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Oxygen Radical Production by Peritoneal Macrophages and Kupffer Cells Elicited with *Lactobacillus casei*

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BALB/c mice were injected intraperitoneally (i.p.) or intravenously (i.v.) with *Lactobacillus casei* YIT9018 (LC 9018). The i.p. injected LC 9018 augmented oxygen radical (OR) production by peritoneal macrophages (PM) and suppressed the production of prostaglandin E_2 by PM. The growth of i.p. inoculated Meth A fibrosarcoma was also inhibited by an i.p. injection of LC 9018. i.v. injection of LC 9018 stimulated OR production by fixed macrophages and inhibited the growth of *Listeria monocytogenes* in the liver. Furthermore, glucose-6-phosphate dehydrogenase activity in the liver was significantly increased (10 to 20 times) by LC 9018 i.v. injection. A significant correlation was observed between the augmentation of OR production by PM or fixed macrophages in the liver and inhibition of growth of Meth A or *L. monocytogenes*. The augmentation of OR production by LC 9018 was more marked and was maintained for a longer period of time than that by other bacterial immunostimulants.

Macrophages can kill or inhibit the growth of certain tumor cells and bacteria. The mechanisms involved are as yet ill defined, but several macrophage products with antitumor or antibacterial properties have been reported, including arginase (12), a serine protease (2), and tumor necrosis factor (24, 33). The contribution of each of the macromolecular mediators to the total antitumor (1, 30) and antibacterial (31, 33) effect of the macrophages is unclear. On the other hand, macrophages produce and release oxygen radicals (OR) which are mediators for tumoricidal activity (30) and inflammatory damage (25). Macrophages can be activated in vivo by *Corynebacterium parvum* (30), *Streptococcus pyogenes* OK-432 (17, 38) *Mycobacterium bovis* (30), and *Lactobacillus casei* (LC 9018) (20, 21). These bacteria have potent antitumor activity in cell-mediated immune responses.

It has been reported that prostaglandin E_2 (PGE_2) regulates several macrophage functions and suppresses the tumoricidal activity of macrophages (39, 40) and that it may interfere, via a cAMP-mediated mechanism, with early events in the activation process leading to the enhanced capacity of these cells to produce OR (26). Although when macrophages were activated in vitro by an immunostimulator such as lipopolysaccharide (40) or yeast glucan (7), the PGE_2 production increased, it was decreased by in vivo activation with *C. parvum* or *M. bovis* (6, 16).

On the other hand, liver has the highest number of fixed macrophages (Kupffer cells) in the body (11, 29) and one of the highest frequencies of metastatic invasion of tumor cells (8, 43). The liver plays a crucial role in the activation, detoxification, and clearance of many endogenous and exogenous substances (32, 44) and has been found to phagocytize tumor cells and modify tumor growth in vivo (33, 36); in particular, Kupffer cells play an important role in the host defense against bacterial infection (27, 28).

Glucose-6-phosphate dehydrogenase (G6PD) activity in the liver is significantly increased by intravenous (i.v.) injection of *C. parvum* (15). ORs are synthesized by the NAD(P)H oxidase present in the cell membranes of macrophages and polymorphonuclear leukocytes (3, 19), and NADPH is supplied from the hexose monophosphate shunt. Therefore, it can be presumed that G6PD activity and OR production in the liver should increase markedly after i.v. injections of immunopotentiators.

It has been reported that LC 9018 has significant antitumor activity due to the activation of macrophages (20, 21). The present study is an investigation of the effect of LC 9018 on OR production by peritoneal macrophages (PM) or Kupffer cells, on G6PD activity in the liver, and on tumor cells and bacterial growth in comparison with that of bacterial immunostimulants such as *C. parvum* or OK-432 in BALB/c mice.

MATERIALS AND METHODS

Animals. Male 8- to 9-week-old BALB/c mice were purchased from Shizuoka Agricultural Cooperative Experimental Animals (Hamamatsu, Japan). Throughout the experimental period, food and water were provided ad libitum, and the animals were maintained in an air-conditioned animal room.

Bacterial preparation. LC 9018 and *Lactobacillus fermentum* YIT0159 (LF) were cultivated on Rogosa medium, washed with distilled water, heated at 100°C for 30 min, and lyophilized. *C. parvum* and OK-432 were supplied commercially by the Institute Merieux, Lyon, France, and Chugai Pharmaceutical Co. Ltd., Tokyo, Japan, respectively.

Preparation of PM. LC 9018, OK-432, or LF (total dose, 300 μg per mouse) was injected into the peritoneal cavity of mice for 3 consecutive days. The peritoneal cells were harvested 1, 2, 3, 4, and 7 days after the last bacterial injection. The peritoneal cells were washed three times with phenol red-free Hanks-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.) solution, suspended in Eagle medium containing 10% fetal calf serum, and transferred to vials. The vials were incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2. Thereafter, the nonadherent cells were washed off with Eagle medium. The percentage of macrophages was more than 95%, as determined morphologically or by phagocytic (latex beads) activity.

Preparation of Kupffer cells (13, 34, 41). Bacteria were injected i.v. for 3 consecutive days (total dose, 300 μg per mouse). On days 2, 5, 7, 10, 14, and 20 after the final injection, livers were perfused with 0.3% pronase E (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) in Hanks-HEPES solution (pH 7.4), removed, chopped into small pieces, and

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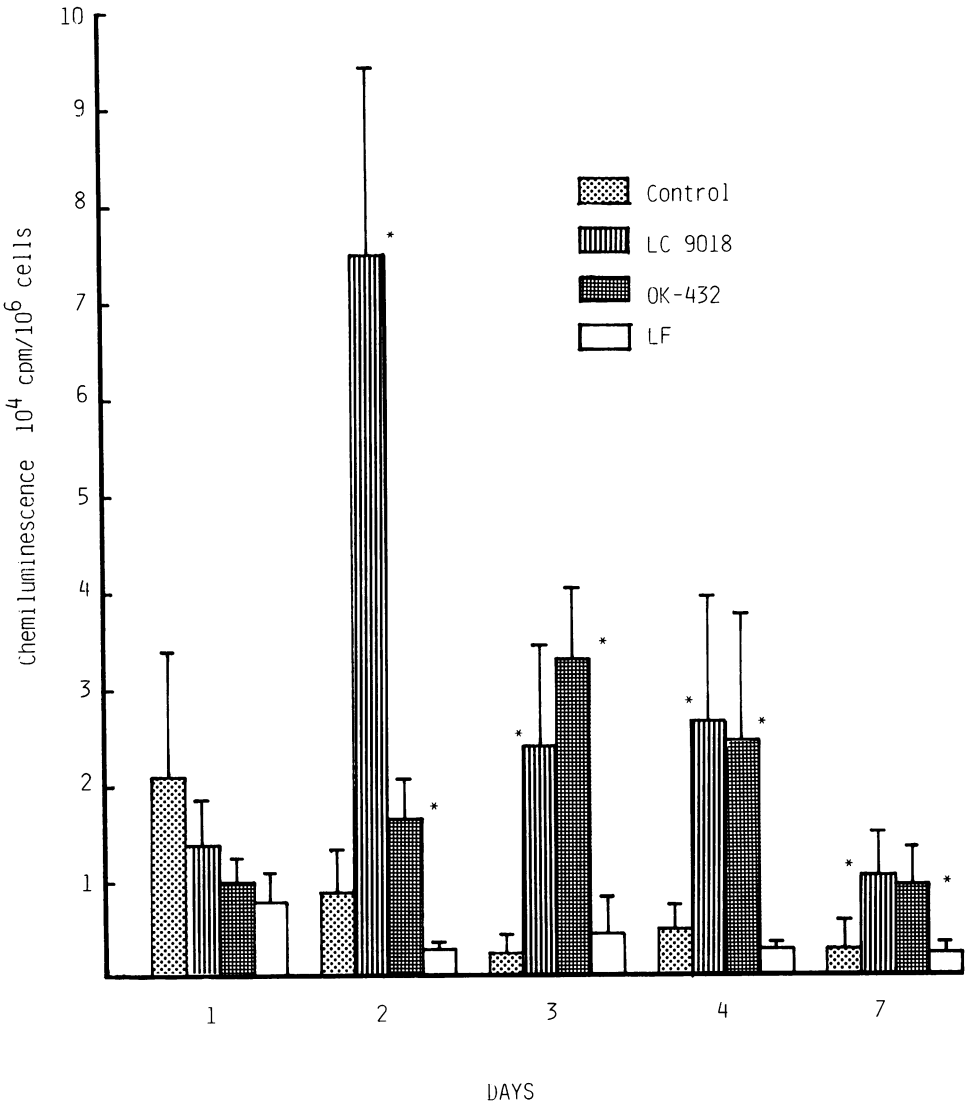

FIG. 1. OR production by LC 9018-, OK-432-, or LF-activated PM. BALB/c mice were injected i.p. with LC 9018, OK-432, LF, or saline for 3 consecutive days (total dose, 300 μg per mouse). The PM were harvested 1, 2, 3, 4, and 7 days after the final injection. The values are the mean ± standard deviation of six mice. *, Significantly different from control; $P < 0.01$.

transferred to a 50-ml flask. The fragments were digested for 60 min in 0.3% pronase E in Hanks-HEPES solution (10 ml/g of liver) at 37°C. After 20 and 40 min of incubation, 0.5 mg of DNase (type I; Boehringer Mannheim GmbH, West Germany) was added to digest the cellular debris. At the end of the incubation period, the suspension was filtered through a stainless steel mesh (80 mesh). The filtrate was centrifuged (1,000 rpm; 10 min), and the precipitate was suspended in ice-cold Dulbecco phosphate-buffered saline (D-PBS; pH 7.4). The precipitate was washed three times with ice-cold D-PBS. After being washed, it was suspended in 2.5 ml of isotonic Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden)-D-PBS solution (density, 1.032), and then the suspension was added to the isotonic Percoll-D-PBS solution (density, 1.065). The samples were centrifuged (3,000 rpm; 60 min; 4°C), and the Kupffer cells in the middle layer were washed three times with phenol red-free Eagle medium supplemented with 20 mM HEPES buffer (pH 7.4). The Kupffer cells were counted with a Coulter counter (model ZBI; Coulter Electronics, Inc., Hialeah, Fla.), and their viability was determined by the trypan blue exclusion method and found to be always above 85%.

Luminol-chemiluminescence assay. The OR production by PM or Kupffer cells was measured by the luminol-chemiluminescence assay method (26, 42). The assay medium consisted of phenol red-free Eagle medium supplemented with 20 mM HEPES buffer (pH 7.4) to which luminol (0.04 μg/ml; Sigma) had been added. The luminol was dissolved in dimethyl sulfoxide (E. Merck, Darmstadt, West Germany) at a concentration of 10 mg/ml and then diluted to 1/100 with the assay medium. The vials containing PM in the assay medium were placed in a 37°C water bath for 2 min and then loaded onto a Pico-lite Luminometer (model 6100; Packard Instrument Co., Inc.). Phorbol myristate acetate (Sigma), dissolved in dimethyl sulfoxide (0.1 μg/ml), was added as a triggering stimulus. The chemiluminescence was subtracted, and the remaining value was expressed as counts per minute. The luminometer was used at 37°C.

PGE_2 production by PM (9). [1-^{14}C]arachidonic acid (57 μCi/mol) was obtained from New England Nuclear Corp.,

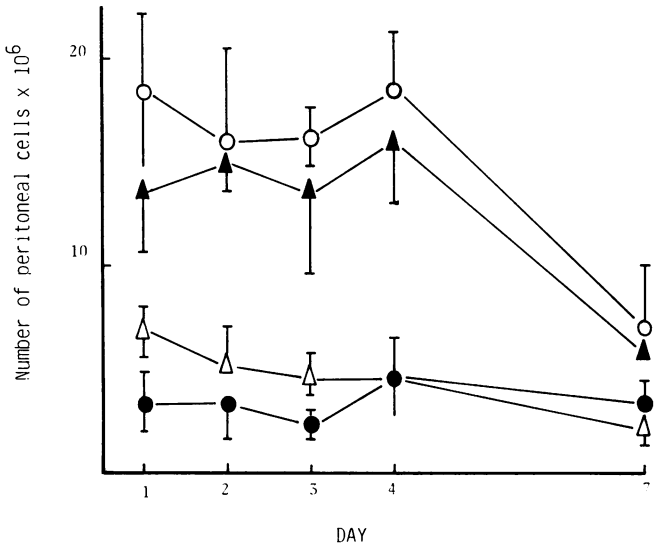

FIG. 2. Effect of LC 9018, OK-432, or LF injection on the number of murine peritoneal cells. BALB/c mice were injected i.p. with LC 9018 (○), OK-432 (▲), LF (△), or saline (●) for 3 consecutive days. The peritoneal cells were harvested on the indicated days after the final injection. The values are the mean ± standard deviation of six mice.

Boston, Mass. Authentic prostaglandin (PG), i.e., PGE_2 and 6-keto PG_1, was purchased from Funakoshi Medicines Co. Ltd., Tokyo, Japan. The silica gel thin-layer chromatographic plates (LK5D) were from Whatman Inc., Clifton, N.J. All other reagents used were of reagent grade. The PM in a 16-mm well of a tissue culture cluster of 24 plates (Costar, Cambridge, Mass.) were incubated for 60 min at 37°C in 15 mM Tris-hydrochloride buffer (pH 8.0) containing 140 mM NaCl, 5.5 mM glucose, and 0.2 μCi of [^{14}C]arachidonic acid. The reaction in the plates was stopped by the addition of an appropriate amount of 2 N HCl to bring the pH of the reaction mixture to 3.0 to 3.5. The mixture was then extracted twice with 2.5 ml of ethyl acetate. The ethyl acetate phase was isolated and evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of chloroform and applied on thin-layer chromatography plates. The plates were developed in the organic phase of a solvent system of ethyl acetate–2,2,4-trimethyl pentane–acetic acid–water (11:5:2:10 [vol/vol]). The standard PGs (10 μg) were detected with 10% phosphomolybdic acid in ethanol. The radioactive peak corresponding to the standard PGE_2 was scraped off the plate, and the radioactivity was counted with a Packard 3255 liquid scintillation counter.

G6PD assays. The liver was perfused with ice-cold 0.15 M KCl solution containing 0.66 mM EDTA (pH 7.4) and homogenized with a Teflon homogenizer in 5 volumes of the same buffer solution. The homogenate was centrifuged at 9,000 × *g* for 20 min, and the supernatant was used to assay G6PD activity (14). Protein was determined by the method of Lowry et al. (23).

Antitumor activity assay. Mice were injected intraperitoneally (i.p.) with LC 9018, OK-432 or LF (total dose, 300 μg per mouse) for 3 consecutive days. The control mice were injected with saline alone (0.1 ml per mouse per day). Meth A fibrosarcoma (10^6 cells per 0.1 ml) was inoculated into the peritoneal cavity at 2 or 4 days after the final bacterial injection, and survival of the mice was monitored for 60 days.

Determination of *Listeria monocytogenes* growth in liver. Mice were injected i.v. with LC 9018 or *C. parvum* for 3 consecutive days (total dose, 300 μg per mouse) and chal-

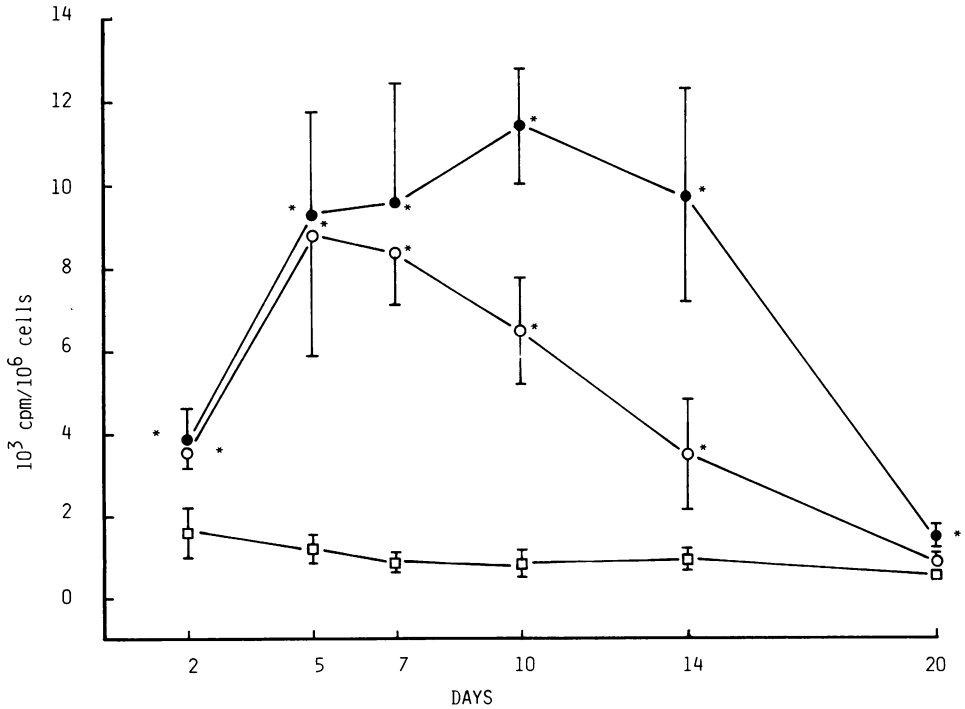

FIG. 3. Effect of LC 9018 and *C. parvum* on OR production by Kupffer cells. LC 9018 (●), *C. parvum* (○), or saline (□) was injected into the tail vein of BALB/c mice for 3 consecutive days (total dose, 300 μg per mouse), and on days 2, 5, 7, 10, 14, and 20 after the final injection OR production by Kupffer cells was measured. Each point represents the mean ± standard deviation of seven to eight mice. *, Significantly different from control; $P < 0.01$.

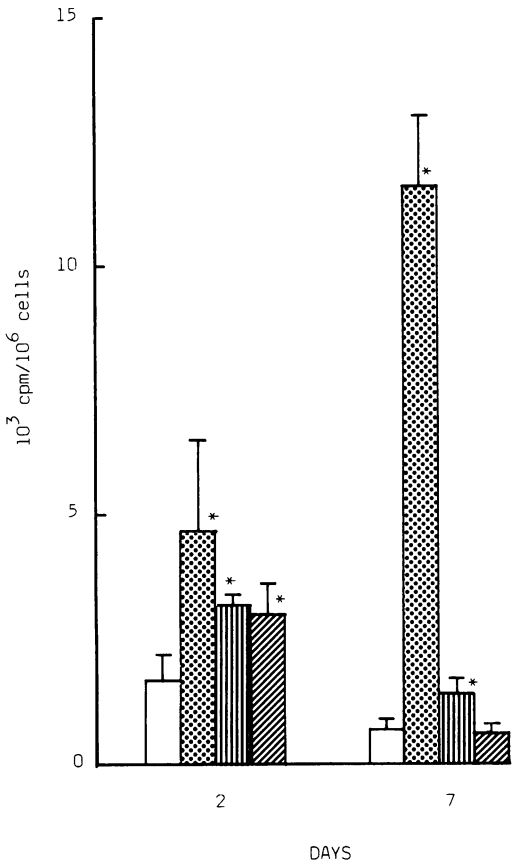

FIG. 4. Effect of LC 9018, OK-432, or LF on OR production by Kupffer cells. LC 9018, OK-432, or LF was injected into the tail vein of BALB/c mice for 3 consecutive days (total dose, 300 μg per mouse), and on days 2 and 7 after the final injection OR production by Kupffer cells was measured. Each bar represents the mean ± standard deviation of six mice. *, Significantly different from control; $P < 0.01$. Symbols: □, control; ⊡, LC 9018; ▥, OK-432; ▨, LF.

lenged i.v. with *L. monocytogenes* EGD (10^5 cells per mouse) 2, 5, 7, 10, and 14 days after the final injection of LC 9018 or *C. parvum*. Twenty-four hours after the challenge, the livers were removed and homogenized with a Teflon homogenizer in Hanks solution. The number of viable bacteria in each of the *L. monocytogenes*-infected mice was determined by plating on nutrient agar containing 0.4% glucose.

RESULTS

OR production by PM. OR production by PM was measured 1, 2, 3, 4, and 7 days after the final i.p. injection of LC 9018, OK-432, or LF. The production of OR by LC 9018-activated PM was highest 2 days after the injection of bacteria. Treatment with LF, which has no antitumor activity (21), caused no increase in the production of OR. On day 2, PM activated by LC 9018 produced approximately 10 times more OR than did LF- or saline-treated mice. OR production by OK-432-activated PM was highest on days 3 and 4 (Fig. 1). The number of peritoneal cells elicited after the i.p. injection of LC 9018, OK-432, or LF is shown in Fig. 2. The number of peritoneal cells in the peritoneal cavity was significantly increased (approximately 10 times) on days 1, 2, 3, and 4 by i.p. injection of LC 9018 or OK-432, but LF did not affect the number of peritoneal cells during the experimental period.

OR production by Kupffer cells. The production of OR by Kupffer cells was measured 2, 5, 7, 10, 14, and 20 days after the final i.v. injection of *C. parvum* or LC 9018 (Fig. 3). Stimulation of OR production by Kupffer cells by i.v. injection of *C. parvum* or LC 9018 was observed on days 7, 10, and 14, and the number of Kupffer cells in the liver was significantly increased (approximately 10 times) on day 10 (data not shown). On day 2, OR production was increased as a result of multiple i.v. injections of OK-432 or LF but showed no effect on OR production by Kupffer cells on day 7 (Fig. 4). The effect of LC 9018 on Kupffer cells remained for a longer period of time than that of *C. parvum* or OK-432.

G6PD activity in the liver. G6PD activity in the liver was measured 2, 5, 7, 10, 14, and 20 days after i.v. injection of *C. parvum* or LC 9018 (Fig. 5). G6PD activity was significantly increased (10 to 20 times) 7 and 10 days after i.v. injection of these bacteria. The increase in G6PD activity in the liver was maintained for more than 20 days after the final injection of LC 9018. OK-432 also increased G6PD activity but to a much lower extent than did LC 9018 or *C. parvum*, and G6PD activity was not changed by i.v. injection of LF (data not shown).

PGE_2 production by PM. PGE_2 production by PM was significantly decreased in mice given multiple injections of LC 9018, OK-432, or LF (Table 1). A single injection of LC 9018 or OK-432 (150 μg per mouse) also resulted in a decrease in production of PGE_2 and other prostaglandins such as PGI_2 and PGF_2 by PM (data not shown).

Inhibition of tumor growth. The effect of LC 9018, OK-432, and LF on the survival of Meth A fibrosarcoma-bearing mice was studied. As shown in Table 2, when mice were injected i.p. with Meth A 2 days after the final injection of LC 9018, their survival was markedly prolonged.

Elimination of *L. monocytogenes* in liver. Mice were inoculated in the tail vein with *L. monocytogenes* 2, 5, 7, 10, and 14 days after the final injection of *C. parvum* or LC 9018. The number of viable *L. monocytogenes* cells in the liver was determined. Elimination of *L. monocytogenes* from the liver was enhanced by *C. parvum* or LC 9018 on day 7, 10, or 14 (Fig. 6). No elimination of *L. monocytogenes* was observed after injection of LF (data not shown).

TABLE 1. PGE_2 production by murine PM elicited with LC 9018, OK-432, or LF[a]

Organism	PGE_2 (ng/10^6 cells/60 min) on the following days:				
	1	2	3	4	7
Control	383.4 ± 87.9	261.2 ± 118.1	346.3 ± 96.9	243.4 ± 100.8	274.8 ± 64.1
LC 9018	32.7 ± 9.2	58.2 ± 15.0	67.6 ± 11.5	31.5 ± 9.7	63.1 ± 11.6
OK-432	57.3 ± 11.9	56.5 ± 14.8	54.6 ± 11.9	49.7 ± 17.4	57.7 ± 11.9
LF	62.9 ± 17.4	64.7 ± 21.4	75.3 ± 23.5	48.4 ± 23.6	192.9 ± 45.5

[a] LC 9018, OK-432, or LF was injected i.p. for 3 consecutive days (total dose, 300 μg per mouse) into BALB/c mice. PM were harvested 1, 2, 3, 4, and 7 days after the final injection of LC 9018, OK-432, or LF. The values are mean ± standard deviation of six mice.

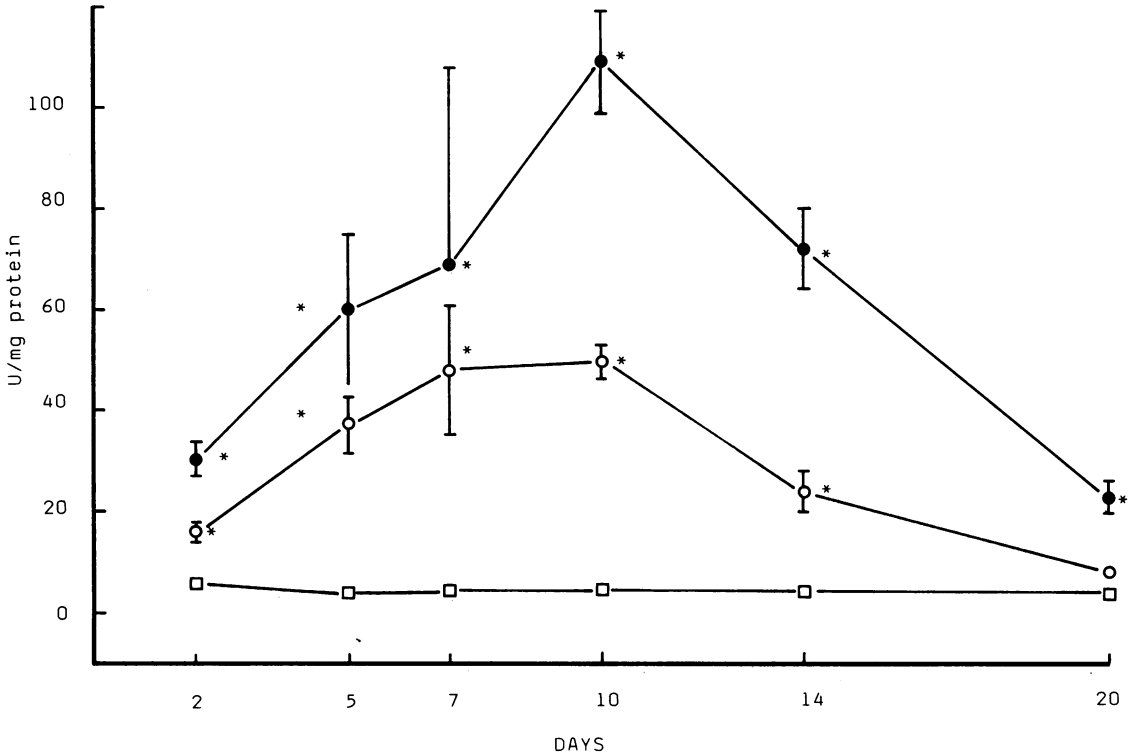

FIG. 5. Effect of LC 9018 and *C. parvum* on G6PD activity in the liver. LC 9018 (●) or *C. parvum* (○) was injected into the tail vein of BALB/c mice for 3 consecutive days (total dose, 300 μg per mouse), and on days 2, 5, 7, 10, 14, and 20 after the final injection the enzyme activity in the liver was measured. Control mice were given saline (□). Each point represents the mean ± standard deviation (seven to eight mice per group were used). *, Significantly different from the control group; $P < 0.01$.

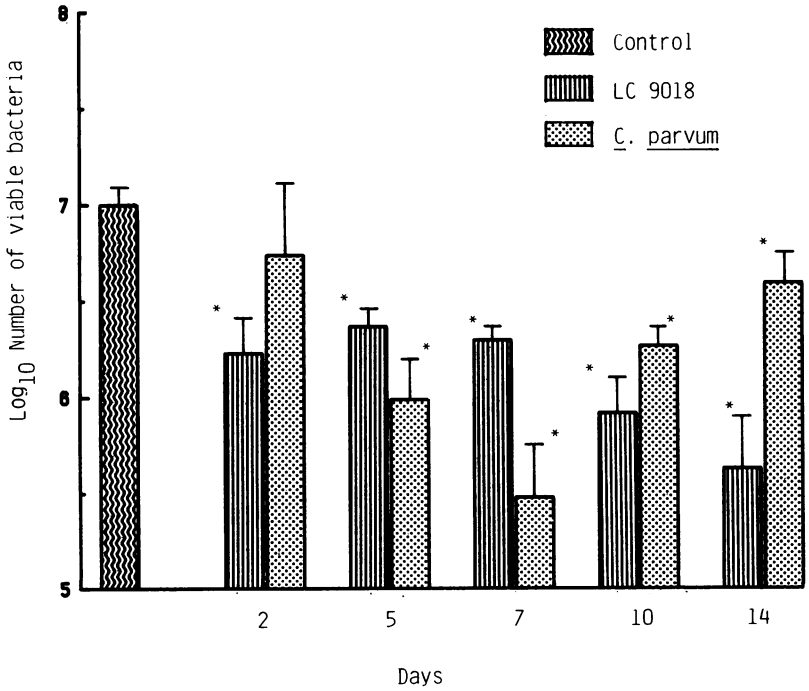

FIG. 6. Effect of LC 9018 or *C. parvum* on the growth of *L. monocytogenes* in the liver. BALB/c mice were injected i.v. with LC 9018 or *C. parvum* for 3 consecutive days (total dose, 300 μg per mouse) and inoculated i.v. with viable *L. monocytogenes* (10^5 cells per mouse) on days 2, 5, 7, 10, and 14 after the final injection of LC 9018 or *C. parvum*. Twenty-four hours after the inoculation of *L. monocytogenes*, the number of viable bacteria was determined. Each bar represents the mean ± standard deviation. *, Significantly different from control; $P < 0.01$.

TABLE 2. Effect of LC 9018, OK-432, and LF on the survival of BALB/c mice inoculated with Meth A[a]

Organism	Days of survival (mean ± SD) after Meth A inoculation on day	
	2	4
Control	14.0 ± 1.2 (0/12)	14.2 ± 2.0 (0/12)
LC 9018	43.5 ± 13.1 (4/12)	19.8 ± 5.5 (0/12)
OK-432	25.5 ± 12.9 (1/12)	20.9 ± 7.6 (0/12)
LF	20.5 ± 12.8 (1/12)	15.3 ± 1.5 (0/12)

[a] LC 9018, OK-432, or LF was injected i.p. for 3 consecutive days (total dose, 300 μg per mouse). Meth A fibrosarcoma cells (10^6 cells per mouse) were inoculated i.p. 2 or 4 days after the final injection of LC 9018, OK-432, or LF, and survival of mice was monitored for 60 days. The numbers in parentheses are the number of mice surviving for more than 60 days/total number of mice.

DISCUSSION

LC 9018 augmented OR production by PM and Kupffer cells according to measurements of chemiluminescence. The chemiluminescence produced has been shown to be due to superoxide anions (22, 42). OR is known to have cytotoxic effects (22, 37, 42), and its production by PM is markedly increased by treatment with immunopotentiators such as *C. parvum*, OK-432, and LC 9018 but not with LF, which has no antitumor activity (21) and does not cause any increase in OR production by PM. This suggests that OR is an important mediator in the antitumor activity of bacterial immunostimulators in vivo.

PGE_2 production by LC 9018-, OK-432-, or LF-treated PM was significantly decreased regardless of whether the mice received a single injection or multiple injections of the bacteria. In mice given a single i.p. injection of LC 9018 or OK-432, survival after Meth A inoculation was not prolonged, nor did OR production by PM increase. PGE_2 production by PM was suppressed for 14 days in mice given three i.p. injections of LC 9018 or OK-432, although OR production did not increase during this time. It has been reported that OR regulates biosynthesis of PGE_2 in vivo (26), but no direct correlation was found between the increase in OR production and biosynthesis of PGE_2 in murine PM in this experimental system.

OR is synthesized from NAD(P)H and O_2 and catalyzed by NAD(P)H oxidase in the cell membrane of macrophages and polymorphonuclear leukocytes (3). NADPH is mainly synthesized in a hexose monophosphate shunt, and G6PD is a limiting enzyme in a hexose monophosphate shunt. G6PD activity in the liver of BALB/c mice was significantly increased by i.v. injection of LC 9018 or *C. parvum* but not markedly so by OK-432 or LF (S. Hashimoto, T. Yokokura, and M. Mutai, in press). Therefore, it may be concluded that the more NADPH synthesized as a result of increased G6PD activity by i.v. injection of *C. parvum* or LC 9018, the more OR is produced by Kupffer cells in the liver and that the effect of *C. parvum* and LC 9018 on the catabolisms of carbohydrate in the liver differs from that of OK-432 and LF. Furthermore, there was a correlation between increased G6PD activity of the liver and the increase in OR production by Kupffer cells after i.v. injection of *C. parvum* or LC 9018. OR production by Kupffer cells was lower than that of PM. This phenomenon may be caused by the isolation of Kupffer cells from the liver by an action such as pronase digestion.

On the other hand, it has been reported that the host defense system against *L. monocytogenes* is strongly reinforced by i.v. or i.p. injection of *C. parvum* and that the effector cells of the system are macrophages (27, 28). In the presence of bacteria and tumor cells, activated macrophages secrete large amounts of OR (1, 4, 5, 18, 22, 25, 30). Cohen et al. (10) suggest that nonparenchymal cells carry out significant spontaneous lysis of tumor cells, and fixed macrophages are most likely the predominant effector cells in consideration of phagocytic and target cell specificity. Therefore, it was suggested that OR is an important mediator of tumoricidal and bactericidal activity in macrophages from the peritoneal cavities and livers of LC 9018-elicited mice. Furthermore, it should be emphasized that augmentation of OR production by PM or Kupffer cells by i.p. or i.v. injection of LC 9018 is more significant and maintained over a longer period of time than that by injection of *C. parvum* or OK-432.

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