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Stimulation of Macrophages by Cord Factor and by Heat-Killed and Living BCG

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Trehalose-6,6'-dimycolate (cord factor; CF) injected into the peritoneal cavity of mice induced stimulation of the peritoneal macrophages, evidenced both by increased activity of the lysosomal enzyme, acid phosphatase, and by increased phagocytosis of *Listeria monocytogenes*. The increase in enzyme activity and phagocytosis was similar to that induced by killed or living BCG. When administered intravenously, CF or BCG did not induce stimulation of peritoneal macrophages. CF when added to tissue cultures of normal peritoneal cells did not induce increased acid phosphatase activity or increased phagocytosis of *L. monocytogenes*.

Many of the biological effects induced by mycobacterial infection can be duplicated by treatment with the mycobacterial glycolipid trehalose-6,6'-dimycolate (cord factor; CF) (16). These shared properties include: (i) induction of a granulomatous reaction (1, 4, 5, 18); (ii) potentiation of the immune response to unrelated antigens (8, 25); and (iii) increased resistance against bacterial infections (2, 4, 29) and antitumor effects (3, 6, 7, 30, 31).

Administration of mycobacteria to rabbits and mice results in formation of granulomas in which the macrophages display a higher content of lysosomal enzymes and increased capacity to phagocytize and kill bacteria (9, 10, 13, 15, 19, 32). In the granulomas induced by CF in mice, epithelioid cells as well as macrophages are present. It was of interest to investigate whether CF is able to affect the activity of macrophages and to compare its effect with that of living and killed BCG.

MATERIALS AND METHODS

Animals. Locally bred Swiss mice and mice of strain ICR were used.

Bacteria. *Listeria monocytogenes*, obtained from our stock, was grown at 37°C in tryptic soy agar (Difco) for 16 to 18 h. *Mycobacterium bovis* BCG, Phipps strain, was maintained by weekly transfer in Dubos broth base (Difco). Heat-killed BCG or *L. monocytogenes* bacilli were prepared by heating for 20 min at 70°C.

Preparation of CF. CFs from *Mycobacterium tuberculosis*, strains Peurois and H37Ra, and their emulsions were prepared as described previously (4). CF from strain Peurois was prepared in the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. CF from H37Ra was prepared in this laboratory by one of us.

PC monolayers. Peritoneal cells (PC) were collected from mice pretreated with the mycobacterial fractions by intraperitoneal (i.p.) injection of 3 ml of medium 199 containing 10% heat-inactivated calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10 U of sodium heparin per ml. Peritoneal fluid was withdrawn through a 19-gauge needle. A sample was removed for cell counting, and the remainder was diluted with medium to a concentration of 1×10^6 to 2×10^6/ml. Two milliliters of cell suspension was distributed without washing to plastic petri dishes (30 mm in diameter; Nunc, Algade, Denmark).

Biochemical determination of acid phosphatase. PC monolayers were incubated for 2 h and decanted, and the cell monolayers were washed three times with a stream of redistilled water. Most of the adherent cells were macrophages. The cells were scraped from the dish into 2 ml of redistilled water with a rubber policeman. The activity of acid phosphatase was determined in this lysate by the method of Torriani (27), using *p*-nitrophenyl phosphate as a substrate. Briefly, the reaction mixtures contained 0.5 ml of macrophage lysate, 1.5 ml of acetate buffer (0.2 M, pH 4.0), and 0.2 ml of substrate (0.04 M). The reaction mixtures were incubated for 1 h at 37°C and stopped by adding 0.5 ml of tris(hydroxymethyl)aminomethane buffer (1 M, pH 8.5) containing K_2HPO_4 (0.4 M). Enzymatic activities were reported in optical density units (420 nm) of nitrophenol released per milligram of protein per hour. The protein content of the monolayer was determined by the method of Lowry (28).

Histochemical determination of acid phosphatase. PC monolayers were incubated for 2 h and decanted, and the cell monolayers were washed three times with 2 ml of saline. The method of Love et al. (17) was used for histochemical determination of acid phosphatase. Since the monolayers were in plastic dishes, the cold acetone fixative that was used by Love et al. (17) was substituted by 1.25% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer,

pH 7.4. The cultures were fixed for 30 min in 4°C and were incubated for 1 h with the substrate at 37°C. The results were graded arbitrarily by microscopic examination of 200 macrophages.

Acid phosphatase determination in macrophages incubated in vitro with CF or BCG. PC monolayers were prepared from each individual normal mouse, and PC suspensions were incubated for 2 h. After that time, the supernatants were decanted and the adherent cells were washed two times with medium without heparin. Then CF or BCG bacilli (living or killed) were added in different concentrations and for different incubation periods. At the end of the incubation time, the cultures were washed three times with redistilled water and the acid phosphatase activity was determined biochemically.

Phagocyte activity assay after CF treatment in vivo. Monolayer cultures of peritoneal macrophages from mice pretreated with CF were prepared by seeding 2×10^6 PC in 2 ml of medium in tissue culture dishes. After 2 h of incubation, the cell layers were washed twice with a strong stream of warm (37°C) medium to remove nonadherent cells. Thereafter, 10^8 heat-killed bacteria (*L. monocytogenes*) were added in 1.5 ml of medium to each culture. After 2 h of incubation, cultures were washed with medium, dried immediately with a stream of air, and, after fixation with methanol, treated with Giemsa stain. The phagocytic activity of the macrophages was determined by counting the bacteria in 200 macrophages.

Phagocytic activity assay after CF treatment in vitro. The PC were harvested in five pools. Each pool contained cells of two ICR mice. From each pool, three cultures of 2×10^6 cells were prepared; one culture served as a control, one for addition of emulsion, and one for CF, as mentioned below. The cell layers were incubated for 1 h and washed twice with a strong stream of warm medium to eliminate nonadherent cells, and incubation was continued for 1 h after the addition of 1.9 ml of medium. Then a total of 0.1 ml of either medium, emulsion, or CF was added to each culture, and incubation was continued for 22 h. The monolayers were washed twice with warm medium, and 10^8 heat-killed bacteria (*L. monocytogenes*) were added; the phagocytic activity was determined as described above.

Statistical evaluation of results. The nonparametric rank test of Wilcoxon (26) was used.

RESULTS

Effect of CF and BCG on acid phosphatase activity of peritoneal macrophages. Groups of five mice were treated i.p. with CF in emulsion or emulsion alone; another group of normal mice served as control. Acid phosphatase activity of peritoneal macrophages was increased 3 days after i.p. administration of 25 μg of CF derived from *M. tuberculosis* strain H37Ra or 20 μg of CF derived from *M. tuberculosis* strain Peurois. In two additional experiments, similar increased activity was found after i.p. administration of living or killed BCG bacilli (Table 1).

Kinetics of acid phosphatase stimulation by CF and BCG. Since CF and BCG bacilli were effective in increasing the acid phosphatase levels in the peritoneal macrophages, the effect of the time interval was investigated.

Twenty-five micrograms of CF H37Ra was injected i.p. into groups of five mice (local strain), and the PC were collected at the following time intervals: 1, 2, 4, and 6 days. In the same type of experiment, the acid phosphatase activity was determined after 14 days. In a second series of experiments, the acid phosphatase activity was determined 1, 2, 3, 7, 8, and 15 days after i.p. administration of living or killed BCG bacilli (Tables 2 and 3). Increased activity of the lysosomal enzyme was evident already after 1 day and after 2 days in the groups treated with CF and BCG, respectively. This increased activity persisted through all the intervals tested.

Effect of different amounts of CF and heat-killed BCG bacilli on the acid phosphatase activity of peritoneal macrophages. The above results raised the question of whether the increased enzyme activity is dose dependent.

Different amounts of CF, H37Ra, and killed BCG were administered to groups of five mice (ICR strain). After 3 days, the peritoneal macrophages were harvested and the acid phosphatase activity was determined (Table 4). Although 10 μg of CF caused a 40% increase in the acid phosphatase activity, two- and fourfold-larger amounts caused a further increase, but of the same magnitude. This seemed to be true also for the killed BCG.

Local effect of CF and BCG bacilli. After i.p. administration into mice, CF enhanced the resistance against i.p. infections of *Salmonella* and inhibited the growth of Ehrlich ascites tumor cells in mice. No such changes were observed after intravenous (i.v.) inoculation of CF (29, 31). It was of interest, therefore, to test whether the route of administration of CF is important in augmenting the acid phosphatase activity.

CF (Peurois) was injected i.p. or i.v. to groups of five mice. In the second experiment, carried out with three mice in each group, living or killed BCG bacilli were injected i.p. or i.v. After 3 days, the peritoneal macrophages were harvested and the acid phosphatase was determined. Table 5 demonstrates the local effect of CF and BCG. Normal levels of enzyme activity were found after i.v. administration of emulsion, CF, and BCG bacilli. In contrast, emulsion injected i.p. caused an increase in

TABLE 1. *Acid phosphatase activity of peritoneal macrophages from mice pretreated intraperitoneally with CF or BCG*

Expt no.	Material injected[a]	Acid phosphatase activity[b]					Avg	Increase in %[c]
1	Control	6.0	6.0	6.2	6.4	6.6	6.2	
	Emulsion	6.0	6.5	6.6	6.8	7.2	6.6	6
	CF (H37Ra), 25 μg	12.2	12.4	13.4	14.0	15.5	13.5	118
2	Control	4.4	5.1	6.4	6.9	8.1	6.2	
	Emulsion	7.1	7.6	7.8	8.2	8.4	7.8	26
	CF (Peurois), 20 μg	8.2	8.2	13.4	13.8	18.6	12.4	100
3	Saline + 0.05% Tween 80	5.6	6.7	6.8	7.1	8.0	6.8	
	BCG (10^8 bacilli), living	12.8	16.8	17.4	18.3	19.9	17.0	150
	BCG (10^8 bacilli), heat killed	14.8	17.0	17.2	17.9	22.3	17.8	162
4	Saline + 0.05% Tween 80	3.8	4.1	4.5	4.7	5.0	4.4	
	BCG (10^8 bacilli), living	15.1	11.3	11.9	12.4	13.4	11.9	170
	BCG (10^8 bacilli), heat killed	15.1	15.4	15.9	16.0	17.0	15.9	261

[a] Emulsion contained 0.4% Bayol F in 0.04% Tween 80 in saline. CF was prepared in the above emulsion. BCG bacilli were washed once and suspended in saline + 0.05% Tween 80. The BCG bacilli were killed at 70°C for 20 min. A final volume of 0.2 ml was injected into each mouse 3 days before the assay was conducted. Swiss mice were used as a control in experiments 1 and 2; ICR mice were used in experiments 3 and 4.

[b] Expressed as optical density units per milligram of protein per hour.

[c] In comparison with control group.

TABLE 2. *Time relationship between administration of CF (H37Ra) and acid phosphatase activity of macrophages*

Expt no.	Material injected[a]	Time interval (days)	Acid phosphatase activity[b]					Avg	Increase in %
1	Control		3.1	4.2	4.6	4.6	5.6	4.4	
	Emulsion	1	5.0	5.3	5.6	6.3	6.7	5.8	32
	CF		7.0	7.1	7.3	7.4	7.7	7.3	66
	Emulsion	2	6.4	7.0	7.0	7.5	7.8	7.1	61
	CF		7.6	7.9	10.3	11.8	11.9	9.9	125
	Emulsion	4	5.4	5.6	6.0	6.2	7.7	6.2	41
	CF		8.3	8.4	8.8	8.9	12.1	9.3	111
	Emulsion	6	5.1	5.6	6.0	6.5	7.0	6.0	36
	CF		8.0	8.0	8.8	9.4	10.5	8.9	102
2	Control		4.6	4.6	5.2	5.3	5.3	5.0	
	Emulsion	14	5.4	6.7	7.4	7.4	7.7	6.9	38
	CF		9.1	9.4	9.6	10.0	10.6	9.7	94

[a] 25 μg of CF (H37Ra) was injected into each mouse. Normal Swiss mice were used as controls. Other details as in legend to Table 1.

[b] Expressed as in Table 1.

enzyme activity (25%), and it was higher after i.p. administration of CF (100%), heat-killed BCG (82%), and living BCG (116%).

Histochemical determination of acid phosphatase in peritoneal macrophages of mice treated with CF or BCG. To differentiate between parts of the peritoneal macrophage population activated by CF and BCG, acid phosphatase activity was assayed histochemically. Emulsion and CF (Peurois) were injected i.p. to groups of five mice. A group of normal mice was used as a control. In a second experiment, en-

TABLE 3. *Time relationship between administration of BCG and acid phosphatase activity of macrophages*

Expt no.	Material injected[a]	Time interval (days)	Acid phosphatase activity[b]					Avg	Increase in %
1	Saline + 0.05% Tween 80	1	6.0	6.6	6.7	7.7	7.9	7.0	
	BCG, living		6.7	7.6	7.9	8.2	8.3	7.7	10
	BCG, heat killed		7.3	7.7	7.8	9.1	10.0	8.4	20
2	Saline + 0.1% Tween 80	1	6.2	7.5	8.2	9.2	10.1	8.2	
	BCG, living		6.8	7.8	8.2	8.8	9.5	8.2	0
	BCG, heat killed		6.2	7.3	7.9	8.0	8.4	7.6	7
	Saline + 0.1% Tween 80	2	7.1	7.4	7.4	7.4	7.7	7.4	
	BCG, living		10.4	11.3	12.9	13.2	13.5	12.3	66
	BCG, heat killed		11.4	11.7	11.8	13.6	13.7	12.4	68
3	Saline + 0.05% Tween 80	2	5.3	5.7	6.2	6.2	7.0	6.1	
	BCG, living		10.2	11.3	11.4	12.6	14.0	11.9	95
	BCG, heat killed		6.8	9.9	11.2	11.7	12.1	10.3	69
4	Saline + 0.05% Tween 80	3	6.5	6.6	6.9	8.7	9.5	7.6	
	BCG, living		7.6	10.3	10.6	10.7	10.8	10.0	32
	BCG, heat killed		7.1	10.9	11.7	12.2	13.0	11.0	45
5	Saline + 0.05% Tween 80	7	5.4	5.7	5.8	6.0	6.0	5.8	
	BCG, living		9.4	9.4	9.7	9.9	11.7	10.0	72
	BCG, heat killed		7.9	8.9	9.8	10.1	10.4	9.4	62
6	Saline + 0.05% Tween 80	8	4.2	4.3	4.4	4.7	7.1	4.9	
	BCG, living		10.3	11.7	11.8	12.8	12.9	11.9	143
	BCG, heat killed		7.9	8.4	8.4	8.8	10.5	8.8	80
	Saline + 0.05% Tween 80	15	5.2	5.3	5.3	5.5	5.6	5.4	
	BCG, living		8.6	8.9	11.9	12.3	12.5	10.8	100
	BCG, heat killed		9.3	9.6	9.9	11.5	13.4	10.7	98

[a] BCG bacilli were washed once and suspended in saline + Tween 80. 10^8 bacilli were injected into each mouse in a final volume of 0.2 ml. Swiss mice were used in the experiments.
[b] Expressed as in Table 1.

TABLE 4. *Acid phosphatase activity in peritoneal macrophages from mice pretreated with different amounts of CF (H37Ra) or killed BCG bacilli*[a]

Expt no.	Material injected	Dosage (μg)	Acid phosphatase activity[b]					Avg	Increase in %
1	Control	–	4.4	4.5	5.1	5.2	6.2	5.1	
	Emulsion	–	5.1	5.3	6.2	6.7	6.8	6.0	18
	CF (H37Ra)	10	7.7	7.9	8.3	9.1	9.9	8.6	69
		20	10.2	10.2	10.4	11.7	12.2	10.9	114
		40	10.4	10.4	10.5	10.6	12.2	10.8	112
2	Saline + 0.05% Tween 80	–	4.7	6.2	6.2	6.4	6.5	6.0	
	BCG, 10^6	–	8.2	8.5	8.6	8.6	9.3	8.6	43
	BCG, 10^7	–	8.3	9.8	11.0	11.9	13.0	10.8	80
	BCG, 10^8	–	10.8	11.6	12.4	12.9	13.7	12.3	105

[a] The mice used were of strain ICR. Other details as in legend to Table 1.
[b] Expressed as in Table 1.

zyme activity was assayed histochemically in BCG (living or killed)-treated mice. Five mice injected with saline + 0.05% Tween 80 served as a control. Three days after the injection, peritoneal macrophages were harvested from each individual mouse and seeded onto petri dishes. After 2 h of incubation, a histochemical assay for acid phosphatase activity was performed. A few activated macrophages were present in the control groups and in the emulsion-treated mice (Tables 6 and 7). A marked increase in the percentage of activated cells appeared after administration of CF or living or killed BCG bacilli.

Influence of CF and BCG on normal peritoneal macrophages in tissue culture. Since peritoneal macrophages of CF-treated mice showed a marked increase in acid phosphatase activity, it was of interest to test whether CF has a direct in vitro action on the macrophages. Peritoneal macrophages from normal mice were inoculated with emulsion containing different concentrations of CF (Peurois). Similar experiments were conducted with killed and living BCG. A biochemical test for acid phosphatase activity was carried out after 18 and 48 h of incubation. CF in the range of concentrations tested did not exert any effect on the macrophages during 18 h of incubation. After 48 h of incubation the activity seemed to decrease, but this was statistically not significant ($P > 0.05$). The results obtained with killed and living BCG were conflicting, and therefore no conclusion could be drawn.

Increased phagocytic activity induced by CF. Activated macrophages that have high lysosomal enzyme activity (9, 24) also show a higher phagocytic activity (21). The ability of CF to increase the phagocytic activity of peritoneal macrophages was studied in vivo and in vitro.

Forty micrograms of CF (Peurois) was ad-

TABLE 5. *Acid phosphatase activity in peritoneal macrophages from mice pretreated with CF (Peurois) or BCG intravenously i.v. or i.p.*

Expt no.	Material injected[a]	Route of injection	Acid phosphatase activity[b]					Avg	Increase in %
1	Control		5.1	5.1	5.6	6.1	6.3	5.7	
	Emulsion	i.p.	5.7	6.6	7.2	7.4	8.4	7.1	25
	Emulsion	i.v.	5.4	5.7	6.1	6.2	6.6	6.0	6
	CF	i.p.	9.7	10.6	11.7	12.1	12.9	11.4	100
	CF	i.v.	5.7	5.9	6.0	6.4	6.5	6.1	7
2	Control		5.2	5.5	6.4			5.7	
	BCG, living	i.p.	11.0	12.7	12.9			12.3	116
	BCG, living	i.v.	4.4	5.0	5.2			4.9	
	BCG, heat killed	i.p.	9.0	9.8	12.5			10.4	82
	BCG, heat killed	i.v.	5.3	5.6	6.1			5.6	

[a] 20 μg of CF (Peurois) or 1.6×10^8 colony-forming units of BCG bacilli was injected into each mouse. Swiss mice were used in the experiments. Other details as in legend to Table 1.

[b] Expressed as in Table 1.

TABLE 6. *Histochemical determination of acid phosphatase in peritoneal macrophages of mice pretreated with CF i.p.*

Material injected[a]	Mouse no.	Percent of macrophages with acid phosphatase activity[b]			Avg ± standard deviation		
		−	+	++	−	+	++
Control	1	96.5	2	1.5			
	2	98	1	1			
	3	99	1	0			
	4	97	2	1			
	5	99	1	0	97.9 ± 1.1	1.4 ± 0.5	0.7 ± 0.7
Emulsion	1	86.5	12	1.5			
	2	99	1	0			
	3	92	7	1			
	4	86.5	11	2.5			
	5	93	7	0	91.4 ± 5.2	7.6 ± 4.3	1 ± 1.1
CF, 20 μg	1	28	47	25			
	2	28	52	20			
	3	23	59	18			
	4	25	47.5	27.5			
	5	35	46	19	27 ± 4.5	50.3 ± 5.4	20.9 ± 4.1

[a] Emulsion contained 0.5% Bayol F in 0.05% Tween 80 in saline. CF (Peurois) was prepared in the above emulsion, and a final volume of 0.1 ml was injected into each mouse 3 days before the assay was conducted. Swiss mice were used in the experiments.

[b] Arbitrary grading for the acid phosphatase activity: −, no activity; +, weak activity (small number of grains); ++, strong activity (numerous grains). In each culture 200 cells were examined.

ministered i.p. to a group of five mice (ICR strain); a control group was injected with emulsion in the same way. After 3 days, tissue cultures of peritoneal macrophages from individual mice were prepared in order to test their phagocytic activity. *L. monocytogenes* bacilli were used as target cells. CF was able to increase the phagocytic activity of the macrophages, whereas the emulsion was without effect (Table 8). Addition of CF (Peurois) (12.5 μg/ml, final concentration) to monolayers of peritoneal macrophages harvested from normal mice of ICR strain did not increase their ability to ingest *L. monocytogenes* (Table 9).

DISCUSSION

It is clear from the present study that 20 to 40 μg of CF was able to stimulate peritoneal macrophages in vivo as judged by increased acid phosphatase and phagocytic activities. The increased enzymatic activity was still evident 2 weeks after administration of CF. The histochemical test of acid phosphatase revealed that about 70% of the peritoneal macrophage popu-

TABLE 7. *Histochemical determination of acid phosphatase in peritoneal macrophages of mice pretreated with BCG i.p.*

Material injected[a]	Mouse no.	Percent of macrophages with acid phosphatase activity[b]			Avg ± standard deviation		
		−	+	++	−	+	++
Saline + 0.05% Tween 80	1	94	5.5	0.5			
	2	98	1.5	0.5			
	3	79	20	1			
	4	91.5	8.0	0.5			
	5	96	4	0	91.7 ±7.5	7.8 ±7.2	0.5 ±0.4
BCG, living	1	15	70.5	14.5			
	2	24	56	20			
	3	12.5	73.5	14			
	4	17.5	68	14.5			
	5	16	74.5	9.5	17.0 ±4.3	68.5 ±7.4	14.5 ±3.7
BCG, heat killed	1	21	68	11			
	2	14	81	5			
	3	16	79	5			
	4	19	71	10			
	5	19.5	63.5	17	17.9 ±2.8	72.5 ±7.4	9.6 ±5.0

[a] The materials were injected in volumes of 0.2 ml into each mouse 3 days before the assay was conducted. Other details as in legend to Table 3.
[b] Arbitrary grading as in Table 6.

TABLE 8. *Phagocytosis of Listeria monocytogenes by peritoneal macrophages from mice pretreated with CF*

Expt no.	Treatment	Avg of percent of macrophages with phagocytized bacteria ± standard deviation				
		0[a]	1-5	6-10	11-20	>20
1	Control	4.4 ± 1.9	38.9 ± 10.1	41.6 ± 4.7	14.0 ± 5.8	1.1 ± 1.2
	Emulsion	4.0 ± 1.7	38.4 ± 12.7	38.7 ± 9.0	17.7 ± 6.3	1.2 ± 1.6
	CF (Peurois)	2.0 ± 1.4	9.4 ± 3.7	25.9 ± 4.7	43.3 ± 5.9[b]	19.3 ± 7.9[b]
2	Control	32.2 ± 12.8	51.6 ± 9.3	14.4 ± 5.3	1.8 ± 1.8	0
	Emulsion	42.8 ± 16.9	46.0 ± 9.6	8.8 ± 7.1	2.2 ± 2.2	0.2 ± 0.5
	CF (Peurois)	14.4 ± 7.0	42.6 ± 6.4	23.8 ± 7.1[b]	15.0 ± 2.9[b]	4.2 ± 4.6
3	Control	67.4 ± 16.1	27.8 ± 13.5	4.2 ± 3.1	0.6 ± 0.6	0
	Emulsion	59.2 ± 12.3	34.6 ± 8.0	4.4 ± 2.9	1.8 ± 2.1	0
	CF (Peurois)	10.6 ± 5.1	33.8 ± 9.5	29.8 ± 3.1[b]	22.6 ± 9.3[b]	3.2 ± 2.4

[a] Number of phagocytized *Listeria* bacilli.
[b] Percentages significantly different from those of the corresponding emulsion groups ($P = 0.005$).

TABLE 9. *Phagocytosis of Listeria monocytogenes by monolayers of peritoneal macrophages from normal mice after incubation in vitro with CF*

Treatment[a]	Avg of percent of macrophages with phagocytized bacteria ± standard deviation				
	0[b]	1-5	6-10	11-20	>20
Control	2.8 ± 1.6	28.3 ± 10.9	36.8 ± 4.1	30.0 ± 10.7	2.0 ± 2.9
Emulsion	3.0 ± 1.6	32.2 ± 12.0	39.0 ± 4.0	24.8 ± 13.9	1.0 ± 1.0
CF	1.6 ± 1.5	22.8 ± 12.1	35.0 ± 4.7	35.6 ± 13.7	5.0 ± 2.7

[a] Final concentrations of Bayol F and Tween 80 in the culture medium were 0.025 and 0.0025%, respectively; that of CF (Peurois) was 12.5 μg/ml. Cultures of PC without addition of emulsion or CF served as control.

[b] Number of phagocytized *Listeria* bacilli.

lation of mice pretreated with CF was active in contrast to 9 and 2% in the emulsion and normal control groups, respectively. Another fact evolving from this study was the local effect of CF. The latter injected i.v. has no effect on the peritoneal macrophages. To exert stimulation of peritoneal macrophages, CF must be administered i.p. This effect is consistent with the local effect of CF and BCG, which had been shown previously in tumor growth inhibition (31) and in nonspecific resistance against *Salmonella* infection (29). The results obtained with CF were comparable to those obtained with living or heat-killed BCG bacilli in all respects. The fact that CF and BCG were able to stimulate macrophages in vivo only, seems to indicate that lymphocytes may be involved in the process of activation. CF is granulomagenic, apparently by virtue of its strong chemotactic activity for macrophages (22). Since CF activates the latter, one can understand why mice with lung granulomas induced by CF were protected against i.v. challenge with the virulent H37Rv strain of *M. tuberculosis*, a protection similar to that observed by others after i.v. injection of intact mycobacterial bacilli (4, 32, 33). Mice pretreated i.p. with CF were protected against an i.p. challenge with *Salmonella typhi* or *S. typhimurium* (29), apparently for the same reason. CF was effective in prevention (6, 30, 31) and in immunotherapy of tumors in mice (E. Yarkoni, M. S. Meltzer, and H. J. Rapp, manuscript in preparation), guinea pigs (3, 7), and patients (H. A. Cohen and A. Bekierkunst, manuscript in preparation), and in combination with nonliving BCG it was used for preparing a tumor vaccine (3). These antitumor activities of CF are also connected with its granulomagenic and macrophage-activating effects at the site of its lodgment in host tissues. Some analogues of CF display similar effects (20, 29, 30). Since macrophages seem to be important in situ effector cells, whose presence may limit the growth of tumor cells (11, 12, 14, 23), CF and its analogues may be useful in immunotherapy of tumors in animals and humans.

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