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Enhancement of Hematopoietic Response of Mice by Subcutaneous Administration of *Lactobacillus casei*

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Mice that had received heat-killed *Lactobacillus casei* (LC 9018) subcutaneously (s.c.) showed enhanced resistance to systemic (i.e., intravenous) infection with *Listeria monocytogenes*, but the antilisterial resistance of mice was less augmented by s.c. administration of *Propionibacterium acnes* ("*Corynebacterium parvum*"). Though there was little change in the total number of splenic leukocytes after s.c. administration of LC 9018, the monocyte-macrophage ratio increased after treatment, reaching its peak on day 5 to 7 after injection. The number of progenitor cells that form macrophage colonies under the stimulus of L-cell-conditioned medium in a semisolid agar culture system increased in the spleens of mice pretreated s.c. with LC 9018, showing a peak response on day 5 after injection. The increase corresponded to the increase in the dose administered, and increased numbers were detected even 10 days after treatment. The number of macrophage colonies in the femurs of mice pretreated s.c. with LC 9018 showed a temporary increase on day 3 after injection but then a decrease until day 10. Colony-stimulating activity was detected in the sera of mice administered LC 9018 s.c. 18 h previously, and the colonies produced were of three types: granulocyte (8%), macrophage (56%), and granulocyte-macrophage (36%). Administration of *C. parvum* s.c. had little effect on these hematopoietic responses of mice.

The macrophage is thought to be one of the most important effector cells in host defense mechanisms against many kinds of organisms and tumor cells, and there are many reports that macrophage-activating agents such as BCG, *Propionibacterium acnes* ("*Corynebacterium parvum*"), and lipopolysaccharide enhance the resistance of mice to infections with pathogenic bacteria or to syngeneic tumors (4–6, 10, 13). Since these agents are also known to augment hematopoietic responses of mice, especially neutrophil and macrophage proliferation, their effect seems to be exerted not only by activating the qualitative function of mature macrophages but also by enhancing the generation and differentiation of their precursor cells (3, 21, 22). We reported previously that heat-killed *Lactobacillus casei* YIT 9018 (LC 9018) augments host resistance to infections with *Pseudomonas aeruginosa* and *Listeria monocytogenes* (11, 16) in mice by way of activating nonspecific scavenger macrophages and that LC 9018 also augments antilisterial and antitumoral resistance of the host systemically by subcutaneous (s.c.) administration (17, 23). In the present study, we determined the effect of s.c. administration of LC 9018 on hematopoietic responses of mice, comparing the effects with those of *C. parvum*, and found that LC 9018 augments monocyte/macrophagepoiesis in these animals.

MATERIALS AND METHODS

Animals. Male BALB/c mice were obtained at 6 weeks of age from Shizuoka Agricultural Cooperative for Experimental Animals, Hamamatsu, Japan, and they were used for experiments at 7 to 10 weeks of age.

Microorganisms. Heat-killed *L. casei* YIT 9018 (LC 9018) was prepared as described previously (8), and the lyophilized preparation was suspended in saline at the desired concentration just before use. A suspension of killed *C. parvum* was obtained commercially from the Institut Merieux, Lyon, France.

Antilisterial effect of macrophage-activating agents in vivo. In one experiment, mice pretreated s.c. with 0.5 mg of LC 9018 or *C. parvum* on day −1, −3, −5, −7, −10, −14, or −21 were infected intravenously (i.v.) with 5×10^4 CFU of *L. monocytogenes* on day 0 and monitored for survival for 14 days after the infection. Each group consisted of 10 mice. In another experiment, mice pretreated intraperitoneally (i.p.), i.v., or s.c. with LC 9018 or *C. parvum* at a dose of 0.5 mg on day −5 were infected i.v. or i.p. with 5×10^6 CFU of *L. monocytogenes* on day 0 and were dissected 24 h later to determine the number of viable bacteria in the spleen (i.v. infection) or peritoneal cavity (i.p. infection). Bacterial growth was determined as described previously (16). Each group consisted of six mice.

Preparation of spleen leukocytes. Single-cell suspensions of spleen were obtained from mice pretreated s.c. with 0.5 mg of LC 9018 or *C. parvum* on day −1, −3, −5, −7, −10, or −14. Each group consisted of three mice. After hemolysis, splenic leukocytes were washed twice with Hanks balanced salt solution and then counted in a hemocytometer. Smear specimens for differential counts were stained with Giemsa solution.

Determination of macrophage colony-forming cells (CFUm). L-cell-conditioned medium (LCM) was prepared as follows. Mouse L 929 cells were seeded in a 75-cm^2 culture flask at 2×10^5 cells in 40 ml of Dulbecco modified Eagle medium supplemented with 10% horse serum (GIBCO Laboratories, Grand Island, N.Y.) and antibiotics, and incubated at 37°C in an atmosphere of 5% CO_2 and air for 7 days. Then the culture medium was removed and centrifuged at $110 \times g$ for 10 min. The supernatant was filtered through a membrane filter (pore size, 0.22 μm) and stored at −20°C. The number of macrophage progenitor cells that would proliferate in response to LCM was determined by the two-layer agar culture method. Briefly, 1.0-ml quantities of

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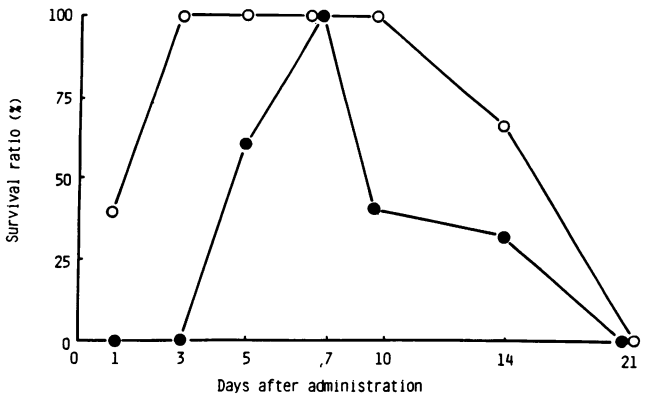

FIG. 1. Effect of s.c. administration of LC 9018 or *C. parvum* on resistance of mice to i.v. infection with *L. monocytogenes*. A 0.5-mg portion of LC 9018 (○) or *C. parvum* (●) per mouse was administered s.c. to BALB/c mice on day −1, −3, −5, −7, −14, or −21. All mice in the untreated control group died within 3 days of i.v. infection with *L. monocytogenes* (5×10^4 CFU per mouse) on day 0.

warmed Dulbecco modified Eagle medium containing 20% LCM, 0.5% agar, and antibiotics were seeded into plastic petri dishes (35 by 10 mm; Falcon no. 3001) and the medium was allowed to harden at room temperature for 15 min. Leukocytes from either spleens or femurs from mice pretreated with LC 9018 or *C. parvum* were suspended at a concentration of 5×10^6 (spleen) or 10^5 (femur) cells per ml in Dulbecco modified Eagle medium containing 0.3% agar and antibiotics. One-milliliter quantities of the single-cell suspension were added on top of the 0.5% underlayers, and the plates were incubated at 37°C in an atmosphere of 10% CO_2 and air. Colonies (more than 50 cells) were scored microscopically on day 7 of incubation, and the results were expressed as the mean of triplicate dishes ± the standard deviation.

Colony-stimulating activity (CSA) of serum. Sera were obtained from mice pretreated with LC 9018 or *C. parvum* and were pooled from groups of three mice, filtered through a membrane filter (pore size, 0.22 μm), and stored at −20°C. Each sample was added to the underlayer of a two-layer semisolid agar culture system, which did not contain LCM, and 10^5 bone marrow cells from untreated mice were added to the upper layer. Then the dishes were incubated at 37°C in an atmosphere of 10% CO_2 and air for 7 days. Colonies were scored and cells in the colonies were identified by Giemsa staining at the end of cultivation. Results were expressed as the mean of triplicate dishes ± the standard deviation.

Statistics. The statistical significance of the data was determined by Student's *t* test. A *P* value of <0.05 was regarded as significant.

RESULTS

Effect of s.c. administration of LC 9018 or *C. parvum* on resistance of mice to systemic infection with *L. monocytogenes*. All untreated mice died within 3 days of i.v. infection with *L. monocytogenes*. Enhanced resistance of the mice was observed in all LC 9018-pretreated groups except for the group treated 21 days previously (Fig. 1). *C. parvum* also had an antilisterial effect, but the effect started later and diminished earlier than that of LC 9018 (Fig. 1).

Effect of route of administration of LC 9018 or *C. parvum* on inhibition of growth of *L. monocytogenes*. LC 9018 and *C. parvum* caused identical inhibition of growth of *L. monocytogenes* in the spleen or peritoneal cavity when administered i.v. or i.p. ($P > 0.05$; Table 1). However, only LC 9018 exerted an antilisterial effect by s.c. administration (Table 1).

Changes in spleen cellularity after s.c. administration of LC 9018 or *C. parvum*. The total number of spleen leukocytes in the LC 9018-treated mice was decreased to about 61.0% of that in the untreated control group 24 h after treatment, but then returned to the normal level on day 3 and showed little change thereafter by day 14. The monocyte/macrophage ratio in spleen leukocytes of the untreated mice was 22.2%. In the LC 9018-treated group, the number of monocytes/macrophages increased gradually after treatment, reaching its peak on day 7 (71.3%), and then decreased to the normal level by day 14. There were only slight changes in both total number and the monocyte/macrophage ratio of spleen leukocytes after s.c. administration of *C. parvum* (Fig. 2).

Changes in number of CFUm in the spleen after s.c. administration of LC 9018 or *C. parvum*. Changes in the number of monocyte-macrophage progenitor cells (CFUm) in the spleen were followed after s.c. administration of LC 9018 or *C. parvum* by using LCM as the specific growth factor for monocytes-macrophages (Fig. 3). The mean number of CFUm in the spleens of untreated control mice was 302 per spleen. In the LC 9018-pretreated group, the number of CFUm in the spleen began to increase on day 3 after treatment and reached its peak on day 5 to about 22.8 times as many as that in the untreated control group, and the elevated level of CFUm was maintained even 10 days after treatment. The ability of LC 9018 to increase the number of splenic CFUm showed dose dependency (Fig. 4). On the other hand, after *C. parvum* administration, though a slight increase in CFUm in the spleen was observed on day 5 (2.8 times as many as that in the untreated control), it disappeared thereafter (Fig. 3). Only a trivial increase was observed when the dose of *C. parvum* was increased to 2.0 mg per mouse (Fig. 4). In these experiments, all of the colonies produced were determined morphologically to be macrophages.

Changes in number of CFUm in the femur after s.c. administration of LC 9018 or *C. parvum*. Administration of LC 9018 s.c. resulted in an increase in the number of CFUm in the femur beginning by 24 h and reaching a peak on day 3 to 4.7 times as many as that in the untreated controls, but the number had decreased to a subnormal level by day 10 (Fig. 5). Administration of *C. parvum* s.c. also increased CFUm in the femur by day 3, but the elevated level was only 1.6 times as high as that of the controls and the number was restored to normal on day 5 to 10 (Fig. 5).

Changes in serum CSA after s.c. administration of LC 9018

TABLE 1. Antilisterial effect of LC 9018 or *C. parvum* administered by various routes

Route		No. of bacteria (log_{10}, mean ± SD)[a]		
Drug[b]	*L. monocytogenes*[c]	Control	LC 9018	*C. parvum*
i.v.	i.v.	7.31 ± 0.09	6.60 ± 0.11[d]	6.73 ± 0.10[d]
s.c.	i.v.	7.31 ± 0.09	6.60 ± 0.23[d]	7.13 ± 0.09
i.p.	i.p.	6.96 ± 0.29	5.82 ± 0.18[d]	5.77 ± 0.26[d]

[a] The number of viable bacteria in the spleen (i.v.) or peritoneal cavity (i.p.) was determined 24 h after infection.
[b] LC 9018 or *C. parvum* at a dose of 0.5 mg was administered i.v., s.c., or i.p. 5 days before infection with *L. monocytogenes*.
[c] *L. monocytogenes* (10^7 CFU per mouse) was injected i.v. or i.p. into BALB/c mice.
[d] $P < 0.01$.

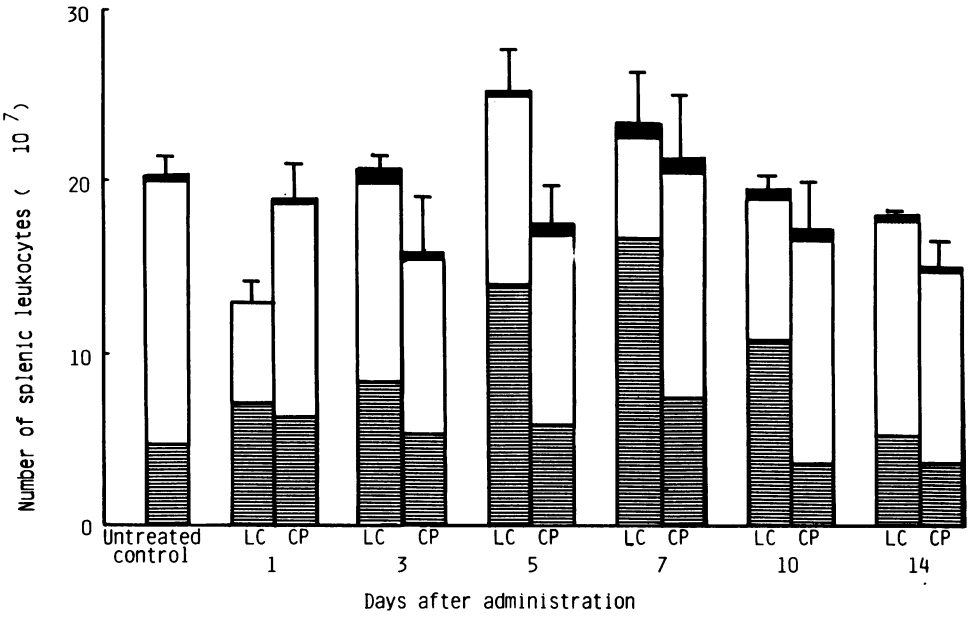

FIG. 2. Changes in spleen cellularity after s.c. administration of LC 9018 (LC) or *C. parvum* (CP). LC 9018 or *C. parvum* at a dose of 0.5 mg was administered s.c. on day 0. Symbols: ▬, macrophage; □, lymphocyte; ▣, polymorphonuclear cells.

or *C. parvum*. CSA of the serum from LC 9018-pretreated mice began to rise by 9 h after treatment, reached its peak in 18 h, and then diminished by 24 h (Fig. 6). Sera from mice that had received *C. parvum* s.c. showed no CSA at all (Fig. 6).

Effect of route of administration of the macrophage-activating agent on CSA of serum. We ascertained in preliminary experiments that i.v. administration of LC 9018 and *C. parvum* increased CSA of the sera of mice to the highest levels by 6 h after administration. Sera from LC 9018-pretreated mice showed marked CSA and the number of colonies was three times higher than in the case of *C.*

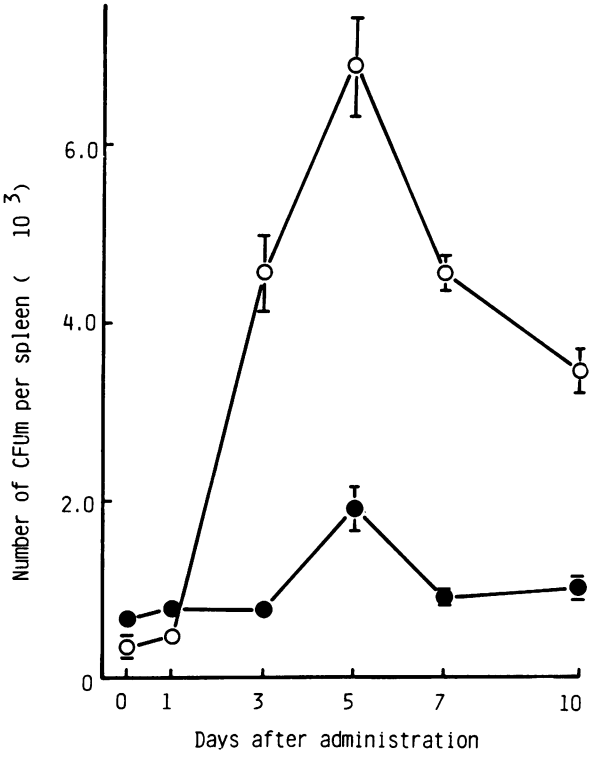

FIG. 3. Changes in number of CFUm in the spleen after s.c. administration of LC 9018 or *C. parvum*. LC 9018 (○) or *C. parvum* (●) at a dose of 0.5 mg was administered s.c. to mice on day 0.

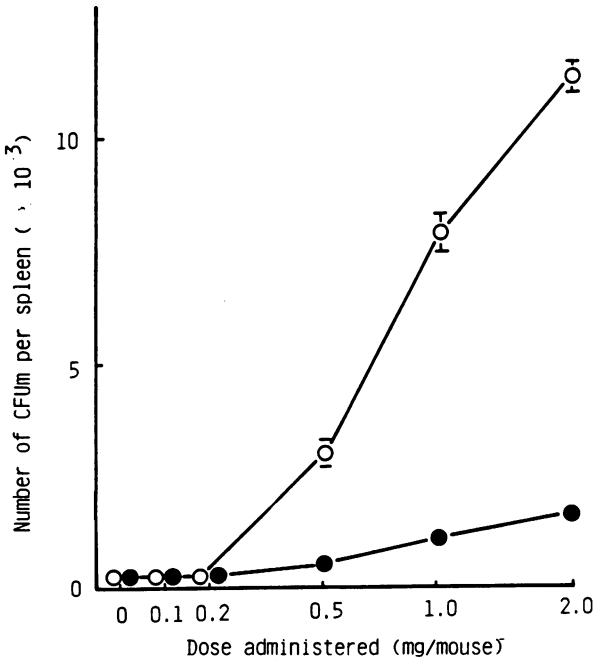

FIG. 4. Dose dependency of the effect of LC 9018 on number of CFUm in the spleen. The doses of LC 9018 (○) or *C. parvum* (●) shown were administered s.c. to mice, and the number of CFUm in the spleen was determined 5 days later.

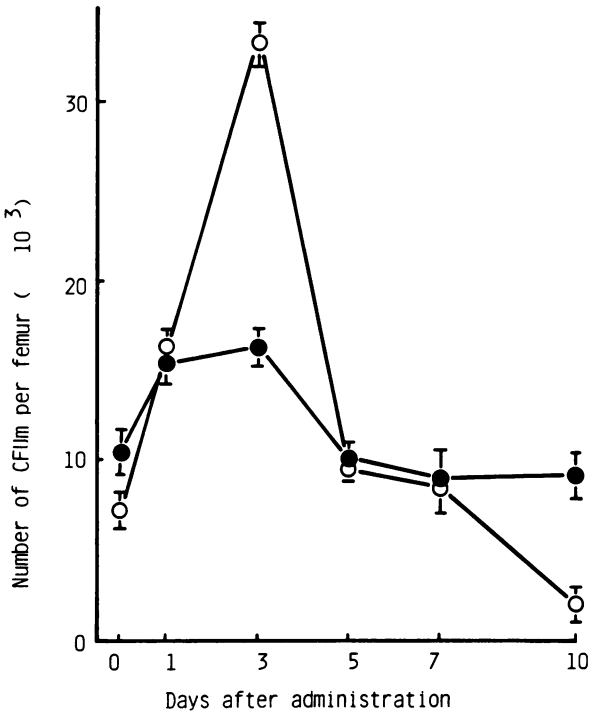

FIG. 5. Changes in number of CFUm in the femur after s.c. administration of LC 9018 or *C. parvum*. LC 9018 (○) or *C. parvum* (●) at a dose of 0.5 mg was administered s.c. to mice on day 0.

parvum-pretreated mice (Table 2). There was no significant difference in the types of colonies according to the route of administration of LC 9018.

DISCUSSION

When a host is burdened with pathogenic bacteria or malignant neoplasms that grow progressively in the host, recruitment of macrophages or polymorphonuclear cells to the sites of growth is thought to be important in stimulation of rapid generation and differentiation of macrophage-polymophonuclear precursor cells. There are several reports about increases in macrophage-polymorphonuclear colony-forming cells in the spleen and bone marrow and about elevated levels of serum CSA during infections with some bacteria or in tumor bearers (2, 9, 20, 22). Therefore, it is reasonable that many macrophage-activating agents of pathogenic bacterial origin such as BCG, *C. parvum*, and lipopolysaccharide enhance the hematopoietic responses of mice (3, 23, 24). In this study, we found that LC 9018, which is nonpathogenic, augmented macrophagepoiesis in the spleen and femur upon s.c. administration but that *C. parvum* had only a slight effect on hematopoietic responses of mice when given by that route.

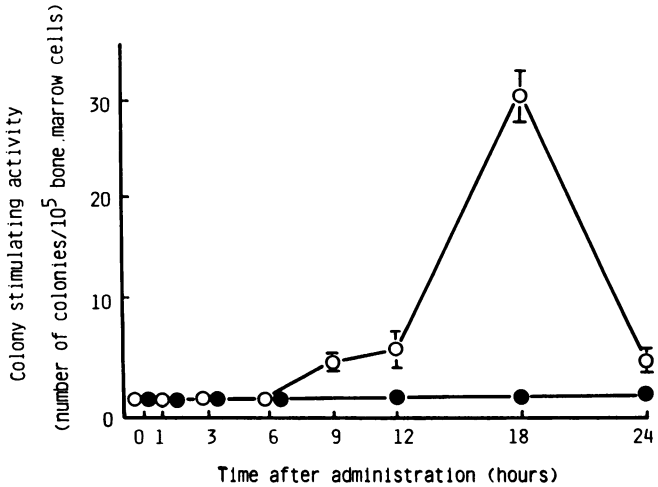

FIG. 6. Changes in serum CSA after s.c. administration of LC 9018 or *C. parvum*. LC 9018 (○) or *C. parvum* (●) at a dose of 0.5 mg was administered s.c. to mice at time zero.

TABLE 2. Effect of route of administration on CSA of serum

Drug[a]	No. of colonies/10^5 bone marrow cells (mean ± SD)	Type of colony (%)		
		Macrophage	Granulocyte-macrophage	Granulocyte
Administered s.c.				
Saline (control)	0 ± 0			
LC 9018	30 ± 3	56	36	8
C. parvum	0 ± 0			
Administered i.v.				
Saline (control)	0 ± 0			
LC 9018	135 ± 5	54	42	4
C. parvum	44 ± 4	64	30	6

[a] LC 9018 or *C. parvum* at a dose of 0.5 mg was administered s.c. or i.v. 18 or 6 h previously, respectively.

Unlike the number of progenitor cells in the spleen, the number in the femur increased early by day 3 after treatment and decreased thereafter to subnormal levels by day 10 (Fig. 5). It is not clear from these data whether CFUm migrated from the femur to the spleen, production of CFUm in the organ was suppressed, or differentiation of immature macrophages was accelerated in the late phase after administration of LC 9018.

Colony-stimulating factor (CSF) has been shown to regulate macrophage-granulocyte proliferation and differentiation in vitro (18, 19) and in vivo (1, 14). We found that CSA increased temporarily in the sera of mice that were injected s.c. with LC 9018 but not with *C. parvum* (Fig. 6). This leads to the possibility that CSF in mice treated s.c. with LC 9018 may play an important role through macrophages in protection from systemic infection with *L. monocytogenes*. How-

That resistance of mice to systemic infection with *L. monocytogenes* was enhanced more and maintained longer by LC 9018 than by *C. parvum* upon s.c. administration (Fig. 1, Table 1) reflects well the differences in the number of splenic macrophages (Fig. 2) and the degree of hematopoietic responses caused by the two agents. It is possible that the increase in macrophages in the spleen caused by s.c. administration of LC 9018 is responsible for the enhanced antilisterial resistance of mice, because the resistance of mice to *L. monocytogenes* in the early phase of infection is thought to be dependent on nonimmune scavenger macrophages (12, 15).

There is a good correlation between increases in splenic macrophages and CFUm in the spleen at 3 to 10 days after administration of LC 9018 (Fig. 2 and 3). The observation that multiplication of *C. parvum* had little effect on the number of splenic CFUm suggests a qualitative difference between the effect of s.c. administration of LC 9018 and that of *C. parvum* (Fig. 4).

ever, further investigation is required before any definitive statements can be made concerning antimicrobial activity of CSF, since it has been reported that killing of intracellular *Leishmania tropica* by macrophages is markedly enhanced by treatment with CSF (7) and that, in contrast, it was impossible to detect any change in proliferation of intracellular *Toxoplasma gondii* after treatment of macrophages with L-cell CSF (21).

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