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Effect of Perorally Administered Lactobacilli on Macrophage Activation in Mice

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The effect of perorally (p.o.) administered *Lactobacillus casei* and *L. bulgaricus* on macrophage activation in mice was studied. *L. casei* and *L. bulgaricus* were administered p.o. to mice for 8 days. The macrophage activation was measured on days 2, 3, 5, and 8 of lactobacillus administration by using biochemical and functional criteria. We measured the release of lysosomal hydrolases, the level of a nonlysosomal enzyme, and in vitro phagocytic activity of mouse peritoneal macrophages. All the assays were performed comparatively with mice inoculated with *L. casei* and *L. bulgaricus* (viable and nonviable cells) intraperitoneally (i.p.) at the same dose as for p.o. administration. The phagocytic activity was significantly higher in mice treated i.p. than in control mice. For p.o. administration, there was an increase only when *L. casei* was used. *L. bulgaricus* had little effect. No differences were found between viable and nonviable cells. The phagocytic function of the reticuloendothelial system was tested by the carbon clearance test, which showed that *L. casei* and *L. bulgaricus* accelerate the phagocytic function in mice treated p.o and i.p., from day 2 onward. These observations show that *L. casei* and *L. bulgaricus* given by p.o. administration are able to activate macrophages in mice and suggest that these bacteria, when passing through the intestinal tract, may be responsible for the enhanced host immune response. This fact is very significant because the diet includes fermented and manufactured products containing lactobacilli.

The beneficial effects of lactobacilli in the intestinal tract (antibiotic production, competitive antagonism, bile deconjugation, source of enzymes) are well known (7, 13, 18). The most-often mentioned as beneficial dietary adjuncts are *Lactobacillus acidophilus*, *L. casei*, and *L. bifidus* (*Bifidobacterium bifidum*). All of these microorganisms possess characteristics that would permit their survival and growth in the intestinal tract. However, another lactobacillus, *L. bulgaricus*, which does not survive in the intestinal tract (it is used as starters for yogurt and cheese elaboration), has been shown to be important as a dietary adjunct (8).

It has also been demonstrated that lactobacilli can confer protection against experimental infection by some pathogens in mice (17) and that they have an adjuvant effect in delayed hypersensitivity and antibody formation (3). It has been shown that *L. casei*, *L. bulgaricus*, and dairy products cultured with lactobacilli exhibit marked antitumor activity in allogeneic and syngeneic murine tumor systems (5, 10, 11, 16, 20) when they are administered intraperitoneally (i.p.). These last investigations suggested that the antitumor activity may be macrophage dependent, as is the immunostimulation exerted by other bacteria such as *Mycobacterium bovis* BCG, *Propionibacterium acnes* (*Corynebacterium parvum*), and *Streptococcus pyogenes*, as used in cancer therapy. Kato et al. also reported the augmentation of mouse natural killer (NK) cell activity by *L. casei* (12) when given i.p.

We previously reported (G. Perdigon et al., in press) that *L. casei* CRL 431 (Centro de Referencia para Lactobaciles) and *L. bulgaricus* CRL 423, administered perorally (p.o.), induce the release of lysosomal and nonlysosomal enzymes from peritoneal macrophages in mice. Since macrophages may be important effector cells in specific and nonspecific host defense, and since other experimental observations (9) have suggested that bacterial flora associated with mucosa can influence the level of macrophage activation, the present study was carried out to elucidate the effect of p.o. administration of lactobacilli on macrophage activation in mice and the possible participation of these organisms in the mechanisms of the enhanced immune response.

MATERIALS AND METHODS

Animals. Swiss albino mice, each weighing 25 to 30 g, were obtained from the random-bred colony kept by our department. The animals were housed in plastic cages and kept at room temperature. Each experimental group consisted of 16 to 20 mice. For the enzymatic and phagocytic activity (in vitro assays), animals were assigned to two groups; in one group they were treated with killed lactobacilli, and in the other they were treated with viable microorganisms. For the clearance test, animals were treated only with viable cells. The group for i.p. injection was fed ad libitum.

Microorganisms. *L. bulgaricus* CRL 423 and *L. casei* CRL 431 were obtained from the Center of Reference for Lactobacilli Culture Collection, Tucumán, Argentina. They were cultured for 8 h at 37°C (final log phase) in *Lactobacillus* MRS broth (Oxoid Ltd.), harvested by centrifugation at 5,000 × *g* for 10 min, and washed several times with sterile saline solution.

Microorganisms were killed by being heated at 100°C for 50 min. For the viable culture, microorganisms were suspended in sterile milk (powdered nonfat milk 10%) or sterile saline solution.

Feeding and inoculation procedure. The groups of animals assigned to be given p.o. administration of lactobacilli were fed for eight consecutive days with 50 μg of protein (1.2 ×

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TABLE 1. Enzymes released from peritoneal macrophages of mice fed or inoculated i.p. with viable lactobacilli

Microorganism and inoculation route	Enzyme[b]	Mean enzymatic activity ± SD on[a]:			
		Day 2	Day 3	Day 5	Day 8
L. bulgaricus					
p.o.	β-Glucuronidase	10.13 ± 3.67	13.95 ± 4.59	46.61 ± 7.50	52.43 ± 4.8
	β-Galactosidase	22.03 ± 6.06	24.28 ± 4.00	69.82 ± 8.50	55.91 ± 3.9
	Lactate dehydrogenase	0.081 ± 0.06	0.040 ± 0.026	0.077 ± 0.027	0.070 ± 0.012
i.p.	β-Glucuronidase	67.18 ± 3.82	106.95 ± 3.4	73.18 ± 5.5	60.67 ± 2.6
	β-Galactosidase	22.74 ± 4.15	45.88 ± 2.84	56.40 ± 4.2	25.84 ± 5.2
	Lactate dehydrogenase	0.192 ± 0.026	0.343 ± 0.018	0.294 ± 0.039	0.252 ± 0.014
L. casei					
p.o.	β-Glucuronidase	49.55 ± 6.8	60.84 ± 7.2	65.51 ± 7.8	65.83 ± 6.3
	β-Galactosidase	37.91 ± 2.67	90.32 ± 5.0	100.71 ± 3.2	121.83 ± 4.8
	Lactate dehydrogenase	0.069 ± 0.003	0.100 ± 0.003	0.175 ± 0.010	0.151 ± 0.003
i.p.	β-Glucuronidase	43.31 ± 1.00	44.22 ± 1.75	41.84 ± 3.2	33.37 ± 1.00
	β-Galactosidase	35.75 ± 2.30	35.26 ± 1.2	29.27 ± 4.05	26.90 ± 1.2
	Lactate dehydrogenase	0.046 ± 0.003	0.164 ± 0.002	0.226 ± 0.001	0.022 ± 0.001

[a] Cumulative doses were as follows: day 2, 100 μg; day 3, 150 μg; day 5, 250 μg; day 8, 400 μg.
[b] β-Galactosidase: control values, 19.90 ± 3.2 nmol of ONP/h per 10^6 cells. β-Glucuronidase: control values, 10.03 ± 2 nmol of PNP/ h per 10^6 cells. Lactate dehydrogenase: control values, 0.045 ± 0.008 μmol of NADH oxidized per 10^6 cells.

10^9 cells) per day. Each viable and nonviable culture was suspended in 5 ml of sterile nonfat milk and given at 20% (vol/vol) in the drinking water. The control group received sterile milk in the drinking water, given under the same conditions as those used for the test group. The groups of mice for i.p. treatment were inoculated at a dose of 50 μg per mouse per day (1.2×10^9 cells) for eight consecutive days. The control group was injected with 0.2 ml of sterile saline solution.

Macrophage collection and culture. The mice were sacrificed by cervical dislocation, and the peritoneal fluid was collected with 5 ml of modified Hanks medium (containing 100 U of penicillin and streptomycin per ml and 0.1% bovine serum albumin but without glucose and stain) after gentle massage of the abdomens of the animals.

Portions of the peritoneal exudate cell suspension containing 10^6 cells per ml were used for in vitro phagocytosis assays. The rest of the exudate was distributed into 35-mm petri dishes and incubated in a humidified atmosphere of 5% CO_2–95% air at 37°C for 2 h, to allow cells to become attached. Nonadherent cells were removed by being washed three times with phosphate-buffered saline. After being washed, the cells were cultured in modified Hanks medium for 18 h. At the end of each incubation period, the activities of various enzymes were assayed.

Enzyme assays. The macrophage activity, as measured by biochemical criteria, was assayed on days 2, 3, 5, and 7 or 8 of lactobacillus injection or administration. For each experiment, four mice were sacrificed and assays were performed in quadruplicate. The enzymes assayed were β-glucuronidase and β-galactosidase with the synthetic substrates were *p*-nitrophenyl-β-D-glucuronide (pNPG; Sigma Chemical Co.) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma), respectively. β-Glucuronidase was determined by the method of Stossel (21), and β-galactosidase was determined by the method of Conchie et al. (4). Lactate dehydrogenase was assayed by the rate of oxidation of NADH at 340 nm.

Phagocytosis assays in vitro. To measure the phagocytic activity, aliquots of peritoneal macrophages (10^6 cells per ml) were incubated for 15 min at 37°C with the same volume

TABLE 2. Percent phagocytosis of peritoneal macrophages in mice[a]

Day	Cumulative dose (μg)	Percent of phagocytosis[b]							
		L. bulgaricus by:				*L. casei* by:			
		p.o. administration		i.p. administration		p.o. administration		i.p. administration	
		w/Ab (1)	Ab (2)	w/Ab	Ab	w/Ab	Ab	w/Ab	Ab
2	100	31.5 ± 5	35 ± 5	76.5 ± 1.5*	80 ± 2	53 ± 5	61 ± 6	59 ± 6	62 ± 5
3	150	38 ± 4	43.5 ± 3.8	76.5 ± 1.5**	83.5 ± 2	55.5 ± 3	59.5 ± 3	61 ± 2**	71.5 ± 3
5	250	39.5 ± 4.5*	51.5 ± 7.2	73.5 ± 2.5	76 ± 1	41 ± 5	47 ± 5.5	55 ± 6	62 ± 5
8	400	36 ± 5*	44 ± 6	72 ± 2*	76 ± 2.5	39 ± 3	43.5 ± 2.5	55.5 ± 5*	64 ± 2

[a] Peritoneal macrophages isolated from the treated mice were incubated with lactobacilli (1) *S. typhi* (1), and opsonized *S. typhi* (2) at 37°C for 5 and 15 min. The macrophages phagocytizing bacteria were counted microscopically after incubation.
[b] Numbers represent mean ± standard deviation for each group of mice ($n = 4$). Asterisks indicate significant difference between opsonzied and nonopsonized bacteria at the levels $P < 0.05$ (*) and $P < 0.01$ (**) (Student test). Normal values: Phagocytosis without antibody, 21%; phagocytosis with antibody, 33%. We did not observe differences between phagocytosis with viable and nonviable cells. w/Ab, Without antibody; Ab, with antibody.

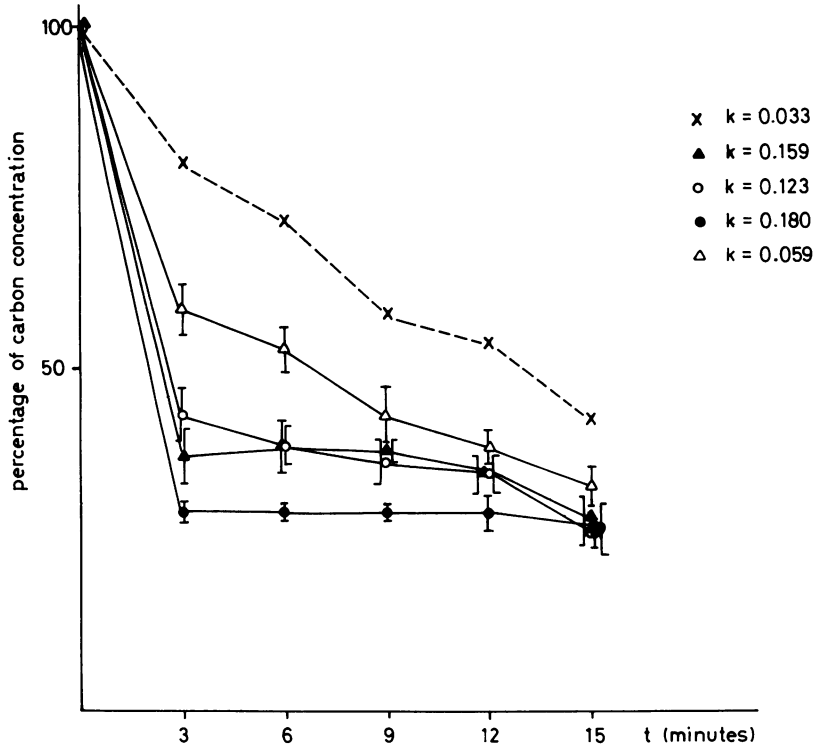

FIG. 1. Kinetics of phagocytosis of colloidal carbon in mice inoculated i.p. with *L. casei* CRL 423 at a dose of 50 μg per day. Symbols: ×, normal mice; ▲, day 2; ○, day 3; ●, day 5; △, day 8. *K* (phagocytic index) was calculated as described in the text.

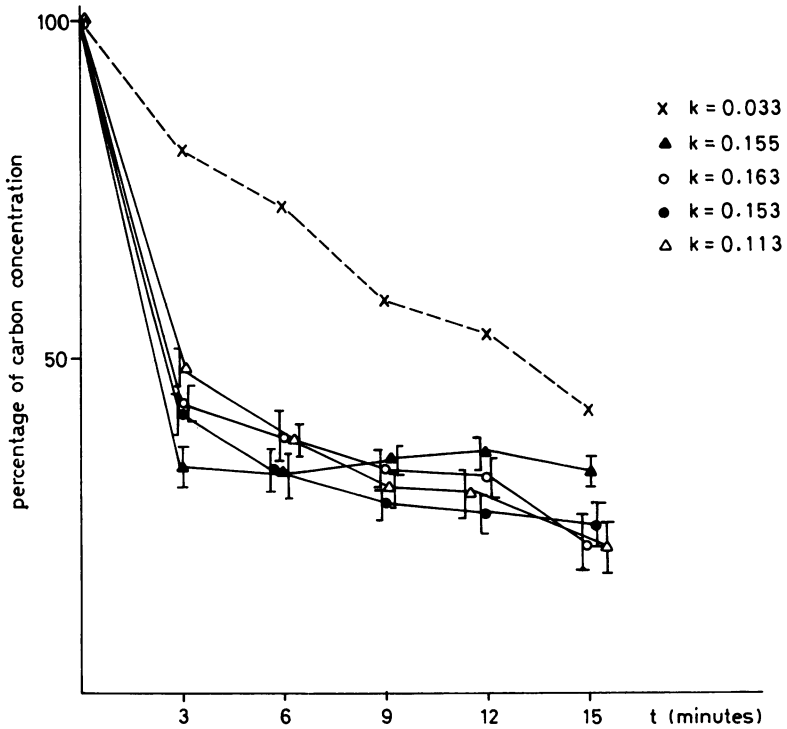

FIG. 2. Kinetics of phagocytosis of colloidal carbon in mice fed *L. casei* CRL 423 at a dose of 50 μg per day. Symbols: ×, normal mice; ▲, day 2; ○, day 3; ●, day 5; △, day 8. *K* (phagocytic index) was calculated as described in the text.

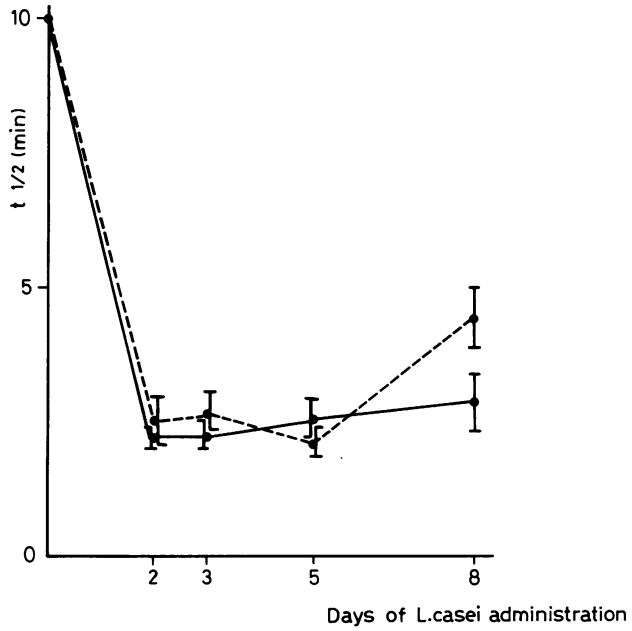

FIG. 3. Effect of *L. casei* CRL 423 on the phagocytic function of the reticuloendothelial system in mice. *L. casei* was administered p.o. (——) or injected i.p. (-----) at a dose of 50 μg per day. Points and bars represent mean ± standard deviation of each group of animals. The clearance rate of carbon ($t_{1/2}$) was calculated as described in the text. Control values, 9.90 ± 0.50.

of bacterial suspension (10^7 cells per ml). The incubation was stopped in an ice-cold bath, the mixture was centrifuged for 5 min at 1,500 × *g*, and the sediment was observed by immersion in a Zeiss microscope. We estimated the percentage of macrophages with ingested bacteria by counting 200 cells. We measured the phagocytic activities of the cells incubated with lactobacilli, *Salmonella typhi*, and *S. typhi* antibody. Opsonization of *Salmonella* strains was carried out by incubation of a bacterial suspension (10^7 cells per ml) with anti-*Salmonella* serum (diluted 1/5,000) for 15 min at 37°C. The bacteria were then centrifuged for 10 min at 1,500 × *g* and washed twice with Hanks modified medium to remove excess serum. Before use, the bacteria were resuspended in the same medium to the initial concentration. The phagocytic activity was performed after 5 and 15 min of incubation at 37°C.

Phagocytosis assays in vivo. Carbon clearance test. Colloidal carbon was injected at a dose of 8 mg/100 ml into the tail vein of each mouse. After injection, 50 μl of blood was taken by capillary from the retroorbital venous plexus at intervals of 0, 3, 6, 9, 12, 15, and 18 min and added to 2 ml of 0.1% Na_2CO_3. The carbon concentration was determined by measuring the optical density at 675 nm in a Gilford spectrophotometer. The phagocytic index (*K*) was calculated by the method of Biozzi et al. (2) by the equation $K = (\log c_1 - \log c_2)/(t_2 - t_1)$, where c_1 and c_2 represent the carbon concentration in the blood at times t_1 and t_2, respectively.

The clearence rate of carbon ($t_{1/2}$) was calculated by the formula described by Kato et al. (12): $t_{1/2} = [(t_2 - t_1)(0.5\ OD_{t_1})]/(OD_{t_1} - OD_{t_2})$, where OD_{t_1} and OD_{t_2} are the carbon concentrations in the blood at times t_1 and t_2, respectively.

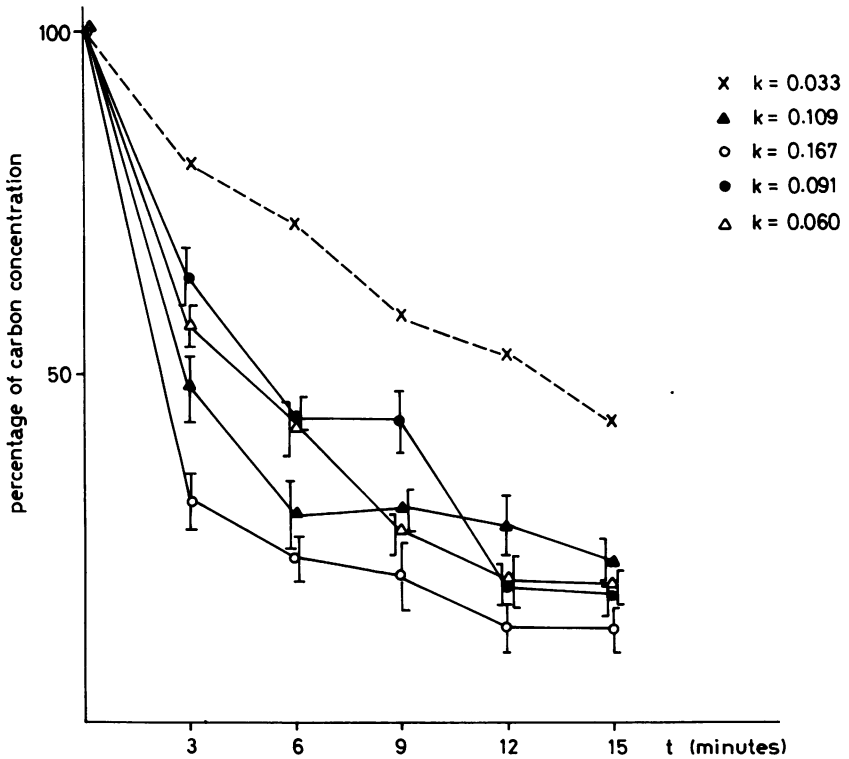

FIG. 4. Kinetics of phagocytosis of colloidal carbon in mice inoculated i.p. with *L. bulgaricus* CRL 431 at a dose of 50 μg per day. Symbols: ×, normal mice; ▲, day 2; ○, day 3; ●, day 5; △, day 8. *K* (phagocytic index) was calculated as described in the text.

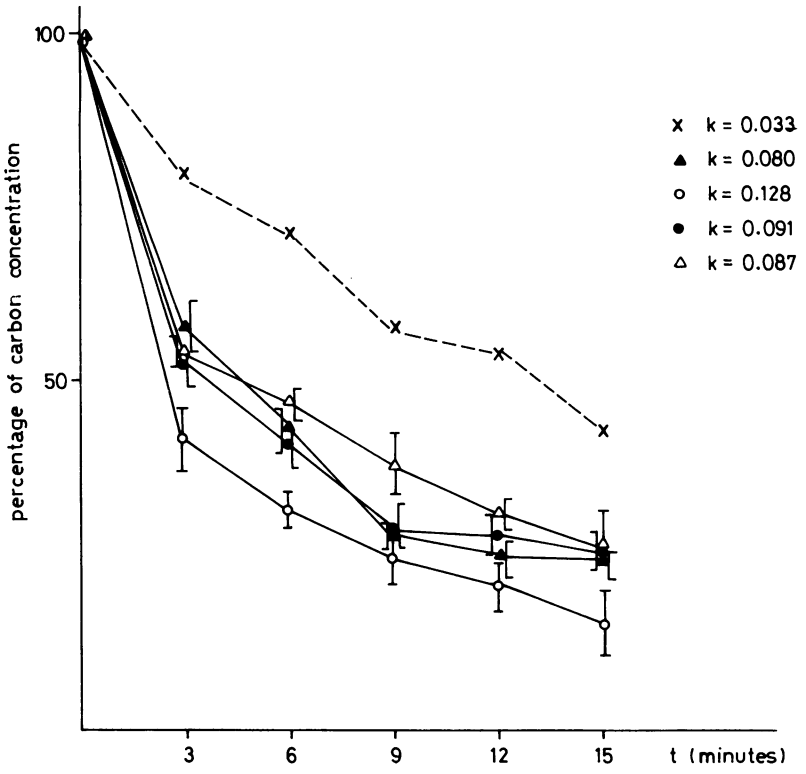

FIG. 5. Kinetics of phagocytosis of colloidal carbon in mice fed *L. bulgaricus* CRL 431 at a dose of 50 μg per day. Symbols: ×, normal mice; ▲, day 2; O, day 3; ●, day 5; △, day 8. *K* (phagocytic index) was calculated as described in the text.

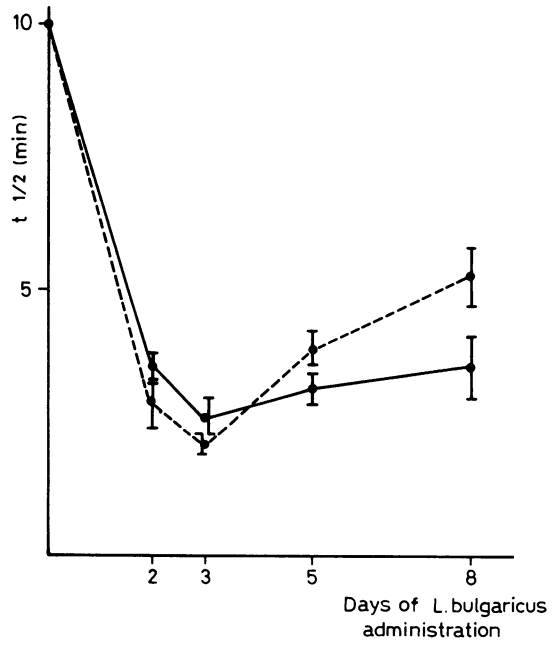

FIG. 6. Effect of *L. bulgaricus* CRL 431 on the phagocytic function of the reticuloendothelial system in mice. *L. bulgaricus* was administered p.o. (——) or injected i.p. (-----) at a dose of 50 μg per day. Points and bars represent mean ± standard deviation of each group of animals.

RESULTS

Increase of enzymatic activities in macrophages. The enzymatic activity of peritoneal macrophages from mice inoculated i.p. with *L. bulgaricus* was 10-fold higher than that of macrophages obtained from normal mice; viable cells were shown to be more active. When this microorganism was administered p.o., the enzymatic activity of macrophages was fivefold higher, and there was no difference between viable and nonviable cells. When *L. casei* was administered i.p., the activity of the lysosomal enzymes were threefold higher than that of the control macrophages on day 2, and there was no difference between viable and nonviable cells. For p.o. administration, the activity of the macrophages was sixfold higher than normal, with higher enzymatic activities for viable cells (Perdigon et al., in press). The results obtained for the enzymatic activities of β-galactosidase, β-glucuronidase, and lactate dehydrogenase are summarized in Table 1.

Enhancement of phagocytic activity. The in vitro phagocytic activity of peritoneal macrophages from mice treated i.p. or p.o. with *L. casei* showed a peak on the days 2 and 3 of lactobacillus administration and continued to remain high. The highest phagocytic activity was obtained for the i.p. inoculation of *L. bulgaricus*, beginning on day 2 and maintaining the same level until day 8. This microorganism, when administered p.o., had little effect on the phagocytic activity of peritoneal macrophages. In these assays, we no found differences between viable and nonviable cells. We did not detect significant differences between the phagocytic function of macrophages incubated with bacteria alone or with opsonized bacteria. The results are summarized in Table 2.

Effect of lactobacilli on the phagocytic function of the reticuloendothelial system in mice. The carbon clearance activity from circulating blood was rapidly enhanced by the i.p. inoculation of *L. casei*; the enhancement was apparent from day 2 onward and decreased slightly on day 8 (Fig. 1). When this microorganism was administered p.o., we obtained slightly closer values of the phagocytic index (*K*) (Fig. 2) and of the clearance rate of carbon ($t_{1/2}$) to those obtained in the i.p. administration (Fig. 3).

In the experiments with *L. bulgaricus*, we observed that the clearance activity was more effective for the i.p. inoculation than for the p.o. administration; we obtained values of *K* two- to threefold higher than those obtained with normal mice (Fig. 4 and 5). Comparative plots of the values of $t_{1/2}$ obtained with i.p. and p.o. administered *L. bulgaricus* are shown in Fig. 6. In all cases, the maximum activity occurred on day 2 and decreased by day 8, but to twofold-higher levels than those in control mice.

DISCUSSION

The enhanced immunity obtained with i.p. administration of lactobacilli has been reported by many investigators. Since macrophages play an important role in the immune response and in the host defense, we studied the effect of two different lactobacilli, administered p.o., on macrophage activation. One of them was *L. casei*, a resident microorganism in the small intestine, and the other was *L. bulgaricus*, a microorganism that does not survive in the intestinal tract.

The term macrophage activation is widely used for the enhanced state of biological activities. Many parameters, such as adherence, phagocytosis, bactericidal activity, and biochemical characteristics, can be taken as indicative of the degree of macrophage activation. In the present report, lysosomal enzyme activities, phagocytosis, and carbon clearance capability were taken as the primary parameters reflecting the degree of macrophage activation.

We observed that the lysosomal enzyme activities of peritoneal macrophages were significantly increased (Table 1) on day 2 by both p.o. and i.p. administration of lactobacilli. Certain differences were obtained with viable cells when *L. casei* was administered p.o., probably as a result of its capability of growing in the intestinal tract. Small amounts of lactate dehydrogenase were detected in all the experiments, because this enzyme is released as a consequence of cell damage.

When we analyzed the phagocytic activity of peritoneal macrophages by using lactobacilli, *S. typhi*, and *S. typhi* antibody, we observed that *L. casei* and *L. bulgaricus* were more effective when administered i.p. This is concordant with the enzyme levels obtained after i.p. administration. This result was expected, because the stimulation was in situ. A few differences were found in the phagocytic activity between macrophages with *S. typhi* and *S. typhi* antibody (Table 2). When *L. casei* and *L. bulgaricus* were administered p.o., *L. casei* proved to be effective. This effect is correlated with the enzyme levels obtained. *L. bulgaricus* was not as effective in increasing the phagocytic activity of peritoneal macrophages, although we have detected high enzyme levels; we do not know the reasons. Probably as a result of antigenic differences, the stimulation by this microorganism induced macrophages capable of responding to T-cell stimuli independent of the increase of phagocytic activity. On other hand, the enzyme release process cannot always be directly related to phagocytosis (19).

The results obtained for peritoneal macrophage activation when the lactobacilli were administered p.o. are in agreement with those of other investigators, who report peritoneal macrophage activation by some antigens administered p.o. Ianello et al. (9) found that a killed polyvalent vaccine administered p.o. enhanced the phagocytic activity from peritoneal macrophages, and Gemsa et al. (6) demonstrated that peritoneal macrophages can be activated by lymphokines from T-cell clones. Namba et al. (15) reported that p.o. administration of lysozyme or digested bacterial cell wall in guinea pigs could increase the humoral and cell-mediated immune response to an antigen administered by the subcutaneous route.

The phagocytic function of the reticuloendothelial system by the carbon clearance test was markedly stimulated by both p.o. and i.p. administration of *L. casei* (Fig. 1 through 3). The differences observed for p.o. administration may be due to the capacity of this lactobacillus to survive in the intestinal tract. *L. bulgaricus* was less effective than *L. casei* by both p.o. and i.p. administration (Fig. 4 through 6), probably because of the structural differences in their cell walls.

The present study demonstrates that the activation of macrophage functions can be originated by *L. casei* CRL 431 and *L. bulgaricus* CRL 423 administered p.o. as well as i.p.

It has been reported that the immune system in germfree animals are poorly developed compared with those of conventional animals and that antigenic stimulation with some bacteria or other antigenic substances is important for the maturation of the immune system (1, 14). This new approach suggests that p.o. administration of lactobacilli may represent a way to produce immunostimulation and lead to stimulation of the host nonspecific immunity. It might also be able to enhance the systemic immune response or to modulate the functions of immunocompetent cells. This fact is important in the immunocompromised host.

Further studies are required to identify the nature of the macrophage-stimulating components of the two lactobacilli used in this work and their mechanisms of macrophage activation.

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