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Role of Reactive Nitrogen and Oxygen Intermediates in Gamma Interferon-Stimulated Murine Macrophage Bactericidal Activity against *Burkholderia pseudomallei*

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We examined the contributions of reactive nitrogen and oxygen intermediates (RNI and ROI, respectively) in macrophage bactericidal activity against *Burkholderia pseudomallei*, the causative agent of melioidosis, in order to understand host defense mechanisms against infection caused by this bacterium. The bacteria multiplied in unstimulated murine macrophage cell line J774.1. However, a strong dose-dependent inhibition of intracellular bacterial growth was observed when gamma interferon (IFN-γ)-activated macrophages were used. The induction of bactericidal activity correlated well with the production of nitric oxide (NO) by IFN-γ-activated macrophages and was markedly suppressed by N^G-monomethyl L-arginine (L-NMMA), a competitive inhibitor of NO synthesis. Superoxide dismutase (SOD) and catalase significantly inhibited macrophage bactericidal activity, and the combined addition of L-NMMA, SOD, and catalase resulted in the complete inhibition of IFN-γ-stimulated activity. The bacteria were susceptible to the killing effects of chemically generated NO and superoxide anion in a macrophage-free system. Our results indicate that IFN-γ-induced macrophage bactericidal activity against *B. pseudomallei* is mediated to a large extent by RNI killing mechanisms and to a lesser extent by ROI-dependent mechanisms.

Burkholderia pseudomallei, formerly designated *Pseudomonas pseudomallei*, is a gram-negative rod bacterium that causes melioidosis, an endemic disease in tropical areas such as Southeast Asia, northern Australia, the Indian subcontinent, Iran, and Central and South America (6, 13, 22, 34). The bacteria reside in water and soil and infect humans and animals through the cutaneous and tracheal routes (13, 34). Although subclinical and asymptomatic infections are the most common forms of this disease (2, 22), in some cases the bacteria are thought to remain latent and to cause clinical manifestations after a long period of time (24). Symptomatic infections are associated with high mortality and relapse rates (28, 33).

Several investigations (10, 18, 26) have recently demonstrated that *B. pseudomallei* survive and multiply in phagocyte cells. Fukuhara et al. (10) found that antibiotics that penetrate macrophages were much more effective than other types of antimicrobial agents in the treatment of pulmonary infection caused by the same organism in an animal model. Host defense against infection with facultative intracellular bacteria such as *Salmonella*, *Listeria*, *Legionella*, and *Mycobacterium* species is predominantly mediated by cellular immune mechanisms (19). Macrophages finally kill the pathogens after being stimulated by gamma interferon (IFN-γ) produced by natural killer cells, γδ T cells, and type-1 helper T cells (19). The two major microbicidal mechanisms for phagocyte cells are reactive oxygen intermediates (ROI), consisting of superoxide anion, hydrogen peroxide, hydroxy radical, and singlet oxygen (3, 17, 20), and reactive nitrogen intermediates (RNI), such as nitric oxide (NO) (14). The killing mechanisms of IFN-γ-activated macrophages are mediated by one or more of these oxidants (1, 3, 9, 11, 12, 14, 16, 17, 20, 21, 23), although the extent of the contributions of ROI and RNI may differ from one pathogen to another. In *Mycobacterium tuberculosis* and *Cryptococcus neoformans*, the RNI pathway is predominantly used, while the ROI pathway regulates the synthesis of another type of microbicidal molecule (4, 31).

Little is known about host defense mechanisms against *B. pseudomallei*, although cell-mediated immunity seems to play a central role, based on the tendency of the microorganism to multiply within the phagocytes. Furthermore, there exist conflicting reports on the susceptibility of *B. pseudomallei* to the bactericidal activity of professional phagocyte cells (18, 26). In the present study, we examined the involvement of the two major microbicidal systems, ROI and RNI, in the killing of this microorganism by IFN-γ-activated murine macrophages.

MATERIALS AND METHODS

Culture medium and reagents. RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco BRL (Grand Island, N.Y.), and fetal calf serum was purchased from Whittaker (Walkersville, Md.). Murine recombinant IFN-γ (specific activity, 5×10^6 U/mg) was purchased from Genzyme Diagnostics (Cambridge, Mass.).

Bacteria. *B. pseudomallei* (H1354), isolated from the blood of a patient with melioidosis, was kindly provided by Prasit Tharvichitkul, Department of Bacteriology, Chiang Mai University, Chiang Mai, Thailand. Bacteria were stored in skim milk at −70°C until use. In every experiment, the microorganisms were cultured in nutrient agar from a fresh stock for 18 h at 37°C. *Staphylococcus aureus* (ATCC 25923) was obtained from the American Type Culture Collection and cultured on nutrient agar for 18 h before use.

Macrophages. A murine macrophage cell line, J774.1 (cell line no. RCB0434), was purchased from Riken Cell Bank (Tsukuba, Japan) and allowed to grow on DMEM supplemented with 10% fetal calf serum. After growth to confluence, the cells were dislodged with a rubber policeman, washed three times in the culture medium, and used for subsequent experiments.

In some experiments, macrophages were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. The fixation was stopped by washing the cells in cold 0.6% glycylglycine (Wako Pure Chemical Industries Ltd., Tokyo, Japan) to block free aldehyde groups, and then unbound PFA was removed by three washes in RPMI 1640 medium.

Assessment of intracellular killing of bacteria by macrophages. Macrophages (1×10^6/well) were precultured with various doses of IFN-γ for 24 h. To examine the growth of bacteria in macrophages, the latter were incubated with *B. pseudomallei* (approximately 1×10^7/well) for 1 h and then washed three times with the culture medium to eliminate bacteria remaining outside the macro-

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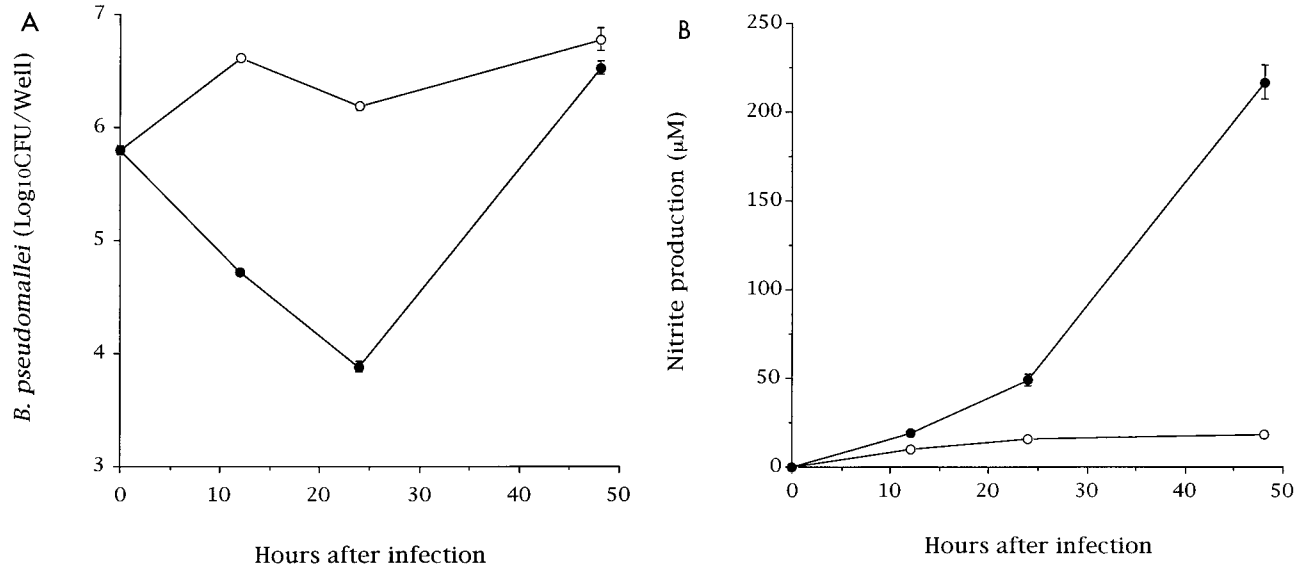

FIG. 1. Bactericidal activity of IFN-γ-stimulated macrophages against *B. pseudomallei*. Macrophages (1×10^6/well) were precultured with (closed circle) or without (open circle) 300 U of IFN-γ per ml for 24 h and then incubated with *B. pseudomallei* (9.8×10^6/well) for 1 h. After the bacteria remaining in the extracellular compartment were washed three times, the cells were further cultured in a medium containing 10 μg of IPM per ml, after which the number of live bacteria (A) and the nitrite concentration in the culture supernatant at various intervals (B) were determined. The dose of IFN-γ was similar to that used in prestimulated macrophage cultures. Each data point represents the mean ± SD of triplicate cultures.

phages. However, preliminary experiments indicated that residual extracellular bacteria multiply quickly following culture, which would prevent us from evaluating the intracellular growth of the bacteria. To avoid this, we added 10 μg of imipenem (IPM) per ml to the culture medium. IPM (MIC, 0.5 μg/ml) is an antibiotic effective against *B. pseudomallei* strains. This treatment successfully suppressed the growth of extracellular bacteria within 24 h, except when longer periods of incubation were allowed (because of loss of antimicrobial activity). After culture for various time intervals, the supernatant in each well was harvested, and each well was washed three times with 1 ml of distilled water to destroy macrophages. Then the culture supernatant and pooled washes were mixed, inoculated at 50 μl onto nutrient agar plates after appropriate dilution with distilled water, and cultured for 1 day, after which the number of colonies was determined.

Culture of *B. pseudomallei* in NO-generating media. *B. pseudomallei* was directly exposed to chemically generated NO in a cell-free system, as previously described by Wang and Casadevall (32). Briefly, the bacteria were suspended at 9.0×10^3/ml in minimal medium consisting of 15 mM D-glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine (Wako Pure Chemical Industries Ltd.), and 3.0 μM vitamin B_1 (Sigma Chemical Co., St. Louis, Mo.) in the presence or absence of 500 μM sodium nitrite (Wako) at various pHs (4.0, 5.0, 6.0, or 7.0) and then cultured at 37°C without CO_2 for 24 h. Media with pHs of 4.0 and 7.0 were prepared by adding appropriate amounts of 25 mM succinic acid and 25 mM sodium 3-(*N*-morpholino)propanesulfonic acid (MOPS), respectively. Media at pHs 5.0 and 6.0 were prepared by adjusting the pHs of the media at 4.0 and 7.0 with 1 N NaOH and 1 N HCl, respectively. The bacteria, harvested and appropriately diluted with distilled water, were inoculated at 50 μl onto nutrient agar plates and cultured for 1 day, followed by determination of the numbers of colonies. NO is generated in cultures with acidic pHs following a chemical reaction with sodium nitrite but not in cultures with neutral pHs (32).

Culture of *B. pseudomallei* in superoxide-generating media. *B. pseudomallei* was directly exposed to oxygen-derived oxidants generated chemically in a cell-free system with the epinephrine oxidative system described by Polacheck et al. (26). Briefly, the bacteria were suspended in 50 mM sodium acetate (pH 5.5) containing 1.0 mM $MgSO_4$ at 2.0 ml. In the next step, ferric ammonium sulfate, H_2O_2, and epinephrine bitartrate (Sigma) were added in that order to final concentrations of 0.5, 1.0, and 1.0 mM, respectively, followed by incubation at 37°C, with continuous shaking. After various intervals of time, the bacteria were plated on nutrient agar plates to determine viability as measured by the number of colonies.

Statistical analysis. Data were expressed as means ± standard deviations (SD). The unpaired Student *t* test was used to compare differences between two groups, with a P of <0.05 considered significant.

RESULTS

Microbicidal activity of IFN-γ-stimulated macrophages against *B. pseudomallei*. Macrophages were allowed to phagocytose *B. pseudomallei* for 1 h, and after extracellular bacteria were washed out, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence of 10 μg of IPM per ml to inhibit the growth of residual extracellular bacteria. Twenty-four hours later, we measured the nitrite concentration in the culture supernatant, using the Griess method. Surprisingly, high levels of nitrite were detected (1.0 mM), even when macrophages were not prestimulated with IFN-γ (data not shown). However, the level of nitrite dropped below the detectable limit when the macrophages were cultured with either bacteria or IPM (data not shown). In contrast, no detectable amount of nitrite was produced in the supernatant of macrophages cultured with *B. pseudomallei* and IPM when DMEM was used in place of RPMI 1640 medium. One possible explanation for this observation is that the 800 μM nitrate in the latter medium was reduced to nitrite by bacterial reductase released from IPM-treated *B. pseudomallei*. Thus, DMEM, which does not contain nitrate, was used in the remaining experiments.

As shown in Fig. 1A, the number of live *B. pseudomallei* organisms in unstimulated macrophages increased during incubation. In contrast, the number of live *B. pseudomallei* organisms in IFN-γ-stimulated macrophages was markedly decreased at 24 h after infection, although the population increased at 48 h to a level similar to that in unstimulated macrophages. It is possible that a reduced viability of macrophages and/or diminished IPM antimicrobial activity during culture allowed the regrowth of bacteria in the IFN-γ-activated macrophages. To identify the mechanism, we examined these two possibilities. The viability of macrophages, determined by a trypan blue exclusion method, was 89.3% ± 1.5% just after the incorporation of *B. pseudomallei*, while 73.0% ± 7.5% and 52.7% ± 7.0% of macrophages were alive 24 and 48 h after the culture with bacteria, respectively. Thus, some reduction in the viability of macrophages was observed during the culture. To examine the possibility of diminished IPM antimicrobial activity, we added IPM to the macrophage cultures at 10 μg/ml 24 h after culture with bacteria was initiated. With this treatment,

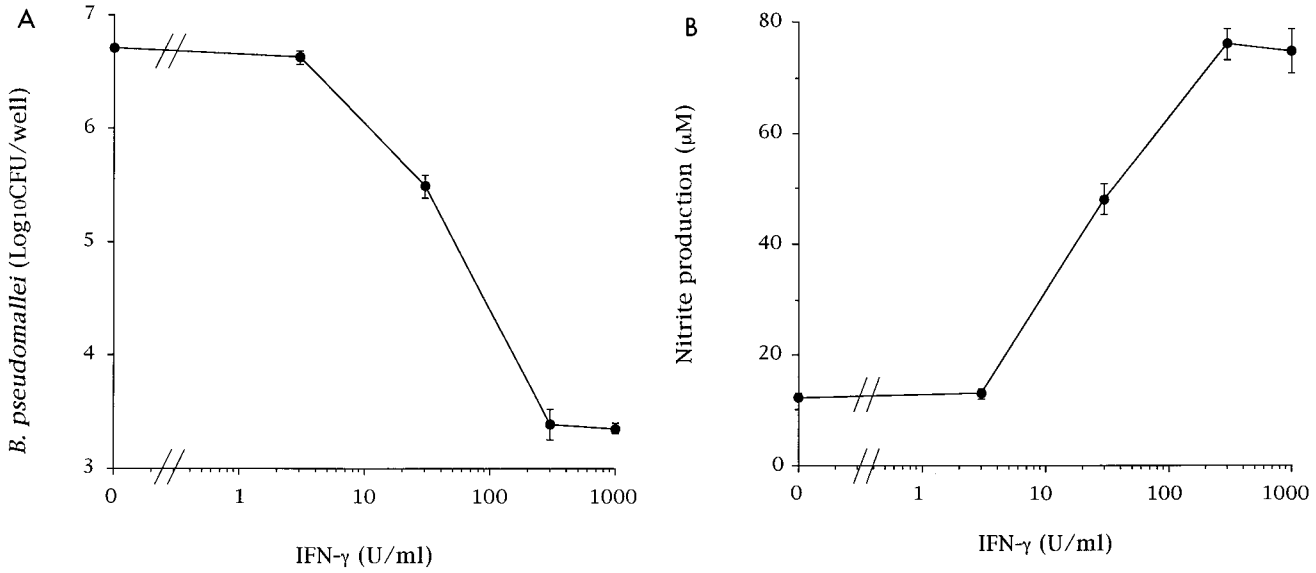

FIG. 2. Dose dependency of IFN-γ. Macrophages (1×10^6/well) were precultured with various doses of IFN-γ for 24 h and then incubated with *B. pseudomallei* (1.4×10^7/well) for 1 h. After the bacteria remaining in the extracellular compartment were washed three times, the cells were further cultured in a medium containing 10 μg of IPM per ml for 24 h, after which the number of live bacteria (A) and the nitrite concentration in the culture supernatant (B) were determined. The dose of IFN-γ was similar to that used in prestimulated macrophage cultures. Each data point represents the mean ± SD of triplicate cultures.

the regrowth of bacteria at 48 h was almost completely abrogated (data not shown). These results indicated that the regrowth of *B. pseudomallei* in IFN-γ-activated macrophages was mostly due to diminished IPM antimicrobial activity, although the possibility of the reduced viability of macrophages was not excluded. Thus, the culture period selected for use in the remaining experiments was 24 h.

To determine whether the bactericidal effect of IFN-γ-activated macrophages was targeted to intracellular or extracellular *B. pseudomallei*, we conducted another control experiment. Unstimulated macrophages (1×10^6/well) were incubated with bacteria (7.8×10^6/well) for 1 h. After the unincorporated bacteria were removed by three washes, macrophages were cultured in the presence of 10 μg of IPM per ml for 24 h. The number of live bacteria increased from $(2.1 \pm 1.0) \times 10^5$ CFU/well at the beginning of the experiment to $(13.5 \pm 2.7) \times 10^5$ CFU/well 24 h after culture. A similar experiment was performed with macrophages fixed with 4% PFA to block the incorporation of bacteria into the cells. In contrast to the results of the former experiment, the number of live bacteria was reduced from $(8.2 \pm 0.5) \times 10^4$ CFU/well at the beginning of the experiment to $(3.7 \pm 0.2) \times 10^4$ CFU/well 24 h after culture. These results indicated that the increase in the number of bacteria in the macrophage cultures was mostly due to the increase in intracellular bacteria and suggested that the main target of the killing by the IFN-γ-activated macrophages in our study was the intracellular bacteria, not the extracellular bacteria.

On the other hand, the concentration of nitrite in the culture supernatants increased with time after the infection of the IFN-γ-stimulated macrophages with *B. pseudomallei*, while a marginal amount of nitrite was produced by the unstimulated macrophages (Fig. 1B).

The microbicidal activity of macrophages against *B. pseudomallei* correlated with the concentration of IFN-γ; a dose-dependent relationship between IFN-γ and the number of bacteria was present, with an optimal IFN-γ dose of 300 U/ml (Fig. 2A). More importantly, the microbicidal activity of macrophages correlated with the concentration of nitrite produced in the culture supernatant (Fig. 2B). These results suggested that the microbicidal activity of macrophages against *B. pseudomallei* was mediated by RNI mechanisms.

Role of RNI in macrophage bactericidal activity against *B. pseudomallei*. To confirm that RNI is an underlying mechanism of macrophage bactericidal activity against *B. pseudomallei*, we examined the effect of N^G-monomethyl L-arginine (L-NMMA), a competitive inhibitor of NO synthesis, on the IFN-γ-induced bactericidal activity of macrophages. As shown in Fig. 3A, the number of live bacteria in macrophages was markedly reduced by IFN-γ, while the addition of L-NMMA inhibited IFN-γ-induced macrophage bactericidal activity. This effect was dose dependent, and the optimal dose was 500 μM. The effect of L-NMMA correlated with the inhibition of IFN-γ-induced NO production by macrophages (Fig. 3B). In contrast, the same dose of L-NMMA did not significantly influence the number of live bacteria in unstimulated macrophages (data not shown). Interestingly, this treatment did not cause the number of live bacteria to return to the control level, even when 1,000 μM L-NMMA was used, suggesting that the RNI mechanisms do not entirely mediate the bactericidal activity of macrophages and that other mechanisms may be involved.

We also investigated the effect of exogenous NO on *B. pseudomallei* in order to examine its susceptibility to RNI mechanisms. The bacteria were exposed to chemically generated NO for 24 h in a macrophage-free system. As shown in Fig. 4, the numbers of live bacteria for groups cultured with and without sodium nitrite at pHs of 7.0 and 6.0 did not differ. In contrast, when the medium was more acidic (pH 5.0), the number of live bacteria was markedly reduced in the presence of sodium nitrite compared with that without sodium nitrite. No comparison could be made at pH 4.0, since the acidic medium resulted in the lysis of the bacteria.

Role of ROI in macrophage microbicidal activity against *B. pseudomallei*. To examine the role of ROI as an underlying mechanism of macrophage bactericidal activity against *B. pseudomallei*, we assessed the effect of superoxide dismutase

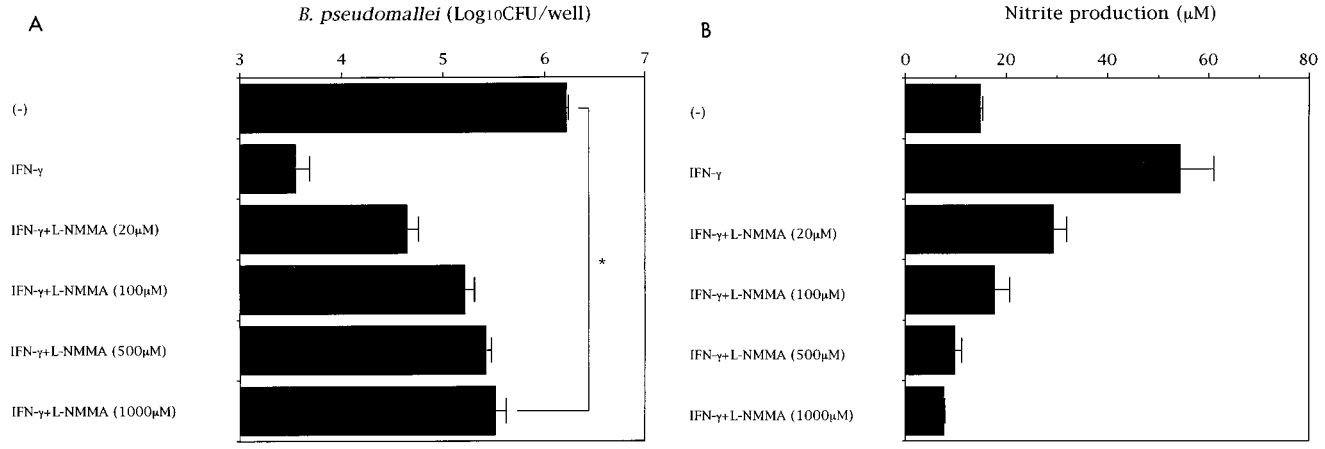

FIG. 3. Effect of L-NMMA. Macrophages (1×10^6/well) were precultured with 300 U of IFN-γ per ml for 24 h in the presence or absence of various doses of L-NMMA and then incubated with *B. pseudomallei* (9.8×10^6/well) for 1 h. After the bacteria remaining in the extracellular compartment were washed three times, the cells were further cultured for 24 h in a medium containing 10 μg of IPM per ml, after which the number of live bacteria (A) and the nitrite concentration in the culture supernatants (B) were determined. The doses of IFN-γ and L-NMMA were similar to those used in prestimulated macrophage cultures. Each bar represents the mean ± SD of triplicate cultures. *, $P < 0.00005$.

(SOD) and catalase on IFN-γ-induced macrophage bactericidal activity. As shown in Fig. 5, the addition of SOD and catalase caused a significant inhibition of the bactericidal activity of macrophages, L-NMMA again showed an inhibitory effect on macrophage microbicidal activity, and when the two sets of inhibitory compounds were combined, IFN-γ-induced macrophage bactericidal activity was completely inhibited. In contrast, the same dose of SOD and catalase did not significantly influence the number of live bacteria in unstimulated macrophages (data not shown). These results indicated that IFN-γ-induced macrophage bactericidal activity is due to a large extent to RNI-mediated killing mechanisms, although it is mediated to a lesser extent by ROI killing mechanisms.

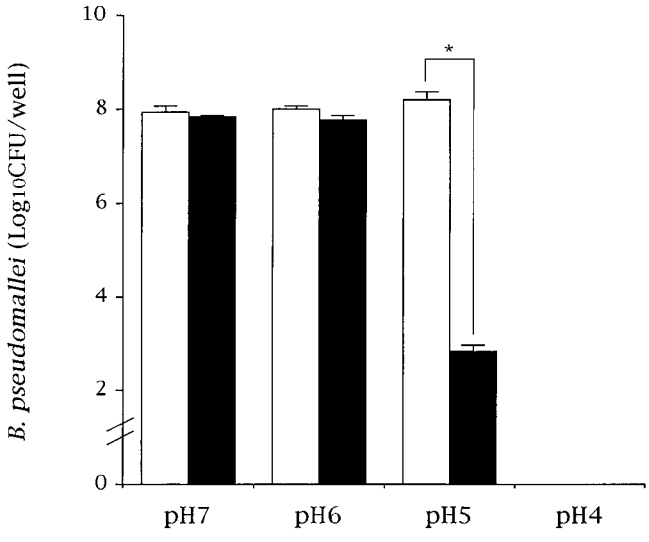

FIG. 4. Susceptibility of *B. pseudomallei* to NO. *B. pseudomallei* (9.0×10^3/ml) was incubated in minimal medium in the presence (closed bars) or absence (open bars) of 500 μM sodium nitrite under various pH conditions for 24 h. The bacteria, harvested and appropriately diluted with distilled water, were inoculated at 50 μl onto nutrient agar plates and cultured for 1 day, followed by counting of the number of colonies. Each bar represents the mean ± SD of triplicate cultures. *, $P < 0.05$.

Finally, we investigated the effect of exogenous superoxide anion on *B. pseudomallei* in order to examine its susceptibility to ROI mechanisms. For this purpose, the bacteria were exposed to chemically generated superoxide anion for various intervals of time in a macrophage-free system. As shown in Fig. 6, the number of live *B. pseudomallei* organisms started to decrease at 30 min, and *B. pseudomallei* was killed completely within 4 h of exposure. In contrast, the microorganism *S. aureus* was more resistant to the chemically generated superoxide anion than *B. pseudomallei*. These results suggested that *B. pseudomallei* was very susceptible to the bactericidal effects of oxygen-derived oxidants.

DISCUSSION

The major finding of the present study was that IFN-γ-activated macrophages inhibited the intracellular growth of *B. pseudomallei* through RNI- and ROI-dependent killing mechanisms. RNI contributed more than ROI, because the inhibitory effect of oxygen radical scavengers on IFN-γ-induced macrophage bactericidal activity was less potent than that of a competitive NO synthesis inhibitor. Our results also showed that *B. pseudomallei* is susceptible to the bactericidal effects of RNI and ROI, as shown by a reduction in the number of live bacteria after in vitro exposure to chemically generated nitrogen- and oxygen-derived oxidants in a macrophage-free system.

B. pseudomallei infection frequently takes a subclinical or an asymptomatic course, resulting in clinical manifestations of the disease only when the infected host is in an immunocompromised state such as those present in patients with diabetes or renal disease (5). The intracellular presence and multiplication of the bacteria have been observed in phagocytes (10, 18, 26). These observations indicate that, similar to other facultative intracellular bacteria, such as *M. tuberculosis*, *Listeria monocytogenes*, *Salmonella typhi*, and *Legionella pneumophila*, *B. pseudomallei* is capable of resisting host defense mechanisms and multiplies within macrophages. In fact, phagocytosed *B. pseudomallei* was found in the present study to multiply even inside unstimulated macrophages. Intracellular bacteria usually possess various mechanisms to evade the microbicidal activity of phagocytes, including interference with phagolysoso-

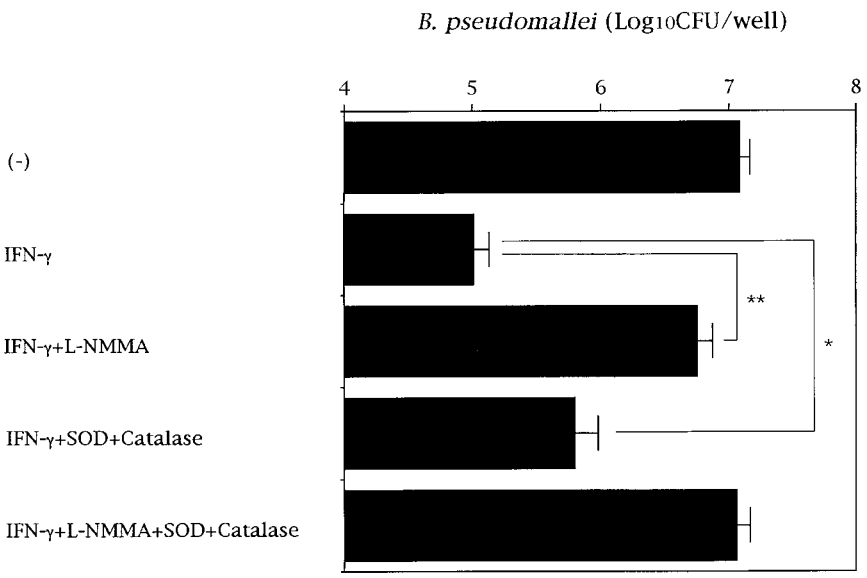

FIG. 5. Effect of oxygen radical scavengers. Macrophages (1×10^6/well) were precultured with 300 U of IFN-γ per ml in the presence or absence of L-NMMA (500 μM), SOD (0.1 mg/ml) plus catalase (0.1 mg/ml), or a compound of the three for 24 h. These cells were then incubated with *B. pseudomallei* (5.4×10^6/well) for 1 h. After the bacteria remaining in the extracellular compartment were washed three times, the cells were further cultured in a medium containing 10 μg of IPM per ml for 24 h, followed by counting of the number of live bacteria. IFN-γ and reagents were added at the same doses used in prestimulated macrophages. *, $P < 0.05$; **, $P < 0.005$.

mal fusion, resistance to bactericidal lysosomal enzymes, and escape from phagosome to cytoplasm (8). Recently, Jones et al. (18) demonstrated that *B. pseudomallei* was also resistant to certain bactericidal compounds known to be present in professional phagocytes, including cationic peptide protamine and purified human defensin. These substances may contribute to the intracellular survival of *B. pseudomallei* by allowing the bacteria to escape killing by macrophages. In our study, however, *B. pseudomallei* was susceptible to the microbicidal effect of chemically generated NO and superoxide anion, and its susceptibility to oxygen radicals was higher than that of *S. aureus*. These results, however, could not explain the intracellular survival of *B. pseudomallei* within the macrophages. Further studies are necessary to define the precise mechanism of the intracellular survival of *B. pseudomallei* in macrophages.

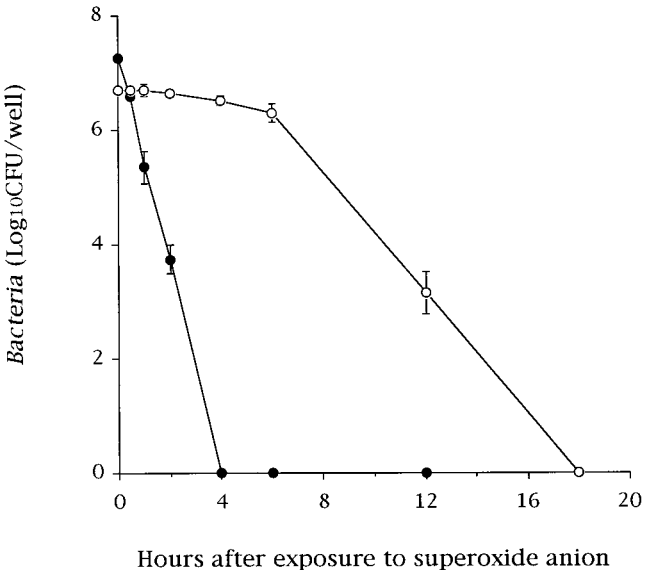

FIG. 6. Susceptibility of *B. pseudomallei* to superoxide anion. *B. pseudomallei* (closed circles) and *S. aureus* (open circles) were suspended at 1.8×10^7 and 4.8×10^6/ml, respectively, in a superoxide-generating medium and incubated at 37°C. After various intervals, the bacteria were plated on nutrient agar plates to determine the viability by counting the number of colonies. Each data point represents the mean ± SD of triplicate cultures.

The activation of macrophages is associated with the induction of their microbicidal activity against various intracellular bacteria, and IFN-γ is one of the most potent activators. In the present study, we demonstrated that IFN-γ-stimulated macrophages acquired bactericidal activity against *B. pseudomallei* multiplying inside the cells. To detect the intracellular growth of bacteria, 10 μg of IPM per ml was added to the cultures. This antibiotic inhibits the growth of residual bacteria outside macrophages and does not penetrate the cells. After 24 h of culture of macrophages with the same dose of IPM, only 5.09% of the dose in the extracellular compartment was detected inside the cells when bactericidal activity against *Bacillus subtilis* (ATCC 6633) was assayed (data not shown). In this regard, it is important to examine the effect of IFN-γ on the rate of IPM incorporation into macrophages, because a higher uptake of the antibiotic may lead to increased killing of intracellular bacteria. The intracellular dose of the antibiotic in macrophages did not differ after stimulation with IFN-γ (unstimulated, 5.09%; stimulated, 4.78%). Thus, we confirmed that IFN-γ did not enhance the bactericidal activity of macrophages against *B. pseudomallei* by increasing the intracellular concentration of the antibiotic.

The contribution of RNI and ROI to macrophage microbicidal activity is not uniform for all pathogens. These two killing mechanisms are known to correlate with each other and in some conditions may even suppress each other by directly influencing xanthine-NADPH oxidase and NO synthase activity (7, 25, 30). When NO and superoxide anions are generated simultaneously, an immediate reaction results in the production of a potent oxidant, peroxynitrite, with a strong microbi-

cidal activity (15, 29). In the present study, both RNI and ROI were found to participate in the bactericidal activity of IFN-γ-stimulated macrophages against *B. pseudomallei*, although the contribution of RNI seemed to be more marked than that of ROI. Our in vitro studies also showed the susceptibility of the bacteria to the killing effects of exogenous NO and superoxide anions.

Our results differ from those of Chan et al. (4), who showed that ROI was not a principal mediator of the antimycobacterial capability of activated macrophages. In the study of Chan et al., the killing activity of ROI-deficient mutant macrophages against *M. tuberculosis* was equivalent to that of ROI-generating parent macrophages. Furthermore, the same researchers showed that the killing activity was not influenced by oxygen radical scavengers such as SOD and catalase. While these differences may point to differences in the effects of the ROI and RNI mechanisms, they may point also to differences in the host response to invasion by different microorganisms. In this regard, we have also recently documented (31) similar results in *C. neoformans*. In that study, oxygen radical scavengers augmented the fungicidal activity of IFN-γ-stimulated macrophages against *C. neoformans* by enhancing the production of NO, suggesting that ROI may negatively regulate the production of RNI during the killing of *M. tuberculosis* and *C. neoformans*. At present, the mechanisms underlying the different effects of oxygen radical scavengers on NO-dependent killing of microorganisms by macrophages remain unclear, and further investigation is necessary.

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