Antimicrobial Actions of the NADPH Phagocyte Oxidase and Inducible Nitric Oxide Synthase in Experimental Salmonellosis. I. Effects on Microbial Killing by Activated Peritoneal Macrophages In Vitro

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Abstract
The contribution of the NADPH phagocyte oxidase (phox) and inducible nitric oxide (NO) synthase (iNOS) to the antimicrobial activity of macrophages for Salmonella typhimurium was studied by using peritoneal phagocytes from C57BL/6, congenic gp91phox2/2, iNOS2/2, and doubly immunodeficient phox2/2iNOS2/2 mice. The respiratory burst and NO radical (NO·) made distinct contributions to the anti-Salmonella activity of macrophages. NADPH oxidase-dependent killing is confined to the first few hours after phagocytosis, whereas iNOS contributes to both early and late phases of antibacterial activity. NO-derived species initially synergize with oxyradicals to kill S. typhimurium, and subsequently exert prolonged oxidase-independent bacteriostatic effects. Biochemical analyses show that early killing of Salmonella by macrophages coincides with an oxidative chemistry characterized by superoxide anion (O2·−), hydrogen peroxide (H2O2), and peroxynitrite (ONOO−) production. However, immunofluorescence microscopy and killing assays using the scavenger uric acid suggest that peroxynitrite is not responsible for macrophage killing of wild-type S. typhimurium. Rapid oxidative bacterial killing is followed by a sustained period of nitrosative chemistry that limits bacterial growth. Interferon γ appears to augment antibacterial activity predominantly by enhancing NO· production, although a small iNOS-independent effect was also observed. These findings demonstrate that macrophages kill Salmonella in a dynamic process that changes over time and requires the generation of both reactive oxidative and nitrosative species.

Key words: phagocyte • Salmonella • innate immunity • nitrosative • oxidative

Introduction
Salmonella pathogenesis commences in the ileal mucosa with invasion of M cells or ingestion by CD18-expressing phagocytes (1, 2). For the duration of the infection, Salmonella can be found principally within mononuclear phagocytes (3), which can serve as a vehicle of extraintestinal dissemination (1) and as a protected site for intracellular bacterial replication (3). The capacity to survive within macrophages is an absolute requirement for Salmonella virulence in vivo (4). Macrophages contribute to resistance to Salmonella by forming granulomas and limiting bacterial growth (5), but the effector mechanisms by which mono-nuclear phagocytes combat this intracellular pathogen are incompletely understood.

Radicals generated by the NADPH oxidase and inducible nitric oxide (NO) synthase (iNOS) are cytotoxic for a variety of microorganisms as phylogenetically diverse as viruses, bacteria, protozoa, and fungi. The NADPH oxidase expressed by myeloid cells catalyzes the univalent reduction of molecular oxygen to O2·−. This radical has only limited

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Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species.
membrane diffusibility and modest antibacterial activity, but also serves as a precursor to more toxic reactive oxygen species (ROS [6]). The critical role of the phagocyte oxidase (phox) is reflected by the enhanced susceptibility of patients with chronic granulomatous disease to a wide range of microbial pathogens, including Salmonella species (6-8). Immunodeficient gp91phox-/- mice, an animal model for X-linked chronic granulomatous disease (CGD), are accordingly very susceptible to experimental Salmonella typhimurium infections (9, 10). The reduced killing capacity of cell lines impaired in their ability to sustain a respiratory burst further attests to the importance of the NADPH oxidase in the anti-Salmonella activity of macrophages (11).

NOS isoforms generate NO in a complex reaction that consumes NADPH, oxygen, and l-arginine (12). NO: by itself possesses only weak antimicrobial activity against Salmonella (13), but congeners resulting from NO: autooxidation such as NO-2, NO-3, and S-nitrosothiols (14) enhance its cytotoxic potential. In addition, concerted actions of the NADPH oxidase and iNOS can synergize to form highly potent antimicrobial species. For example, NO: reacts at a rate of 6.7 × 10^6 M/s with O2- - to form ONOO- (15, 16), an oxidant capable of damaging lipids, proteins, and DNA; ONOO- has been associated with enhanced killing of S. typhimurium, Esheridha coli, and Candida albicans (13, 17, 18). NO: can also act in concert with H2O2 to kill E. coli in vitro by a mechanism that appears to be at least partially iron dependent (19).

The contribution of NO: is of particular significance in resistance to intracellular pathogens. Development of a Th1 immune response dominated by IFN-γ, IL-2, and IL-12 synthesis in combination with NO: -mediated effector functions has been correlated with resistance to L. pneumophila and mycobacterial infections (20, 21). Similarly, IFN-γ, IL-12, and TNF-α production is associated with resistance to S. typhimurium (22, 23). However, the contribution of NO: in innate immunity to Salmonella is still a matter of active debate. Evidence obtained from several laboratories using several nitrogen oxide donors such as S-nitrosoglutathione, acidified NO-2, and ONOO- has unequivocally established that S. typhimurium is susceptible to reactive nitrogen species (RNS) in vitro (13, 24-26). Furthermore, S. typhimurium deficient in DNA repair systems (e.g., umuC and recB), small thiol molecules (e.g., homocysteine and glutathione), or detoxifying enzymes (e.g., flavohemoprotein or copper zinc superoxide dismutase) is hypersusceptible to NO: congeners in vitro, and shows reduced macrophage resistance and virulence in vivo (13, 25, 27-29). Yet, despite this compelling evidence favoring distinct NO: actions against Salmonella, some studies have failed to demonstrate that NO: plays a role in macrophage inhibition or killing of wild-type S. typhimurium (9, 24, 30, 31).

In this work, we employed macrophages from C57BL/6 mice and their congenic in NO-/-, gp91phox-/-, and doubly immunodeficient in NO-/- gp91phox-/- derivatives to elucidate the contributions of the NADPH phagocyte oxidase and iNOS to antibacterial actions of macrophages for wild-type S. typhimurium.

Materials and Methods

Bacterial Strains. Wild-type S. typhimurium strains American Type Culture Collection 14028s (1) and M525P (10, 32) were used for this study. For immunofluorescence microscopy, rpm::fp was moved by P22-mediated transduction from strain SM O22 (1) into S. typhimurium 14028s to yield S. typhimurium AF991 (gfp+).

Macrophages. Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Congenic in NO-/- (33), gp91phox-/- (34), and doubly immunodeficient in NO-/- gp91phox-/- (9) mice were bred in our animal facility according to Institutional Animal Care and Use Committee guidelines. The in NO-/- mice were the progeny (N2-N3) of mice backcrossed onto a C57BL/6 background for 10 generations (gift of C. Nathan, Cornell University, New York, N.Y.). The gp91phox-/- and in NO-/- gp91phox-/- mice were progeny (N2-N3) of mice described previously (9, 34). gp91phox-/- and in NO-/- gp91phox-/- mice were maintained on drinking water containing 15 mg/ml itraconazole, 0.2 mg/ml trimethoprim, and 40 mg/ml sulfamethoxazole up to 4 d before experimentation to prevent spontaneous infections.

Macrophages. Peritoneal macrophages from C57BL/6 and congenic in NO-/- (33), gp91phox-/- (34) and doubly immunodeficient in NO-/- gp91phox-/- (9) mice were harvested 4 d after intraperitoneal inoculation of 1 mg/ml sodium periodate as described (25). The peritoneal exudate cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Gemini Bioproducts), 1 mM sodium pyruvate, 10 mM Hepes, and 2 mM l-glutamine (all reagents from Sigma-Aldrich). The macrophages were selected by adherence in a 96-well plate and cultured for 48 h at 37°C in a 5% CO2 incubator. Unless otherwise indicated, adherent macrophages were treated in vitro overnight with 20 U/ml of IFN-γ (Life Technologies) from a 105 U/ml stock containing 0.8 ng/ml LPS.

Macrophage Killing Assays. Periodate-elicited macrophages were challenged with S. typhimurium opsonized with 10% normal mouse serum at a 10:1 multiplicity of infection, allowed to internalize the bacteria for 15 min, and washed with warmed medium containing 6 μg/ml gentamicin (25). At several time points after infection, the macrophages were lysed with 0.5% sodium deoxycholate, and surviving bacteria were enumerated on Luria-Bertani agar plates. The results are expressed as percentage survival.

Chemiluminescence. Macrophage chemiluminescence was estimated by the reduction of 25 μM lucigenin (bis-methylacridinium) and the oxidation of 100 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) with a Lumistar chemiluminescence reader (Bergen Biotechs, Inc.). The macrophages were challenged with wild-type S. typhimurium American Type Culture Collection 14028s as described in the macrophage killing assays above. Extracellular bacteria were removed by washing and the addition of 6 μg/ml gentamicin. At specified time intervals, medium was harvested and replaced by fresh medium for performance of the assays. Lucigenin and luminol were used as indicators of O2- - and ONOO- production (35, 36), respectively. 1 mM uric acid (37, 38) inhibited ~80% of luminol-dependent chemiluminescence generated by IFN-γ-activated periodate-elicited macrophages 1 h after bacterial challenge, whereas 1,000 U/ml catalase (gift of Dr. S. Libby, North Carolina State University, Raleigh, NC) inhibited only 20%. These observations, along with the dependence of chemiluminescence on both the NADPH oxidase and iNOS, indicate that the majority of this luminol chemiluminescence is mediated by ONOO-.
minor proportion reflects $\text{H}_2\text{O}_2$. Periodate-elicited macrophages from wild-type, in $\text{OS}^{--}$, and phox$^{--}$ mice were compared.

Superoxide A nion D eterrination. $\text{O}_2^{--}$ was quantified by the superoxide dismutase–inhibitable reduction of ferricytochrome $c$ (39). At different time points after infection with Salmonella, medium was removed from cultured IFN-$\gamma$-treated 48 h-aged periodate-elicited macrophages and replaced with fresh medium containing 60 $\mu$M ferricytochrome $c$ in phenol red-free Earle's balanced salt solution. After 1 h incubation in 5% CO$_2$ at 37°C, the OD of the supernatants was determined spectrophotometrically at 550 nm. The concentration of $\text{O}_2^{--}$ was calculated by using an extinction coefficient of 2.1 $\times$ $10^3$ M$^{-1}$ cm$^{-1}$. All reagents were purchased from Sigma-Aldrich.

Hydrogen Peroxide D eterrination. $\text{H}_2\text{O}_2$ was measured by the horseradish peroxidase–dependent oxidation of phenol red (39). The macrophages were challenged with Salmonella as described above, and extracellular bacteria were removed by washing and the addition of gentamicin before incubation in Earle's balanced salt solution containing 0.56 mM phenol red and 20 U/ml horseradish peroxidase. At indicated time intervals, medium was harvested and replaced with fresh medium. After 1 h incubation at 37°C in a 5%-CO$_2$ atmosphere, the absorbance of the supernatants was read at 600 nm after mixing with 10 $\mu$L of 1 N NaOH per well. $\text{H}_2\text{O}_2$ was quantified by comparison with a standard curve prepared with known concentrations of $\text{H}_2\text{O}_2$. All reagents were purchased from Sigma-Aldrich.

NO$\cdot$ D eterrination. NO synthesis by periodate-elicited macrophages challenged with Salmonella as described above was estimated by measuring the accumulation of nitrite ($\text{NO}_2^{--}$) and nitrate ($\text{NO}_3^{--}$), stable metabolites of the reaction of NO with oxygen, using the Griess reaction. The NO$\cdot$ present in supernatants of Salmonella-infected macrophages was measured spectrophotometrically at 550 nm after mixing with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethenediamine hydrochloride in 2.5% acetic acid). The NO$\cdot$ concentration was determined from a standard curve prepared with NaH$_2$O$_2$. The NO$\cdot$ accumulated in the supernatants was estimated by the Griess reaction after enzymatic reduction of NO$\cdot$ to NO$\_2^{--}$ (40, 41) in pH 7 sodium phosphate buffer containing 1.6 U/ml nitrate reductase, 16 U/ml glucose dehydrogenase, 10 $\mu$L NADPH, and 10 mM glucose-6-phosphate. NO$\cdot$ concentration was calculated as the difference between NO$\_2^{--}$ accumulated in the presence and absence of nitrate reductase. At designated time points, the NO$\cdot$ concentrations were determined from S. typhimurium–challenged macrophages cultured for 1 h in serum-free IMDM (Sigma-Aldrich) supplemented with 7.5% minimal essential amino acid solution (Life Technologies), 1% minimal nonessential amino acid solution, 1.1 mM sodium pyruvate (Life Technologies), 1 mg/ml streptomycin sulfate (Sigma-Aldrich), 0.5 mg/ml gentamicin (Sigma-Aldrich), 0.6 mg/ml penicillin G (Life Technologies), 0.75% wt/vol dextrose (Sigma-Aldrich), 0.85% wt/vol NaH$_2$CO$_3$ (Sigma-Aldrich), and 1% nitritidasa–SP (Boehringer). Low background levels of nitrate (~1 $\mu$L) detected in parallel wells were subtracted to exclude a contribution by residual levels of nitrate present in the culture medium.

Immunocytochemistry. Macrophages plated onto sterile coverslips were incubated for 48 h at 37°C in a 5%-CO$_2$ atmosphere. The macrophages were stimulated with IFN-$\gamma$ during the last 20 h and challenged with S. typhimurium strain 14028s as described above. After 90 min of infection, the coverslips were washed with PBS, and the cells were fixed with 2% paraformaldehyde in PBS for 20 min. After extensive washing with 0.1% Tween in PBS, the macrophages were incubated with a 3% normal goat serum solution in Tris balanced solution, pH 7.3. The cells were stained with 5 $\mu$g/ml of a rabbit antinoriyrosine polyclonal antibody (42) or a rabbit antinitrophenol (Zymed Laboratories) polyclonal control antibody for 1 h, followed by a rhodamine-conjugated goat anti–rabbit polyclonal antibody (Jackson Immunoresearch Laboratories) for 1 h. After washing, the coverslips were mounted with Vectashield® (Vector Laboratories) and examined with an Olympus IX70 inverted microscope, a Photometrics PXL camera with Kodak KAF1400 chip (6.7 $\times$ 6.7 $\mu$m physical pixels giving 67 nm per image pixel with a $\times$100 oil immersion objective), and a Silicon Graphic O$_2$ computer with DeltaVision deconvolution software (Applied Precision).

Results

NADPH O xidase– and iN O S–derived Chemical Species C ontribute to the A ntimicrobial A ctivity of M acrophages for Salmo nella. Macrophages from in $\text{OS}^{--}$ or gp91phox$^{--}$ mice exerted less antimicrobial activity to S. typhimurium strain 14028s than macrophages from congenic wild-type control animals (Fig. 1). Macrophages lacking the NADPH oxidase were less effective in their ability to contain Salmonella than congenic cells lacking in NOS, suggesting that $\text{O}_2^{--}$ or its derivatives play a greater role than NO–congeners in Salmonella killing (Fig. 1). The addition of IFN-$\gamma$ enhanced bacterial activity of wild-type macrophages (Fig. 1), and this was correlated with enhanced NO–production (Fig. 2). IFN-$\gamma$ did not increase $\text{O}_2^{--}$ production by wild-type macrophages as measured by reduction of cytochrome $c$ (Fig. 2), and only very modestly increased the antimicrobial activity of inNOS$^{--}$ macrophages (Fig. 1). Similar results were obtained for wild-type S. typhimurium American T ype Culture Collection strain 14028s and wild-type strain M 525P (data not shown).

Temporad D ifferences in iN O S$^{--}$ and NADPH O xidase–mediated M acrophage C ytotoxicity. To study the relative con-
distribution of iNOS and the NADPH oxidase to phagocyte-mediated killing of S. typhimurium in more detail, the cytotoxicity of IFN-γ-activated macrophages from wild-type, iNOS−/−, and gp91phox−/− mice was studied over a period of 14 h (Fig. 3). Macrophages from wild-type C57BL/6 mice exhibited pronounced bactericidal activity towards Salmonella during the first 6 h after challenge. In fact, these macrophages eliminated >99% of the original inoculum during the first 6 h of infection. At later time points, macrophages exhibited cytostatic behavior, confining the bacterial burden to a steady level. Although the NADPH oxidase contributed more than iNOS to overall macrophage antimicrobial activity, substantial temporal differences were observed in the contribution of these systems. Macrophages deficient in the NADPH oxidase did not reduce the original inoculum, but were still able to maintain the bacterial burden at a steady level over time (Fig. 3 A). In contrast, macrophages deficient in iNOS considerably reduced the initial inoculum but were unable to control bacterial replication at later time points (Fig. 3 B). The contribution of iNOS to macrophage antibacterial activity was detectable as early as 2 h after the initial challenge (P < 0.05), but became more substantial over time.

In accord with these observations, macrophages deficient in both the NADPH oxidase and iNOS did not reduce the inoculum at early time points nor achieve subsequent control of bacterial replication (Fig. 3 C). A comparison of macrophages from phox−/− and iNOS−/− gp91phox−/− mice revealed an NO−-dependent antimicrobial activity at later time points that is independent of the NADPH oxidase.

Production of ROS and RNS in Response to Salmonella. Production of ROS and RNS by IFN-γ-activated macrophages from wild-type, iNOS−/−, and gp91phox−/− mice was determined over a 10-h period by chemiluminescence using lucigenin and luminol, and by spectrophotometry using the Griess reagent (Fig. 4). Production of ROS by macrophages from wild-type mice was initiated immediately after phagocytosis, decreasing to undetectable levels by 6 h thereafter (Fig. 4 A). Lucigenin-dependent chemiluminescence was three times lower than that mediated by luminol (Fig. 4, A and B), suggesting that a substantial proportion of the O2− formed by the NADPH oxidase reacts with NO− to form ONOO−. As anticipated, macrophages from phox−/− mice exhibited neither lucigenin- nor luminol-dependent chemiluminescence. Macrophages from iNOS−/− mice showed a considerable diminution in
luminol-dependent chemiluminescence. However, these macrophages generated a prolonged lucigenin-dependent chemiluminescence that persisted for the duration of the experiment.

The rate of NOX production by macrophages from wild-type mice infected with S. typhimurium increased over time (Fig. 4 C). In agreement with previous observations (9), macrophages from gp91phox−/− mice consistently produced more NOX than wild-type controls, likely reflecting a lack of NO scavenging by O2−. Macrophages from iNOS−/− mice appeared to produce very low levels of NOX that almost exclusively consisted of NO3− (Fig. 5, C and E), suggesting that a significant proportion of the NO− attributed to constitutive NOS might react with O2− to form ONOO− (Fig. 4 B). Macrophages from gp91phox−/− mice did not produce detectable quantities of O2−, H2O2, or chemiluminescence. Even at the earliest time point, ~0.4 nmol/h/10^5 macrophages of NOX was produced (Fig. 5 C). The majority of the NO− was oxidized to NO3− (~0.3 nmol/h/10^5 macrophages), although a third was metabolized to NO2− (Fig. 5, D and E). After 10 h of infection, the macrophages increased their production of both NO3− and NO2−. NO3− production greatly exceeded that of NO2− in wild-type, iNOS−/−, and gp91phox−/− macrophages. NO− synthesized by constitutive NOS appear to have a negligible contribution to macrophage antimicrobial activity, as the NOS inhibitor NG-monomethyl l-arginine did not reduce the inhibition of Salmonella by macrophages from iNOS−/− mice (data not shown).

Nitrotyrosine Formation in Salmonella-infected Macrophages. To determine whether ONOO− produced by macrophages targets intracellular Salmonella, formation of nitrotyrosine, a product that can be formed from the reaction of ONOO− with tyrosine residues, was investigated by immunofluorescence microscopy (Fig. 6). In agreement with the biochemical data (Fig. 4), nitrotyrosine was present in wild-type macrophages infected with wild-type S. typhimurium, but absent from NADPH oxidase–deficient control macrophages (Fig. 6, A and C). The presence of nitrotyrosine was markedly reduced, but not totally absent, in macrophages from iNOS−/− mice (Fig. 6 B). Nitrotyrosine labeling failed to colocalize with green fluorescent protein (GFP)-tagged Salmonella in any instance (Fig. 6, A and B), suggesting that ONOO− is formed but may not contribute to bacterial killing. In further support of this notion, the scavenger uric acid did not diminish, but rather enhanced by twofold the bactericidal activity of wild-type macrophages (Fig. 7), suggesting that formation of ONOO− is actually detrimental to macrophage anti-Salmonella activity.

Discussion

Macrophages can kill or limit the replication of intracellular bacteria by producing antimicrobial peptides, lysoso-
mal enzymes, ROS, and RNS. The importance of ROS for macrophage killing of S. typhimurium has been demonstrated (9, 11, 30); however, the participation of RNS has been less clear (9, 24, 30, 31). In this work, we demonstrate that although the NADPH oxidase is more essential than iNOS for Salmonella killing by peritoneal macrophages, iNOS nevertheless contributes to macrophage antibacterial activity against Salmonella in distinct and important ways. The NADPH oxidase is required for rapid initial Salmonella killing by macrophages (Fig. 3A), and iNOS provides a subsequent sustained bacteriostatic effect (Fig. 3B). The failure of previous investigators to demonstrate macrophage NO\textsuperscript{-}\textsuperscript{dependent anti-Salmonella activity may be attributable to a reliance on phagocyte killing assays of relatively brief duration (9, 24, 30). Effector functions of macrophage-derived nitrogen oxides may explain why iNOS is required for resistance to Salmonella infection (10, 43), despite the apparent immunosuppressive actions of NO\textsuperscript{-} on T cells (43, 44).

N O\textsuperscript{-}-dependent anti-Salmonella activity was demonstrable in both untreated and IFN-\gamma-treated macrophages, suggesting that bacterial products such as LPS and DNA (45) can trigger sufficient NO\textsuperscript{-} synthesis to exert antimicrobial activity, at least in partially activated peritoneal cells elicited by sodium periodate. Nevertheless, the addition of IFN-\gamma enhances macrophage anti-Salmonella activity predominantly by increasing NO\textsuperscript{-} production (Figs. 1 and 2).

Although ROS and RNS can exert synergistic antimicrobial actions (14, 17–19, 25), they principally act in sequential fashion in assays of macrophages infected with Salmonella. An early phase of rapid oxidative killing occurs during the peak respiratory burst, followed by a nitrosative bacteriostatic phase (Figs. 3 and 4). The functional separation of these two phases of antimicrobial activity is illustrated by the capacity of macrophages deficient in the NADPH oxidase to maintain their bacterial load at a steady level despite an inability to reduce the original inoculum. In contrast, congenic iNOS-deficient macrophages retained early bacterial killing but were unable to maintain control of subsequent bacterial replication over time. These temporal differences in the antimicrobial behavior of macrophages were paralleled by the early detection of oxidative products followed by a later rise in nitrogen oxides (Fig. 4). The respiratory burst of infected macrophages peaked shortly after phagocytosis, decreasing rapidly thereafter. The brief duration of the respiratory burst may result in part from direct inhibition of NADPH oxidase assembly by NO\textsuperscript{-} congeners (46–49), as macrophages from iNOS\textsuperscript{-/-} mice sustained a more prolonged respiratory burst. Additionally, the increasing abundance of nitrogen oxides over time may be quenching the oxidative chemistry of O2\textsuperscript{-} and ONOO\textsuperscript{-} (50, 51).

The sequential, functionally distinct, and essential roles of ROS and RNS in Salmonella killing or inhibition by macrophages contrast with earlier studies of Leishmania, in which ROS did not appear to play a role (52). Studies of Listeria killing by macrophages have yielded somewhat conflicting results, with various investigators reporting that antilisterial activity is RNS independent (9, 53), ROS de-
S. typhimurium killing by macrophages appears to be completely dependent upon the NADPH oxidase. H₂O₂ is likely to make a major contribution to bacterial killing, as it diffuses rapidly through membranes and reacts with transition metals to form highly toxic hydroxyl radicals. O₂⁻ has also been implicated by observations that sodC mutant bacteria deficient in periplasmic Cu,Zn-superoxide dismutase are hypersusceptible to macrophage killing and exhibit reduced virulence (25, 55, 56).

NO⁻ makes a relatively minor contribution to early macrophage oxidative killing (Fig. 3). O₃O⁻, a reactive molecule capable of mediating 1- and 2-electron oxidations (16) with potent in vitro microbicidal activity for E. coli, S. typhimurium, and C. albicans (17, 18, 25), might account for this activity. However, intracellular bacteria fail to colocalize with nitrotyrosine (Fig. 6), a molecular signature that can be associated with ONOO⁻ synthesis (15, 42), suggesting that ONOO⁻ may not be responsible for early bactericidal effects. The scavenger uric acid actually potentiates Salmonella killing (Fig. 7), further indicating that ONOO⁻ production by Salmonella-containing macrophages may be responsible for host cell autotoxicity rather than antimicrobial activity. Synergistic actions of metal ions, NO·-redox congeners, and H₂O₂, or the production of singlet oxygen from the reaction of NO· and H₂O₂, are alternative mechanisms by which NO·-derived species may potentiate NADPH oxidase-dependent macrophage killing (19, 57, 58).

At this time, the identity of the nitrogen oxides responsible for the sustained NO·-dependent inhibition of bacterial growth are not known, but possibilities include NO· itself, N₂O₃, and S-nitrosothiols, which have been shown to exert bacteriostatic activity for S. typhimurium in vitro (13, 14). The predominant accumulation of NO₃⁻ rather than NO₂⁻ at late time points in the macrophage assay when ONOO⁻ is no longer detectable suggests that dioxygenase activity (59) might be involved in NO· oxidation.

Of the three NOS isoforms that can catalyze the enzymatic production of NO·, iNOS is most closely associated with antimicrobial activity. The contribution of cNOS to the antibacterial activity of macrophages in this study was negligible. The small quantity of NO· attributed to cNOS was oxidized to NO₃⁻ (~0.12 nmol/h/10⁶ macrophages). A small amount of nitrotyrosine staining and NOx production detectable in iNOS-deficient macrophages infected with Salmonella suggests that NO· derived from cNOS can react with O₂⁻, as has been demonstrated in murine M yо-
plasma pulmonis infection (60). These results might indicate that murine mononuclear phagocytes are able to express both inducible and constitutive NOS, as has been suggested by others (61, 62), although functional cNOS has not yet been definitively demonstrated in primary murine peritoneal macrophages.

In conclusion, both the NADPH phagocyte oxidase and iNOS contribute to the ability of macrophages to inhibit or kill S. typhimurium. Analysis of murine peritoneal macrophages reveals a temporally coordinated action of ROS and RNS. Rapid bacterial killing coinciding with production of O$_2^-$ by the NADPH phagocyte oxidase is followed by a prolonged period of inhibition of bacterial growth associated with the production of iNOS-derived nitrosative species. The sequential roles of oxidative and nitrosative phagocyte antimicrobial mediators in vitro are mirrored by the temporal relationship of early NADPH oxidase-dependent and late iNOS-dependent antimicrobial effector mechanisms observed during murine Salmonella infection in vivo (10).

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