Oxidative and Phagocytic Functions of Macrophages during Infections Induced in Mice by *Mycobacterium intracellulare* and *Listeria monocytogenes*

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The oxidative metabolism (chemiluminescence and H₂O₂ release) and phagocytic activity of mouse peritoneal macrophages during chronic infections induced by *Mycobacterium intracellulare* and more acute infections due to *Listeria monocytogenes* were studied. In *M. intracellulare* infections, macrophage chemiluminescence in response to phorbol myristate acetate (PMA) was greatest at around 2 weeks, with a 1 week lag phase after infection, while the PMA-triggered H₂O₂ release was markedly enhanced even 1 d after challenge, and remained high thereafter for up to 10 weeks. The pattern of changes in the phagocytic activity of host macrophages in response to latex beads during this infection resembled the pattern seen with macrophage H₂O₂ release. In the *L. monocytogenes* infections, the PMA-triggered chemiluminescence of the host macrophages increased 4 d (in a sublethal infection) and 2 d (in a lethal infection) after bacterial challenge, whereas the PMA-triggered H₂O₂ release was markedly enhanced as early as 1 d after infection and the elevated level persisted until either the bacteria were eliminated or the animals died. The patterns of changes in phagocytic activity of the host macrophages during *L. monocytogenes* infection at sublethal and lethal doses differed. In the former, phagocytosis was most active in the early phase of infection, with a peak around day 2, followed by a rapid decrease; in the latter, the phagocytic ability increased more slowly, and remained elevated until the animals died. The results suggest that the macrophages induced by *M. intracellulare* are in a more activated state than are those induced by *L. monocytogenes*.

INTRODUCTION

Certain macrophage cell functions are modulated during bacterial, fungal and protozoal infections. Stimulation and/or activation of the host macrophages, based on their phagocytic or antimicrobial activities, occur in cases of infection due to *Brucella abortus* (Cheers & Pagram, 1979), *Mycobacterium tuberculosis* (North, 1974; King *et al.*, 1975), *Blastomyces dermatitidis* (Brummer *et al.*, 1980) and *Trypanosoma cruzi* (Nogueira *et al.*, 1977). In contrast, suppression of some macrophage functions, such as pinocytic activity, chemotactic response and bactericidal activity, are seen in BCG infections (Wing, 1981) and *Toxoplasma* infections (Jones & Len, 1976) in mice, and in active pulmonary tuberculosis in humans (Campbell, 1979). There are few reports dealing with detailed changes in macrophage function during infection, i.e. from the onset of infection until recovery or death of the animal host. Here, we report changes in macrophage oxygen metabolism (chemiluminescence and H₂O₂ release) and phagocytic ability during infections induced in mice by the intracellular parasites *Listeria monocytogenes* (acute infection) and *Mycobacterium intracellulare* (chronic infection).

Abbreviations: PMA, phorbol myristate acetate; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; MEM, Eagle's minimal essential medium; PC, peritoneal cells; PMNL, polymorphonuclear leucocytes; FBS, foetal bovine serum; p.i., post-infection.

METHODS

Animals. Female ddY strain mice (6 to 8 weeks) were purchased from the Shizuoka Union for Experimental Animals, Shizuoka, Japan.

Experimental infections. *Listeria monocytogenes* EGD (obtained from K. Takeya, Kyushu University, Fukuoka, Japan) and *Mycobacterium intracellulare* 31F093 TD (obtained from F. Kuse, Kyoto University, Kyoto, Japan) were cultured in tryptose broth (Eiken Chemical Co.) at 37 °C for 18 h and in Dubos Tween-albumin medium (Eiken) at 37 °C for 7 d, respectively. Bacteria were harvested by centrifugation at 2000 g for 15 min, washed twice with phosphate-buffered (pH 7.0) saline (PBS) and finally suspended in PBS. The bacterial suspension was injected intraperitoneally into mice (see figure legends for details). At various intervals, the number of organisms in the liver, spleen and lungs of the infected animals was determined by plating tenfold serial dilutions of the tissue homogenates on tryptose agar and Middlebrook 7H10 agar (Difco) for *L. monocytogenes* and *M. intracellulare*, respectively.

Assay for chemiluminescence of host peritoneal cells. The peritoneal cells (PC) of the host mice, with or without infection, were harvested with Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co.) containing 100 units penicillin G ml⁻¹, 100 µg streptomycin ml⁻¹, and 5 or 10 units heparin ml⁻¹. The PC were treated with distilled water for 15 s to lyse any contaminating erythrocytes, washed twice with PBS, and suspended in Hanks' balanced salt solution (HBSS). For measurement of chemiluminescence, the PC (10⁶ cells in 0.9 ml) were added to a glass vial (12 mm diameter, 15 mm length) with 0.1 ml 10⁻³ M-luminol and the basal light emission was recorded using a Lumicounter ATP-237 (Toyo Kagaku Ind.) over 10 s periods at 37 °C. To this preparation was added either between 1 and 100 ng phorbol myristate acetate (PMA) (Sigma) in 10 µl dimethylsulphoxide, or 100 µg latex beads (0.8 µm; Sigma) in 10 µl HBSS, and light emission was recorded for 10 s periods at intervals for up to 10 min after addition.

Assay for release of H₂O₂ from macrophages. PC were prepared from normal and infected mice, as described above, except that any contaminating erythrocytes were lysed by treatment with 0.83% (v/v) NH₄Cl in 0.17 M-Tris/HCl buffer (pH 7.6) and the subsequent washing of the PC was done with PBS supplemented with 1% foetal bovine serum (FBS) (M. A. Bioproducts). The resultant PC were suspended in MEM supplemented with 10% (v/v) FBS, 100 units penicillin G ml⁻¹, and 100 µg streptomycin ml⁻¹. To prepare macrophage monolayers, 4 ml of the cell suspension (2 × 10⁶ cells) was allowed to settle onto the surface of a 50 mm plastic culture dish (Nunc). After incubation at 37 °C for 2 h in a CO₂ incubator (5% CO₂/95% humidified air), the dish was thoroughly rinsed with PBS. Macrophage H₂O₂ release was measured using the resultant macrophage monolayer culture (6.8 × 10⁵ to 1.3 × 10⁶ cells per dish), as described by Tomioka & Saito (1980). Briefly, the macrophage monolayer was overlaid with 2.4 ml HBSS containing 0.1% (w/v) glucose, horseradish peroxidase (50 µg ml⁻¹; type II; Sigma), and 5 nmol scopoletin (reduced type; Sigma), and the dish incubated in the presence (1 to 100 ng ml⁻¹) or absence of PMA at 37 °C for 20 min in a CO₂ incubator. The medium was removed and measured for its fluorescence at 460 nm by setting the excitation light at 350 nm in an Aminco Bowman TM spectrophotofluorometer. Macrophage H₂O₂ release was expressed as pmol min⁻¹ per 10⁶ adhered cells.

Macrophage phagocytosis. Phagocytosis of latex particles by macrophages was measured according to Tomioka & Saito (1980), with minor modifications. Briefly, the macrophage monolayer, prepared as described above, was overlaid with 2 ml 10% FBS-MEM containing 100 µg latex beads (0.8 µm) ml⁻¹, with or without addition of 10 ng PMA in 10 µl dimethylsulphoxide. After 20 min incubation in a CO₂ incubator at 37 °C, the macrophage monolayer was rinsed three times with PBS, fixed with methanol and stained with Giemsa's solution, and the number of latex beads ingested per macrophage was counted microscopically.

RESULTS

Changes in oxidative metabolism of macrophages during M. intracellulare infection

Fig. 1 shows changes in the chemiluminescence of the host PC in response to PMA-triggering or to latex phagocytosis. The infection was chronic and there was a very slow decrease in the number of viable organisms in the spleen during the 6 weeks post-infection (p.i.) (Fig. 1*d*). In this infection, the chemiluminescence of the PC triggered by PMA showed a transient increase at 1 d p.i. (Fig. 1*a*). This may have been attributable to the accumulation of polymorphonuclear leucocytes (PMNL) in the peritoneal cavity due to acute inflammation elicited by the bacterial inoculum (Fig. 1*b, c*). PMNL have a high ability to generate active oxygen, of which [•]OH and/or ¹O₂ are thought to cause light emission (Johnston, 1978), as in the case of activated macrophages (Nathan & Root, 1977). A marked increase in the PMA-elicited chemiluminescence of the PC was noted around 2 weeks p.i. This was probably due to activation of the host peritoneal macrophages, because the proportion of PMNL in the PC at this stage of the infection was not high enough to produce such an increase (the PC at 1 d p.i. consisted of 5% PMNL and showed

Fig. 1. Changes in PMA-triggered and phagocytosis-associated chemiluminescence of host PC after intraperitoneal infection with *M. intracellulare* (1×10^6 c.f.u. per mouse). PC were harvested from five mice and pooled. A separate cell culture was prepared for chemiluminescence. (a) Chemiluminescence triggered by 1 ($\circ$), 10 ($\triangle$), and 100 ($\square$) ng PMA ml^{-1} or elicited by phagocytosis of latex beads ($100 \mu\text{g ml}^{-1}$) ($\bullet$). The results are means $\pm$ range; $n = 2$. (b) Number of PC (mean; $n = 5$). (c) Cell population in PC: $\circ$, macrophages; $\triangle$, PMNL; $\bullet$, lymphocytes. (d) Number of c.f.u. of organisms in the spleen (means $\pm$ range, $n = 5$).

Fig. 2. Time-course of light emission from host PC after infection with *M. intracellulare*. (a) Chemiluminescence triggered with PMA at 100 ng ml^{-1} ; (b) chemiluminescence associated with phagocytosis of latex beads. —, Chemiluminescence at 1 d; ---, 3 d; ----, 7 d; ·····, 2 weeks; —·—, 4 weeks; - - - - - , 6 weeks p.i. Other details are as described in Fig. 1.

Fig. 3. Changes in PMA-triggered H_2O_2 release from host macrophages after intraperitoneal infection with *M. intracellulare* (1×10^6 c.f.u. per mouse) (a) H_2O_2 release without PMA (○) and in response to 1 (△), 10 (◐), 100 (●) ng PMA ml⁻¹. (b) Cell population in the macrophage monolayer culture tested: ○, macrophages; △, PMNL; ●, lymphocytes. (c) Number of c.f.u. of organisms in the spleen (○) and lung (△). The results are means $\pm$ range (in a and c); $n = 2$ in (a) and 5 in (c); a horizontal bar indicates that the results were identical.

much less chemiluminescence than did the PC at 2 to 6 weeks p.i. with a similar proportion of PMNL). The PMA-triggered chemiluminescence of the PC during 2 to 6 weeks p.i. was dose-dependent. It is noteworthy that the chemiluminescence of the PC in response to phagocytosis of latex beads (Fig. 1a) gradually increased from 2 weeks p.i. until a peak was reached at 4 to 6 weeks p.i.

Fig. 2 shows the time course of PMA-triggered and phagocytosis-associated chemiluminescence of the PC used in the experiment shown in Fig. 1. The peak value of PMA-triggered chemiluminescence of a given PC preparation correlated well with the cumulative intensity of light emission. However, such a clear relationship was not noted in the case of phagocytosis-associated chemiluminescence.

Fig. 3 shows the changes in the spontaneous and PMA-triggered H_2O_2 -releasing ability of macrophages during *M. intracellulare* infection (a separate experiment from that presented in Figs 1 and 2). In this case, the H_2O_2 -releasing ability of the macrophages in response to triggering by PMA at 10 and 100 ng ml⁻¹ was elevated as early as 1 d p.i. This enhanced state continued for at least 6 weeks. Also, macrophage H_2O_2 release in response to 1 ng ml⁻¹ PMA and without PMA was significantly enhanced around 1 to 2 weeks p.i. The increase in the high dose PMA-triggered H_2O_2 release from 'macrophages' obtained at 1 d p.i. may have been partly attributable to a high rate of contamination of PMNL in the macrophage monolayer culture (Fig. 3b).

Changes in the oxidative metabolism of host macrophages during L. monocytogenes infection

Fig. 4 shows the changes in PMA-triggered and phagocytosis-associated chemiluminescence of PC from mice infected with sublethal and lethal doses of *L. monocytogenes*. In the sublethal infection a relatively small number of the organisms were retained in the host liver for 1 to 7 d

Fig. 4. Changes in PMA-triggered and phagocytosis-associated chemiluminescence of host PC after intraperitoneal infection with *L. monocytogenes* at doses of 1.5×10^6 (sublethal) (a, c, e, g) and 2.5×10^7 (lethal) (b, d, f, h) organisms per mouse. (a, b) Chemiluminescence; (c, d) number of PC; (e, f) cell population in PC: ○, macrophages; △, PMNL; ●, lymphocytes; (g, h) number of c.f.u. of organisms in the liver. In the lethal infection 70% of the mice survived at 5 d p.i. See Fig. 1 for other details.

p.i. and were thereafter eliminated (Fig. 4g), while in case of the lethal infection, the organisms in the liver continued to increase until the death of the animals 5 d p.i. (Fig. 4h). In the sublethal infection (Fig. 4a, c, e, g), a slight enhancement in PMA-triggered chemiluminescence, mainly due to macrophages (Fig. 4e), was observed around 4 d p.i. (Fig. 4a). It is noteworthy that there was no elevation of PMA-triggered chemiluminescence 1 d p.i., in spite of a significant increase in the ratio of PMNL in the PC preparation (Fig. 4a, e). In the lethal infection (Fig. 4b, d, f, h), PMA-triggered chemiluminescence was sharply elevated around 2 d p.i. and rapidly declined thereafter (Fig. 4b). Marked enhancement of phagocytosis-associated chemiluminescence was also noted around 2 d p.i. In this case, the PC of mice from 1 to 4 d p.i. contained a high proportion of PMNL (Fig. 4f), which probably contributed considerably to the elevated chemiluminescence seen around 2 d p.i.

Fig. 5 shows the changes in macrophage H_2O_2 -releasing ability during sublethal and lethal *L. monocytogenes* infection. The H_2O_2 release in response to $100 \text{ ng PMA ml}^{-1}$ markedly increased even 1 d p.i., regardless of the infecting dose, and remained high until elimination of the infection (Fig. 5a) or the death of the animals (Fig. 5b). Since the PC preparation at 1 d p.i. contained a high proportion of PMNL (Fig. 4) and the macrophage monolayer was thus highly contaminated with PMNL (Fig. 5c, d), the H_2O_2 release at 1 d p.i. may have been partly generated by PMNL.

Fig. 5. Changes in PMA-triggered H_2O_2 release from host macrophages after intraperitoneal infection with *L. monocytogenes* at doses of 1.5×10^6 (sublethal) (a, c, e) and 2.5×10^7 (lethal) (b, d, f) organisms per mouse. (a, b) H_2O_2 release; (c, d) cell population in the macrophage monolayer culture tested: ○, macrophages; △, PMNL; ●, lymphocytes; (e, f) number of c.f.u. of organisms in the liver. In the lethal infection, 60 and 0% of the mice survived at 4 and 6 d p.i., respectively. See Fig. 3 for other details.

Fig. 6. Changes in macrophage phagocytic activity against latex beads in the presence (●) or absence (○) of 10 ng PMA ml⁻¹ after infection due to *M. intracellulare* (a) and *L. monocytogenes* at sublethal (b) or lethal (c) doses. The macrophages were harvested from the animals tested in the experiments shown in Figs 1 and 4.

Changes in the phagocytic ability of host macrophages against latex particles after infection with M. intracellulare or L. monocytogenes

In *M. intracellulare* infection (Fig. 6a), macrophage phagocytic activity against latex beads was markedly enhanced even 1 d p.i. and gradually increased thereafter. The addition of 10 ng PMA ml⁻¹ to the phagocytosis incubation medium led to a slight (1.1- to 2-fold) increase in the phagocytic activity of the host macrophages. It is noteworthy that the phagocytic capacity of non-infected macrophages (resident peritoneal macrophages) was markedly enhanced by PMA.

In the listerial infections (Fig. 6*b, c*), the phagocytic patterns in the macrophage differed markedly, depending on the infection dose. In the case of sublethal infection, macrophage phagocytic activity in the absence of PMA was markedly increased at 1 d p.i., remained elevated until 4 d p.i. and then rapidly decreased (Fig. 6*b*). The pattern was similar in the presence of PMA, but with a sharp peak at 1 d p.i., when the enhancement by PMA was most marked. In contrast, in the case of lethal infection (Fig. 6*c*), the onset of enhanced phagocytic activity of the host macrophages was somewhat slower (with or without PMA). In the absence of PMA, phagocytic activity reached a maximum 3 d p.i., which was retained until the death of the animals. With PMA, phagocytic activity continued to increase until the death of the animals.

DISCUSSION

In *M. intracellulare* infection, PMA-triggered chemiluminescence of macrophages rapidly decreased between the second and sixth week, whereas the PMA-elicited H_2O_2 release was retained at a high level for 10 weeks. Similar differences were noted in the patterns of the alterations of chemiluminescence and H_2O_2 -releasing ability of the host macrophages during *L. monocytogenes* infection, regardless of the infection dose. If macrophage chemiluminescence originates from $^1\text{O}_2$ or ^1OH (Cheson *et al.*, 1976; Miles *et al.*, 1978) rather than O_2^- , the discrepancy between the patterns of change in PMA-triggered chemiluminescence and H_2O_2 release is not so enigmatic. In macrophages, $^1\text{O}_2$ or ^1OH are generated mainly through the Harber-Weiss reaction, catalysed by iron and tryptophan (Halliwell, 1978; Fong *et al.*, 1978), from O_2^- and H_2O_2 , and this reaction is thought to be regulated by a different cellular mechanism from that related to regulation of PMA-triggering of NADPH oxidase, conversion of O_2^- to H_2O_2 , and the H_2O_2 -releasing process itself. In macrophages of mice infected with *M. intracellulare*, the Harber-Weiss reaction step was probably most accelerated at 2 weeks p.i., following a 1 week lag, although the PMA-triggered oxidative burst was enhanced even 1 d after infection and a high level of activity was retained thereafter. This may explain differences between the patterns of change in the chemiluminescence and H_2O_2 release after infection.

As reported by Nathan & Root (1977) and by our group (Tomioka & Saito, 1980; Saito *et al.*, 1981), the PMA responsiveness of a given macrophage cell correlates well with its stimulated and/or activated state. That is, immunologically activated macrophages show a higher responsiveness to PMA-triggering for H_2O_2 and O_2^- release than do chemically stimulated macrophages (Tomioka & Saito, 1980; Johnston *et al.*, 1978) and resident macrophages lack such a responsiveness (Nathan & Root, 1977; Saito *et al.*, 1981). Therefore, host macrophages are thought to be stimulated and/or activated during infections due to *M. intracellulare* and *L. monocytogenes*. It is noteworthy that the responsiveness of macrophage to PMA at 100 ng ml^{-1} was markedly elevated as early as 1 d after bacterial challenge, in both infections, although a considerable part of the apparent H_2O_2 release from the macrophage culture may have originated from contaminating PMNL because PMNL produce amounts of H_2O_2 similar to activated macrophages (Nathan & Root, 1977). Since the T-cell mediated immune response to the infected organisms had barely developed at this stage, the increase in PMA-responsiveness of the host macrophages is more likely to have been due to a direct stimulation or activation of their cell functions by contact with component(s) or product(s) of the infecting bacteria or mediator(s) derived from the inflammatory process (Pabst & Johnston, 1980; Diaz *et al.*, 1979). Host macrophages from animals infected with *M. intracellulare* could respond to $10 \text{ ng PMA ml}^{-1}$, whereas the macrophages of mice with *L. monocytogenes* infection did not respond to this dose of PMA (Figs 3 and 5). Thus the degree of stimulation (or activation) of the host macrophages in the former infection is evidently much higher than that in the latter. In separate experiments, macrophages induced by a single intraperitoneal injection of thioglycollate or Zymosan A 4 d before harvesting showed a significant chemiluminescent response to PMA, but it was much lower (thioglycollate-induced macrophages, $3.2, 5.8$ and $7.6 \times 10^3 \text{ c.p.m. per } 10^6 \text{ cells}$; Zymosan A-induced macrophages, $3.1, 6.4$ and $5.9 \times 10^4 \text{ c.p.m. per } 10^6$ at 1, 10 and $100 \text{ ng PMA ml}^{-1}$, respectively) than that of macrophages of mice 2 weeks after *M. intracellulare* infection (1.9 to $3.4 \times 10^5 \text{ c.p.m. per } 10^6 \text{ cells}$). The macrophages at this stage of *M. intracellulare*

infection thus seem to have a higher ability for producing an oxidative burst than do chemically elicited macrophages. Furthermore, macrophage H_2O_2 -releasing ability without triggering by PMA or in response to a low concentration of PMA (1 ng ml^{-1}) increased at around the same stage of the infection. These findings suggest the generation of immunologically activated macrophages which are highly responsive to PMA (Tomioka & Saito, 1980). Generation of activated macrophages is evident in infections with other mycobacteria, such as *M. tuberculosis* (North, 1974) and *M. bovis* (Mackanness, 1969). The possibility that an increase in the number or affinity of PMA receptors during the infections caused the increase in the PMA responsiveness of the host macrophages may be excluded, since there is evidence that *in vivo* 'activation' of macrophages in mice infected with BCG is not associated with a change in the cell's receptors for phorbol diesters (Weinberg & Misukonis, 1983). As they proposed, the increase in PMA responsiveness for an oxidative burst in the host macrophages may be associated with 'postreceptor' changes such as the induction of the synthesis of NADPH oxidase, an increase in the affinity of the enzyme for its substrate, or 'linking' of the NADPH oxidase system with the phorbol diester receptor.

Campbell and his colleagues (Czuprynski *et al.*, 1983, 1984) proposed that, in a *L. monocytogenes* infection, monocytes and PMNL, but not monocyte-derived 'activated' macrophages, play a central role in the resistance of the host to the parasites. They demonstrated that increased mobilization of PMNL and mononuclear phagocytes into the site of infection is of prime importance in resistance to listeriosis (Czuprynski *et al.*, 1984). This is consistent with our findings in sublethal *L. monocytogenes* infections, in which chemiluminescence of host macrophages was less elevated than in lethal infections (Fig. 4a), and that the host macrophages did not acquire responsiveness to a low dose of PMA during the course of *L. monocytogenes* infection until the infecting organism had been eliminated (Fig. 5a).

Macrophage phagocytic ability was markedly enhanced even 1 d p.i. in both experimental infections, indicating a rapid stimulation of macrophage cell function, presumably due to acute inflammation. In *M. intracellulare* infection (chronic infection), the enhanced state persisted for up to 6 weeks. This is consistent with the change seen in PMA-triggered H_2O_2 -releasing ability of the macrophages. On the other hand, in case of *L. monocytogenes* infection at a sublethal dose, the enhanced state rapidly declined, almost in parallel with the elimination of the organisms (Figs 4 and 6). Differing from *M. intracellulare* infection, this pattern resembled that of PMA-triggered chemiluminescence of the host macrophages infected with *L. monocytogenes*. This indicates essential differences between changes in macrophage cell functions during the chronic and acute infections, with sublethal doses. Interestingly, in *L. monocytogenes* infection induced by a lethal dose (acute infection), the phagocytic ability of the host macrophages increased almost continuously until the death of the animals.

Our study has revealed that two macrophage functions related to oxidative burst, namely chemiluminescence and H_2O_2 release, can be differentiated on the basis of their patterns of changes after *M. intracellulare* and *L. monocytogenes* infections. It is important to know which function relates to the activated or suppressed state of the host macrophages during infection, because, at present, both functions are used as measures of macrophage activation.

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