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Cytotoxic factor production by Kupffer cells elicited with *Lactobacillus casei* and *Corynebacterium parvum*

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Summary. The ability of Kupffer cells, spleen macrophages, pulmonary macrophages, and peritoneal macrophages (PM) to produce cytotoxic factor (CTF) was investigated in vitro. The production of CTF by Kupffer cells elicited with *Corynebacterium parvum* (CP) or *Lactobacillus casei* YIT9018 (LC9018) was higher than that of spleen, pulmonary macrophages, or PM. In addition, oxygen radical (OR) production by Kupffer cells or PM was measured. The production of OR by Kupffer cells or PM was significantly augmented by i.v. or i.p. injection of LC9018 or CP. No significant correlation was observed between the increase in OR production by Kupffer cells or PM and CTF production by Kupffer cells or PM elicited with either organism. It was suggested that activated Kupffer cells may be one important source of CTF production in serum and that the CTF-producing macrophages may be different from the OR-producing macrophages.

Introduction

The extracellular cytotoxic activities mediated by macrophages have been atributed to a number of products of those cells such as thymidine [18], complement fragment C3a [4], toxic oxygen radicals (OR) [8, 16], arginase [3], protease [1], and tumor necrosis factor (TNF) [14].

Mice and rats are made hypersensitive to a lethal dose of lipopolysaccharide (LPS) by pretreatment with immunomodulating agents like Bacillus Calmette-Guerin (BCG), *Corynebacterium parvum* (CP) or zymosan [2]. The serum from a hypersensitive mouse or rat undergoing LPS-induced desensitization, sometimes referred to as "tumor necrosis serum", (TNS) has strong antitumor activity in vitro [6, 19] and in vivo [7]. TNF is cytotoxic to some tumor cell lines but not to normal cell lines in vitro [9], the cytotoxic activity is species independent [6, 7, 9], and the macrophages from hypersensitive mice or rats produce cytotoxic factor (CTF) upon in vitro incubation in the presence of LPS [12].

Macrophages have been accepted as the source of TNF [17]. However, macrophages are found in different tissues throughout the host and reflect a population of cells with significant physical and functional heterogeneity [11]. The cellular source of TNF in the mouse is assumed to be the macrophage because the agents used to prime for TNF production cause massive hyperplasia of macrophages in liver or spleen [2, 15]. The idea that macrophages are at least one cell type in the mouse capable of producing TNF comes from studies with cloned lines of mouse histiocytomas [11], but it is still not known whether the cellular source of TNF is macrophages or not. Williamson et al. reported that TNF was produced by a cloned B-cell line after stimulation with phorbolmyristate acetate [19].

The purposes of this paper are, first, to describe a convenient and controlled system to generate CTF using Kupffer cells, or pulmonary, spleen or peritoneal macrophages (PM) activated with CP or *Lactobacillus casei* YIT 9018 (LC9018) in vitro, in which the various macrophages from CP- or LC9018-primed mice are compared for production of CTF in vitro, and second, to report our investigation on whether macrophages capable of producing CTF are the same as TNF- or OR-producing macrophages or not.

Materials and methods

Animals. Male 7 to 8-week-old ddY 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.

Bacteria. Lactobacillus fermentum YIT 0159 (LF) and LC9018 were cultivated on Rogosa's medium, washed with distilled water, heated at 100 °C for 30 min, and lyophilized; CP was supplied commercially by the Institut Merieux, Lyon, France.

Preparation of Kupffer cells, spleen macrophages, pulmonary macrophages and PM. LC9018, LF or CP were injected into mice i.v. (800 μg/mouse) and on days 5, 7, 10, 12, 14, and 20 after the injection, the livers were perfused with 0.2% pronase E (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) in Hanks-HEPES (Sigma Chemical Co., St. Louis, Mo., USA) solution (pH 7.4), then removed, chopped into small pieces, and transferred to a 50 ml flask. The fragments were digested for 45 min in 0.2% pronase E in Hanks-HEPES solution (10 ml/g of liver) at 37 °C. After 15 and 30 min of incubation, 0.5 mg of DNase (type I; Boehringer Mannheim GmbH, FRG) was added to digest

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the cellular debris. At the end of the incubation period, the suspension was filtered through nylon mesh (200 mesh). The filtrate was centrifuged (1000 rpm; 10 min) and the precipitate suspended in ice-cold Hanks balanced salt solution (HBSS), then washed three times with ice-cold HBSS. LC9018, CP, or LF (800 μg/mouse) was injected into the intraperitoneal cavity of ddY mice. The peritoneal cells were harvested 1, 2, 3, 4, 5, 7, and 10 days after the injection and washed with HBSS. After being washed, the liver cells and peritoneal cells were suspended in 2.5 ml of isotonic Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden)-HBSS solution (density 1.035), and then the suspension was added to an isotonic Percoll-HBSS solution (density 1.070). The samples were centrifuged (3000 rpm; 20 min; 4 °C), and the Kupffer cells or PM in the middle layer were collected and washed three times with HBSS. The viability of Kupffer cells and PM was determined by the trypan blue exclusion method and always found to be above 85%. The percentage of PM was more than 99%, as determined morphologically. LC9018, CP or LF was injected i.v. and on days 5, 7, 10, 12, 14, and 20 after the injection spleen and pulmonary macrophages were harvested. The spleen was chopped into small pieces, and filtered through nylon mesh (200 mesh). The filtrate was centrifuged (1000 rpm; 10 min) and the precipitate was suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). Also pulmonary macrophages, PM, and Kupffer cells were suspended in RPMI 1640 medium containing 10% FCS and transferred to 35-mm tissue culture dishes (Bioquest, Div. of Becton, Dickinson and Co, Cockeysville, Md., USA). The dishes were incubated for 120 min at 37 °C in a humidified atmosphere of 5% CO_2. Then the nonadherent cells were washed off with RPMI 1640 medium. The percentages of spleen and pulmonary macrophages were 85% and 98%, respectively, as determined morphologically.

Preparation of tumor necrosis serum (TNS). LPS (12.5 μg/mouse; *Escherchia coli* strain 0127 : B8 Difco Laboratories, Detroit, Mich., USA) was injected i.v. into ddY mice 5, 7, 10, 12, 14, or 20 days after the injection of LC9018, CP, or LF (800 μg/mouse). For determination of TNS activity, 2 h after the LPS injection the mice were anesthetized with sodium pentobarbital and blood was obtained by cardiac puncture.

Preparation of cytotoxic factor (CTF) by macrophages. Kupffer cells, spleen and pulmonary macrophages, and PM were stimulated with LPS to secrete CTF. The macrophages were diluted to 1×10^6/ml in RPMI 1640 medium containing 10% FCS. All cultures were incubated with LPS (25 μg/ml) in 35-mm plastic tissue culture dishes at 1×10^6 cells/2 ml per dish at 37 °C in a humidified atmosphere of 5% CO_2. After 3 h of incubation, the culture medium was collected and centrifuged for 10 min at 1000 rpm to remove the cells. The medium was then frozen at −25 °C, and used within 2 weeks for assay of CTF activity.

Cell line and culture. The L929 murine tumorigenic fibroblast line was obtained from the American Type Culture Collection (CCL 1.2; Rockville, Md., USA). The cells were grown in tissue culture flasks (Corning Glass Works, Corning, NY, USA) in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. The culture medium was Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co. Ltd., Tokyo Japan) containing 5% FCS.

Assay for cytotoxic activity of CTF or TNS in vitro. The samples serially diluted in MEM containing 5% FCS were added to microtiter wells containing 2×10^4 growing adherent L929 cells and actinomycin D (Cosmegen, Merck Sharpe and Dohme, West Point, Pa., USA; final concentration 1 μg/ml) for assay of CTF activity or without a actinomycin D for assay of TNS activity [12, 13]. The cells were incubated at 37 °C for 20 h, the supernatant was discarded and the number of viable cells determined by the neutral red uptake method (0.06% neutral red in HBSS; incubation at 37 °C for 60 min). The number of cells which took up the dye was proportional to the number of viable cells in the well, and was measured photometrically using an Immunoreader NJ 2000 multiscan photometer (Japan Intermed Ltd., Tokyo, Japan). The titer (defined as dilution causing 50% cytotoxicity) was calculated from the probit transformation of the cytotoxicity vs $\log_{10}$ dilution using a computer (Professional 350; Digital Equipment Co., Maynard, Mass., USA).

Luminol-chemiluminescence assay. The OR production by PM or Kupffer cells was measured by Luminol-chemiluminescence assay methods as described alsewhere [8].

Results

TNS activity in vitro

The effect of CP, LC9018, and LF on TNF production in ddY mice was investigated for 20 days after injection of the bacteria (Fig. 1). LPS was injected i.v. 5, 7, 10, 12, 14, or 20 days after the i.v. injection of CP, LC9018, or LF, and 2 h later the serum was collected from the mice and its

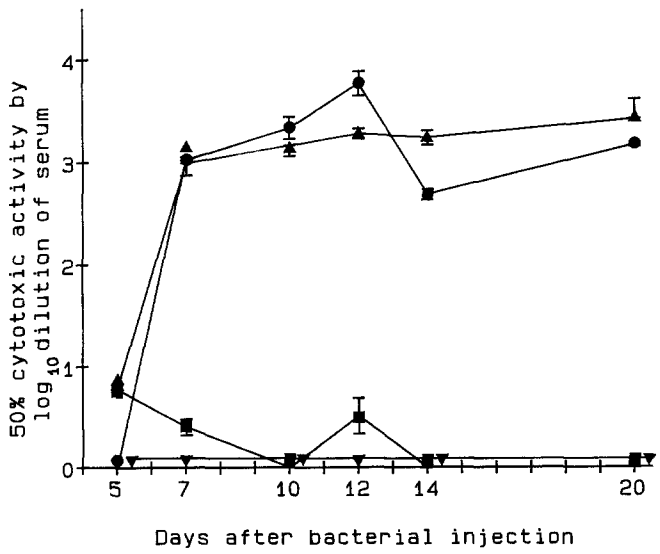

Fig. 1. Cytotoxic activity of the serum in vitro. LPS (12.5 μg/mouse) was injected i.v. into ddY mice 5, 7, 10, 12, 14, and 20 days after the injection of LC9018 (●), CP (▲), LF (■) (800 μg/mouse), or saline as control (▼). The serum was harvested 2 h after the LPS injection for determination of cytotoxic activity against L929 cells. The values are the means ± SE (50% cytotoxic activity by $\log_{10}$ dilution of the serum) of triplicate cultures

Table 1. Cytotoxic activity in culture medium of spleen cells

Bacterial strain	Days after injection of bacteria					
	5	7	10	12	14	20
LC9018	2.73 ± 0.05	2.43 ± 0.13	2.62 ± 0.16	2.56 ± 0.07	2.46 ± 0.02	2.51 ± 0.06
CP	2.71 ± 0.01	2.54 ± 0.09	2.69 ± 0.11	2.71 ± 0.07	2.59 ± 0.04	2.71 ± 0.04
LF	2.18 ± 0.05	1.98 ± 0.05	< 1.0	1.75 ± 0.12	2.03 ± 0.02	1.72 ± 0.08
Control	1.98 ± 0.17	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

The spleen cells were harvested 5, 7, 10, 12, 14, and 20 days after the bacterial injection and cultured (1×10^7 cells) with 25 μg of LPS/ml for 3 h at 37°C and the cytotoxic activity in the culture medium was assayed against L929 cells in the presence of actinomycin D (1 μg/ml). The values are the means ± SE (50% cytotoxic activity by $\log_{10}$ dilution of the culture medium) of triplicate cultures

Table 2. Cytotoxic activity in culture medium of spleen adherent and nonadherent cells

Bacterial strain	Spleen cells	Spleen nonadherent cells	Spleen adherent cells
LC9018	2.01 ± 0.02	1.75 ± 0.04	2.94 ± 0.07
CP	2.31 ± 0.08	1.89 ± 0.02	2.61 ± 0.04
LF	1.64 ± 0.09	0.89 ± 0.03	1.99 ± 0.05
Control	1.45 ± 0.10	0.72 ± 0.01	1.92 ± 0.02

The spleen cells were harvested 12 days after the injection of LC9018, CP, LF, or saline as control and the spleen cells (1×10^7), nonadherent cells (1×10^7), or adherent cells (1×10^6) were cultured and the cytotoxic activity in the culture medium was assayed. Experimental conditions are the same as shown in Table 1. The values are the means ± SE (50% cytotoxic activity by $\log_{10}$ dilution of the culture medium) of triplicate cultures

cytotoxic activity against L929 cells was measured. The injection of CP or LC9018 augmented cytotoxic activity 1000 times above that of the control or LF in ddY mouse, but treatment with LF caused no induction of TNF. TNF activity in the serum of BALB/c mice treated with LC9018 or CP was lower (1/10–1/100) than that in the serum of ddY mice (data not shown).

CTF production by spleen cells

The ability of spleen cells to produce CTF was measured on days 5, 7, 10, 12, 14, and 20 after the i.v. injection of CP, LC9018,or LF in ddY mice (Table 1). The CTF activity in mice injected with CP or LC9018 was higher (more than 10 times) than that of mice injected with LF or the

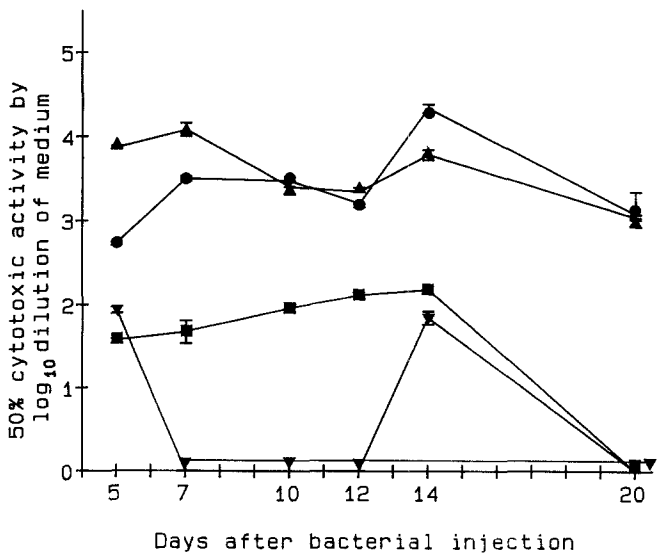

Fig. 2. Cytotoxic activity in culture medium of Kupffer cells. ddY mice were injected i.v. with LC9018 (●), CP (▲), LF (■) (800 μg/mouse), or saline as control (▼). Experimental conditions are the same as shown in Table 3. The values are the means ±SE (50% cytotoxic activity by $\log_{10}$ dilution of the culture medium) of triplicate cultures

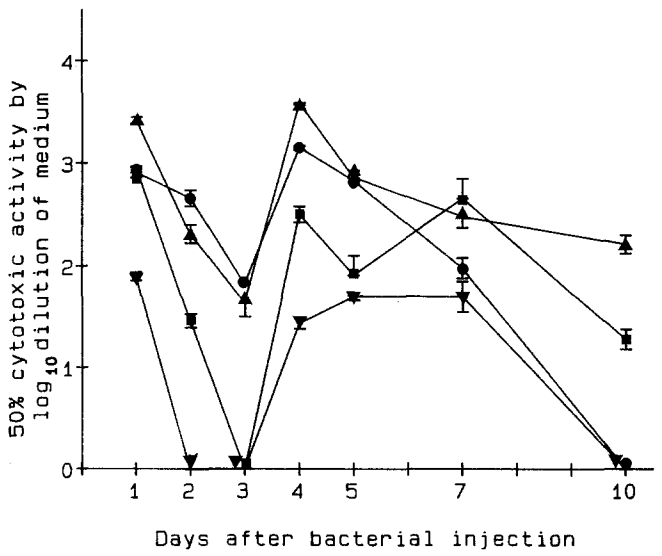

Fig. 3. Cytotoxic activity in culture medium of PM. ddY mice were injected i.p. with LC9018 (●), CP (▲), LF (■) (800 μg/mouse), or peptone as control (▼) (10 mg/mouse). PM were harvested on days 1, 2, 3, 4, 5, 7, and 10 after the injection. Experimental conditions are the same as shown in Table 3. The values are the means ±SE (50% cytotoxic activity by $\log_{10}$ dilution of the culture medium) of triplicate cultures

Table 3. Cytotoxic activity in culture medium of pulmonary macrophages

Bacterial strain	Days after injection of bacteria					
	5	7	10	12	14	20
LC9018	2.38 ± 0.17	2.75 ± 0.04	2.69 ± 0.02	2.06 ± 0.04	1.95 ± 0.07	1.78 ± 0.06
CP	1.99 ± 0.11	2.64 ± 0.08	2.91 ± 0.05	2.08 ± 0.04	2.11 ± 0.08	1.85 ± 0.10
LF	< 1.0	2.14 ± 0.05	2.51 ± 0.19	1.99 ± 0.06	1.86 ± 0.08	< 1.0
Control	1.92 ± 0.02	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

The pulmonary macrophages were were harvested 5, 7, 10, 12, 14, and 20 days after the i.v. injection of LC9018, CP, LF (800 μg/mouse), or saline as control and cultured (1×10^6 cells) with 25 μg of LPS/ml for 3 h at 37°C and the cytotoxic activity in the culture medium against L929 cells was assayed in the presence of actinomycin D (1 μg/ml). The values are the means ± SE (50% cytotoxic activity by $\log_{10}$ dilution of the culture medium) of triplicate cultures

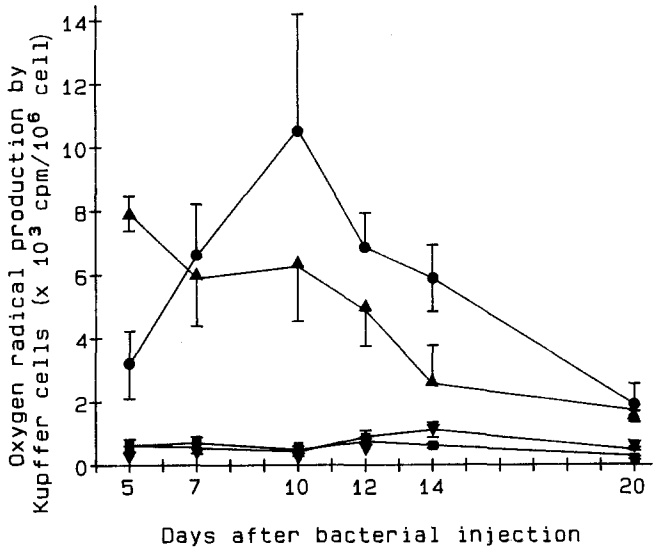

Fig. 4. OR production by Kupffer cells. ddY mice were injected i.v. with LC9018 (●), CP (▲), LF (■) (800 μg/mouse), or saline as control (▼). The Kupffer cells were harvested 5, 7, 10, 12, 14, and 20 days after the injection and then the OR production by these cells was measured. The values are the means ±SD ($\times 10^3$ cpm/10^6 cells) of the counts of 5 to 10 mice

controls. The production of CTF in the spleen cells was mainly via adherent cells (Table 2) and the cells were morphologically shown to be macrophages.

CTF production by macrophages

Kupffer cells and pulmonary macrophages were examined for production of CTF on days 5, 7, 10, 12, 14, and 20 after the i.v. injection of CP, LC9018, or LF. The production of CTF by Kupffer cells (Fig. 2) was augmented by injection of CP or LC9018, and the CTF-producing ability of Kupffer cells was about 10 times higher than that of the spleen and pulmonary macrophages (Table 3). On the other hand, the production of CTF by pulmonary macrophages was not augmented by injection of CP or LC9018. The CTF-producing ability of CP- or LC9018-treated Kupffer cells was higher (30–100 times) than that of LF-treated cells or the control. And peroxidase-positive cells in the liver were increased 20 times by injection of LC9018 as compared with the control (data not shown). The production of CTF by PM was measured 1, 2, 3, 4, 5, 7, and 10 days after i.p. injection of CP, LC9018, or LF (Fig. 3). The production of CTF by CP- or LC9018-activated PM was highest on day 4 and lowest on day 3 after injection of the bacteria, and the same phenomenon was observed after treatment with LF.

OR production by Kupffer cells and PM

The production of OR by Kupffer cells was measured 5, 7, 10, 12, 14, and 20 days after i.v. injection of CP or LC9018 (Fig. 4) and the production of OR by PM was measured 1, 2, 3, 4, 5, 7, and 10 days after i.p. injection of the bacteria (Fig. 5). Although OR production by Kupffer cells was enhanced 10 days after the injection of LC9018 and 5–10 days after the injection of CP, LF had no effect on the OR production by Kupffer cells during the experimental period. On the other hand, stimulation of OR produc-

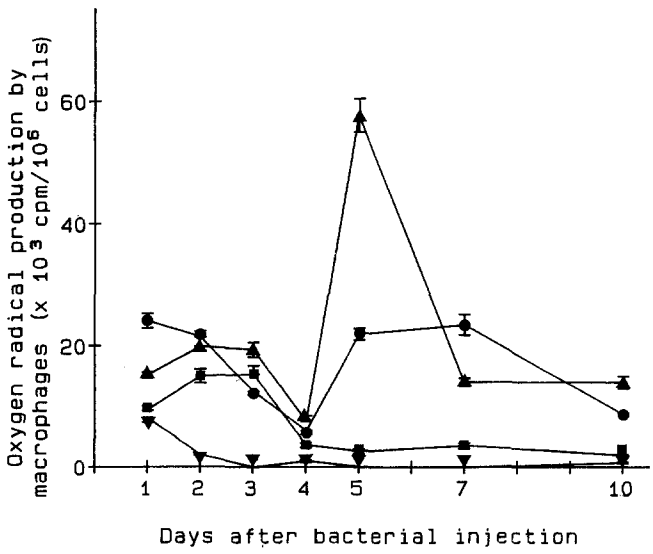

Fig. 5. OR production by PM. ddY mice were injected i.p. with LC9018 (●), CP (▲), LF (■) (800 μg/mouse), or peptone (10 mg/mouse) as control (▼). The PM were harvested 1, 2, 3, 4, 5, 7, and 10 days after the injection and then the OR production by the PM was measured. The values are the means ±SD ($\times 10^3$ cpm/10^6 cells) of the counts of 5 to 10 mice

tion by CP-activated PM was observed on day 5 and by LC9018-activated PM on days 1, 2, 5, and 7. The production of OR by CP-activated PM was higher than that by LC9018-activated PM on day 5. The production of OR by PM was increased on day 3 after treatment with LF but was lower than that after treatment with CP or LC9018.

Discussion

As stated in the introduction, the cellular source of TNF in rodents has been assumed to be the macrophages in the spleen and liver. On the other hand, TNF production by macrophages is not influenced by the injection of silica, which has selective toxicity for macrophages in vivo [5], and the cellular source of TNF in vivo is still not clear. However, our results suggest that CTF is not produced by nonadherent cells but by adherent cells, i.e., macrophages in the spleen.

The ability of LPS to induce CTF in the serum was enhanced by treatment with CP or LC9018 from 7 to 20 days after priming with CP or LC9018 and the levels of activity were about the same during that period. The priming ability of LC9018 was almost equal to that of CP. On the other hand, macrophages from various sites can produce CTF upon challenge with LPS in vitro. In CP- or LC9018-injected mice, Kupffer cells and PM were the most potent producers of CTF during our experimental period. The production of CTF by Kupffer cells was maintained for a longer period than that by PM. Since the total production of CTF by Kupffer cells in the mouse was augmented about 200,000 times by i.v. injection of LC9018 compared with the control, the Kupffer cells in the liver may be a more important source of CTF and/or TNF in the serum than spleen or pulmonary macrophages and PM.

The production of OR by Kupffer cells or PM was elicited by injection of CP or LC9018. Those macrophages have strong cytotoxic activity against tumor cells and bacteria, therefore, OR is used as one of the markers of cytotoxic activity of macrophages. In our experiment, CTF production by Kupffer cells continued from 7 to 20 days after the injection of CP or LC 9018, while OR production by Kupffer cells was most stimulated 10 days after injection of the bacteria, CTF production by PM was increased 4 days after the injection of CP or LC9018 but OR production by PM was reduced on day 4. No significant correlation was observed between the augmentation of OR production and stimulation of CTF production by Kupffer cells or PM. Therefore CTF producing-macrophages may be different from OR-producing macrophages. This may be due to a difference in the degree of maturation and/or a difference in the subset of activated macrophages. LF is known to be a nonpotent immunostimulant and has been shown not to increase host resistance to some bacterial infection and tumors [8, 10], therefore LF may be not have the CTF priming ability in vivo.

The molecular weights of Kupffer cells producing CTF in vitro and of TNF in the serum were in the same range (41,000 to 43,000) as determined by HPLC and CTF activity is stable at 56 °C for 30 min. However, since the characteristics of CTF have not been determined, it is not clear whether CTF has the same characteristics as TNF or not [6].

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