Antimicrobial Actions of the NADPH Phagocyte Oxidase and Inducible Nitric Oxide Synthase in Experimental Salmonellosis. II. Effects on Microbial Proliferation and Host Survival In Vivo

By Pietro Mastroeni,*‡ Andrés Vazquez-Torres,§‖¶ Ferric C. Fang,§‖¶ Yisheng Xu,§‖¶ Shahid Khan,* Carlos E. Hormaeche,*** and Gordon Dougan‡

*From the *Centre for Veterinary Science, University of Cambridge, Cambridge CB3 0ES, United Kingdom; the ‡Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom; the §Department of Medicine, the ‖Department of Pathology, and the ¶Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262; and the **Department of Microbiology and Immunology, The Medical School, University of Newcastle, Newcastle upon Tyne, NE2 4HH, United Kingdom*

Abstract

The roles of the NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS) in host resistance to virulent *Salmonella typhimurium* were investigated in gp91*phox*$^{-/-}$, *iNOS*$^{-/-}$, and congenic wild-type mice. Although both gp91*phox*$^{-/-}$ and *iNOS*$^{-/-}$ mice demonstrated increased susceptibility to infection with *S. typhimurium* compared with wild-type mice, the kinetics of bacterial replication were dramatically different in the gp91*phox*$^{-/-}$ and *iNOS*$^{-/-}$ mouse strains. Greater bacterial numbers were present in the spleens and livers of gp91*phox*$^{-/-}$ mice compared with C57BL/6 controls as early as day 1 of infection, and all of the gp91*phox*$^{-/-}$ mice succumbed to infection within 5 d. In contrast, an increased bacterial burden was detected within reticuloendothelial organs of *iNOS*$^{-/-}$ mice only beyond the first week of infection. Influx of inflammatory CD11b^{+} cells, granuloma formation, and serum interferon γ levels were unimpaired in *iNOS*$^{-/-}$ mice, but the iNOS-deficient granulomas were unable to limit bacterial replication. The NADPH phagocye oxidase and iNOS are both required for host resistance to wild-type *Salmonella*, but appear to operate principally at different stages of infection.

Key words: *Salmonella* • virulence • innate immunity • oxidative • nitrosative

Introduction

Salmonella infections afflicting animals and humans pose a serious medical and veterinary problem worldwide. Despite considerable progress in understanding the genetic determinants of *Salmonella* virulence, rational vaccine design is still hampered by an inadequate understanding of the essential elements of host resistance to *Salmonella*. Much of the current knowledge of mechanisms of natural resistance and acquired immunity in salmonellosis has been generated in the mouse typhoid model (1–3), in which polymorphonuclear and mononuclear phagocytes play a critical role by limiting initial bacterial replication (4–10). In immunocompetent mice, bacterial growth in the reticulendothelial system is suppressed by the end of the first week of infection, resulting in a plateau phase (11). This requires an influx of bone marrow–derived mononuclear cells and coincides with the onset of hepatosplenomegaly and granuloma formation, but does not require functional CD4^{+} or CD8^{+} T cells (11). Soluble factors including TNF-α, IFN-γ, IL-12, and IL-18 are essential for the suppression of bacterial growth during sublethal *Salmonella* infections (9, 12–22), and are specifically required for granuloma formation (9, 12, 17) and macrophage activation (14, 20). Secondary infection in immunized mice additionally involves *Salmonella*-immune CD4^{+} and CD8^{+} T cells and *Salmonella*-specific antibodies (3, 17).

Address correspondence to Dr. Pietro Mastroeni, Centre for Veterinary Science, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK. Phone: 44-1223-339868; Fax: 44-1223-337610; E-mail: pm274@cm.ac.uk

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2000/07/237/11 $5.00
Volume 192, Number 2, July 17, 2000 237–247
http://www.jem.org/cgi/current/full/192/2/237

The mechanisms by which phagocytes inhibit or kill virulent salmonellae are incompletely characterized, but the NADPH phagocyte oxidase has been strongly implicated (23, 24). The NADPH phagocyte oxidase catalyzes the production of superoxide ($O_2\cdot^-$) that can be metabolized to a variety of toxic reactive oxygen species (ROS).[1] Patients deficient in the NADPH phagocyte oxidase are susceptible to recurrent microbial infections, including salmonellosis (25), in a clinical condition known as chronic granulomatous disease (CGD; for a review, see reference 26). The gp91*phox* gene encoding an essential component of the NADPH phagocyte oxidase has been targeted in mice, resulting in the elimination of oxidative burst activity in neutrophils and macrophages and an increased susceptibility to microorganisms including *Salmonella typhimurium* (24), *Aspergillus fumigatus*, *Staphylococcus aureus* (26), and *Mycobacterium tuberculosis* (27).

The role of the inducible nitric oxide (NO) synthase (iNOS) in the ability of macrophages to inhibit or kill *Salmonella* has been somewhat less clear (28, 29). Dimeric iNOS catalyzes the conversion of l-arginine to l-citrulline with the production of NO·, which can be further metabolized to a variety of congeners (reactive nitrogen species [RNS]) with antimicrobial activity (30). Activated macrophages display increased expression of iNOS and elevated production of NO· derivatives (for a review, see reference 30). The role of NO· derivatives in host resistance to microbes has been documented in vitro and in vivo in a large number of infection models (for a review, see reference 31).

Generation of $O_2\cdot^-$ and NO· derivatives in response to *Salmonella* infection has been documented both in vivo (20, 32) and in vitro (24, 33–35). The activity of iNOS is positively regulated by several cytokines (IL-12, IFN-γ, TNF-α) known to be essential for host resistance to *Salmonella* (12, 20, 30). Evidence obtained using metabolic inhibitors and immunodeficient knockout mice suggests that oxygen radicals mediate resistance to *Salmonella* in mice and are required for maximal bacterial killing by macrophages (24, 29). Furthermore, data obtained using NO synthase (NOS) inhibitors L-NMMA or aminoguanidine support a role for NO· derivatives in host resistance to *Salmonella* (32, 33, 36). However, some evidence from in vitro models has suggested that the respiratory burst oxidase, but not iNOS, is essential to kill virulent *Salmonella*, whereas both ROS and RNS are involved in the killing of some mutant *Salmonella* strains (24, 28, 29). Moreover, observations using the NOS-inhibitor aminoguanidine have suggested that iNOS may regulate infiltration of inflammatory cells in the tissues, rather than exert direct antimicrobial activity against *Salmonella* (36).

Many of the published studies examining phox-dependent and iNOS-dependent host defenses in salmonellosis have used attenuated bacterial strains and innately susceptible mice, making it somewhat difficult to ascertain the role of these defenses in infections with virulent *Salmonella* (29, 32, 33, 36). The goal of this study was to clarify the roles of the NADPH phagocyte oxidase and iNOS in limiting bacterial replication during experimental infection with virulent *S. typhimurium*, using gp91*phox*$^{-/-}$ mice in a *Salmonella*-susceptible C57BL/6 (*Nramp1*^{s}) genetic background, and *iNOS*$^{-/-}$ mice in both C57BL/6- (*Nramp1*^{s}) and *Salmonella*-resistant 129Sv (*Nramp1*^{r}) genetic backgrounds. Sublethal inocula of wild-type *Salmonella* were administered to facilitate the detection of phox-dependent and iNOS-dependent antimicrobial actions.

Materials and Methods

Animals. C57BL/6 mice (*Nramp1*^{s}) and 129Sv mice (*Nramp1*^{r}) were purchased from Harlan Olac, Ltd. Mice homozygous for a targeted mutation in the gp91 subunit of the NADPH oxidase (gp91*phox*$^{-/-}$) on a C57BL/6 background (26) were bred at the University of Colorado School of Medicine Center for Laboratory Animal Care. Mice homozygous for a targeted mutation in the iNOS gene (*iNOS*$^{-/-}$ [37, 38]) on a C57BL/6 or 129Sv background were bred at either BαK Universal, Ltd. or the Imperial College animal unit.

Bacteria. *S. typhimurium* M525P, a variant of *S. typhimurium* M525 (18), is a wild-type strain of intermediate virulence. *S. typhimurium* C5 is a highly virulent strain (6). For intravenous inoculation, bacteria were grown at 37°C as stationary overnight cultures in Luria-Bertani (LB) broth (Difco). Aliquots were snap frozen and stored in liquid nitrogen. The inoculum was diluted in PBS and injected in a lateral tail vein. The number of viable bacteria in each inoculum was checked by dilution and pour plating onto LB agar plates.

Bacterial Enumeration in Organ Homogenates. Mice were killed by cervical dislocation. Spleens and livers were aseptically removed and homogenized in a Colworth Stomacher in 10 ml of cold distilled water (11). Viable counts were determined using pour plates of LB agar.

LD_{50} Determinations. Groups of mice were injected intravenously with 10-fold decreasing doses of *S. typhimurium* M525P or *S. typhimurium* C5. Parallel groups of age-matched uninfected mice were observed in parallel during each experiment. Mortality was scored over a 30-d period. LD_{50} values were calculated according to the method of Reed and Muench (39).

IFN-γ ELISA. Mice were bled from a lateral tail vein. Sera were collected and stored at −70°C. IFN-γ was measured by capture ELISA using antibody pairs and cytokine standards purchased from BD PharMingen.

For IFN-γ determinations, 96 multiwell ELISA plates (MaxiSorp Immuno Plate; Nunc) were coated overnight at 4°C with 50 μl/well of a rat anti–mouse IFN-γ IgG1 monoclonal capture antibody (clone R4-6A2) in 0.1 M $NaHCO_3$ buffer, pH 9.5 at 2 μg/ml. After blocking with PBS supplemented with 10% FCS at 37°C for 1 h, twofold serum dilutions were loaded in 50 μl in triplicate, and the plates were incubated at 37°C for 2 h. Serial twofold dilutions of rIFN-γ ranging from 20 ng/ml to 40 pg/ml were included as standards. Biotinylated rat anti–mouse IFN-γ IgG1 mAb (clone XMG1.2; 100 μl/well) at 1 μg/ml in PBS supplemented with 10% FCS was added for 1 h at 37°C, after which 100 μl/well of peroxidase-labeled streptavidin at 2.5 μg/ml (Sigma-Aldrich) in PBS supplemented with 10% FCS was added for 45 min at room temperature. *Ortho*-phenylenediamine

[1]*Abbreviations used in this paper:* iNOS, inducible nitric oxide synthase; LB, Luria-Bertani; NO, nitric oxide; NOS, nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

(OPD; 1 mg/ml in 0.2 M Na_2HPO_4, 0.1 M citrate buffer) in the presence of H_2O_2 was used to develop the plates. The reaction was stopped by adding 15 μl/well of 3 M H_2SO_4. OD was read at 490 nm. IFN-γ values (ng/ml) were determined by comparison with the standard curve. 80 pg/ml was considered to be the lower limit of sensitivity of this IFN-γ ELISA test.

Histology. 8-μm thick sections were prepared from tissues fixed by immersion in 10% formalinized saline, and were stained with hematoxylin and eosin. The number of focal lesions in several nonserial liver sections was counted at low-power magnification (×10) using a Nikon Labophot 2 microscope. The results were obtained by counting 15 fields per organ.

Staining for Flow Cytometry Analysis. Single cell suspensions prepared from mouse spleens were washed once in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS containing 2 mM EDTA (Sigma-Aldrich) by centrifugation at 300 *g* and incubation in Gey's solution to lyse the red cells. White cells were washed twice more before being resuspended at a concentration of 10^7/ml in PBS containing 1% FCS, 0.1% NaN_3, and 2 mM EDTA. All further steps were performed on ice. Antibodies for flow cytometry were purchased from BD PharMingen, with the exception of the anti–I-A^b MHC class II FITC-conjugated antibody, which was obtained from Caltag Laboratories. FcγIII/II receptors were blocked by incubating cells with the rat anti–mouse CD16/CD32 (IgG2b; clone 2.4G2) mAb for 10 min. The following antibodies were used: FITC–anti-CD4 (IgG2a; clone RM4-4), FITC–anti-CD8 (IgG2a; clone 53-6.7), PE–anti-CD11b (IgG2b; clone M1/70), FITC–anti-B220 (IgG2a; clone RA3-6B2), PE–anti-CD25 (IgG1; clone PC61), and FITC–anti–I-A^b MHC class II (IgM; clone 28-16-8S). Similarly conjugated isotype control antibodies were used in parallel. Cells were stained for 30 min with the relevant antibodies, washed with PBS, fixed with 1% paraformaldehyde, and kept in the dark until use. 1,000 events were acquired on a Becton Dickinson FACScan™.

Statistical Analysis. Student's *t* test was used to determine the significance of differences between controls and experimental groups. Differences between experimental groups were considered significant for P values <0.05.

Results

Mortality of S. typhimurium Infection in Wild-type, gp91 phox$^{-/-}$, and iNOS$^{-/-}$ Mice. gp91*phox*$^{-/-}$ mice and congenic C57BL/6 wild-type control mice were infected intravenously with ~600 CFU of *S. typhimurium* M525P. All gp91*phox*$^{-/-}$ mice were dead by day 4–5 of infection, despite the modest inoculum, whereas no deaths were observed among the C57BL/6 controls (Fig. 1 A). Thus, NADPH phagocyte oxidase–dependent antimicrobial mechanisms are required for host resistance in the early stages of *Salmonella* infection in mice.

Groups of five to seven *iNOS*$^{-/-}$ mice on the *Salmonella*-susceptible C57BL/6 or *Salmonella*-resistant 129Sv strain backgrounds were infected intravenously with 10-fold decreasing doses ranging from 10^7–10^3 CFU of moderately virulent *S. typhimurium* M525P or the highly virulent *S. typhimurium* C5 strain, respectively. Congenic wild-type C57BL/6 or 129Sv control mice were infected in parallel experiments. Log_{10} LD_{50} values 1 mo after infection were found to be 3.35 ± 0.06 in C57BL/6 *iNOS*$^{-/-}$ mice and 4.29 ± 0.19 in 129Sv *iNOS*$^{-/-}$ mice, compared with 5.42 ± 0.22 and 5.28 ± 0.07 in the respective control groups. Thus, *iNOS*$^{-/-}$ mice show increased susceptibility to *Salmonella*, but can still survive infection with low bacterial inocula over a 30-d observation period. This contrasts with the gp91*phox*$^{-/-}$ mice, for whom even a very modest inoculum was rapidly lethal.

On the basis of the LD_{50} determinations, seven C57BL/6 *iNOS*$^{-/-}$ mice and seven congenic wild-type controls were infected intravenously with ~10^5 CFU of *S. typhimurium* M525P (Fig. 1 B). Similarly, nine 129Sv *iNOS*$^{-/-}$ mice and nine congenic wild-type mice were infected with ~10^5 CFU of *S. typhimurium* C5 (Fig. 1 C). All of the C57BL/6 *iNOS*$^{-/-}$ mice died within 18 d after inoculation

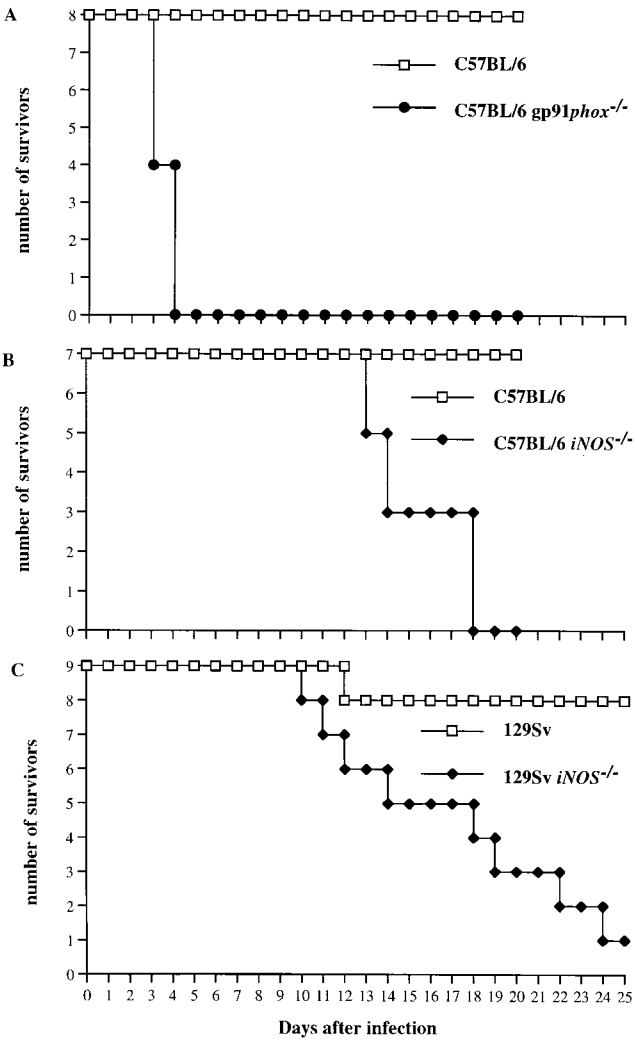

Figure 1. Survival of gp91*phox*$^{-/-}$, *iNOS*$^{-/-}$, and congenic wild-type mice infected with *S. typhimurium*. (A) Eight gp91*phox*$^{-/-}$ mice and eight congenic C57BL/6 controls (*Nramp1*^{s}, *Salmonella* susceptible) were infected intravenously with ~600 CFU of *S. typhimurium* M525P, a strain of intermediate virulence. (B) Seven *iNOS*$^{-/-}$mice and seven congenic C57BL/6 controls (*Nramp1*^{s}) were infected intravenously with ~10^5 CFU of *S. typhimurium* M525P. (C) Nine *iNOS*$^{-/-}$ mice and nine congenic 129Sv controls (*Nramp1*^{r}, *Salmonella* resistant) were infected with ~10^5 CFU of the highly virulent *S. typhimurium* C5 strain. Results are expressed as number of survivors at times after infection.

(with deaths beginning on day 13), whereas all of the C57BL/6 wild-type mice survived the infection. Similarly, eight out of nine (89%) *iNOS*$^{-/-}$ mice in the 129Sv background died within 24 d after inoculation (with deaths beginning on day 10), whereas only one of nine (11%) wild-type control mice died throughout the course of the experiment. Thus, *iNOS*$^{-/-}$ mice infected with *S. typhimurium* show increased susceptibility to infection that leads to death of the animals during the second and third week of infection. No deaths were observed in parallel groups of five uninfected *iNOS*$^{-/-}$ or wild-type mice of either strain.

S. typhimurium Organism Burden After Infection of Wild-type, gp91phox$^{-/-}$*, and iNOS*$^{-/-}$ *Mice.* Hepatic and splenic bacterial burden was quantified in gp91*phox*$^{-/-}$ and congenic C57BL/6 wild-type control mice after intravenous infection with ~600 CFU of *S. typhimurium* M525P. Bacterial counts by day 1 of infection were already significantly higher in the NADPH phagocyte oxidase–deficient mice compared with control animals, and were dramatically higher by day 3 of infection (Fig. 2, A and B).

In parallel experiments, *iNOS*$^{-/-}$ and congenic C57BL/6 wild-type mice were infected with 6.5×10^4 CFU of *S. typhimurium* M525P, whereas *iNOS*$^{-/-}$ and congenic 129Sv wild-type controls were infected with 8.5×10^4 CFU of *S. typhimurium* C5. Fig. 2, C and D shows that spleen and liver bacterial counts were similar in C57BL/6- and *iNOS*$^{-/-}$-derivative mice during the first 5 d of infection, despite the administration of higher inocula than those administered to the gp91*phox*$^{-/-}$ mice. However, by days 8 and 12, the counts in the livers of *iNOS*$^{-/-}$ mice were significantly higher than those of C57BL/6 control mice; by day 21, the bacterial counts in both spleens and livers of C57BL/6 *iNOS*$^{-/-}$ mice were considerably higher in comparison to controls. The course of infection and differences in bacterial counts between mutant and wild-type mice were similar when a lower inoculum (6.5×10^3 CFU) was administered, except for lower absolute values and an absence of deaths observed throughout the 30-d experiment (data not shown). Fig. 2, E and F shows the course of the infection with *S. typhimurium* C5 in 129Sv and congenic *iNOS*$^{-/-}$ mice. Bacterial counts in *iNOS*$^{-/-}$ mutant and wild-type mice were similar until day 8 of the infection. Thereafter, both spleen and liver counts were significantly higher in the *iNOS*$^{-/-}$ mice. Repeat experiments gave similar results (data not shown).

Histopathology of S. typhimurium Infection in Wild-type, gp91phox$^{-/-}$*, and iNOS*$^{-/-}$ *Mice.* Spleen weight was monitored at specified times in the experiments described in Fig. 2. The spleens of infected gp91*phox*$^{-/-}$ increased in size more rapidly ($P < 0.01$) than those of congenic

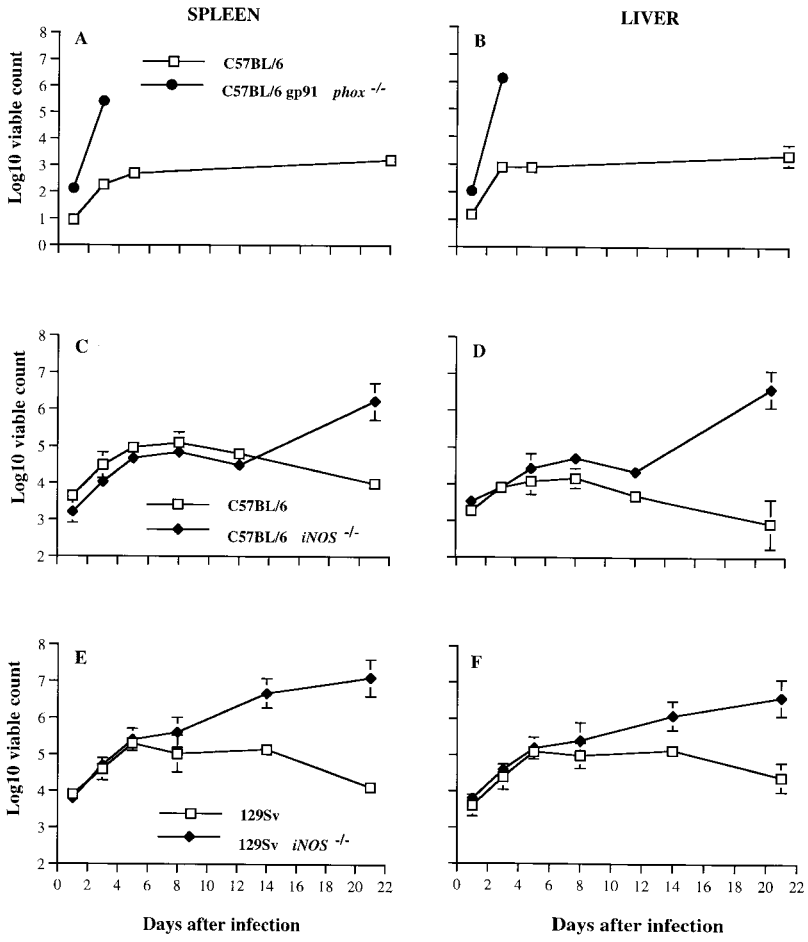

Figure 2. Bacterial counts in liver and spleen of gp91*phox*$^{-/-}$, *iNOS*$^{-/-}$, and congenic wild-type mice infected with *S. typhimurium*. (A and B) gp91*phox*$^{-/-}$ mice and congenic C57BL/6 controls (*Nramp1*^{s}) were infected intravenously with ~600 CFU of *S. typhimurium* M525P, a wild-type strain of intermediate virulence. All *Salmonella*-infected gp91*phox*$^{-/-}$ mice were dead by day four. (C and D) *iNOS*$^{-/-}$ mice and congenic C57BL/6 controls (*Nramp1*^{s}) were infected with 6.5×10^4 CFU of *S. typhimurium* M525P. (E and F) *iNOS*$^{-/-}$ mice and congenic 129Sv controls (*Nramp1*^{r}) were infected with 8.5×10^4 CFU of the highly virulent wild-type *S. typhimurium* C5 strain. Spleen and liver counts were determined at time points thereafter. The results are expressed as Log_{10} viable count (mean ± SD) obtained from groups of four mice per data point.

C57BL/6 wild-type control mice (Fig. 3 A) after infection with 600 CFU *S. typhimurium* M525P, in parallel with organism burden. As shown in Fig. 3 B, a similar increase in spleen weight was observed during the first 8 d of the infection in C57BL/6 $iNOS^{-/-}$ mice and C57BL/6 mice infected with 6.5×10^4 CFU of *S. typhimurium* M525P. The mean spleen weight was slightly higher in C57BL/6 mice than in C57BL/6 $iNOS^{-/-}$on day 12 ($P = 0.02$), but no significant differences in spleen weight between $iNOS^{-/-}$ mutant and wild-type mice were observed by day 21 of the infection. A similar experiment performed with a lower challenge dose (6.5×10^3 CFU per mouse) showed similar spleen weights in $iNOS^{-/-}$ mutant and C57BL/6 wild-type mice at all time points (data not shown). Fig. 3 C shows that significant ($P = 0.02$) differences in spleen weight between $iNOS^{-/-}$ mice and congenic 129Sv wild-type mice infected with 8.5×10^4 CFU of *S. typhimurium* C5 appeared by day 8 after infection and persisted until at least day 14.

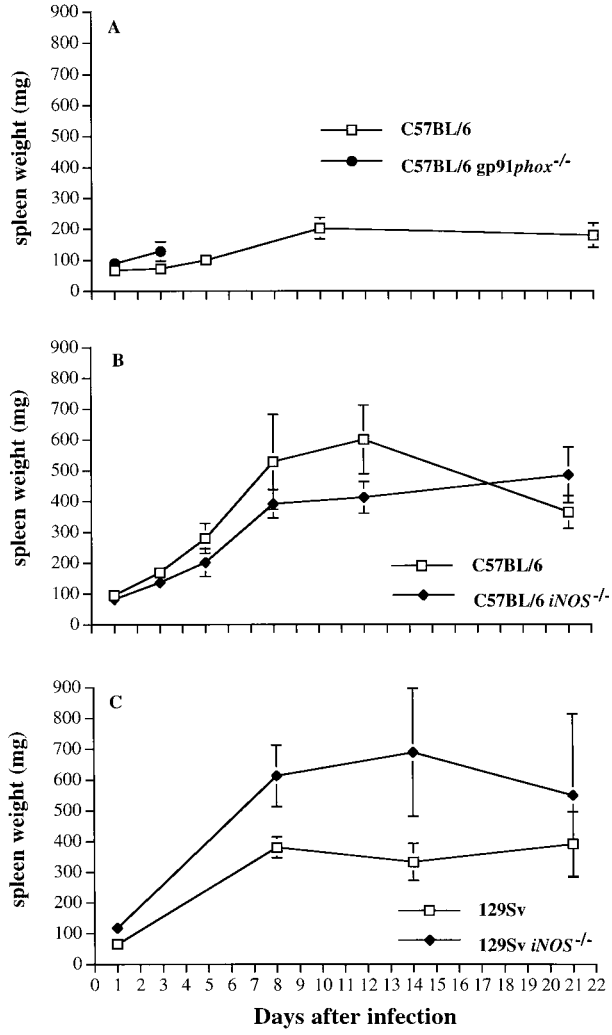

Figure 3. Spleen weights of gp91*phox*$^{-/-}$, $iNOS^{-/-}$, and congenic wild-type mice infected with *S. typhimurium*. Mice were infected as described in the legend to Fig. 2. Spleen weights were measured from (A) infected gp91*phox*$^{-/-}$ and congenic C57BL/6 mice, (B) $iNOS^{-/-}$ and congenic C57BL/6 mice, and (C) $iNOS^{-/-}$ mice and congenic 129Sv mice at designated time points. Results are expressed as mean spleen weight ± SD for four mice per data point.

Microscopic examination of livers of C57BL/6 and congenic gp91*phox*$^{-/-}$ and $iNOS^{-/-}$ mice revealed the early formation of microabscesses rich in polymorphonuclear neutrophils by day 3 of infection (Fig. 4, A–C), with more extensive lesions noted in the gp91*phox*$^{-/-}$ animals. On days 8, 11, and 14, the focal lesions in the spleens and livers of the infected C57BL/6 and $iNOS^{-/-}$ mice consisted mainly of mononuclear cells and were surrounded by normal tissue. This evolution of the inflammatory response (10, 18) occurred similarly in both $iNOS^{-/-}$ mice (Fig. 4, D and F) and C57BL/6 mice (Fig. 4, E and G), showing no iNOS dependence on the formation of mononuclear cell–rich granulomas. In several $iNOS^{-/-}$ mice it was visually evident that the average size of focal granulomas was larger than in congenic wild-type control mice. On day 21 of infection, granulomatous lesions present in the livers and spleens of $iNOS^{-/-}$ mice in the C57BL/6 background showed a variable degree of central necrosis (Fig. 4, H–K); macroscopically visible abscesses were present in the livers and occasionally in the spleens. The histological picture in organs of wild-type 129Sv and congenic $iNOS^{-/-}$ mice infected with *S. typhimurium* C5 was similar to that described in the C57BL/6 strain background, except for the absence of central necrosis in the hepatic and splenic lesions.

The focal lesions in several liver sections were counted at low-power magnification. As shown in Fig. 5, similar numbers of inflammatory foci were present in the livers of $iNOS^{-/-}$ and congenic C57BL/6 mice on day 3 (predominantly neutrophils) and day 8 (predominantly mononuclear cells) of infection, but the numbers of mononuclear cell–rich lesions were significantly higher in C57BL/6 $iNOS^{-/-}$ mice by days 12 and 21. Similar numbers of inflammatory foci were found within the livers of $iNOS^{-/-}$ and congenic 129Sv mice on day 3 of infection, but thereafter the number of lesions was significantly higher in the livers of the $iNOS^{-/-}$ mice. Thus, both $iNOS^{-/-}$ mice and congenic wild-type mice develop splenomegaly and form granulomas in the spleen and liver during experimental salmonellosis.

Flow Cytometry of Splenocyte Populations in Salmonella-infected Wild-type and $iNOS^{-/-}$ Mice. Studies in mice treated with iNOS inhibitors have suggested that iNOS might be required for the recruitment of inflammatory cells to the spleen (36). The percentages of $CD11b^+$ cells and I-A^{b+} $CD11b^+$ cells were determined on days 3, 8, and 15 or 16 in the spleens of *Salmonella*-infected wild-type and $iNOS^{-/-}$ mice in experiments parallel to those depicted in Fig. 2. $CD11b^+$ cells and I-$A^{b+}CD11b^+$ cells were recruited to infected spleens of $iNOS^{-/-}$ mice and wild-type mice to a similar degree and, at some time points, higher percentages of inflammatory cells were present in the spleens of $iNOS^{-/-}$ mice compared with wild-type mice (Table I). The percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD25^+$ cells, and $B220^+$ cells in the spleens of wild-type and

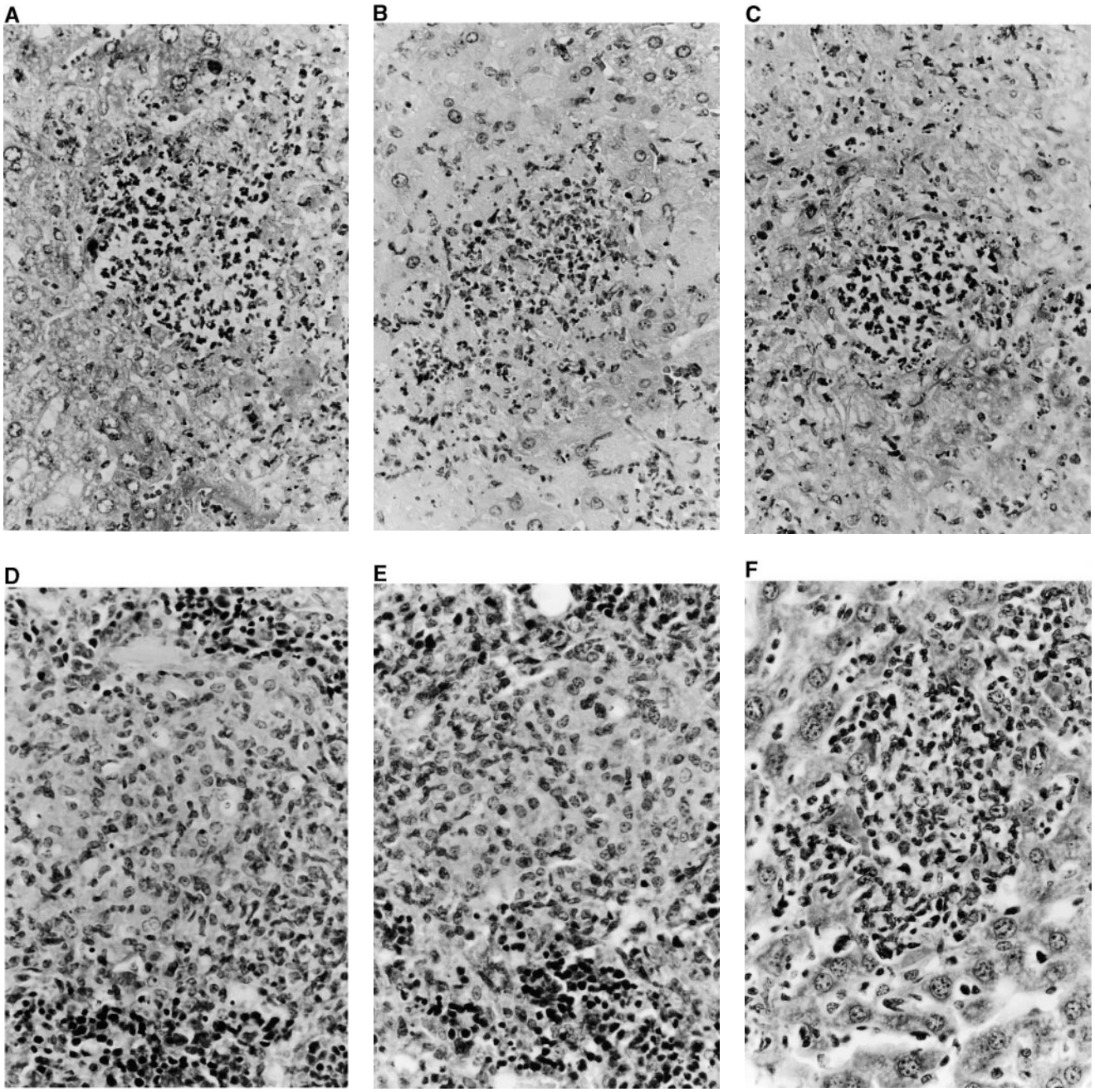

Figure 4. *(continues on facing page).* Microscopic appearance of livers and spleens from gp91*phox*$^{-/-}$, *iNOS*$^{-/-}$, and congenic wild-type mice infected with *S. typhimurium*. C57BL/6, gp91*phox*$^{-/-}$, and *iNOS*$^{-/-}$ were infected as described in the legend to Fig. 2. Hematoxylin and eosin–stained sections were prepared from spleens and livers harvested at time points thereafter. Microabscesses were seen in the livers of (A) gp91*phox*$^{-/-}$, (B) *iNOS*$^{-/-}$, and (C) wild-type animals on day 3. Granulomatous lesions were seen within the (D and E) spleens and (F and G) livers of (D and F) *iNOS*$^{-/-}$ and (E and G) wild-type C57BL/6 mice on day 8 (original magnification: ×200). The histopathology from days 11 and 14 closely resembled that observed on day 8. Necrotic lesions were detected in the (H and I) livers and (J and K) spleens of *iNOS*$^{-/-}$ mice on day 21. Approximate original magnifications: A–G, ×400; H, ×60; I, ×200; and J–K, ×400.

iNOS$^{-/-}$ mice were also comparable (not shown). The results shown are representative of three individual experiments that yielded similar results. Thus, the greater bacterial burden observed in *iNOS*$^{-/-}$ mice (Fig. 2, C–F) cannot be attributed to an impairment in the infiltration of inflammatory cells.

Serum IFN-γ Levels in Salmonella-infected Wild-type and iNOS$^{-/-}$ *Mice.* Serum IFN-γ was measured by ELISA in the sera of six *Salmonella*-infected wild-type and six congenic *iNOS*$^{-/-}$ mice at days 3, 7, 11, 16, and 21 of infection. No statistically significant differences ($P > 0.05$) in serum IFN-γ levels between *iNOS*$^{-/-}$ and C57BL/6 wild-

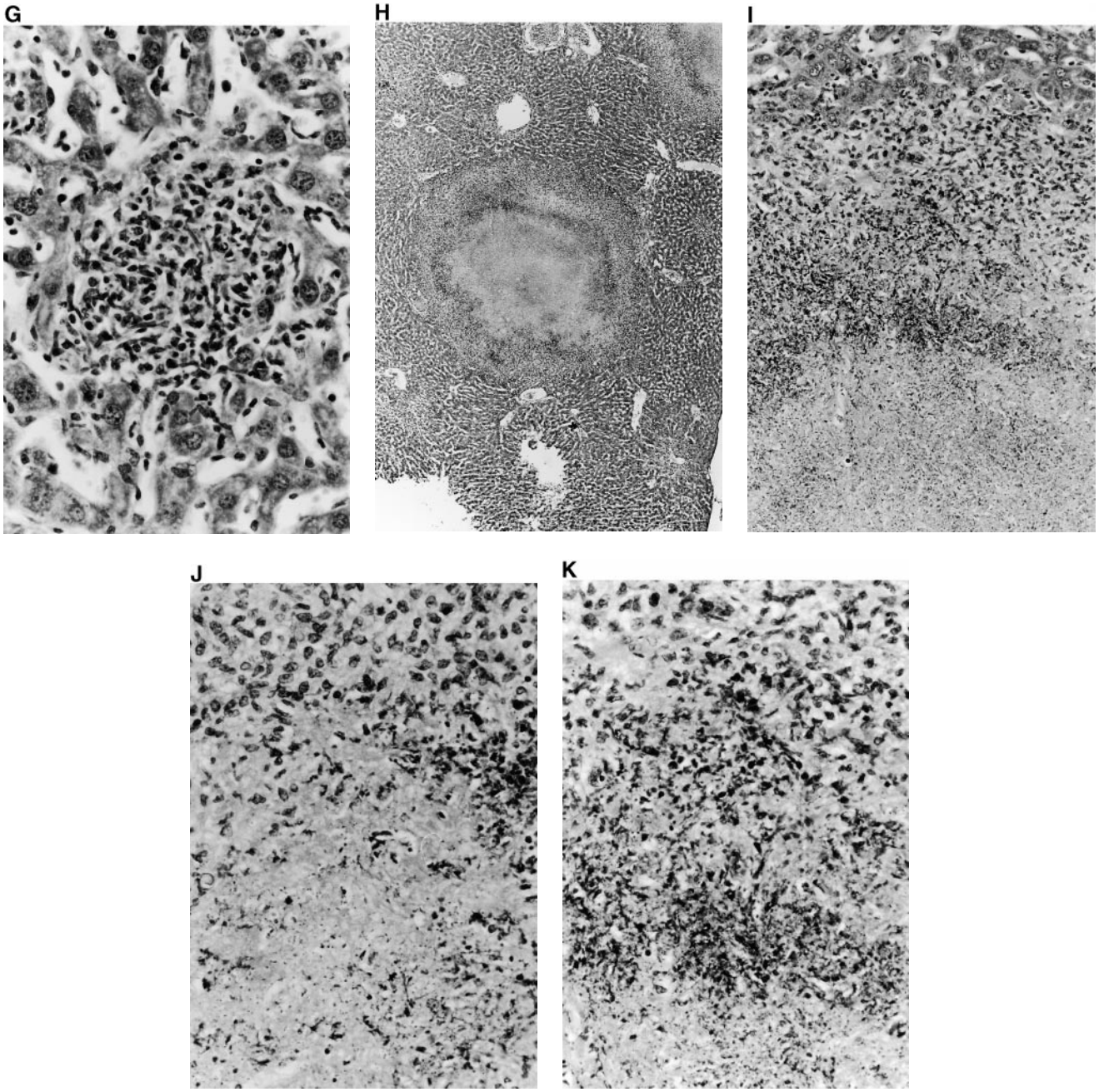

Figure 4. *(continued).*

type mice after infection with *S. typhimurium* M525P were detected on days 3, 7, and 11. Serum IFN-γ levels at later time points were statistically higher ($P < 0.05$) in $iNOS^{-/-}$ mice (243 ± 109 pg/ml on day 16; 430 ± 44 pg/ml on day 21) than in C57BL/6 wild-type controls (82 ± 62 pg/ml on day 16; 75 ± 9 pg/ml on day 21). Similarly, no differences in serum IFN-γ levels between $iNOS^{-/-}$ and congenic 129Sv wild-type mice infected with *S. typhimurium* C5 were detected on days 3, 7, and 11, but IFN-γ levels on days 16 and 21 of infection were statistically higher ($P < 0.05$) in the $iNOS^{-/-}$ mice (500 ± 99 pg/ml on day 16; 710 ± 144 pg/ml on day 21) than in the 129Sv wild-type controls (156 ± 130 pg/ml on day 16; 200 ± 49 pg/ml on day 21). Thus, iNOS deficiency does not impair circulating levels of IFN-γ during *Salmonella* infection. Higher levels of IFN-γ at later time points are probably attributable to increased organism burden, but may also reflect regulatory effects of NO· on cytokine production.

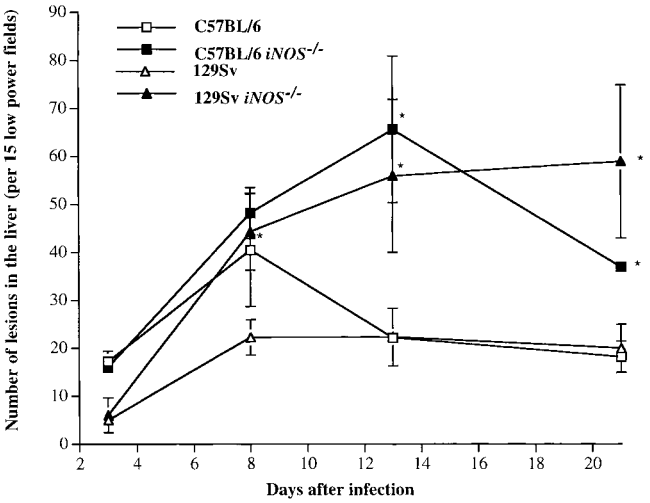

Figure 5. Quantitation of focal hepatic lesions. Lesions were enumerated from $iNOS^{-/-}$ and congenic C57BL/6 mice infected with *S. typhimurium* M525P or $iNOS^{-/-}$, and from congenic 129Sv mice infected with *S. typhimurium* C5 as described in the legend to Fig. 2. Results are shown as numbers of lesions in 15 low-power fields (mean ± SD from groups of four mice). $*P \leq 0.01$.

Table I. *Flow Cytometry*

	$CD11b^+$	$I\text{-}A^{b+}CD11b^+$
Day 3		
C57BL/6	12.45	5.0
C57BL/6 $iNOS^{-/-}$	6.4	5.5
129	8.82	5.3
129 $iNOS^{-/-}$	12.8	8.2
Day 8		
C57BL/6	12.11	7.69
C57BL/6 $iNOS^{-/-}$	15.1	7.3
129Sv	14.4	11.3
129Sv $iNOS^{-/-}$	25.1	22.7
Days 15–16		
C57BL/6	12.8	6.83
C57BL/6 $iNOS^{-/-}$	21.3	9.05
129Sv	14.0	8.6
129Sv $iNOS^{-/-}$	23.0	12.7

Percentages of $CD11b^+$ and $CD11b^+I\text{-}A^{b+}$ double positive cells in the spleens of $iNOS^{-/-}$ and congenic C57BL/6 mice infected with *S. typhimurium* M525P, or $iNOS^{-/-}$ and congenic 129Sv mice infected with *S. typhimurium* C5. Mice were infected as described in the legend to Fig. 2. Results are expressed as percentages of total cells. The data are representative of three individual experiments that yielded similar results.

Discussion

Detailed study of the course of *Salmonella* infection in gp91$phox^{-/-}$ or $iNOS^{-/-}$ mice has revealed major differences in the temporal contribution of the NADPH phagocyte oxidase and iNOS to host defense in salmonellosis. Enhanced bacterial proliferation was observed in gp91 $phox^{-/-}$ mice as early as day 1 after infection. In contrast, $iNOS^{-/-}$ mice effectively controlled bacterial growth in the tissues during the first week of infection, but a lethal increase in bacterial burden appeared later in the course of infection.

Early killing of bacteria by resident phagocytes and acute inflammatory cells during the first 24 h of infection appears to be heavily dependent on the presence of an intact NADPH phagocyte oxidase. These in vivo observations correlate well with in vitro studies, suggesting a crucial role for the oxidative burst in the rapid killing of ingested salmonellae by macrophages (28, 29, 35). The accelerated replication of *Salmonella* in gp91$phox^{-/-}$ mice that already possess an innately susceptible $Nramp1^s$ (G169D) allele (40) reveals a degree of independence between Nramp1 and oxidative burst–dependent host defenses, and shows that $Nramp1^s$ mice still retain potent antimicrobial mechanisms during the early phases of *Salmonella* infection. Indeed, Nramp1 (*Ity*) is believed to limit bacterial growth rather than enhance bacterial killing in vivo (41), whereas NADPH-mediated oxidative mechanisms have been shown to mediate killing of *Salmonella* by mononuclear cells (28, 29, 35). iNOS expression in the tissues can be detected very early in the course of a *Salmonella* infection (within 24 h) (32, 34; our unpublished observations), raising the possibility of synergistic antimicrobial interactions between ROS and RNS, as have been shown for *Escherichia coli* and *S. typhimurium* in vitro (35, 42) and for *sodC*-deficient *S. typhimurium* in vivo (24). However, such interactions are unlikely to be essential for the early killing of wild-type *S. typhimurium* in this experimental model, because iNOS was dispensable for the control of bacterial replication during the initial days of infection.

The early bactericidal role of the NADPH phagocyte oxidase followed by a late essential bacteriostatic role of iNOS mirrors our observations in elicited peritoneal phagocytes (35). Although high levels of superoxide production during initial phagocyte–pathogen interactions (with or without the presence of smaller quantities of NO·) can generate a variety of oxidant species, the subsequent predominant production of NO· over time would be predicted to lead to a progressive conversion to nitrosative chemistry (35, 43). The tenuous stability of the activated NADPH phagocyte oxidase complex (44), and perhaps even its direct inhibition by nitrogen oxides (45), might contribute to the evolution of the host response from ROS to RNS dependence. This temporal progression of host defense may also be accounted for by the greater reliance of acute inflammatory neutrophils and resident mononuclear cells on oxidative killing mechanisms, succeeded by an influx of NO·-producing activated macrophages. Although oxidative bacterial killing by neutrophils is highly effective (46), organisms persisting within mononuclear cells (10) may require slower iNOS-dependent mechanisms for their eventual clearance.

In murine typhoid, iNOS is positively modulated by IFN-γ and TNF-α (12, 20); therefore, it could be hypothesized that IFN-γ and TNF-α mediate the suppression of bacterial growth in the tissues of infected mice via NO·-dependent mechanisms. However, there are important distinctions among the courses of infection in cytokine-deficient, gp91*phox*$^{-/-}$, and *iNOS*$^{-/-}$ mice. In contrast to *iNOS*$^{-/-}$ mice, whole body X-irradiated mice, IFN-γ$^{-/-}$ mice, IFN-γR$^{-/-}$ mice, TNFR55$^{-/-}$ mice, and mice treated with anti–IFN-γ or anti–TNF-α neutralizing antibodies fail to suppress the early growth of salmonellae in the reticuloendothelial system (11–13, 16; our unpublished observations), and succumb within 7–8 d after challenge with wild-type *Salmonella* under experimental conditions similar to those used in this study (our unpublished observations). These observations indicate that IFN-γ and TNF-α do not exclusively enhance innate immunity via NOS activation. Later stages of *Salmonella* infection are controlled by T cell–mediated immunity, and mouse strains lacking T cells such as *nu/nu* mice, TcR-α/β$^{-/-}$ mice, recombination activating gene (*rag*)*1*$^{-/-}$ mice, or *scid* mice fail to clear *Salmonella* and succumb to infection despite initial suppression of early bacterial replication (13, 47; our unpublished observations). At this time, we cannot exclude that some degree of impairment in T cell responses to *Salmonella* might occur in *iNOS*$^{-/-}$ gene-targeted mutant mice, but recent evidence from mouse models of *Trypanosoma brucei* and *Mycobacterium avium* infection shows that iNOS-deficient mice can mount effective T cell responses despite the absence of high-output NO· production (48, 49).

Early histopathological examination of *Salmonella*-infected mice revealed microabscesses containing neutrophils in all strains, but lesions were most extensive with surrounding necrosis in gp91*phox*$^{-/-}$ animals. *iNOS*$^{-/-}$ and wild-type mice progressed to develop macrophage-rich granulomas by day 8, but all gp91*phox*$^{-/-}$ had succumbed to uncontrolled infection by this time point. We observed the development of central necrosis in some granulomas during later stages of infection in *Nramp1*^{s} C57BL/6 *iNOS*$^{-/-}$ mice but not in wild-type mice or *Nramp1*^{r} 129Sv *iNOS*$^{-/-}$ mice, possibly reflecting increased bacterial proliferation leading to the death of the phagocytic and parenchymal cells in the more susceptible mouse strain. Dysregulation of cytokine production in the absence of RNS may also be contributing to the development of necrosis (50, 51).

Results obtained after chemical iNOS inhibition during *Salmonella* infection have suggested that impaired RNS production can lead to a reduction of macrophage tissue infiltration and granuloma formation (32, 36). Nitrogen oxides are known to play a role in the positive regulation of macrophage inhibitory protein (MIP)-1α, a powerful chemoattractant for macrophages (51), and have additional important immunoregulatory properties (52). However, in this study we found the morphology of granulomatous lesions to be very similar in the tissues of *iNOS*$^{-/-}$ and congenic wild-type mice infected with *S. typhimurium*. In fact, focal lesions and numbers of CD11b^{+} cells were even more numerous in *iNOS*$^{-/-}$ mice during late stages of infection compared with wild-type mice. Increased granuloma formation, which has also been noted in *iNOS*$^{-/-}$ mice infected with *M. avium* (53), may reflect inhibitory effects of NO· on leukocyte recruitment (54). We have also shown that the influx of inflammatory cells in the spleen as measured by flow cytometry, the development of splenomegaly, the elevation of serum IFN-γ levels, and the activation of inflammatory cells as measured by I-A^{b} expression (12, 55) are not impaired in *iNOS*$^{-/-}$ mice. Elevation of serum IFN-γ levels has also been described in *iNOS*$^{-/-}$ mice infected with *T. brucei* or *Leishmania major* (38, 56). The discrepant findings in *iNOS*$^{-/-}$ mice and animals administered iNOS inhibitors may reflect NOS-independent actions of inhibitors such as aminoguanidine (57), or residual or compensatory mechanisms in the mice (58). Our results indicate that NO· production plays an important antimicrobial effector role during salmonellosis. This conclusion is further supported by observations in murine listeriosis, in which iNOS inhibition exacerbates infection and bacterial burden without apparent effects on hepatosplenic macrophage influx and granuloma formation (59).

In conclusion, the course of experimental salmonellosis in congenic mice lacking a functional NADPH phagocyte oxidase or iNOS has revealed critical temporal differences in the roles of these host immune effector mechanisms. The early induction of the NADPH phagocyte oxidase results in rapid bacterial killing, but this phase is rapidly succeeded by a prolonged plateau phase with eventual bacterial clearance dependent upon the induction of NO· production. This coordinated response may maximize antimicrobial actions of the innate immune system while limiting collateral tissue injury from exposure to reactive oxidants.

This work was supported by grants from The Wellcome Trust, the Biotechnology and Biological Sciences Research Council, the National Institutes of Health (AI39557, AI44486, and AI10181), and the James Biundo Foundation.

Submitted: 21 January 2000
Revised: 25 April 2000
Accepted: 5 May 2000

References

1. Collins, F.M. 1974. Vaccines and cell mediated immunity. *Bacteriol. Rev.* 38:371–402.
2. Hormaeche, C.E., C.M.A. Khan, P. Mastroeni, B. Villarreal, G. Dougan, and S.N. Chatfield. 1994. *Salmonella* vaccines: mechanisms of immunity and their use as carriers of recombinant antigens. *In* Molecular and Clinical Aspects of Vaccine Development. D. Ala, D. Aldeen, and C.E. Hormaeche, editors. John Wiley & Sons, Chichester, UK. 19–153.
3. Mastroeni, P., J.A. Harrison, and C.E. Hormaeche. 1994. Natural resistance and acquired immunity to *Salmonella*. *Fund. Clin. Immunol.* 2:83–95.
4. Fields, P.I., R.V. Swanson, C.G. Haidaris, and F. Heffron. 1986. Mutants of *Salmonella typhimurium* that cannot survive within macrophages are avirulent. *Proc. Natl. Acad. Sci. USA.* 83:5189–5193.
5. Hoiseth, S.K., and B.A.D. Stocker. 1981. Aromatic depen-

dent *Salmonella typhimurium* are non virulent and effective as live vaccines. *Nature*. 291:238–239.
6. Hormaeche, C.E. 1979. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology*. 37: 311–318.
7. Lissner, C.R., R.N. Swanson, and A.D. O'Brien. 1983. Genetic control of the innate resistance of mice to *Salmonella typhimurium*: expression of the *Ity* gene in peritoneal and splenic macrophages isolated *in vitro*. *J. Immunol*. 131:3006–3013.
8. Maskell, D.J., C.E. Hormaeche, K.E. Harrington, H.S. Joysey, and F.Y. Liew. 1987. The initial suppression of bacterial growth in a *Salmonella* infection is mediated by a localised rather than a systemic response. *Microb. Pathog*. 2:295–305.
9. Mastroeni, P., J.N. Skepper, and C.E. Hormaeche. 1995. Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary *Salmonella* infections. *Infect. Immun*. 63: 3674–3682.
10. Richter-Dahlfors, A., A.M.J. Buchan, and B. Finlay. 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J. Exp. Med*. 186:569–580.
11. Hormaeche, C.E., P. Mastroeni, A. Arena, J. Uddin, and H.S. Joysey. 1990. T cells do not mediate the initial suppression of a *Salmonella* infection in the RES. *Immunology*. 70: 247–250.
12. Everest, P., M. Roberts, and G. Dougan. 1998. Susceptibility to *Salmonella typhimurium* infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. *Infect. Immun*. 66:3355–3364.
13. Hess, J., C. Ladel, D. Miko, and S.H.E. Kaufmann. 1996. *Salmonella typhimurium aroA*$^-$ infection in gene targeted immunodeficient mice. *J. Immunol*. 156:3321–3326.
14. Kagaya, K., K. Watanabe, and Y. Fukazawa. 1989. Capacity of recombinant gamma interferon to activate macrophages for *Salmonella*-killing activity. *Infect. Immun*. 57:609–615.
15. Kincy-Cain, T., J.D. Clements, and L. Bost. 1996. Endogenous and exogenous interleukin-12 augment protective immune response in mice orally challenged with *Salmonella dublin*. *Infect. Immun*. 64:1437–1440.
16. Mastroeni, P., A. Arena, G.B. Costa, M.C. Liberto, L. Bonina, and C.E. Hormaeche. 1991. Serum TNFα in mouse typhoid and enhancement of a *Salmonella* infection by anti-TNFα antibodies. *Microb. Pathog*. 11:33–38.
17. Mastroeni, P., B. Villarreal-Ramos, and C.E. Hormaeche. 1992. Role of T-cells, TNFα and IFNγ in recall of immunity to oral challenge with virulent salmonellae in mice immunised with live attenuated *aroA Salmonella* vaccines. *Microb. Pathog*. 13:477–491.
18. Mastroeni, P., B. Villarreal-Ramos, and C.E. Hormaeche. 1993. Effect of late administration of anti-TNFα antibodies on a *Salmonella* infection in the mouse model. *Microb. Pathog*. 14:473–480.
19. Mastroeni, P., J.A. Harrison, J.A. Chabalgoity, and C.E. Hormaeche. 1995. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infect. Immun*. 64:189–196.
20. Mastroeni, P., J.A. Harrison, J.H. Robinson, S. Clare, S. Khan, D.J. Maskell, G. Dougan, and C.E. Hormaeche. 1998. Interleukin 12 is required for the control of the growth of attenuated aromatic-compound dependent salmonellae in BALB/c mice: role of gamma interferon and macrophage activation. *Infect. Immun*. 66:4767–4776.
21. Mastroeni, P., S. Clare, S. Khan, J.A. Harrison, C.E. Hormaeche, H. Okamura, M. Kurimoto, and G. Dougan. 1999. IL18 (IGIF) contributes to host resistance and IFNγ production in mice infected with virulent *S. typhimurium*. *Infect. Immun*. 67:478–483.
22. Tite, J.P., G. Dougan, and S.N. Chatfield. 1991. The involvement of tumor necrosis factor in immunity to *Salmonella* infection. *J. Immunol*. 147:3161–3164.
23. Buchmeier, N., C.J. Lipps, M.H.Y. So, and F. Heffron. 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol. Microbiol*. 7:933–936.
24. De Groote, M.A., U.A. Ochsner, M.U. Shiloh, C. Nathan, J.M. McCord, M.C. Dinauer, S.J. Libby, A. Vazquez-Torres, Y. Xu, and F. Fang. 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc. Natl. Acad. Sci. USA*. 94:13997–14001.
25. Mouy, R., A. Fischer, E. Vilmer, R. Seger, and C. Griscelli. 1989. Incidence, severity, and prevention of infections in chronic granulomatous disease. *J. Pediatr*. 114:555–560.
26. Pollock, J.D., D.A. Williams, M.A.C. Gifford, L.L. Li, X. Du, J. Fisherman, S.H. Orkin, C.M. Doershuck, and M.C. Dinauer. 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat. Genet*. 9:202–209.
27. Adams, L.B., M.C. Dinauer, D.E. Morgenstern, and J.K. Krahenbuhl. 1997. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to *Mycobacterium tuberculosis* using transgenic mice. *Tuber. Lung. Dis*. 78:237–246.
28. Shiloh, M.U., J. Ruan, and C. Nathan. 1997. Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. *Infect. Immun*. 65:3193–3198.
29. Shiloh, M.U., J.D. MacMiking, S. Nicholson, J.E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity*. 10:29–38.
30. Fang, F. 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest*. 99:2818–2825.
31. DeGroote, M.A., and F.C. Fang. 1999. Antimicrobial properties of nitric oxide. *In* Nitric Oxide and Infection. F.C. Fang, editor. Kluwer Academic/Plenum Publishers, New York. 231–261.
32. Umezawa, K., T. Akaike, S. Fujii, M. Suga, K. Setoguchi, A. Ozawa, and H. Maeda. 1997. Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against *S. typhimurium* infection in mice. *Infect. Immun*. 65:2932–2940.
33. De Groote, M.A., T. Testerman, Y. Xu, G. Stauffer, and F.C. Fang. 1996. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. *Science*. 272:414–417.
34. Khan, S.A., P. Everest, S. Servos, N. Foxwell, U. Zahringer, H. Brade, E.T. Rietschel, G. Dougan, I.G. Charles, and D.J. Maskell. 1998. A lethal role for lipid A in *Salmonella infections*. *Mol. Microbiol*. 29:571–579.
35. Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulous, and F.C. Fang. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide syn-

thase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med.* 192:227–236.
36. MacFarlane, A.S., M.G. Schwacha, and T.K. Eisenstein. 1999. *In vivo* blockage of nitric oxide with aminoguanidine inhibits immunosuppression induced by an attenuated strain of *Salmonella typhimurium*, potentiates *Salmonella* infection, and inhibits macrophage and polymorphonuclear leukocyte influx into the spleen. *Infect. Immun.* 67:891–898.
37. Laubach, V.E., E.G. Shesely, O. Smithies, and P.A. Sherman. 1995. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc. Natl. Acad. Sci. USA*. 92:10688–10692.
38. Wei, X., I.G. Charles, A. Smith, J. Ure, G. Feng, F. Huang, D. Xu, W. Muller, S. Moncada, and F.Y. Liew. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*. 375:408–411.
39. Reed, L.J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493–497.
40. Vidal, S.M., D. Malo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell*. 73:469–485.
41. Hormaeche, C.E. 1980. The *in vivo* division and death rates of *Salmonella typhimurium* in the spleens of naturally resistant and susceptible mice measured by the superinfecting phage technique of Meynell. *Immunology*. 41:973–979.
42. Pacelli, R., D.A. Wink, J.A. Cook, M.C. Krishna, W. DeGraff, N. Friedman, M. Tsokos, A. Samuni, and J.B. Mitchell. 1995. Nitric oxide potentiates hydrogen peroxide–induced killing of *Escherichia coli*. *J. Exp. Med.* 182:1469–1479.
43. Wink, D.A., J.A. Cook, S.Y. Kim, Y. Vodovotz, R. Pacelli, M.C. Krishna, A. Russo, J.B. Mitchell, D. Jourd'heuil, A.M. Miles, and M.B. Grisham. 1997. Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. Chemical aspects involved in the balance between oxidative and nitrosative stress. *J. Biol. Chem.* 272:11147–11151.
44. Tamura, M., M. Takeshita, J.T. Curnutte, D.J. Uhlinger, and J.D. Lambeth. 1992. Stabilization of human neutrophil NADPH oxidase activated in a cell-free system by cytosolic proteins and by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. *J. Biol. Chem.* 267:7529–7538.
45. Iha, S., K. Orita, T. Kanno, T. Utsumi, E.F. Sato, M. Inoue, and K. Utsumi. 1996. Oxygen-dependent inhibition of neutrophil respiratory burst by nitric oxide. *Free Radic. Res.* 25: 489–498.
46. Vassiloyanakopoulos, A.P., S. Okamoto, and J. Fierer. 1998. The crucial role of polymorphonuclear leukocytes in resistance to *Salmonella dublin* infections in genetically susceptible and resistant mice. *Proc. Natl. Acad. Sci. USA*. 95:7676–7681.
47. Sinha, K.A., P. Mastroeni, J. Harrison, R.D. de Hormaeche, and C.E. Hormaeche. 1997. *Salmonella typhimurium aroA, htrA* and *aroD htrA* mutants cause progressive infections in athymic (nu/nu) BALB/c mice. *Infect. Immun.* 65:1566–1569.
48. Gomes, M.S., M. Florido, T.F. Pais, and R. Appelberg. 1999. Improved clearance of *Mycobacterium avium* upon disruption of the inducible nitric oxide synthase. *J. Immunol.* 162:6734–6739.
49. Millar, A.E., J. Stenberg, C. McSharry, X.Q. Wei, F.Y. Liew, and C.M. Turner. 1999. T-cell responses during *Trypanosoma brucei* infections in mice deficient in inducible nitric oxide synthase. *Infect. Immun.* 67:3334–3338.
50. Everest, P., J. Allen, A. Papacostantinopoulou, P. Mastroeni, M. Roberts, and G. Dougan. 1997. *Salmonella typhimurium* infections in mice deficient in interleukin-4 production: role of IL-4 in infection associated pathology. *J. Immunol.* 159: 1820–1827.
51. Muhl, H., and C.A. Dinarello. 1997. Macrophage inflammatory protein-1α production in lipopolysaccharide-stimulated human adherent blood mononuclear cells is inhibited by the nitric oxide synthase inhibitor N^G-monomethyl-l-arginine. *J. Immunol.* 159:5063–5069.
52. McInnes, I.B., and F.Y. Liew. 1999. Immunomodulatory actions of nitric oxide. *In* Nitric Oxide and Infection. F.C. Fang, editor. Kluwer Academic/Plenum Publishers, New York. 199–213.
53. Ehlers, S., S. Kutsch, J. Benini, A. Cooper, C. Hanhn, J. Gerdes, I. Orme, C. Martin, and E.T. Rietschel. 1999. NOS2-derived nitric oxide regulates the size, quantity and quality of granuloma formation in *Mycobacterium avium*-infected mice without affecting bacterial loads. *J. Immunol.* 98: 313–323.
54. Hickey, M.J., K.A. Sharkey, E.G. Sihota, P.H. Reinhardt, J.D. Macmicking, C. Nathan, and P. Kubes. 1997. Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. *FASEB J.* 11: 955–964.
55. Trinchieri, G. 1997. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). *Curr. Opin. Immunol.* 9:17–23.
56. Hertz, C.J., and J.M. Mansfield. 1999. IFN-gamma-dependent nitric oxide production is not linked to resistance in experimental African trypanosomiasis. *Cell. Immunol.* 192:24–32.
57. Wray, G., and C. Thiemermann. 1999. Nitric oxide in sepsis. *In* Nitric Oxide and Infection. F.C. Fang, editor. Kluwer Academic/Plenum Publishers, New York. 265–280.
58. Niedbala, W., X.Q. Wei, D. Piedrafita, D. Xu, and F.Y. Liew. 1999. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* 29:2498–2505.
59. Boockvar, K.S., D.L. Granger, R.M. Poston, M. Maybody, M.K. Washington, J.B. Hibbs, and R.L. Kurlander. 1994. Nitric oxide produced during murine listeriosis is protective. *Infect. Immun.* 62:1089–1100.