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Phagocytic and Chemiluminescent Responses of Mouse Peritoneal Macrophages to Living and Killed *Salmonella typhimurium* and Other Bacteria

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In the presence of luminol, resident as well as thioglycolate-induced and immunized macrophages emitted chemiluminescence more efficiently when the cells were exposed to living *Salmonella typhimurium* than when they were exposed to the same bacterium killed by ultraviolet light or heat. This phenomenon was observed whether or not the bacterium was opsonized. The different response to living and killed bacteria was also found with *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus morganii*, and *Enterobacter aerogenes*, but not with *Shigella sonnei*, *Klebsiella pneumoniae*, and *Propionibacterium acnes*. The results suggest that macrophages respond better to living, motile bacteria than to nonmotile or killed bacteria. The experimental results obtained with motility mutants of *S. typhimurium*, *E. coli*, and *P. aeruginosa* confirm that macrophages exposed to the motile bacteria emit chemiluminescence more efficiently and ingest the motile bacteria at a much faster rate than the nonmotile bacteria.

The nature of potentially ingested particles is known to influence the rate of phagocytosis, as well as metabolic changes accompanied by phagocytosis. DeChatelet et al. (1) first reported that heat-killed bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* were phagocytized at a much slower rate by human polymorphonuclear leukocytes than were living bacteria. They also showed that hexose monophosphate shunt activity was enhanced efficiently upon phagocytosis of living bacteria but less efficiently upon phagocytosis of heat-killed bacteria. Recently, Blumenstock (E. Blumenstock, Ph.D. thesis, Freiburg University, 1979) showed that mouse resident peritoneal macrophages emitted more chemiluminescence when the cells were incubated with living *Salmonella typhimurium* than when incubated with the same bacterium killed by ultraviolet light (UV) or heat. The killed bacteria adsorbed to the cells more slowly.

Chemiluminescence is considered to be indicative of the generation of reactive species of oxygen, such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen (4, 11). The reactive oxygen produced by phagocytes seems to be directly related to the killing effect (6, 11). Such reactive oxygen may be released to the outside of the cells, as well as to the inside of phagosome (2). Since generation of chemiluminescence is one of the earliest responses of phagocytes upon exposure to stimuli, chemiluminescence measurement is considered to be a useful aid for investigation of earlier events in phagocytic recognition.

In this paper, we show that the higher response of chemiluminescence and phagocytosis of macrophages observed when the cells contact with certain living bacteria is caused mainly by motility of such bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used were listed in Table 1. Motility mutants and chemotaxis mutants of *S. typhimurium*, *E. coli*, and *P. aeruginosa* were obtained from T. Iino of this university. An Mot^+ derivative of *E. coli* IID953 was obtained after phage P1 transduction with strain W3110 as donor. Mot^+ revertants and Mot^+ transductants were isolated on L-broth agar plates containing 0.35% agar.

All bacterial strains, except *Propionibacterium acnes*, were grown aerobically at 37°C in L-broth without glucose (9). *P. acnes* was cultivated anaerobically at 37°C under CO_2 atmosphere in brain heart infusion broth (Difco Laboratories) supplemented with 0.075% cysteine and 0.4% sodium carbonate.

Preparation of killed bacteria. Bacteria were killed by dipping the vessel in boiling water for 3 min (heat-killed bacteria), exposure to UV for 30 min (UV-killed bacteria), or treating with 0.5% Formalin for 24 h (Formalin-killed bacteria). No viable bacteria grew on L-broth plates after the treatments.

Opsonization of bacteria. For opsonization, homologous normal serum or antiserum obtained from C3H/He mice was used. Two hundred microliters of

TABLE 1. *Bacterial strains*

Strains	Character	Source
S. typhimurium		
LT2	Wild type	T. Iino
SJ442	*motA* mutant of LT2	T. Iino
SJ603	*motB* mutant of LT2	T. Iino
TT2	Mot^+ revertant of SJ442	This study
TT1	Mot^+ revertant of SJ603	This study
ST23	*his, thy* derivative of LT2	T. Iino
ST104	*cheA* mutant of ST23	T. Iino
ST109	*cheB* mutant of ST23	T. Iino
E. coli		
IID952	O 44, K 74	This institute
IID953	O 86a, K 61	This institute
TT3	Mot^+ transductant of IID953	This study
IID861	Mot^- strain	This institute
TT4	Mot^+ revertant of IID861	This study
W3110	F^- derivative of K-12	T. Iino
TH282	*mot* mutant of W3110	T. Iino
TT5	Mot^+ revertant of TH282	This study
P. aeruginosa		
IFO3455		This institute
PAO2003	*argH32, str39, rec2,* FP^-	T. Iino
MT519	*mot* mutant of PAO2003	T. Iino
P. morganii IID602		This institute
E. aerogenes ATCC 13048		This institute
S. sonnei IID969		This institute
K. pneumoniae IID875		This institute
P. acnes Habazaki		This institute

bacterial suspension in Dulbecco phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$ were mixed either with 50 μl of normal serum or antiserum and incubated at 37°C for 20 min. The opsonized bacteria were kept in ice until use. Under the conditions, no agglutination of bacteria was observed.

Preparation of macrophages. Eight to ten week-old, female C3H/He mice (this institute) were used throughout the experiments. Peritoneal cells were obtained by washing the peritoneal cavity with Eagle minimal essential medium (Nissui Seiyaku) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.4. Five or two milliliters of cell suspension containing 3×10^6 cells was incubated at 37°C in a sterilized plastic liquid scintillation vial or in a plastic dish (35-mm-diameter, Corning Glass Works), respectively. After 2 h, nonadherent cells were removed by changing medium, and adherent cells predominantly consisting of macrophages were kept at 37°C before use.

Thioglycolate-induced or immunized macrophages were obtained as follows: 2 ml of thioglycolate broth or 2×10^4 to 3×10^4 viable *S. typhimurium* LT2 was injected intraperitoneally, and peritoneal exudated cells were collected after 4 or 8 days, respectively.

Measurement of chemiluminescence. Chemiluminescence was measured at 37°C in a temperature-stabilized liquid scintillation spectrometer (Beckman LS 233) in the out-of-coincidence mode. The experiments were started by the addition of luminol. Readings on individual vials were recorded at 1-min intervals. When background counts became constant, bacteria were added, and counting was continued.

Luminol, purchased from Tokyo Kasei, was suspended in water (2 mg/ml), and, after addition of triethylamine (1 μl/ml), the suspension was sonicated and then filtered through a membrane filter (Millipore Corp.; pore size, 0.45 μm).

Phagocytosis test for bacteria. Monolayers of cells predominantly consisting of macrophages (in a plastic dish as described above) received the indicated number of bacteria opsonized with normal serum. After incubation at 37°C, ingestion was terminated by the addition of 2 ml of ice cold phosphate-buffered saline containing 1 μg of cytochalasin B per ml, and the monolayers were washed twice with 10 ml of ice cold phosphate-buffered saline to remove free and loosely bound bacteria. The number of ingested bacteria was determined either by viable counting or by morphology.

(i) Viable counting of ingested bacteria. The cells ingesting bacteria were lysed by scraping with silicon rubber. Samples of the lysate were plated on L-broth agar plates for counting of bacterial viability. No significant intracellular killing took place within 10 min, since counting of bacteria in a Petroff-Hauser counting chamber (Erma Optical Co. Ltd., Tokyo) gave similar results.

(ii) Morphological assessment of phagocytosis. The cells were fixed with methanol and stained with Giemsa stain. The number of phagocytized bacteria was determined under a microscope by counting the number of bacteria per cell for at least 100 macrophages.

RESULTS

Chemiluminescent response of macrophages to living and UV-killed *S. typhimurium*. Efficient chemiluminescence emission was observed in the presence of luminol from mouse peritoneal macrophages upon exposure to living *S. typhimurium* LT2 opsonized with normal serum. In contrast, when the same bacteria killed by UV, Formalin, or heat were given to macrophages, much lower chemiluminescence was observed (Fig. 1A). The different response to living and killed bacteria was also detected with thioglycolate-induced or immunized macrophages, although the level of chemiluminescence generation from immunized or thioglycolate-induced macrophages was higher or lower than from resident macrophages, respectively (Fig. 1B and C).

The difference is not due to an altered ability of the killed bacterium to interact with normal

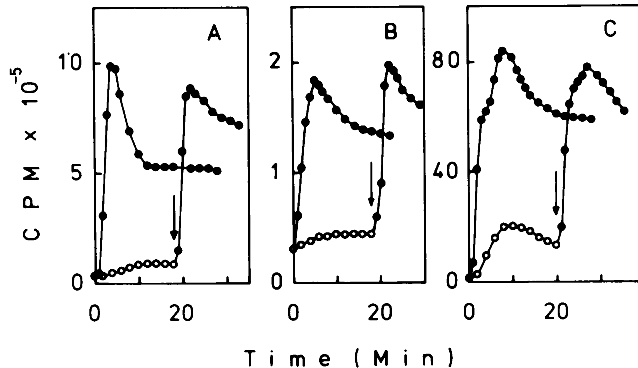

FIG. 1. *Chemiluminescent response of macrophages to living and killed S. typhimurium. Fifty microliters of luminol solution was added to each vial approximately 10 min before addition of bacteria. At time zero, each vial which contained resident (A), thioglycolate-induced (B), or immunized (C) macrophages (approximately 2×10^6 cells per 5 ml) received 1.7×10^7 of S. typhimurium, either living (●) or UV-killed (○). Photon counts were recorded at 1-min intervals. At the time indicated by arrows, vials which had received killed bacteria again received the same number of living bacteria, to show that macrophages were still intact.*

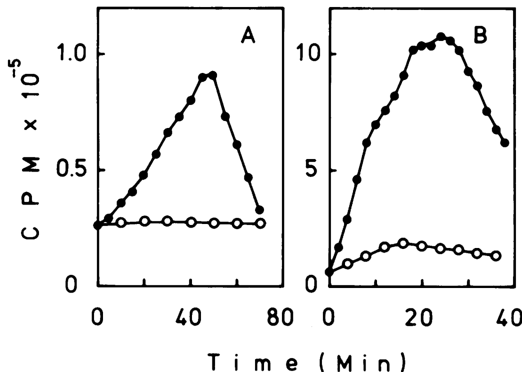

FIG. 2. *Emission of chemiluminescence by macrophages responding to nonopsonized, living, and killed S. typhimurium. Living (●) or UV-killed (○), nonopsonized S. typhimurium LT2 (2.0×10^7) was added to the vials containing resident (A) or immunized (B) macrophages at time zero. Other conditions were the same as described in the legend for Fig. 1.*

serum, since a similar difference was also observed with nonopsonized *S. typhimurium* (Fig. 2), as well as the bacterium opsonized with antiserum (data not shown).

Comparison of response of macrophages to living and killed bacteria of various species. Table 2 is a summary of the results of chemiluminescent response of resident macrophages to various species of bacteria opsonized with normal serum. A different response to living and killed bacteria was observed when *S. typhimurium, E. coli, P. aeruginosa, Proteus morganii,* and *Enterobacter aerogenes* were employed but not when *Shigella sonnei, Klebsiella pneumoniae,* and *Propionibacterium acnes* were used. Only a low level of chemiluminescence emission was observed upon exposure of macrophages to the latter group of bacteria, except *P. acnes.* These results suggest that living, motile bacteria induce more chemiluminescence generation from macrophages than do nonmotile or killed bacteria.

Response of macrophages to motility mutants and chemotaxis mutants of *S. typhimurium.* The preceding results enable us to examine whether motility of bacteria really influences chemiluminescent response of macrophages. Generation of chemiluminescence with *motA* (Fig. 3) and *motB* mutants (data not shown) of *S. typhimurium,* both of which possess flagella but lack motility, was much lower than with parent or Mot^+ revertant strains.

The susceptibility of parent, *motA* mutant, and Mot^+ revertant bacteria to phagocytosis by macrophages was assessed by viable counting of ingested bacteria (Table 3) and by morphological technique (Fig. 5 and 6). The results indicate that the *mot* mutant was phagocytized much more slowly than the parent and the revertant strains. Ingestion of killed bacteria was also found to be at a low level, similar to that of *mot* mutant strains (data not shown).

Bacterial chemotaxis seems not to be concerned with this difference, since similar level of chemiluminescence production was observed with the mutants which lost chemotactic ability (*cheA* and *cheB*; Fig. 4).

Response of macrophages to motile and nonmotile bacteria of *E. coli* and *P. aeruginosa.* The influence of bacterial motility upon the response of macrophages was further investigated using *mot* mutants of *E. coli* and *P. aeruginosa.* Chemiluminescent (Table 4) and phagocytic (data not shown) responses of mac-

TABLE 2. *Chemiluminescent response of macrophages to living and killed bacteria of various species*[a]

Bacteria	Motility	Living or killed by	Production of chemiluminescence: Total counts in 30 min ($\times 10^{-5}$)	Maximum response: Peak ht (cpm $\times 10^{-5}$)	Maximum response: Time (min)
S. typhimurium (LT2)	+	Living	163	9.5	4
		UV	15	0.9	12
E. coli (IID952)	+	Living	146	9.3	6
		Heat	9	0.7	5
E. coli (IID953)	−	Living	40	3.0	18
		Heat	12	1.1	23
P. aeruginosa	+	Living	186	11.3	5
		UV	11	0.9	20
P. morganii	+	Living	58	7.4	5
		Heat	18	1.3	10
E. aerogenes	+	Living	139	6.4	22
		Heat	16	1.1	12
S. sonnei	−	Living	1	0.3	9
		UV	1	0.3	10
K. pneumoniae	−	Living	4	0.4	18
		UV	6	0.5	10
P. acnes	−	Living	58	3.2	54
		Heat	61	3.3	56

[a] Bacteria opsonized with normal serum (2.0×10^7) and resident macrophages were used.

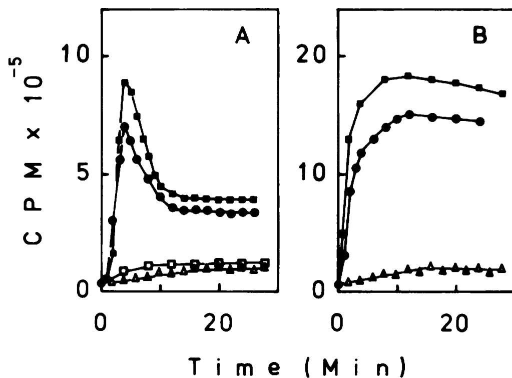

FIG. 3. *Chemiluminescent response of macrophages to motility mutants of S. typhimurium. Experimental results of the following combinations were shown: (A) resident macrophages and opsonized bacteria and (B) immunized macrophages and nonopsonized bacteria. Experimental conditions were the same as described in the legend for Fig. 1 except that living bacteria used were LT2 (●), SJ442 motA (▲), and TT2 Mot⁺ revertant (■). As comparison, the results obtained with heat-killed SJ442 (△) and TT2 (□) were also shown.*

TABLE 3. *Phagocytic test of macrophages for motility mutants of S. typhimurium*

S. typhimurium strain	Motility	No. of bacteria: Added ($\times 10^7$)	No. of bacteria: Ingested[a] ($\times 10^5$)	% Ingestion
LT2 (wild type)	+	4.3	5.5	1.3
SJ442 (*motA* mutant)	−	5.2	0.1	0.02
TT2 (Mot^+ revertant)	+	4.9	8.8	1.8

[a] After a 10-min incubation.

rophages were much higher when the cells were exposed to living, motile bacteria than when they were exposed to nonmotile or killed bacteria.

In Table 2, a somewhat different response was observed between living and killed *E. coli* IID953, although this bacterium was not motile. The difference was only observed when the bacterium was opsonized, and the response induced was not different whether the bacterium was killed or not, if the bacterium was not opsonized (Fig. 7B). The results may indicate that alteration of bacterial surface, which influenced opsonization, had taken place during heat treatment, and consequently, living bacteria adsorbed to macrophages more easily than killed bacteria in this case. Furthermore, isolated Mot^+ transductant of this strain induced chemiluminescence much more than did Mot^-, live parent (*E. coli* IID953; Fig. 7A), indicating that bacterial motility is the more important determinant of phagocytic and chemiluminescent responses.

DISCUSSION

In this paper we showed that phagocytic as well as chemiluminescent responses of macrophages were much greater when the cells were exposed to living, motile bacteria than when

exposed to the killed bacteria. The response to living, nonmotile bacteria was low and was the same as the response to killed bacteria. The simplest explanation of these phenomena may be that they are caused by the higher collision frequency of motile bacteria at the surface of macrophages. Moving bacteria have a better chance to hit the cell surface of macrophages. However, a higher collision rate itself seems not to be enough to explain all of the events, since a similar level of chemiluminescent response evoked by living, motile *S. typhimurium* LT2 was never induced, even by 200 times the number of killed *S. typhimurium* LT2 (ratio of the bacteria to single cell was 2,000; unpublished data). Since the surfaces of macrophages and bacteria are charged negatively, Brownian movement may not be enough for bacteria to reach to the cell surface of macrophages and to induce chemiluminescent response. It is possible that bacterial motility plays some role in overcoming the barrier of electric repulsion.

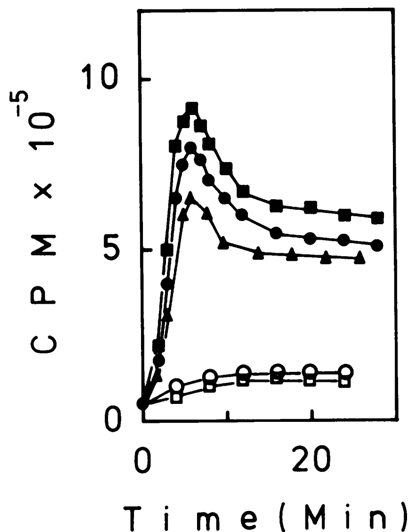

FIG. 4. *Chemiluminescent response of macrophages to chemotactic mutants of S. typhimurium. The bacteria used were living or heat-killed S. typhimurium ST23 (●; ○), ST104 cheA (■; □), and ST109 cheB (▲). Solid and open symbols represent living and heat-killed bacteria, respectively. Experimental conditions were the same as described in the legend for Fig. 1.*

Griffin et al. (3) showed that simple attachment of a particle to the macrophage surface was not sufficient to trigger ingestion of the particle. They suggested that ingestion requires the sequential, circumferential interaction of particle-bound ligands with membrane receptors different from the initial attachment site. The findings that small particles neither evoke chemiluminescence (unpublished data) nor induce oxygen uptake (8) may also indicate the requirement of more than two sites for phagocytic and chemiluminescent responses. Therefore, if that is the case, bacteria attached to one of the surface receptors must reach other receptor sites to be recognized by macrophages. Living, motile bacteria which initially contact with one site of the surface of macrophages may have a better chance to attach to the other receptor sites, before dissociating and becoming free from the cell surface.

Our preliminary experiment showed that the lethal dose to mice of motility mutants of *S. typhimurium* (*motA* and *motB*) was approximately 10-fold lower than the dose of motile *S. typhimurium* when injected intraperitoneally. This indicates that even in vivo, motile bacteria

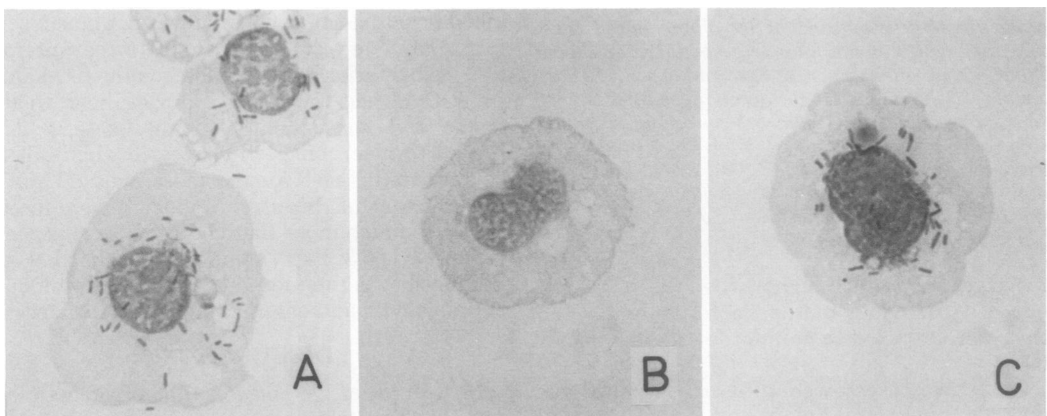

FIG. 5. *Ingestion of S. typhimurium LT2 (A), SJ422 motA (B), or TT2 Mot⁺ revertant (C) by monolayered macrophages. Experimental conditions are described in the text.*

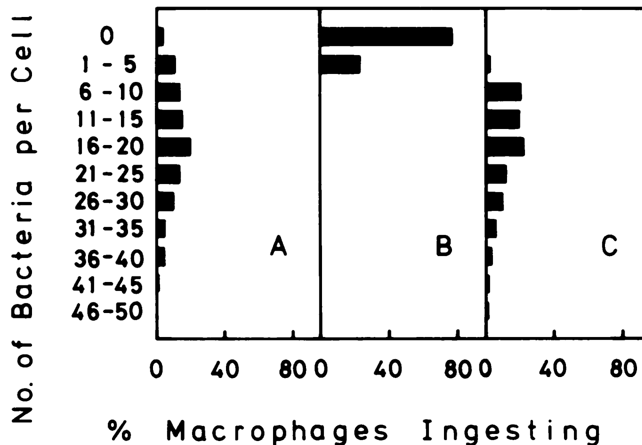

FIG. 6. *Numbers of bacteria ingested by macrophages. After 30 min of incubation with various strains of bacteria (7 × 10^7 to 8 × 10^7), the monolayer of macrophages was washed and stained as described in the text. The percent distribution of cells which ingested the indicated numbers of S. typhimurium LT2 (A), SJ442 motA (B), or TT2 Mot^+ revertant (C) is shown. The mean numbers of ingested bacteria per cell were 17.3, 0.4, and 18.9 for S. typhimurium LT2, SJ442, and TT2, respectively.*

TABLE 4. *Chemiluminescent response of macrophages to motile and nonmotile bacteria*

Bacterial strain	Motility	Living or killed by	Production of chemiluminescence		
			Total counts in 20 min ($\times 10^{-5}$)	Maximum response	
				Peak ht (cpm $\times 10^{-5}$)	Time (min)
E. coli					
W3110 (parent)[a]	+	Living	62.4	5.3	9
		Formalin	7.5	0.6	18
TH282 (*mot* mutant)[a]	−	Living	2.3	0.6	13
		Formalin	2.1	0.6	15
TT5 (Mot^+ revertant)[a]	+	Living	76.9	7.5	8
IID861 (Mot^- strain)[b]	−	Living	4.6	0.9	15
		Heat	4.7	0.9	14
TT4 (Mot^+ revertant)[b]	+	Living	77.0	5.4	5
		Heat	9.2	1.3	15
P. aeruginosa					
PAO2003 (parent)[b]	+	Living	201.0	14.2	12
MT519 (*mot* mutant)[b]	−	Living	23.1	1.8	10

[a] Nonopsonized bacteria (8.6×10^7) and resident macrophages were used.
[b] Bacteria opsonized with normal serum (2.3×10^7) and resident macrophages were used.

are ingested or killed more easily than the same bacteria which have lost their motility.

Motility of bacteria may also explain the observation reported by DeChatelet et al. (1) that heat-killed *P. aeruginosa* and *E. coli* were phagocytized by human polymorphonuclear leukocytes and enhanced hexose monophosphate shunt activity of the cells less efficiently than the living bacteria and the finding described by Kihlström and Edebo (7) that heat- or UV-killed, rough *S. typhimurium* interact with HeLa cells less efficiently than the living bacterium.

Although bacterial motility is, thus, the most important factor that influences response of macrophages, a slight difference was observed between living and killed, nonmotile bacteria in the case of *E. coli* IID953. Living *E. coli* IID953, which does not have motility, induced more chemiluminescence than the heat-killed bacteria. In this case, difference was only observed when the bacterium was opsonized. The difference was thus explained by surface alteration during heating. It is known that the capsule of *E. coli* is removed by heat treatment (10) and that the removal of the capsule reduces complement fixation (5). Therefore, in certain cases

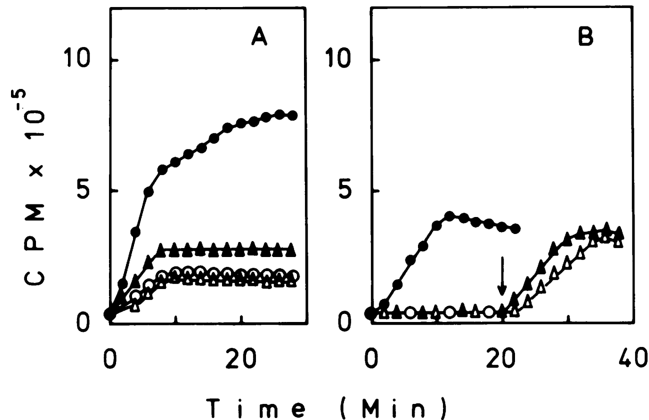

FIG. 7. *Chemiluminescent response of macrophages to nonmotile E. coli IID953 and its Mot⁺ transductant. Living or Formalin-killed E. coli IID953 (▲; △) and Mot⁺ transductant TT3 (●; ○) opsonized with normal serum (A) or without opsonization (B) were used. Solid and open symbols represent living and Formalin-killed bacteria, respectively. Numbers of bacteria added were 1.8 × 10^7 (A) and 7.2 × 10^7 (B). At the time indicated by arrow, live Mot⁺ transductants (7.2 × 10^7) were added. Other conditions were the same as described in the legend for Fig. 1.*

alterations of bacterial surface by heating might also influence the response of macrophages.

Finally, although molecular species of oxygen intermediates generated in the chemiluminescence reaction are not precisely known, emission of chemiluminescence was reduced (to around 20%) by the addition of various scavengers, such as superoxide dismutase (200 μg/ml), histidine (4 mM), or benzoate (4 mM), but not by the addition of catalase (200 μg/ml) (unpublished data), suggesting that superoxide, singlet oxygen, and hydroxyl radical, but not hydrogen peroxide, may be involved in the chemiluminescence reaction.

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