INFECTION AND IMMUNITY, Dec. 1996, p. 5403–5405 Vol. 64, No. 12
0019-9567/96/$04.00+0

Production of Human Tumor Necrosis Factor Alpha, Interleukin-6, and Interleukin-10 Is Induced by Lactic Acid Bacteria

MINJA MIETTINEN,[1]* JAANA VUOPIO-VARKILA,[1] AND KARI VARKILA[2]

National Public Health Institute, 00300 Helsinki,[1] and Orion, 02101 Espoo,[2] Finland

Received 12 February 1996/Returned for modification 17 May 1996/Accepted 30 September 1996

To investigate the role of cytokines in interactions between lactic acid bacteria and the immune system, we measured production of tumor necrosis factor alpha, interleukin-6 (IL-6), and IL-10 from human peripheral blood mononuclear cells after stimulation with live or glutaraldehyde-fixed bacteria. Production of tumor necrosis factor alpha, IL-6, and, in some cases, IL-10 was induced in amounts even greater than those obtained with lipopolysaccharide as a stimulant. Our results suggest that lactic acid bacteria can stimulate nonspecific immunity.

Strains of the genera *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* are commonly referred as lactic acid bacteria (LAB). LAB are considered nonpathogenic and are believed to be beneficial to human health (7, 8, 14). So-called probiotic effects of LAB could include stimulation of the immune system. LAB have been shown to induce proliferation of immune cells and to enhance synthesis of antibodies to microbial pathogens (5, 15, 16, 29). The fact that they are nonpathogenic and safe for humans when administered orally, as shown by their use in many dairy products, makes LAB attractive as live vectors for oral or local vaccines (11, 19).

Lipopolysaccharide (LPS) of gram-negative bacteria induces production of proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), as well as IL-10, which is known to inhibit the synthesis of the former two cytokines (20, 32). These cytokines contribute to defense mechanisms of the host in response to bacterial colonization or invasion, and when secreted in excess, they may induce immunopathological disorders. Many components of gram-positive bacterial cell wall, e.g., capsular polysaccharides, peptidoglycans, and lipoteichoic acids, have been shown to be involved in cytokine induction. However, studies have been focused mostly on pathogenic streptococci and staphylococci (2, 10, 17, 21, 24, 28, 31). The ability of LAB and their cell wall components to induce cytokine release has been previously poorly studied. Only production of alpha interferon and TNF-α from murine cells and gamma interferon from human peripheral blood lymphocytes have been reported (5, 9, 12, 13, 23, 25).

Since LAB have been suggested to stimulate the immune system, we investigated whether LAB exert their effects through induction of the cytokines TNF-α, IL-6, or IL-10 from human peripheral blood mononuclear cells (PBMCs). We compared the abilities of 10 different LAB strains to induce cytokine release. To assess the role of bacterial surface structures in these interactions, we used both live and glutaraldehyde-fixed whole bacterial cells.

Bifidobacterium longum E505 and *Lactobacillus paracasei* subsp. *paracasei* E506 were obtained from C. Hansen A/S; *Lactobacillus acidophilus* E507 and *Bifidobacterium animalis* E508 were from Norske Meierier and C. Hansen A/S; *Lactobacillus rhamnosus* E509 and *L. paracasei* subsp. *paracasei* E510 were from Arla R&D; *L. rhamnosus* GG E522 (ATCC 53103) and *Lactococcus lactis* subsp. *cremoris* E523 (ARH 74) were from Valio, Ltd., R&D; and *Lactococcus lactis* subsp. *lactis* E414 and *Lactobacillus plantarum* E98 were from VTT. *Escherichia coli* HB101 (3) was used as a control.

For stimulation assays, bacteria were passaged three times before testing. Strains were first grown on MRS agar (Difco, Detroit, Mich.) at 37°C for 48 h and then were inoculated in MRS broth and grown for 24 h, diluted 1/100, and grown to the logarithmic growth phase as measured by an A_{600} of 0.5. The number of viable bacteria was confirmed by plating of serial dilutions. To obtain nonviable cells, logarithmic-growth-phase bacteria were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in phosphate-buffered saline (PBS) [pH 7.4] at a concentration of 10^{10} CFU/ml for 2 h at room temperature. Bacterial cells were washed twice with PBS, aliquoted, and stored at −20°C.

Human peripheral blood mononuclear leukocytes were isolated from buffy coats obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland) by centrifugation over Ficoll-Paque ET (Pharmacia, Uppsala, Sweden). After washing, the cells were resuspended in RPMI 1640 medium (Sigma, St. Louis, Mo.) containing 10% heat-inactivated fetal calf serum (Integro, Zaandam, Holland), 2 mM L-glutamine (Sigma), 100 U of penicillin per ml, and 100 μg of streptomycin (Gibco BRL, Paisley, Scotland) per ml at a final concentration of 10^6 cells per ml. Purified leukocytes (10^6 cells per ml) were incubated in a volume of 2 ml in 24-well plates (Nunc, Roskilde, Denmark) in 5% CO_2 at 37°C for 24, 48, 72, or 96 h with stimulators. Final concentrations of live and fixed bacteria used in the assays were 10^5, 10^6, or 10^7 CFU/ml. Purified LPS from *E. coli* HB101 (kindly provided by I. Helander, National Public Health Institute, Helsinki, Finland) was used as a control at a concentration of 10 μg/ml. The optimal amount of LPS used in stimulations was determined by titration. Every sample was assayed in triplicate, and each experiment was performed with mononuclear cells from two to five donors. Cell culture supernatants were harvested and stored at −20°C.

The cytokines present in cell culture supernatants were quantitated by a sandwich enzyme-linked immunosorbent assay (ELISA) method. The substrate for peroxidase–*Z*-avidin

* Corresponding author. Mailing address: Department of Bacteriology, National Public Health Institute, Mannerheimintie 166, 00300 Helsinki, Finland. Phone: 358-9-4744378. Fax: 358-9-4744238. Electronic mail address: minja.miettinen@ktl.fi.

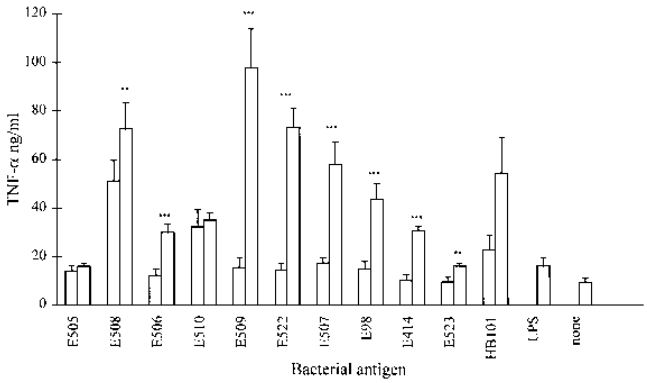

FIG. 1. TNF-α production induced by fixed (open bars) and live (shaded bars) bacteria after 24 h of stimulation. Concentrations of 10^6 CFU of bacteria per ml, 10 μg of LPS per ml, and 10^6 PBMCs per ml were used. Results are means ± standard errors of four assays, each done with PBMCs from a different donor. $P < 0.01$ (**) and $P < 0.001$ (***) for fixed versus live bacterial strains as determined by Student's two-tailed paired *t* test.

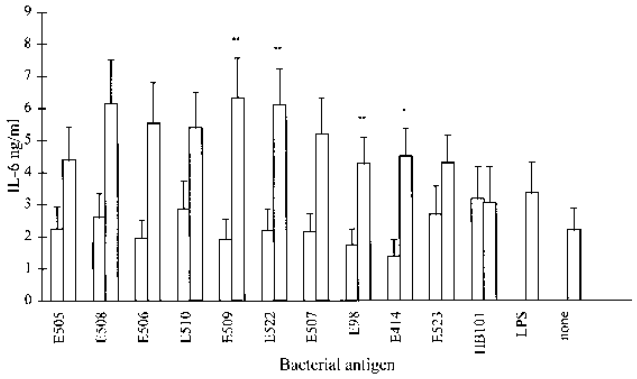

FIG. 2. IL-6 production induced by fixed (open bars) and live (shaded bars) bacteria after 24 h of stimulation. Concentrations of 10^6 CFU of bacteria per ml, 10 μg of LPS per ml, and 10^6 PBMCs per ml were used. Results are means ± standard errors of four assays, each done with PBMCs from a different donor. $P < 0.05$ (*) and $P < 0.01$ (**) for fixed versus live bacterial strains as determined by Student's two-tailed paired *t* test.

(Zymed, San Francisco, Calif.) used in all ELISAs was *o*-phenylenediamine dihydrochloride (Sigma), and A_{492} was measured. Antibodies and standards used in TNF-α and IL-10 ELISA tests were from Pharmingen, San Diego, Calif. An ELISA specific for human TNF-α was set up and was sensitive to 20 pg/ml. Monoclonal mouse anti-human TNF-α antibody clone MAb1 was used as the capturing antibody, and biotinylated mouse anti-human TNF-α antibody clone MAb11 was used as the detection antibody. Recombinant human TNF-α from *E. coli* was used as a standard. An ELISA set up for human IL-10 was sensitive to 10 pg/ml. Monoclonal rat anti-human IL-10 antibody clone JES3-9D7 was used as the capture antibody, and biotinylated rat anti-human and viral antibody clone JES3-12G8 was used as the detection antibody. Recombinant human IL-10 from *E. coli* was used as a standard. The Duoset kit for human IL-6 (Genzyme, Cambridge, Mass.) was used according to the manufacturer's instructions. The sensitivity of the assay was 20 pg/ml.

The results are expressed as means ± standard errors. Statistical comparisons were made with Student's two-tailed paired *t* test. Differences were considered significant at $P < 0.05$.

Live bacteria stimulated TNF-α production better than glutaraldehyde-fixed bacteria (Fig. 1). Also, LAB strains differed in their abilities to stimulate TNF-α production (Fig. 1). Four of the weakest inducers, *B. longum* E505, *L. lactis* subsp. *cremoris* E523, *L. paracasei* subsp. *paracasei* E506, and *L. lactis* subsp. *lactis* E414 differed significantly ($P < 0.01$) from four of the best inducers, *L. rhamnosus* E509, *L. rhamnosus* E522, *B. animalis* E508, and *L. acidophilus* E507. All live strains, with the exception of *B. longum* E505 and *L. lactis* subsp. *cremoris* E523, were significantly better ($P < 0.01$ or $P < 0.001$) than LPS in inducing TNF-α release. The induction of TNF-α production from PBMCs was time and concentration dependent, with production being optimal after 24 h of stimulation with 10^6 CFU of bacteria per ml (data not shown).

Baseline production of IL-6 showed slight variability in repeated experiments, and the amounts of IL-6 released after stimulation with LAB strains varied accordingly. Most live bacteria showed a trend toward being better inducers of IL-6 release than fixed bacteria (Fig. 2). Live bacteria induced the release of IL-6 after 24 h in nearly the same or larger amounts than that induced by LPS, but the differences were not significant. Three of the best inducers, *L. rhamnosus* E509, *L. rhamnosus* E522, and *B. animalis* E508, differed significantly ($P < 0.05$) from three of the weakest inducers, *L. plantarum* E98, *L. lactis* subsp. *cremoris* E523, and *B. longum* E505. A slight increase in IL-6 release as a function of time was observed (data not shown).

IL-10 production varied depending on the donor of the PBMCs. Most PBMC donors were unresponsive to both LAB or LPS stimulation; however, with good responders, the same strains that were good inducers of TNF-α and IL-6 emerged as good inducers of IL-10, with several strains being better than LPS in inducing IL-10 release (data not shown).

Our results show that several strains of live nonpathogenic LAB induced significantly higher amounts of TNF-α to be released from human PBMCs than did LPS. A similar finding has previously been reported for streptococci (21). However, significant differences between LAB strains in their ability to induce TNF-α release were seen. Our results further suggest, similarly to other gram-positive bacteria, that surface structures of LAB are important in determining immune responses (2, 10, 17, 18, 21, 24, 26, 28, 31). In our experiments, live bacteria were better inducers of TNF-α release than fixed bacteria. With 8 of 10 LAB strains studied, significant differences in TNF-α induction between live and fixed cells were detected. Since glutaraldehyde is a cross-linking agent, it may denature proteins and change their immunological properties (30). The observed inferiority of glutaraldehyde-fixed LAB to induce TNF-α could thus be the result of changes in their immunostimulatory surface structures, which could, as a consequence, diminish their capacity to interact with PBMCs. The cell wall compositions of different LAB strains may vary. Most but not all LAB contain teichoic acid, and their peptidoglycans can be different (27). For lactobacilli, differences between strains in their ability to confer immunopotentiating effects have been attributed to differences in cell wall structures (26). The size and tertiary configuration of peptidoglycan are important in induction of TNF-α release (31), and the presence of a capsule could result in greater TNF-α induction by increasing bacterial cell size or by other physical factors (17). Differences in TNF-α induction seen between LAB strains in our study might be due to variability in surface structures. Because three of the best inducers of TNF-α, *L. rhamnosus* E509 and E522 and *L. acidophilus* E507, contain the same peptidoglycan type as two of the weakest inducers, *L. paracasei* subsp. *paracasei* E506 and E510 (27), other components be-

sides peptidoglycan are also probably of importance in the phenomenon. In a preliminary study, some LAB broth culture supernatants showed induction of cytokine release, and studies are under way to learn more about the possible soluble bacterial factors involved in cytokine induction.

Despite variability in the baseline production of IL-6, the same three strains that were good inducers of TNF-α appeared to induce large amounts of IL-6 as well. Similarly, the same two strains were among the weak inducers for both cytokines. IL-6 release is known to be primed by monocyte adherence (22) and is suggested to be affected by methods of cell separation (22) as well as by duration of monocyte culture (4) and the donor (1). The variability observed in our study is likely to be explained partly by the reasons mentioned above and more specifically by differences between PBMCs from separate donors and by the time before the blood samples were processed. We noticed differences among PBMC donors also in terms of IL-10 production. These differences did not correlate with variability in production of IL-6 or TNF-α, since PBMCs that were unable to respond by producing measurable IL-10 after stimulation with bacteria or LPS did produce IL-6 and TNF-α. At the moment, this finding has no clear explanation, since at least after LPS stimulation of PBMCs, IL-10 (6), as well as IL-6 and TNF-α, is mainly produced by macrophages. Future studies are warranted to elucidate the mechanisms of the blockade in IL-10 release seen in this study in certain individuals.

In conclusion, the present work demonstrates that several live LAB but not glutaraldehyde-fixed LAB are potent inducers of TNF-α release from human PBMCs in quantities even higher than those induced with LPS. Production of IL-6 is also induced by LAB, with live cells being more potent than fixed cells. Induction of proinflammatory cytokines TNF-α and IL-6 by LAB could indicate that LAB can stimulate nonspecific immune responses. The finding that live bacteria induce the release of TNF-α and IL-6 better than fixed bacteria could be important with regard to using live nonpathogenic LAB as vaccine vectors or as probiotics for the purpose of stimulating nonspecific immunity.

This work was supported by the Nordic Industrial Fund, project number P 93176.

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Editor: J. R. McGhee