-1α produced by macrophages in response to stimulation with equivalent amounts (identical optical density units/ml) of heat-killed bacterial cultures was determined by enzyme-linked immunosorbent assay. Values are means of triplicates; vertical bars, SD.

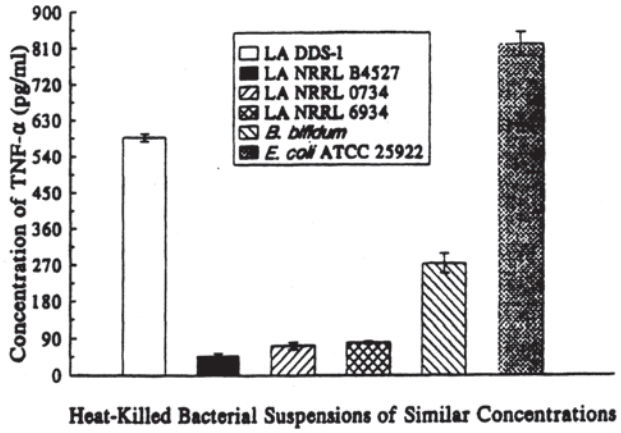

Figure 4. Production of TNF-α by different bacterial cultures. Amount of TNF-α produced by macrophages in response to stimulation with equivalent amounts (identical optical density units/ml) of heat-killed bacterial cultures was determined by enzyme-linked immunosorbent assay. Values are means of triplicates; vertical bars, SD.

To confirm further that the cytokine-inducing ability of LA1 was due to its non-LPS component(s), we compared the cytokine-inducing ability of LA1 with that of LPS at concentrations equivalent to the concentration of endotoxin in LA1, as well as 10-fold and 100-fold greater than that of LA1. As shown in Figure 5, LPS at concentrations equivalent to the endotoxin concentration of LA1 induced negligible amounts of the cytokines. Even at a 100-fold greater concentration, LPS induced only a very small amount of IL-1α or TNF-α.

Discussion

Several epidemiologic studies have suggested that the low incidence of cancer in certain populations may be due to the regular consumption of fermented milk products by

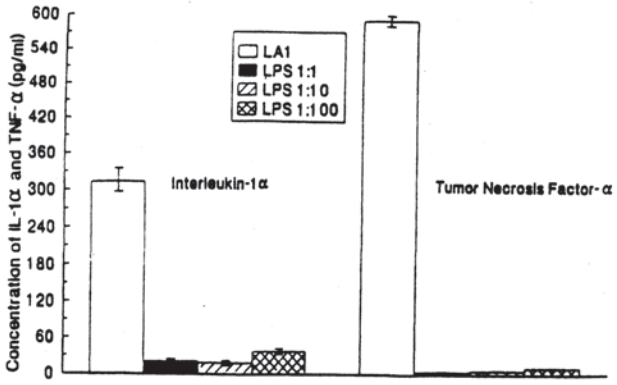

Figure 5. Role of lipopolysaccharide (LPS) in production of IL-1α and TNF-α by macrophages. Amount of IL-1α and TNF-α produced by macrophages in response to LA1 and LPS was determined by enzyme-linked immunosorbent assay. LPS was used at concentrations equivalent to endotoxin concentration of LA1 and at concentrations 10- and 100-fold higher. Values are means of triplicates; vertical bars, SD.

the individuals in these populations (1,7). However, it has not been demonstrated that the observed beneficial effects are in fact due to the modulation of the immune system by specific components of lactic acid bacteria. Previous studies in our laboratory indicated that LA1 exhibited antitumor activity in chemically induced tumors in rats (8). To gain insight into the possible mechanism of the observed prophylactic role of LA1 in tumor prevention, we investigated the ability of LA1 to stimulate macrophages, since it has been suggested that the antitumor activity of LA1 may be macrophage dependent (9). The antitumor activity of macrophages is mediated by direct contact with the tumor cells as well as via the production of several cytokines. We decided to study the IL-1α and TNF-α production first, since in addition to the direct cytostatic and cytocidal effects, these cytokines have indirect effects on cytotoxic T lymphocytes and natural killer cells via the activation of T-helper cells (13).

It is clear from our results that LA1 induces the production of IL-1α and TNF-α and that heat killing of the bacterial cells does not abrogate this stimulatory activity. It has been reported earlier that heat-killed bifidobacteria, lactobacteria, and enterococci augmented the immunologic response in experimental salmonellosis infection in mice (21) and heat-killed *Lactobacillus gasseri* induced the production of IFN-α by macrophages (22). Hence, we used heat-killed cultures in our experiments. The use of heat-killed bacteria also circumvented the problem of multiplication of bacteria and competition for nutrients during incubation with the macrophages. The macrophages were incubated with LA1 for 24 hours, since our preliminary studies indicated that the cytokine production was maximal at 24 hours poststimulation (data not shown).

LA1 induced the production of IL-1α and TNF-α in a dose-dependent manner, with the maximum production stimulated by heat-killed bacteria derived from $\log_{10}$ 6.6 and $\log_{10}$ 5.0 of the cfu equivalent per milliliter of live cells, respectively. Of all the bacteria tested, LA1 induced production of the highest amounts of cytokines, except *E. coli*, which induced the highest concentrations of IL-1α and TNF-α, respectively. This finding is not surprising in light of the fact that the cell wall of Gram-negative bacteria such as *E. coli* contains high amounts of LPS (310 EU/ml), which is known to induce cytokine production (23). However, little is known about the immunogenic structure of Gram-positive bacteria, which includes the lactic acid bacteria (24). The comparison of the IL-1α and TNF-α inducing ability of LA1 with that of LPS at concentrations equivalent to that of LA1 and severalfold higher (10- and 100-fold) clearly indicated that the cyto- kine-inducing ability of LA1 was not due to its endotoxin content but to certain other components of the cell. It has been reported that molecules such as peptidoglycan present in lactic acid bacteria can induce cytotoxic effects against tumor cells (25). Cell-free extracts of *L. acidophilus* and *Bifidobacterium longum* were found to stimulate phagocytic activity in an *in vitro* macrophage system (26). It is possible, therefore, that cell-free extracts of LA1 could induce the production of IL-1α. However, this needs to be confirmed experimentally. In summary, our results clearly indicate that LA1 stimulates macrophages and induces the production of IL-1α and TNF-α. This stimulatory effect is mediated by non-LPS component(s) of LA1.

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