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The Contributions of Reactive Oxygen Intermediates and Reactive Nitrogen Intermediates to Listericidal Mechanisms Differ in Macrophages Activated Pre- and Postinfection

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The contribution of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) to the killing of *Listeria monocytogenes* by macrophages activated by addition of spleen cells from listeria-immune mice plus specific antigen was examined. When macrophages were infected with *L. monocytogenes* and then spleen cells were added, there was not as big a difference in listericidal activity between macrophages cultured with normal spleen cells and those cultured with immune spleen cells as expected. In this culture system, RNI was mainly involved in the macrophage intracellular killing. In macrophages first activated and then infected, a significant level of enhanced killing was observed. Blockade of ROI production drastically affected the enhanced killing ability, while inhibition of RNI production had a negligible effect. Thus, the contributions of ROI and RNI to listericidal mechanisms of macrophages were different between macrophages activated at pre- and postinfection stages.

Listeria monocytogenes is a facultative intracellular pathogen capable of escaping the killing mechanism of macrophages (24, 25). Mice that have survived a primary infection with *L. monocytogenes* experience protective immunity and are able to eliminate the bacteria much faster than in the primary infection (18). The protective immunity is believed to be mediated by both $CD4^+$ and $CD8^+$ T cells in mice (14).

The enhanced bacterial elimination expressed in the immune mice depends mainly on the enhanced killing of macrophages activated by various cytokines, especially gamma interferon (IFN-γ). As we have shown in the previous studies, the main source of IFN-γ during a secondary infection is antigen-specific $CD4^+$ T cells (29, 31). In contrast, in primary infection IFN-γ is secreted mainly by natural killer (NK) cells stimulated with interleukin-1α (IL-1α), IL-12, and tumor necrosis factor alpha, which are secreted by macrophages (32). Although IFN-γ is endogenously produced in both the primary and secondary infections, the ultimate bacterial elimination is quite different.

Reactive nitrogen intermediates (RNI), including nitric oxide, are involved in the antimicrobial activity of activated macrophages against a variety of intracellular microorganisms, e.g., *Leishmania major* (17), *Toxoplasma gondii* (1, 15), *Legionella pneumophila* (3), *Mycobacterium tuberculosis* (4), *Mycobacterium bovis* BCG (9), and *L. monocytogenes* (2), as are reactive oxygen intermediates (ROI). There have been conflicting results on the involvement of RNI in the course of *Listeria* infection (11). Recent reports show that mice deficient in inducible nitric oxide synthase (iNOS) were not able to eliminate *L. monocytogenes* as efficiently as did control mice during a primary infection (19); however, acquired resistance against the secondary infection was not impaired, suggesting that the immune resistance is not dependent on RNI (28).

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In the present study, we attempted to analyze the involvement of ROI and RNI in the listericidal mechanisms used by macrophages during primary and secondary infections in vitro. In addition, we investigated the kinetics of IFN-γ and nitric oxide production in primary and secondary infection to elucidate the mechanism of enhanced killing in the secondary infection.

MATERIALS AND METHODS

Experimental animals. Male C3H/He mice (Charles River Japan, Atsugi, Japan), raised and maintained under specific-pathogen-free conditions, were used at the age of 7 to 9 weeks.

Preparation of bacteria. *L. monocytogenes* EGD, a virulent and highly immunogenic strain, was used throughout the study. The bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 16 h, washed repeatedly, suspended in phosphate-buffered saline, and stored at −70°C until used. Killed cells of *L. monocytogenes* were prepared by heating the viable bacterial suspension of a known concentration at 74°C for 90 min (29).

Reagents. Superoxide dismutase (SOD), N^G-monomethyl-L-arginine (L-NMMA), and N^G-monomethyl-D-arginine (D-NMMA) were purchased from Wako Pure Chemicals, Inc. (Osaka, Japan). Gentamicin reagent solution was purchased from Life Technologies, Inc. (Grand Island, N.Y.). SOD and NMMA were added to the cultures at final concentrations of 100 U/ml and 1 mM, respectively.

Preparation of macrophages. Peritoneal exudate cells (PEC) were recovered from C3H/He mice 3 days after an intraperitoneal injection of 1.5 ml of 10% Proteose Peptone (Difco). The PEC were washed with Hanks' balanced salt solution (HBSS) and suspended in medium consisting of RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% heat-inactivated fetal bovine serum, 10 μg of gentamicin/ml, 5 g of HEPES/liter, and 2 g of $NaHCO_3$/liter. PEC (10^6) were cultured in a 24-well flat-bottom tissue culture plate (Costar, Cambridge, Mass.) for 2 h at 37°C, nonadherent cells were removed by gentle washing with warm HBSS, and the culture medium was replaced with 1 ml of fresh medium per well. Adherent cells thus prepared were used as macrophages.

Immunization of mice and preparation of immune spleen cells and normal spleen cells. Mice were immunized by an intravenous injection with 2×10^3 viable *L. monocytogenes* cells. One week after the immunization, spleens were removed and single-cell suspensions were prepared. The cells were suspended at 5×10^6 cells per ml in the medium and were used as immune spleen cells. Normal spleen cells were prepared from nonimmunized mice.

Activation of macrophages. Adherent macrophages (10^6) were infected with *L. monocytogenes*, and extracellular bacteria were removed by washing and killed with gentamicin-containing medium. Then immune spleen cells (5×10^6) or normal spleen cells (5×10^6) were added to activate macrophages (postinfec-

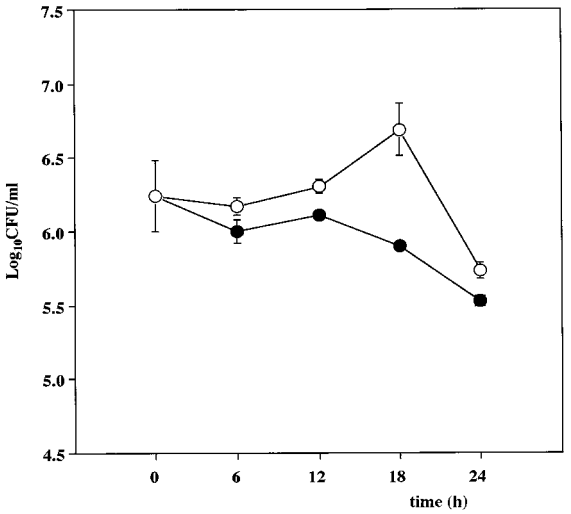

FIG. 1. Kinetics of intracellular killing of *L. monocytogenes* by macrophages cultured in the presence of spleen cells after infection. Macrophages (10^6) were infected with *L. monocytogenes* at a multiplicity of infection of 20. After elimination of extracellular bacteria, 5×10^6 spleen cells from immune mice (●) or normal mice (○) were added to the infected macrophages. Bacterial counts were determined by serial dilution and plating after incubation for the indicated times. Data are representative of three consecutive experiments and are expressed as the means of triplicate cultures ± standard deviations.

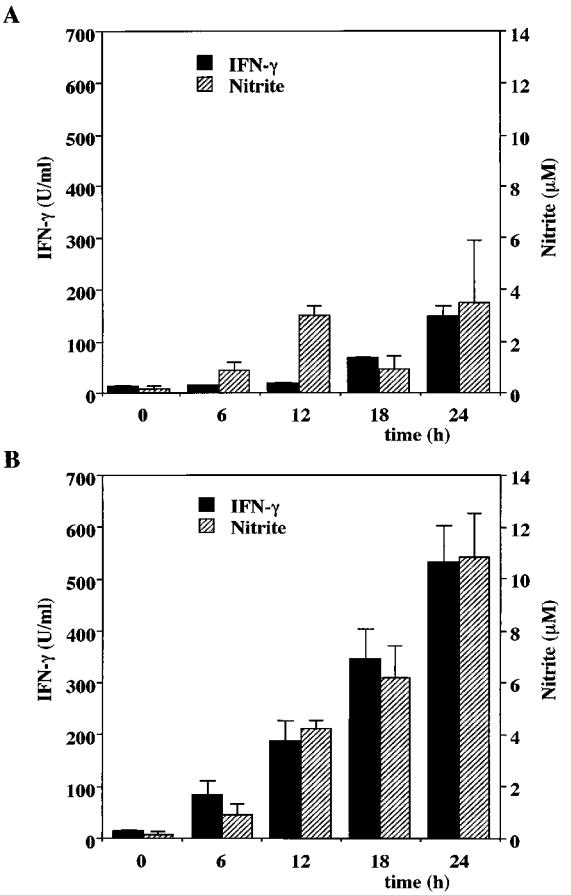

FIG. 2. Kinetics of IFN-γ production and nitrite accumulation in the supernatants of cultures shown in Fig. 1. Normal spleen cells (A) or immune spleen cells (B) were added to infected macrophages. Culture supernatants at the indicated time points were subjected to IFN-γ titration by enzyme immunoassay and nitrite determination by using Griess reagent. Data are representative of three consecutive experiments and are expressed as the means of triplicate cultures + standard deviations.

tion). For preinfection activation, macrophages were incubated with immune spleen cells (5×10^6) and killed *L. monocytogenes* cells (2×10^7) for 12 h, washed to remove spleen cells and killed *L. monocytogenes* cells, and used for the intracellular killing assay.

Intracellular killing assay. The intracellular killing assay was performed by the method described recently (23). In brief, adherent macrophages were infected with *L. monocytogenes* at a 20:1 ratio of bacteria to cells and the plates were centrifuged at 450 × *g* for 5 min to enhance the attachment of bacteria to macrophages and incubated at 37°C for 60 min to facilitate the ingestion of bacteria. After phagocytosis, the cells were washed seven times with 1 ml of warm HBSS, and 5 μg of gentamicin/ml was added to the culture medium. The cells were incubated for 4 h to kill extracellular bacteria. It was confirmed that this was sufficient to kill the extracellular bacteria without affecting the intracellular killing (23). Several hours later, the cells were disrupted with sterile distilled water to release the intracellular bacteria. The number of bacteria inside the macrophages was determined by serial dilution and plating on brain heart infusion agar (Eiken Chemical Co., Ltd., Tokyo, Japan).

Nitrite determination. The nitrite concentration in culture, a measurement of nitric oxide synthesis, was assayed by a standard Griess reaction adapted to microplates as described previously (31). The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 5% H_3PO_4) and naphthylethylene diamine dihydrochloride (0.1% in H_2O). A 100-μl volume of reagent was mixed with an equal volume of supernatant and incubated at room temperature for 10 min. The absorbance of the chromophore formed was measured at 540 nm in an automated microplate reader. Nitrite was quantitated with $NaNO_2$ as a standard, and the data were expressed as micromoles of nitrite per liter.

IFN-γ assay. The IFN-γ titer in the supernatant was determined by an enzyme-linked immunosorbent assay as described previously (13). Briefly, the supernatant and recombinant mouse IFN-γ (a gift from Central Research Institute, Daiichi Seiyaku Co. Ltd.) were placed in the wells of enzyme immunoassay plates precoated with rat anti-mouse IFN-γ monoclonal antibody (Lee Biomolecular Research Inc., San Diego, Calif.) and 0.5% bovine serum albumin in carbonate-bicarbonate buffer (pH 9.6). After incubation for 90 min, the plates were washed and incubated with rabbit anti-mouse IFN-γ polyclonal antibody for 90 min. After the plates were washed, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, Inc., San Francisco, Calif.) was added, and the mixture was incubated for 90 min. The plates were washed, and then orthophenylenediamine in phosphate buffer (pH 5.0) with 0.03% H_2O_2 was added as a substrate solution. The reaction was terminated by adding 2.5 M H_2SO_4, and the absorbance was measured at 490 nm.

Luminol-dependent chemiluminescence. Luminol-dependent chemiluminescence was determined by using a lumiphotometer (TD-4000; Labo Science, Tokyo, Japan) as described previously (34). PEC were washed with buffer II, consisting of 10 mM HEPES, 5 mM KCl, 145 mM NaCl, and 5.5 mM glucose (pH 7.4), scraped with a cell scraper, and suspended to yield 10^7 cells/ml in 50 μl of buffer II. Then 50 μM luminol sodium salt (Wako), 100 μl of buffer I (consisting of buffer II supplemented with 1 mM $CaCl_2$), and 50 μl of phorbol myristate acetate (20 μg/ml) were added to the cells. Chemiluminescence was monitored by the lumiphotometer for 10 min and expressed in relative light units.

RNA extraction. Total cellular RNA was extracted by a previously described method (31). The cells were washed, and 1 ml of solution D (4 M guanidinium isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, 0.05 mM 2-mercaptoethanol) was added to the cell pellet. Cells were disrupted by being passed through a 21-gauge needle. Subsequently, 0.1 ml of 2 M sodium acetate (pH 4), 1 ml of water-saturated phenol, and 0.2 ml of chloroform-isoamyl alcohol were added to the mixture, with thorough mixing by inversion after the addition of each reagent. The final suspension was vigorously vortexed for 20 s and then cooled on ice for 15 min. Samples were centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the aqueous phase containing RNA was transferred to a new tube, mixed with the same volume of isopropanol, and held at −20°C overnight to precipitate RNA. After centrifugation at 10,000 × *g* for 20 min, the RNA pellet was dissolved in solution D and precipitated with the same volume of isopropanol at −20°C for 2 h. RNA was collected by centrifugation for 15 min at 4°C, washed once with 75% ethanol, dried, and dissolved in 20 μl of distilled water. The RNA concentration was measured by monitoring the absorbance at 260 nm with a spectrophotometer (GeneQuant; Pharmacia LKB Biochem Ltd., Cambridge, United Kingdom).

RT-PCR and gel electrophoresis. cDNA was produced by reverse transcription (RT) as follows (31). The total RNA extracted (5 μg) was mixed with 4 μl of RT buffer, 2 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (Promega, Madison, Wis.), 1 μl of 10 mM deoxynucleoside triphosphates (Pharmacia), 2 μl of random primer (Pharmacia), 0.5 μl of reverse transcriptase (Gibco-BRL Life Technologies Inc., Gaithersburg, Md.), and distilled water to give a total volume of 20 μl. The mixture was incubated at 42°C for 60 min and then boiled at 95°C for 3 min. The samples were kept at −20°C until used. The PCR mixture consisted of 2 μl of sample cDNA, 5 μl of PCR amplification buffer, 2 μl of 25 mM $MgCl_2$, 4 μl

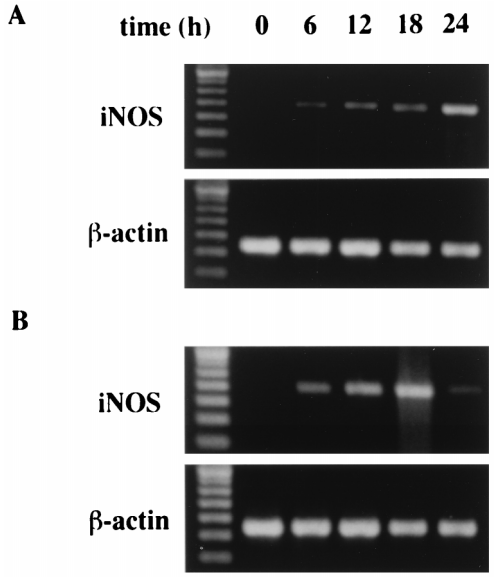

FIG. 3. Kinetics of iNOS gene expression in macrophages cultured with spleen cells after infection. Normal spleen cells (A) or immune spleen cells (B) were added to infected macrophages. The cells were incubated, and the total cellular RNA was extracted at the indicated time points. Total RNA was subjected to RT-PCR to detect iNOS gene expression.

of 2.5 mM deoxynucleoside triphosphates, 0.3 μl of *Taq* DNA polymerase (5 U/μl; Promega), 2 μl of 20 μM primer, and 32.7 μl of double-distilled water to give a final volume of 50 μl. The sequences of the oligonucleotide primers used are as follows: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTG TCAGAGCCTCGTGGCTTTGG-3′ for iNOS and 5′-TGGAATCCTGTGGC ATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ for β-actin. The predicted sizes of the amplified products for iNOS and β-actin were 497 and 348 bp, respectively. PCR amplification was performed with a TP cycler 100 (Toyobo, Osaka, Japan). The PCR program was one cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by amplification for 23 cycles for β-actin and 25 cycles for iNOS according to the most appropriate cycle number determined by a preliminary experiment. The reaction was terminated by incubation at 72°C for 7 min, and the products were kept at 4°C in the cycler. The PCR products were analyzed by gel electrophoresis with a 1% low-melting-point agarose gel (Wako) in 1× TAE (Tris-acetate-EDTA) buffer supplemented with 0.005% ethidium bromide. A 10-μl volume of PCR products and 2 μl of marker dye were applied to each well. The bands were visualized by a UV transilluminator and photographed.

RESULTS

Kinetics of intracellular killing of macrophages cultured with normal or immune spleen cells. To test the listericidal activity of macrophages in primary and secondary infections, we first evaluated the enhancement of listericidal activity of macrophages cultured with spleen cells from mice immunized with *L. monocytogenes* or not immunized. Macrophages were infected with *L. monocytogenes*, washed with HBSS, and incubated for 4 h in gentamicin-containing medium to kill extracellular bacteria, and then normal or immune spleen cells were added to infected macrophages (time zero). Every 6 h, intracellular viable bacteria were enumerated on brain heart infusion agar (Fig. 1). When infected macrophages were cultured with immune spleen cells, the number of *L. monocytogenes* cells decreased after 12 h of incubation. In cultures with normal spleen cells, the number of bacteria increased during the first 18 h and decreased thereafter. The difference in bactericidal activity between cultures with normal and immune spleen cells was not as significant as we had expected.

Next, we examined the IFN-γ titer and nitrite concentration

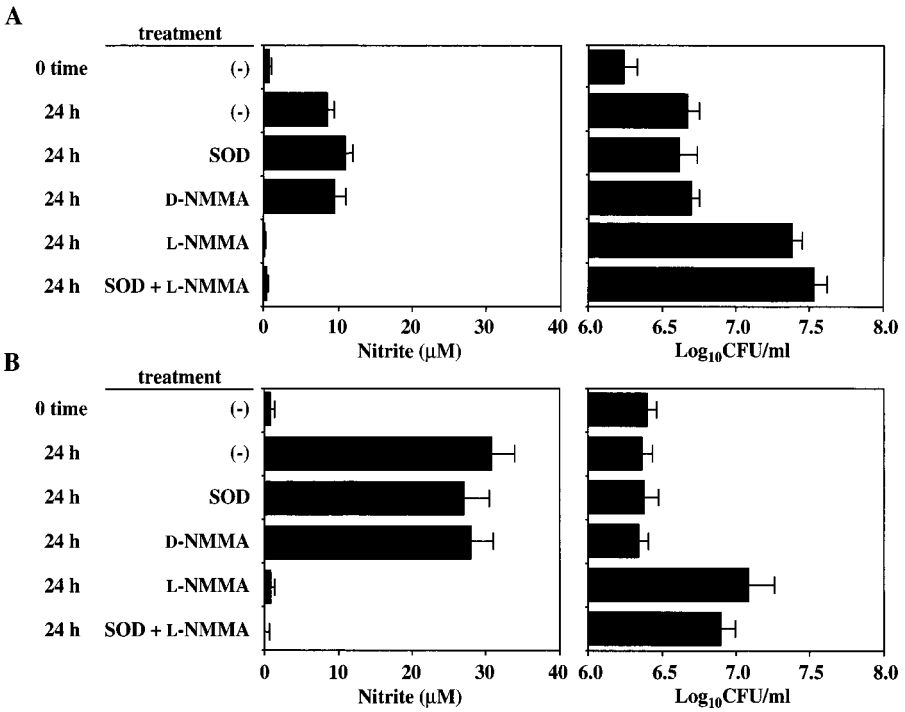

FIG. 4. Effects of SOD and NMMA on the intracellular killing of *L. monocytogenes* by macrophages cultured in the presence of spleen cells after infection. Normal spleen cells (A) or immune spleen cells (B) were added to infected macrophages. SOD and/or L- or D-NMMA was added to infected macrophages simultaneously with the addition of spleen cells. Bacterial counts were determined after incubation for 24 h by serial dilution and plating. Data are representative of two consecutive experiments and are expressed as the means of triplicate cultures + standard deviations.

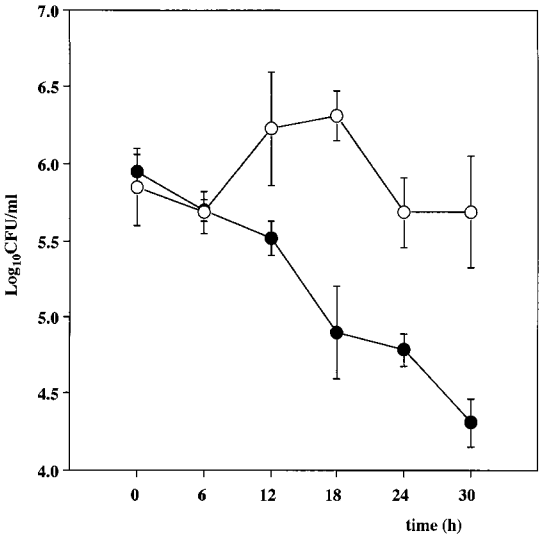

FIG. 5. Kinetics of intracellular killing of *L. monocytogenes* by macrophages activated at the pre- or postinfection stage. Macrophages (10^6) were cultured with (●) or without (○) immune spleen cells (5×10^6) plus killed *L. monocytogenes* cells (2×10^7) for activation preinfection for 12 h. Then the macrophages were washed and infected with *L. monocytogenes* at a multiplicity of infection of 20. After elimination of extracellular bacteria, immune spleen cells (5×10^6) and killed *L. monocytogenes* cells (2×10^7) were added to both macrophage cultures. Bacterial counts were determined after incubation for the indicated times by serial dilution and plating. Data are representative of three consecutive experiments and are expressed as the means of triplicate cultures ± standard deviations.

in the supernatant in this assay system (Fig. 2). There was a significant difference in the production of IFN-γ and nitrite between cultures of infected macrophages with normal or immune spleen cells. Interestingly, the IFN-γ titer and nitric oxide production showed a tendency to increase even in the culture with normal spleen cells. RT-PCR detection of iNOS gene expression in this assay system has shown that iNOS mRNA in macrophages cultured with immune spleen cells reached a peak at around 18 h and that the mRNA level in a culture with normal spleen cells reached a peak at 24 h or later (Fig. 3).

Involvement of ROI and RNI in listericidal mechanisms used by macrophages cultured with normal or immune spleen cells. Next we determined the involvement of ROI and RNI in the killing of *L. monocytogenes* by macrophages in this assay system. SOD and L-NMMA were employed to inhibit O_2^- and nitric oxide production, respectively. SOD and/or L-NMMA was added to infected macrophages simultaneously with the addition of spleen cells. Addition of L-NMMA to give 1 mM in culture resulted in complete abolition of nitrite production, and SOD did not affect the nitrite level (Fig. 4). It was found that the listericidal activity of macrophages cultured with immune spleen cells was impaired after the addition of L-NMMA (Fig. 4B). The specific inhibition of nitric oxide production by L-NMMA could be confirmed by the ineffectiveness of D-NMMA, employed as a specificity control. It was interesting that the listericidal activity of macrophages cultured with normal spleen cells was also affected after addition of L-NMMA (Fig. 4A). In contrast, SOD did not affect the listericidal activity of macrophages in this assay system (Fig. 4). These results suggested that nitric oxide played an important role in the killing of *L. monocytogenes* by macrophages which were first infected and then supplemented with normal or immune lymphocytes.

From the results obtained in the above-described experiments, it was difficult to explain the well-known difference of bacterial elimination between the primary and secondary infections in vivo, since there was no evident difference in bacterial killing by and killing mechanisms of macrophages in primary- and secondary-infection models in vitro.

Kinetics of intracellular killing by macrophages activated pre- and postinfection. We reported previously that ROI but not RNI was involved in the listericidal mechanisms of macrophages activated by IFN-γ and lipopolysaccharide (LPS) (23), which is not consistent with the results obtained in the present study. The activation of macrophages by IFN-γ and LPS was completed preinfection, whereas macrophages cultured with immune spleen cells in the present assay system were activated postinfection. Therefore, we compared the macrophages activated pre- or postinfection for their listericidal activity. Macrophages were cultured with immune spleen cells (5×10^6) plus killed *L. monocytogenes* cells (7×10^6) for 12 h to activate them preinfection. For comparison, a macrophage culture with killed *L. monocytogenes* cells in the absence of immune spleen cells was prepared. Then the cells were washed and infected with viable *L. monocytogenes*. After elimination of extracellular bacteria, immune spleen cells and killed *L. monocytogenes* cells were added again to both types of macrophage culture (time zero). The number of intracellular bacteria was assessed every 6 h (Fig. 5). It was found that preactivated macrophages could kill intracellular bacteria soon after infection, whereas the cells activated postinfection allowed the number of intracellular bacteria to increase for 18 h and then began to kill these bacteria. This assay system indicated a significant difference in the intracellular killing activity between macrophages activated pre- or postinfection.

Involvement of ROI and RNI in listericidal mechanisms used by macrophages activated pre- or postinfection. Next we tried to determine whether ROI and RNI are involved in the killing of *L. monocytogenes* by macrophages in this assay system. We found that the listericidal activity of macrophages activated preinfection was impaired after the addition of SOD but not after the addition of NMMA (Fig. 6). This result was the exact opposite of the results shown in Fig. 4. It was suggested that ROI was involved mainly in the killing of bacteria by macrophages activated preinfection, as in macrophages activated preinfection by IFN-γ and LPS (23). In contrast, in macrophages activated postinfection, RNI was essential in the killing of *L. monocytogenes* and ROI did not appear to contribute.

We also examined nonactivated and activated macrophages cultured with normal or immune spleen cells for their ability to produce ROI by means of luminol-dependent chemiluminescence. Macrophages were cultured with killed *L. monocytogenes* cells only or killed *L. monocytogenes* cells plus normal or immune spleen cells for 12 h, and O_2^- production was determined (Fig. 7). It was found that macrophages cultured with killed *L. monocytogenes* cells and immune spleen cells produced 10 times more O_2^- than did nonactivated macrophages. Interestingly, macrophages cultured with normal spleen cells were also capable of producing O_2^- to a level half that produced by macrophages cultured with immune spleen cells. Addition of SOD to give 100 U/ml completely abolished the O_2^- production in these cultures (data not shown). These results suggested that nonactivated macrophages, as well as macrophages activated postinfection, allowed the number of intracellular bacteria to increase because of the low level of O_2^--producing ability.

Kinetics of IFN-γ and nitric oxide production of macrophages activated pre- and postinfection. The above results

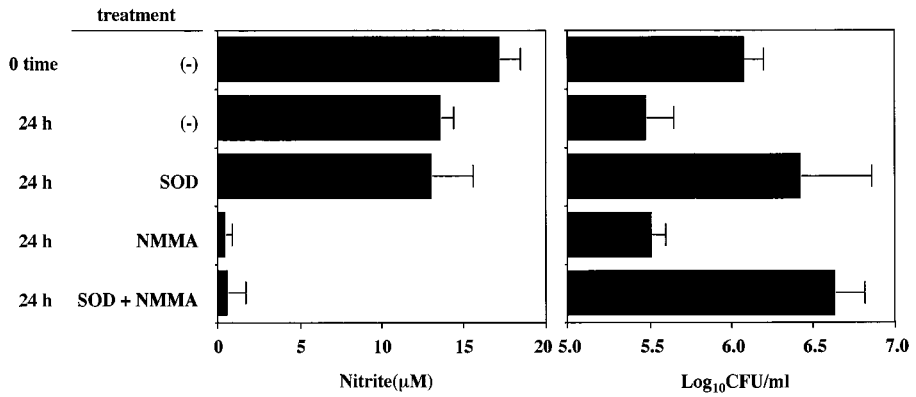

FIG. 6. Effects of SOD and NMMA on the intracellular killing of *L. monocytogenes* by macrophages activated at the preinfection stage. Macrophages were cultured with immune spleen cells plus killed *L. monocytogenes* cells for activation and then washed and infected with *L. monocytogenes* at a multiplicity of infection of 20. SOD and/or NMMA was added to preactivated and infected macrophages at the time of readdition of the spleen cells. Bacterial counts were determined after incubation for 24 h by serial dilution and plating. Data are representative of two consecutive experiments and are expressed as the means of triplicate cultures + standard deviations.

showed that when macrophages were activated preinfection, ROI was involved in the listericidal mechanism and RNI was not. When macrophages were activated postinfection, the number of intracellular bacteria increased for 20 h after infection, probably because nonactivated macrophages were not able to produce enough ROI. The killing observed at a later stage appeared to be attributable to the generation of RNI.

Lastly we investigated whether nitric oxide was actually produced in this assay system. We found that nitric oxide was produced at the early stage of infection in macrophages activated preinfection (Fig. 8A) but that RNI was not involved in the listericidal activity. In macrophages activated postinfection, the kinetics of nitric oxide production was coincident with the bactericidal activity (Fig. 8B).

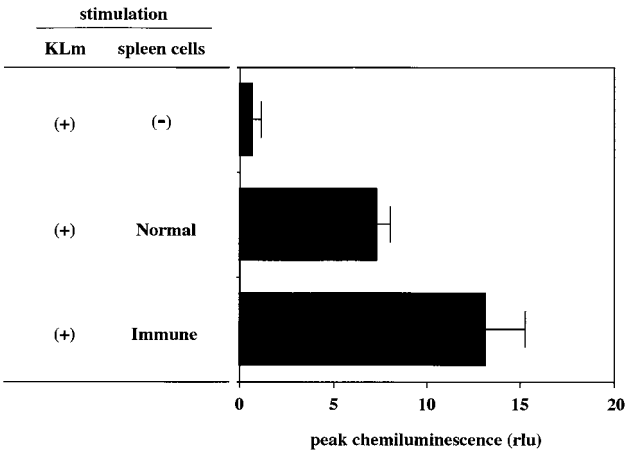

FIG. 7. Chemiluminescent response of macrophages cultured in the presence of spleen cells plus killed *L. monocytogenes* cells (KLm). Cells were cultured for 12 h, and then adherent macrophages were scraped off with a cell scraper and suspended to yield 10^7 cells/ml in reaction buffer. Macrophages were stimulated with phorbol myristate acetate, and chemiluminescence was monitored for 10 min with a lumiphotometer. The results are expressed in relative light units (rlu). Data are representative of two consecutive experiments, and peak chemiluminescence is expressed as the means of triplicate cultures + standard deviations.

DISCUSSION

Recent reports showed that bacterial elimination in *L. monocytogenes* infection is mediated by macrophages (27) and neutrophils (5) in mice. It is generally accepted that IFN-γ is one of the most important cytokines involved in the activation of macrophages. When macrophages are stimulated with IFN-γ and other cytokines, ROI production is enhanced (20, 21) and RNI production is induced (8, 22). However, whether these effector molecules are involved in the killing during primary and secondary infection has not been completely elucidated. In particular, the involvement of nitric oxide seems to be controversial. Several studies support the critical role of nitric oxide in host defense against *L. monocytogenes* in vivo in primary infection (2, 19). We also reported that nitric oxide is an important mediator of nonspecific antilisterial activity induced by viable *M. bovis* BCG (33). In the model of secondary infection, Samsom et al. showed that the resistance was not dependent on RNI (28). It is suggested that the secondary infection is different from the primary infection in the killing mechanisms of *L. monocytogenes*. In several in vitro assays, when macrophages were activated by IFN-γ and LPS or tumor necrosis factor alpha preinfection, no contribution of RNI to the listericidal mechanisms was observed (12, 16, 23). It appears that the artificial in vitro conditions obtained by using recombinant cytokines or LPS which is not present in *L. monocytogenes* do not always represent the actual infection in vivo.

In the present study, we developed an in vitro assay system which may reproduce the actual *L. monocytogenes* infection without using recombinant cytokines or LPS but using immune spleen cells to clarify the mechanisms of killing in the infection. We first examined the kinetics of intracellular killing and IFN-γ production by macrophages cultured with normal or immune spleen cells after infection. The difference in the bactericidal activity of macrophages cultured with normal or immune spleen cells was not as significant as expected (Fig. 1). It appeared that a time lag in IFN-γ production in culture (Fig. 2) resulted in the time lag in macrophage activation and a difference in bacterial killing. It was interesting that RNI but not ROI played a critical role in antilisterial defense induced by both normal and immune spleen cells in this assay system (Fig. 4). In both cultures, macrophages were activated postinfection; therefore, we next examined the activity and mecha-

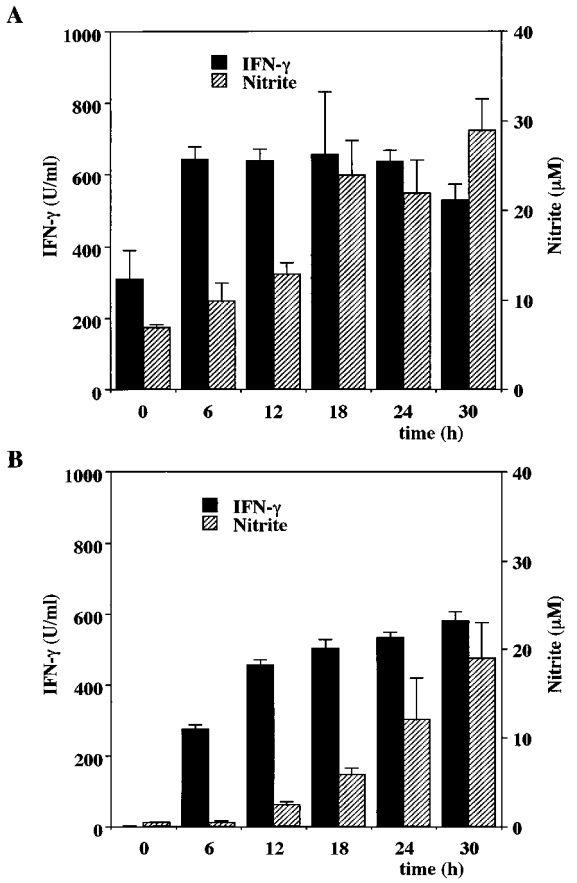

FIG. 8. Kinetics of IFN-γ production and nitrite accumulation in the supernatants of the cultures in Fig. 5. Macrophages were activated preinfection (A) or postinfection (B) by the addition of immune spleen cells plus killed *L. monocytogenes* cells. Data are representative of three consecutive experiments and are expressed as the means of triplicate cultures + standard deviations.

nisms of killing used by macrophages activated by the addition of killed *L. monocytogenes* antigen and immune spleen cells pre- and postinfection. It was evident that the killing activity of macrophages activated preinfection differed from that of macrophages activated postinfection (Fig. 5) and that ROI played a critical role in antilisterial mechanisms of macrophages activated preinfection (Fig. 6). These results are consistent with those of our previous study, in which macrophages were activated preinfection (23). It became clear that there are apparent differences in the killing mechanisms between macrophages activated at the pre- and postinfection stages.

There may be an argument about whether the in vitro situation employed in the present study actually takes place in vivo. During the course of primary infection in mice, infected macrophages may be activated at some later time by IFN-γ secreted by NK cells or in association with the development of antigen-specific T cells (activation postinfection), and immunologically activated macrophages may engulf the bacteria upon secondary infection (activation preinfection). Therefore, we believe that the present experimental data obtained in vitro are relevant to the in vivo phenomena.

A virulent strain of *L. monocytogenes* is able to secrete listeriolysin O, which has been shown to be a major virulence factor involved in the escape of this bacterium from the phagosomal compartment to the cytoplasm of macrophages (6, 10, 25). de Chastellier and Berche determined the percentages of *L. monocytogenes* in the cell compartments by quantitative assessment at different times after infection (7). They reported that about 14% of bacteria were found in the cytoplasm within 1 h after infection and that the proportion of bacteria reached about 50% 4 h after infection. These data suggest that the bacteria begin to escape from the phagosome immediately after infection and that a considerable number of them reach the cytoplasm before the macrophages are activated by cocultivation with spleen cells in our assay. It is conceivable that the bacteria in the cytoplasm are free from the attack by ROI, since superoxide-forming NADPH oxidase is localized in phagocytic vesicles but not in the cytosol (30), whereas RNI can contribute to the killing of cytoplasmic bacteria. On the other hand, the access of *L. monocytogenes* to the cytoplasm inside activated macrophages is limited, as Portnoy et al. reported (26). Accordingly, bacteria are not able to escape from the phagosome when macrophages are activated preinfection, and so they are killed mainly by ROI.

Thus, the present study has revealed that the contributions of ROI and RNI to listericidal mechanisms of macrophages are different between macrophages activated at the pre- and postinfection stages. Instead of activating macrophages by recombinant cytokines or LPS, we have employed the addition of immune spleen cells plus killed *L. monocytogenes* cell antigens to mimic the in vivo infection. Therefore, it is likely that ROI contributes mainly to the defense against secondary *L. monocytogenes* infection whereas RNI contributes mainly to the defense against primary infection. The present in vitro assay system may provide a tool for a further analysis of the killing mechanisms used by macrophages operating in an in vivo situation.

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