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Analysis of Nitric Oxide-Dependent Antimicrobial Actions in Macrophages and Mice

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Abstract

Nitric oxide (NO•) is a critical component of mammalian host defense that is produced in macrophages and other cells comprising the innate immune system. Isolated mammalian macrophages have been utilized to measure the kinetics of NO production and to demonstrate NO-related antimicrobial actions. Some microorganisms possess enzymes to detoxify nitrogen oxides, and mutant strains lacking these enzymes can be used to demonstrate the importance of these mechanisms for intracellular bacterial survival. This chapter describes techniques with which to analyze the antimicrobial actions of nitric oxide in murine and human macrophages and in laboratory mice.

Nitric oxide (NO•) produced by host phagocytic cells plays a major role in innate immunity, in large part because of the ability of NO• to inhibit or kill a broad range of microorganisms (Fang, 2004). By targeting protein thiols and metal centers (Stamler *et al.*, 2001), NO• can block essential microbial physiological processes, including respiration (Stevanin *et al.*, 2000) and DNA replication (Schapiro *et al.*, 2003). This chapter discusses methods for the examination of NO•-dependent antimicrobial actions using murine or human macrophages and laboratory mice. The protocols described have specifically been employed to investigate the actions of host cell-derived NO• to inhibit the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium. However, these methods should be generally applicable with minor modifications to investigate the role of NO• in infections caused by other microbes as well.

1. NO•-DEPENDENT ANTIMICROBIAL ACTIONS of MURINE MACROPHAGES

The contribution of NO• to the anti-*Salmonella* activity of interferon (IFN)γ-activated macrophages is well accepted (McCollister *et al.*, 2005; Vazquez-Torres *et al.*, 2000, 2004; Webb *et al.*, 2001). However, the importance of NO• production by nonactivated macrophages is less certain (Bang *et al.*, 2006; Chakravorty *et al.*, 2002; Ekman *et al.*, 1999; Saito *et al.*, 1991; Shiloh *et al.*, 1997, 1999; Vazquez-Torres *et al.*, 2000, 2004). This largely reflects the greater NO• production by activated macrophages (Ding *et al.*, 1988; Vazquez-Torres *et al.*, 2004). An important determinant of the outcome of such assays is the ability of the microbe to detoxify NO•. For *Salmonella*, the major enzyme responsible for NO• detoxification is the flavohemoglobin Hmp (Bang *et al.*, 2006). Hmp increases the survival of *Salmonella* in unstimulated macrophages, suggesting that this flavohemoprotein can detoxify the quantity of

NO• synthesized by these cells. A comparison of the anti-*Salmonella* activity of unstimulated macrophages isolated from C3H/HeN and congenic Tlr4-deficient C3H/HeJ mice has revealed that the innate ability of macrophages to produce NO• in response to *Salmonella* requires LPS/Tlr4 signaling (Vazquez-Torres *et al.*, 2004). Increased killing of *hmp* mutant *Salmonella* is abrogated in iNOS-deficient macrophages, demonstrating that Hmp-mediated NO• detoxification increases *Salmonella* intracellular survival. However, the Hmp flavohemoprotein is unable to counteract the fourfold higher NO• fluxes¹ produced by IFN γ -activated macrophages (Bang *et al.*, 2006; McCollister and Vazquez-Torres, unpublished results). The use of NO•-resistant and -susceptible bacteria in combination with cells producing various quantities of NO• can elucidate both NO•-dependent antimicrobial actions and mechanisms of microbial resistance. The following section describes a protocol used to examine the role of iNOS in the anti-*Salmonella* activity of macrophages and the role of the Hmp flavohemoglobin in antagonizing this activity.

1.1. Equipment

Centrifuge equipped with holders for microtiter plates
 CO₂ incubator
 Inverted and upright microscopes
 Hemocytometer
 Flow cytometer (optional)

1.2. Reagents

Frozen bacterial stock cultures
 Microbial culture medium (liquid and solid)
 Antibiotics as indicated
 Phosphate-buffered saline (PBS)
 Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺ or Mg²⁺
 Hanks' balance salt solution (HBSS)
 RPMI 1640 tissue culture medium
 Sodium *m*-periodate
 Fetal bovine serum
 Normal mouse serum
 HEPES
 L-Glutamine
 Sodium pyruvate
 Trypan blue
 Penicillin/streptomycin

¹The production of NO• was estimated by measuring accumulation of the stable NO• oxidation product NO₂⁻. Total NO₂⁻ concentrations were measured spectrophotometrically at 550 nm after mixing sample supernatants with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylethylenediamide hydrochloride in 2.5% phosphoric acid). Standard curves were prepared with NaNO₂.

Dimethyl sulfoxide (DMSO)
 Recombinant murine IFN γ
 Antibodies to macrophage surface markers
 Deoxycholic acid
 Collagenases I and IV
 EDTA
 Glucose dehydrogenase
 NADPH
 Glucose-6-phosphate
 Topro-3
 5-ml polystyrene round-bottom tubes
 15-ml polypropylene tubes
 Syringes, 10 ml
 Needles, 18 and 25 gauge
 Microcentrifuge tubes (0.65 and 1.5 ml)
 Flat-bottom 96-well microtiter plates
 Surgical scissors and forceps

1.3. Protocol

1.3.1. Isolation of murine macrophages—

- 1 C57BL/6 mice and congenic *iNOS*^{-/-} controls² (MacMicking *et al.*, 1995) are inoculated intraperitoneally with 1 ml of 5 mM sodium *m*-periodate (Sigma-Aldrich, St. Louis, MO) prepared in PBS. The solution is drawn into the peritoneal cavity of hand-held mice using a 23-gauge needle mounted in a 3-ml syringe.
- 2 Inflammatory cells can be harvested 4 days after sodium *m*-periodate is injected. The peritoneum of mice euthanized in a CO₂ chamber is exposed following sterile technique. Ten milliliters of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD), 15 mM HEPES, 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO) (RPMI⁺ medium) is injected using an 18-gauge needle mounted in a 10-cc syringe. The medium is immediately drawn back with the same syringe.
- 3 Peritoneal exudate cells (PEC) are concentrated following a 5-min centrifugation at 200 g in a Centra CL3R centrifuge (Thermo Fisher Scientific Inc., Waltham, MA). The cell pellet is resuspended in 1 ml of RPMI⁺ medium.
- 4 Cells present in the suspension are counted in a hemocytometer after 1:50 dilution in 0.5% Trypan blue prepared in PBS (w/v). Cells are adjusted to 2×10^6 PCE/ml in RPMI⁺ medium containing 100 U·ml⁻¹/ 100 mg·ml⁻¹ of penicillin/streptomycin (Mediatech Inc. Herndon, VA). For macrophage killing assays and NO₂⁻

²The contribution of NO• to innate Tlr4-dependent host defenses of macrophages can be best shown in the presence of a functional Nramp1 (Slc11a1) cation transporter. The antimicrobial activity of peritoneal macrophages isolated from wild-type C3H/HeN can therefore be compared to controls isolated from congenic Tlr4-deficient C3H/HeJ mice.

determination, 2×10^5 PCE/100- μ l well are seeded into tissue culture grade, flat bottom 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). Selected groups of macrophages are treated with 200 U/ml of IFN γ (Life Technologies, St. Paul, MN) during the last 20 h of culture prior to *Salmonella* infection.

- 5 Macrophages contained in the PEC are selected after 48 h of culture at 37° in a 5% CO₂ incubator by gently washing out nonadherent cells with 2 volumes of prewarmed RPMI⁺ medium.³

1.3.2. Phenotypic analysis of murine macrophages (optional)—To characterize the population of macrophages isolated using this protocol, adherent macrophages are selected as described earlier in 24-well plates. Five hundred microliters of cells is added per well of 24-well plates after PEC are adjusted to 2×10^6 cells/ml in RPMI⁺ medium containing 100 U·ml⁻¹/100 mg·ml⁻¹ of penicillin/streptomycin as described previously. After adjusting for differences in volume, the procedure is as described for the selection of macrophages in 96-well microtiter plates.

- 6 Adherent cells are detached upon replacement of RPMI⁺ medium with 500 μ l of prewarmed DPBS without Ca²⁺ or Mg²⁺ (Sigma-Aldrich) containing 0.2% collagenases I and IV (Sigma-Aldrich) and 2% heat-inactivated fetal calf serum (FCS). Cells are incubated for 5 min in a humidified, 5% CO₂ incubator at 37°. Culture plates are placed on ice for 15 min after the addition of 500 μ l of 10 mM EDTA diluted in DPBS containing 2% heat-inactivated FCS.
- 7 Detached macrophages are recovered by pipetting, and the wells are further washed with 500 μ l of DPBS supplemented with 2% FCS. EDTA is removed by centrifugation, and cells are resuspended in ice-cold HBSS with 5% FCS.
- 8 After nonspecific labeling is blocked using purified antibodies to FcR γ II and FcR γ III, cells are stained with the macrophage phenotypic markers FITC-labeled F4/80 (FL1) (Caltag, Burlingame, CA) and PE-labeled CD11b Mac-1 (FL2) (eBioscience, San Diego, CA). Dead cells are excluded from the analysis after labeling with 1 nM Topro-3, a fluorescent vital stain (Invitrogen, Molecular Probes, Eugene, OR).
- 9 Cells are analyzed on a FACSCalibur flow cytometer. The population of adherent PEC is highly enriched for mononuclear phagocytes, as indicated by the fact that over 95% of the cells are F4/80⁺ CD11b⁺ macrophages (Fig. 26.1).

1.3.3. Bacterial cultures—

- 10 *Salmonella enterica* serovar Typhimurium strain ATCC 14028s and isogenic bacterial strains of interest [e.g., *hmp* mutant strains IB3 and AV0468 (Bang *et al.*, 2006; McCollister *et al.*, 2005)] are used for infection of macrophages. Bacterial cultures are grown from a -80° frozen stock prepared in DMSO. A crystal scraped from bacterial frozen stocks is grown in Luria-Bertani (LB) broth to stationary phase for 16 h at 37° in a shaker incubator set at 315 rpm.⁴

³Typically, about 10⁵ macrophages are seeded per well, as quantified in a hemocytometer after adherent cells are released by collagenase treatment. Macrophage-like cell lines such as J774.1 or RAW264.7 cells can be very useful in the characterization of NO•-related antimicrobial activity. As for primary macrophages, macrophage-like cells are seeded at densities of 10⁵ cells/well of 96-well tissue culture plates and cultured in the presence or absence of 200 U/ml IFN γ for 16–20 h prior to infection.

⁴The majority of the iNOS-dependent anti-*Salmonella* activity of IFN γ -activated macrophages is associated with repression of SPI2 transcription (8). The bacterial culture conditions described herein have been selected because in our experience they consistently induce expression of a functional SPI2 phenotype as indicated by the increased intracellular survival of wild-type *Salmonella* compared to an *AspiC::FRT* isogenic control.

1.3.4. Macrophage killing assays—

- 11 Macrophages are challenged at a multiplicity of infection (MOI) of 2 bacteria:1 macrophage with *Salmonella* that have been opsonized previously for 20 min in RPMI⁺ medium containing 10% normal mouse serum⁵ as described (McCollister *et al.*, 2005). Briefly, 15 μ l of overnight bacterial cultures diluted 1:5 (v/v) in PBS is added to 435 μ l RPMI⁺ medium and 50 μ l of normal mouse serum.⁶ Bacteria are then cultured in a 5% CO₂ incubator at 37° for 20 min.
- 12 Macrophages are challenged with 50 μ l opsonized bacteria that have been diluted with 3 volumes of prewarmed RPMI⁺ medium. Plates are centrifuged at 3200 g for 1 min, and cells are cultured at 37° in a 5% CO₂ incubator.
- 13 Extracellular bacteria are removed 25 min after challenge by washing the monolayers with 2 volumes of prewarmed RPMI⁺ medium containing 6 μ g/ml gentamicin (Sigma). To estimate the number of intracellular bacteria at time zero (t_0), selected groups of macrophages are lysed 25 min after challenge with 0.5% deoxycholic acid prepared in PBS (w/v). The role of NO• in the antimicrobial activity of macrophage populations expressing iNOS can be studied by adding inhibitors such as monomethyl-L-arginine (250 μ M) at time zero of infection. To demonstrate NO• synthesis or its pharmacological inhibition, accumulation of its oxidation product NO₂⁻ (nitrite) in the supernatant can be quantified with the Griess reagent after 8–20 h of infection. If quantitation of NO₃⁻ is also desired, the Griess reaction can be used to determine total NO_x after enzymatic reduction of NO₃⁻ to NO₂⁻ in pH 7 sodium phosphate buffer containing 1.6 U/ml *Aspergillus* nitrate reductase, 16 U/ml glucose dehydrogenase, 10 μ M NADPH, and 10 mM glucose-6-phosphate. NO₃⁻ can be calculated as NO_x – NO₂⁻ (Fig. 26.2).
- 14 Samples are serially diluted and spotted in LB agar plates. The rest of the *Salmonella*-infected macrophages are lysed at various time points after challenge (t_n), and surviving bacteria are enumerated as described earlier by serial culture on LB agar plates. Results are expressed as % survival = (t_n/t_0)100 (Fig. 26.3).

2. NO-Dependent Antimicrobial Actions of Human Macrophages

Although NO• production by murine macrophages can be demonstrated readily in response to inflammatory stimuli, the production of NO• by human monocyte-derived macrophages is much more difficult to elicit under experimental conditions. Numerous publications have shown that macrophages from patients with infections or other inflammatory diseases exhibit expression of inducible NO• synthase and production of NO• (Weinberg, 1998), but the quantities of NO• produced *in vitro* by human macrophages from healthy subjects are modest compared to those produced by murine cells. This increases considerably the challenge of demonstrating NO•-dependent antimicrobial actions. Evidence that NO•-related killing occurs in human macrophages includes the observation that NO• detoxification mechanisms of both *Neisseria meningitidis* (Stevanin *et al.*, 2005) and *Salmonella* (Stevanin *et al.*, 2002) protect these organisms from killing by 10- to 12-day cultured human monocytes-derived macrophages, an effect that is eliminated by the presence of iNOS inhibitors.

⁵Various MOI may be used in macrophage killing assays. An MOI of 2:1 has been chosen in this case to diminish nonspecific killing associated with gentamicin included in the culture medium. At this low MOI, a functional NADPH phagocyte oxidase enzymatic complex accounts for all early *Salmonella* killing by control and IFN γ -primed macrophages (B. D. McCollister and A. Vazquez-Torres, unpublished observations).

⁶Normal mouse serum (Sigma-Aldrich) is stored in 100- μ l aliquots at –80°.

2.1. Equipment

Category 2 laminar flow hood
CO₂ incubator
Inverted and upright microscopes
Hemocytometer
Orbital shaker centrifuge
Spectrophotometer
Flow cytometer (optional)
Sievers NO• analyzer NOA 280i (optional)

2.2. Reagents

Frozen bacterial stock cultures
Microbial culture medium (liquid and solid)
Antibiotics as indicated
PBS
RPMI 1640 tissue culture medium
Fetal bovine serum
L-Glutamine
CPDA-anticoagulant
Trypan blue
DMSO
L-N^G-monomethyl arginine (L-NMMA)
Saponin (1%)
Antibodies to macrophage surface markers
5-ml polystyrene round-bottom tubes
15-ml polypropylene tubes
Microcentrifuge tubes (0.65 and 1.5 ml)
24-well flat-bottom plates
Sterile glass beads (1 mm diameter)
Cell scraper (25 cm) (optional)
Hamilton syringes, 50 and 100 μ l (optional)

2.3. Protocol

2.3.1. Isolation of human monocyte-derived macrophages—The GE-Healthcare Ficoll-Paque Plus offers a quick and reliable way of isolating primary human mononuclear cells from whole blood.

- 1 Peripheral blood is collected from healthy volunteers after informed consent is obtained. Universal precautions to protect the worker from blood-borne virus infection must be implemented. The worker must be vaccinated against hepatitis B.
- 2 The blood specimen is collected into a blood transfusion bag containing 63 ml of CPDA-anticoagulant. The amount of blood needed will vary from experiment to experiment, but 1×10^8 MDM can usually be derived from one unit (450 ml) of whole blood. Mononuclear cells are then isolated by density centrifugation by Ficoll–Paque and subsequently purified on Percoll density gradients to separate monocytes from lymphocytes (as per manufacturer's instructions). Because of differential density, monocytes aggregate at the interface between the plasma and the Ficoll–Paque.
- 3 The layer containing monocytes is gently removed to a fresh tube and washed twice with PBS. After the second wash, the pellet is resuspended in RPMI 1640 medium containing 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. Monocytes are plated at a density of 1×10^6 cell/well in flat-bottom 24-well plates. Plates are then incubated at 37°, 95% air, 5% CO₂.
- 4 After 24 h incubation, nonadherent cells are gently washed out, and adherent cells are cultured in RPMI 1640 with fresh medium and allowed to differentiate into macrophages over a 12-day period, with media being aspirated and replenished at 48-h intervals.

2.3.2. Bacterial cultures—

- 5 Human MDM have been inoculated with a wide variety of bacteria, including category 2 and category 3 (biosafety level 3) organisms. An important consideration in planning experiments is the MOI, which will vary with the organism under examination and the nature of the experiment. The following description refers to our experience with *S. enterica* serovar Typhimurium and its isogenic *hmp* derivative. Strain ATCC 14028s and an isogenic strain carrying an antibody resistance cassette inserted in the *hmp* gene (14028 *hmp* or MCS2A) (Crawford and Goldberg, 1998) are used for infection of macrophages. Bacterial cultures are grown from a –80° frozen stock prepared in DMSO onto nutrient agar (NA) plates and incubated overnight at 37°. Transfer three to four colonies from the NA plate to 10 ml LB broth in a shaker set at 200 rpm at 37° until it reaches an OD₆₀₀ of 0.025 nm (midexponential phase). This was identified in viability studies as the optimum OD₆₀₀ to obtain an MOI of approximately 10 (10 bacteria/MDM) in a 250-μl suspension.
- 6 Bacteria are harvested and washed three times in PBS. The pellet should be resuspended in RPMI 1640 in the presence of two or three 1-mm glass beads to break up clumps of bacteria.

2.3.3. Macrophage killing assays—

- 7 MDM are challenged with 250 μl/well suspensions of nonopsonized bacteria. There is no need to centrifuge plates at this stage. At the recommended MOI, nonopsonized *Salmonella* binds at approximately four bacterial cells/MDM (Stevanin *et al.*, 2002). Plates are incubated at 4° for 60 min to allow binding of bacteria to MDM. (This is an optional method that can be used to prevent phagocytosis by cells if there is a possibility that the strains being tested are differentially internalized. MDM will only internalize bacteria at a temperature >17°.) Plates should be transferred to an incubator at 37° and incubated for a total of 4 h, taking samples every 30 min for determination of viability.

- 8 After the first 30 min at 37°, the medium is removed and all wells are washed twice with PBS. To some wells, 250 μ l of saponin (1%) will be added to disrupt the MDM, which will subsequently be incubated for 12 min. This control is important when comparing small differences in MDM killing between different strains and allows one to confirm that the numbers of bacteria associated with MDM have remained similar for all strains used in the experiment. This can be considered as time zero relative to gentamicin treatment.
- 9 To the remaining wells, a minimum of 1 ml fresh RPMI 1640 containing gentamicin is added to kill any bacteria that have not been internalized by MDM. It is important to add enough antibiotic-containing medium to completely cover the wells, as residual viable extracellular bacteria may confound the results. The minimum bactericidal concentration for gentamicin must be established for each test strain or when varying experimental conditions such as inoculum size. The plates should be returned to the incubator for an additional 30 min.
- 10 To confirm that noninternalized bacteria have been killed, the supernatant is collected into a centrifuge tube prior to the addition of saponin to the wells. The wells are to be rinsed twice with PBS to remove the antibiotic before plating samples for viable counts. The remaining wells are rinsed twice with PBS before replacement with fresh medium.
- 11 After 12 min with saponin, the supernatant is collected into a centrifuge tube. The wells are washed with 500 μ l PBS by pipetting up and down a few times to ensure that as many bacteria as possible are collected. Bacteria are harvested with resuspension of the pellet in 150 μ l PBS. Fifty to 100 μ l of 1×10^0 , 1×10^{-1} , and 1×10^{-2} dilutions of the bacterial suspension is spread onto agar plates for enumeration of viable counts.

Generally speaking, it is difficult to study iNOS activation in human macrophages *in vitro*, but it is nevertheless possible to show the importance of NO• for the antimicrobial activity of human macrophages by inhibiting iNOS using L-NMMA.

- 12 One millimolar of L-NMMA may be added to the MDM 48 h prior to challenging with bacteria, with subsequent protocol steps as described earlier.
- 13 Supernatants from MDM that have or have not been treated with L-NMMA are collected to measure nitrite accumulation in MDM supernatants and to confirm a decrease in NO• production by L-NMMA-treated MDM. The Griess reagent used to measure NO₂⁻ generation by murine macrophages is insufficiently sensitive to detect variations in NO• production by human macrophages. A more sensitive chemiluminescence-based method should therefore be employed (see Chapter 7 in volume 436).

To study MDM killing of *Salmonella* for longer than 4 h, the medium should be replaced with RPMI containing 20 μ g/ml gentamicin to suppress extracellular bacterial growth.

3. NO•-Dependent Antimicrobial Actions in Laboratory Mice

As for studies with murine macrophages, the basic approach to examining NO•-dependent antimicrobial actions in laboratory mice involves a comparison between wild-type and isogenic iNOS knockout mice or between mice with and without administration of an NO• synthase inhibitor. The availability of iNOS^{-/-} mice has greatly facilitated analysis of the role of NO• in infection and other conditions (MacMicking *et al.*, 1995; Mastroeni *et al.*, 2000). However, studies using iNOS^{-/-} mice are generally performed in a C57BL/6 background. C57BL/6 mice, like a number of inbred mouse strains, carry a mutation at the Slc11a1 (Nramp1 G169D) locus

that enhances susceptibility to certain pathogens, including *Mycobacterium bovis* BCG (bacillus Calmette-Guerin), *S. enterica* serovar Typhimurium, and *Leishmania donovani*. The Slc11a1 locus encodes a cation transporter that appears to limit concentrations of manganese and iron within the phagosome (Jabado *et al.*, 2000; Zaharik *et al.*, 2004). The mutant Slc11a1 locus of C57BL/6 mice may mask the ability to detect NO•-dependent antimicrobial actions *in vivo* (Bang *et al.*, 2006). Thus, it may also be informative to perform infections of inbred mouse strains carrying a functional Slc11a1 locus (e.g., C3H/HeN, 129Sv), in which case an NO• synthase inhibitor must be administered to abrogate NO• production. A nonselective NO• synthase inhibitor cannot be used for this purpose, as the inhibition of eNOS can have deleterious effects on tissue perfusion and cardiac output (Cobb *et al.*, 1995). Because of its relative selectivity for iNOS and low cost, aminoguanidine has been employed in a number of studies. However, adverse effects of aminoguanidine during infection of iNOS^{-/-} mice (Zhou *et al.*, 2002) indicate that nonspecific actions of aminoguanidine may complicate the interpretation of experimental results. Therefore, the use of more specific and costly iNOS-selective inhibitors (e.g., *N*-imino-ethyl L-lysine or L-NIL) is preferred (Stenger *et al.*, 1996).

Several complementary end points may be utilized to determine the effects of host NO• production during infection. Historically, virulence was measured by the determination of mortality following different inocula and calculating the 50% lethal dose (LD₅₀). This is no longer regarded as ethically acceptable under most circumstances, but one may still monitor infected animals and euthanize moribund animals humanely, using the number of days until euthanasia as a surrogate indicator of mortality. Alternatively, animals may be sacrificed after a designated time interval, and the organism burden in specific organs may be determined by dilution and plating (Mastroeni *et al.*, 2000). A variation on this method involves the coinoculation of equal quantities of wild-type and mutant bacteria to determine whether the mutant is at a competitive disadvantage in the host. This can be useful in evaluating whether a specific genetic locus is involved in resistance to host-derived NO•. The competitive disadvantage of a strain carrying a mutation in the locus of interest could be measured in the presence or absence of host NO• production. The following protocol was developed for *S. enterica* serovar Typhimurium but may be adapted for use with other organisms.

3.1. Equipment

- Spectrophotometer
- Tissue homogenizer with disposable probes

3.2. Reagents

- Frozen bacterial stock culture
- Microbial culture medium (liquid and solid)
- Antibiotics as indicated
- PBS
- 70% ethanol
- Sterile wooden stick applicators
- Sterile toothpicks
- Cuvettes for spectrophotometer
- 5-ml polystyrene round-bottom tubes
- 15-ml polypropylene tubes

Syringes

Needles, 18 and 25 gauge

Microcentrifuge tubes (0.65 and 1.5 ml)

96-well microtiter plates

Ice bucket

Scissors

Forceps

Dissecting board

3.3. Protocol

- 1 The relationship between OD₆₀₀ (optical density at 600 nm) and colony-forming units (cfu) can be determined by growing an overnight culture, preparing 1:10 serial dilutions, measuring the OD₆₀₀ on a spectrophotometer, and plating dilutions onto solid medium.
- 2 Bacteria are inoculated into culture medium, with antibiotic selection as appropriate, the night prior to infecting the mice (or longer as required for slowly growing organisms).
- 3 The OD₆₀₀ of a 1:10 dilution of cultures is measured on a spectrophotometer. Based on the calculated colony-forming units per milliliter, the culture can be diluted into PBS so that the precise desired inoculum will be contained in 500 μ l of the diluted culture. Aliquots of the diluted culture are plated onto solid medium for quantitation of the actual inoculum.
- 4 The inoculum is administered intraperitoneally to each mouse using a syringe and 25-gauge needle. Typically, at least 5–10 mice are included in each experimental group, with a minimum of two experimental replicates. A control group inoculated only with PBS should be included for each mouse strain to be tested. Gloves should be worn when handling mice. For intraperitoneal inoculation, mice can be restrained by grasping the scruff of the neck between the thumb and fingers to control the head; the tail and right hind leg may then be restrained using the fifth finger of the same hand. With the ventral side of the mouse facing the experimenter, intraperitoneal injection should be made into the right lower quadrant of the abdomen, lifting the needle against the abdominal wall after penetration of the musculature to minimize the risk of visceral perforation. Alternative routes of infection that might be appropriate for selected organisms include oral, intragastric, intravenous, subcutaneous, intradermal, or intramuscular.
- 5 Mice must be monitored twice daily for the duration of the experiment (typically 7 to 28 days, depending on the virulence of the bacterium and intrinsic resistance of the mouse strain). Moribund animals (i.e., animals not eating or drinking, lacking spontaneous movement, or exhibiting labored respiration) or animals that have lost 10% or more of their body weight should be euthanized humanely. A Kaplan–Meier plot of survival for each experimental group provides a useful representation of the contribution of NO• to host resistance (Fig. 26.4).

3.3.1. Quantitation of bacterial burden—

- 6 For enumeration of bacteria in the liver and spleen, mice must be euthanized by an approved method, such as a CO₂ chamber (AVMA Panel on Euthanasia, 2001). Mice

should be removed from the chamber and secured to a dissecting board. The ventral surface should be disinfected with 70% ethanol prior to cutting the skin along the midline to expose abdominal viscera. The spleen is removed by retracting the organ with forceps and cutting the associated vessels and membrane with scissors. Subsequently the esophagus can be severed to allow retraction of the stomach and intestines, thereby facilitating removal of the liver. As with the spleen, the liver is removed by cutting associated membranes with the scissors. Organs are placed in tubes containing 1 ml PBS on ice prior to homogenization with a disposable probe. Homogenates are serially diluted in a microtiter plate, followed by the plating of samples for quantitation of cfu.

3.3.2. Competition assays—

- 7 For competition assays, mixed infections are performed using two strains distinguishable by antibiotic markers. Bacteria should be recovered from the initial inoculum and from the liver and spleen as in step 6. One hundred colonies from the initial inoculum and from each organ are patched onto nutrient agar plates containing antibiotics as indicated to distinguish the infecting strains. The competitive index (CI) can be calculated as

$$CI = \frac{\text{strain \#1 (input) / strain \#2 (input)}}{\text{strain \#1 (organ) / strain \#2 (organ)}}$$

If strain #1 is wild type and strain #2 is a mutant strain, then a $CI < 1$ would indicate that the mutant strain has attenuated virulence. Restoration of a $CI \sim 1$ by the abrogation of $NO\bullet$ production would be consistent with a role of the mutated locus in providing resistance to $NO\bullet$.

3.3.3. Inhibition of inducible NO synthase—

- 8 If L-NIL is to be employed as an iNOS inhibitor, L-NIL should be provided in drinking water at a final concentration of 4 mM prior to and for the duration of the experiment.

3.3.4. Measurement of host NO production (optional)—

- 9 The excretion of NO_x ($NO_2^- + NO_3^-$) as a measure of host $NO\bullet$ production may be measured by collecting 24-h urine samples on isopropanol, centrifuging at 6000 g for 10 min to remove debris, and diluting 1:10 in HEPES (0.2 M, pH 7.4) (Boockvar *et al.*, 1994). Samples may then be treated with nitrate reductase and assayed for nitrite using the Griess reagent as described in the first procedure.

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Figure 26.1. Phenotypic analysis of C57BL/6 murine macrophages by flow cytometry. More than 95% of the adherent peritoneal exudate cells isolated by this protocol are F4/80⁺ CD11b⁺.

Figure 26.2.

NO_x(NO₂⁻+NO₃⁻) production by IFNγ-activated murine peritoneal macrophages over time. Macrophages were infected with *Salmonella* at *t*=0. Each data point represents NO_x production over a 1-h sampling period. Reprinted with permission from Vazquez-Torres *et al.* (2000).

Figure 26.3.

Salmonella survival after 20 h in wild-type and iNOS-deficient C57BL/6 murine peritoneal macrophages. Reduced survival of the *hmp* mutant is observed in NO $\bullet$ -producing macrophages. Interferon- γ enhances NO $\bullet$ -dependent antimicrobial activity but has a minimal effect on NO $\bullet$ -independent antimicrobial activity. Reprinted with permission from Bang *et al.* (2006).

Figure 26.4.

Survival of C3H/HeN mice after intraperitoneal challenge with wild-type or *hmp* mutant *Salmonella*. Addition of the iNOS inhibitor L-NIL to the drinking water restores virulence to the *hmp* mutant strain, suggesting that the Hmp flavohemoglobin promotes virulence by detoxifying host-derived NO•. Reprinted with permission from Bang *et al.* (2006).