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Evaluation of Bacterial Survival and Phagocyte Function with a Fluorescence-Based Microplate Assay

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To compare antibacterial function in macrophages from mice deficient in the respiratory burst oxidase or inducible nitric oxide synthase, we developed a fluorescence-based microplate assay of bacterial survival. As bacteria grow, they convert a formulation of resazurin termed AlamarBlue from its nonfluorescent oxidized state to its fluorescent reduced state. The time required to achieve a given fluorescence is inversely proportional to the number of viable bacteria present when the dye is added. This relationship allows a precise, accurate assessment of bacterial numbers with greater sensitivity and throughput and at less cost than conventional assays. The assay facilitated quantification of the killing of *Escherichia coli* by *S*-nitrosoglutathione and hydrogen peroxide and of *Salmonella typhimurium* by human neutrophils and mouse macrophages. Mouse macrophages lacking the 91-kDa subunit of the respiratory burst oxidase were deficient in their ability to kill *S. typhimurium*, while those lacking inducible nitric oxide synthase were unimpaired.

High-output pathways for production of reactive oxygen intermediates via the respiratory burst and reactive nitrogen intermediates via iNOS are presently the major molecularly defined means by which activated macrophages are thought to inhibit facultative or obligate intracellular pathogens (4, 15). Mice with induced genetic deficiencies in these ($gp91^{phox}$ deficient; iNOS deficient) (9, 10, 23, 29) and other gene products provide a powerful new approach to the study of macrophage antimicrobial pathways.

Our initial efforts to compare the rate of killing of *S. typhimurium* by macrophages from wild-type, iNOS-deficient, and $gp91^{phox}$-deficient mice in the same experiments were complicated by the cumbersome nature of existing assays of bacterial survival. Most methods for the quantitation of viable microorganisms, such as dilution and plating (3), disc diffusion (2), microbroth dilution (24), tetrazolium reduction (8, 20, 26, 27), thymidine incorporation (8), chemiluminescence (12), and optical density (28), are limited by sensitivity, precision, complexity, time, cost, and/or the use and disposal of regulated materials. Herein we describe a simple assay that avoids such problems by using a fluorescent indicator to enumerate viable bacteria accurately and semiautomatically over the range of 10^2 to 10^8 per ml in individual wells of 96-well trays. Application of the assay demonstrates that mouse macrophages require the respiratory burst oxidase but not iNOS to kill *Salmonella typhimurium* in vitro.

MATERIALS AND METHODS

Abbreviations used in this paper. FSU, fluorescence standard units; $gp91^{phox}$, 91-kDa subunit of the NADPH oxidase; GSNO, *S*-nitrosoglutathione; iNOS, inducible nitric oxide synthase; KRPG, Krebs-Ringer phosphate buffer with glucose; LB, Luria-Bertani broth; M9, minimal medium; PBS, phosphate-buffered saline; PMN, neutrophils.

Bacterial strains and growth media. *E. coli* HB101 and *S. typhimurium* ATCC 14028, provided by F. C. Fang and M. A. De Groote (University of Colorado, Denver, Colo.), were grown in LB broth or M9 or on LB plates. *Listeria monocytogenes* serotype 3b, provided by R. J. North (Trudeau Institute, Saranac Lake, N.Y.), was grown in tryptose phosphate broth or plates.

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Reagents. Tryptone, yeast extract and tryptose phosphate were from Difco Laboratories (Detroit, Mich.). Sodium periodate, sodium deoxycholate, ammonium chloride, and hydrogen peroxide were from Sigma; sodium chloride and sodium phosphate dibasic were from J. T. Baker; potassium phosphate was from Fisher; and dextrose was from EM Science (Gibbstown, N.J.). RPMI (JRH Bioscience, Lenexa, Kans.) was supplemented with 2 mM L-glutamine (JRH Bioscience) and heat-inactivated fetal bovine serum (Hyclone, Logan, Utah). GSNO was from Alexis Corp. (San Diego, Calif.).

To determine the nature and concentration of the indicator sold as AlamarBlue (Sensititre/Alamar; AccuMed International Co., Westlake, Ohio), we ascertained from the U.S. Government Patent Office (http://patents.uspto.gov/) that this is a proprietary mixture of stabilizing and poising agents whose primary constituent is a redox-sensitive dye, resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) in equilibrium with its fluorescent, reduced form, resorfin. By using the molar extinction coefficients of resazurin and resorfin, we determined that their concentrations in the AlamarBlue stock solution provided by the manufacturer were 175 ± 5 μM and 3 ± 1 μM, respectively (means ± standard deviations of three determinations).

The manufacturer's recommended wavelengths for monitoring the fluorescence of AlamarBlue are 530 to 560 nm for excitation and 590 nm for emission. Since our goal was to use the dye in a microplate format and since most microplate reader filters are not tunable, we identified the acceptable excitation-emission wavelength range by spectral analysis. From an overnight culture of *S. typhimurium*, 100 μl was added to 4.4 ml of LB broth plus 0.5 ml of AlamarBlue in a culture tube. An identical tube was prepared without bacteria. The tubes were shaken at 200 rpm and 37°C for 3 h until the bacterium-containing culture turned red (reduced). The samples were centrifuged at $10{,}000 \times g$ for 5 min to pellet the bacteria. Then 2.5 ml of supernatant was transferred to cuvettes to obtain three-dimensional excitation-emission spectra (Aminco Bowman series 2 luminescence spectrometer; SLM Instruments, Inc., Urbana, Ill.). The use of excitation filters ranging from 490 to 560 nm and emission filters ranging from 590 to 610 nm should allow a clear distinction between oxidized and reduced AlamarBlue (data not shown).

Fluorescence curves of bacteria in LB-AlamarBlue. Bacteria from an overnight culture were diluted to approximately 10^9/ml in the indicated growth medium supplemented to 10% (vol/vol) AlamarBlue stock solution (final molar concentration of resazurin, ~17.5 μM). In all subsequent experiments, medium-AlamarBlue denotes medium supplemented to 10% (vol/vol) AlamarBlue stock solution. Serial 10-fold dilutions were made into medium-AlamarBlue, and the dilutions were stored on ice. Aliquots (100 μl) from each dilution were plated in triplicate in 96-well flat-bottom polystyrene plates (Corning Glass Works, Corning, N.Y.). Additionally, 100 μl of medium-AlamarBlue was aliquoted in triplicate to serve as the reagent blank. The number of CFU in each dilution was determined by plating on LB plates. The 96-well plate was immediately placed in a Cytofluor 2350 plate-reading fluorometer (Millipore), and the fluorescence was recorded (excitation, 530 nm; emission, 590 nm; sensitivity, 2) in arbitrary units (FSU) for each well. Reading each plate required less than 1 min. The plate was shaken at 75 rpm in a 37°C air incubator, and the fluorescence was recorded every 15 to 30 min until all bacterium-containing wells reached maximum fluorescence.

Calculations. Fluorescence data from each well and time point were compiled in a computer after subtraction of the fluorescence of the reagent blank (nor-

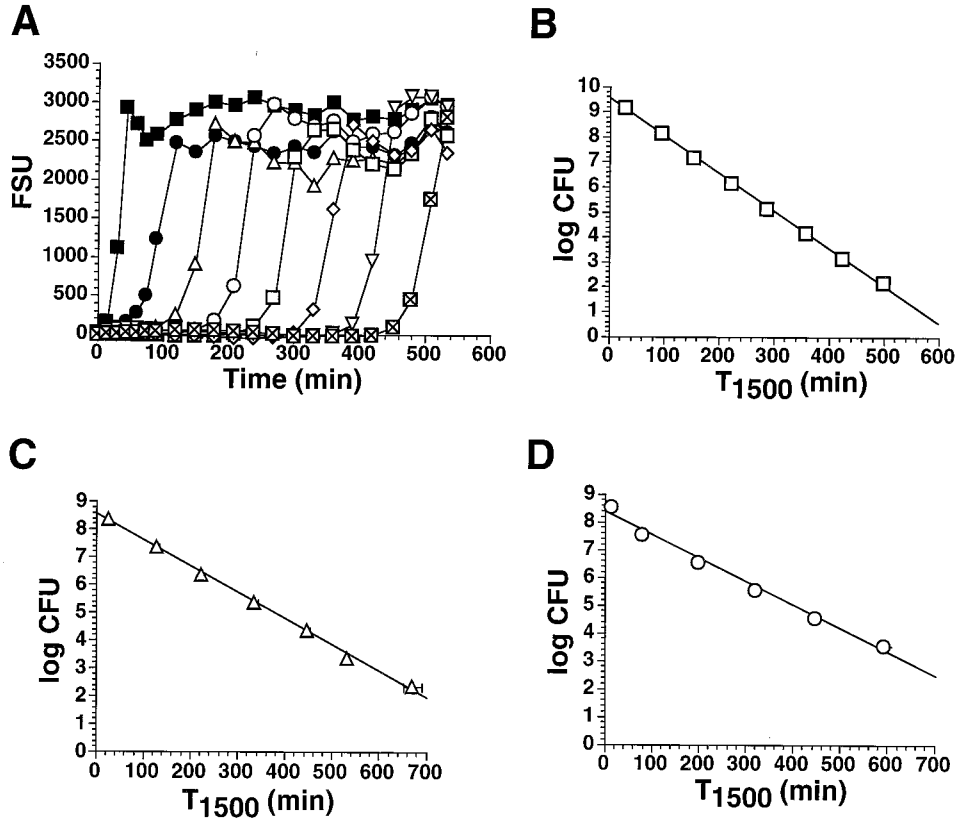

FIG. 1. Standard curves of $T_{1,500}$ versus log CFU. (A) Tenfold serial dilutions of *S. typhimurium* ATCC 14028 were plated in triplicate in LB-AlamarBlue™ in 96-well plates. The fluorescence was determined with a Millipore Cytofluor 2350 fluorometer (excitation, 530 nm; emission, 590 nm) every 15 to 30 min for 10^8 (■), 10^7 (●), 10^6 (△), 10^5 (○), 10^4 (□), 10^3 (◇), 10^2 (▽), and 10^1 (☒) bacteria/well. (B to D) By using linear regression analysis, $T_{1,500}$ was determined for each bacterial dilution and $T_{1,500}$ versus log CFU per milliliter was plotted for *S. typhimurium* ATCC 14028 (B), *E. coli* HB101 (C), and *L. monocytogenes* (D). The data shown are the mean of triplicate samples ± standard error. In this and all subsequent figures, many of the bars denoting standard errors fall within the symbols. One of three similar experiments is shown for each bacterium. The lines drawn to fit the data are based on linear regression analysis and represent $y = -0.015x + 9.53$, $r^2 \geq 0.99$ (B), $y = -0.009x + 8.53$, $r^2 \geq 0.99$ (C), and $y = -0.008x + 8.34$, $r^2 \geq 0.99$ (D), where y is log CFU per milliliter and x is time to 1,500 FSU.

malized FSU). Where the curves of FSU versus time were linear, they were analyzed by linear regression. By using the calculated slope and y intercept, the time to 1,500 FSU ($T_{1,500}$) (~half-maximal fluorescence) was calculated for each dilution. $T_{1,500}$ was plotted against log CFU (determined on LB plates as described above), and the relationship was analyzed by linear regression.

Bactericidal effects of GSNO and H_2O_2. Fresh overnight cultures of *E. coli* were diluted to an optical density at 600 nm of 0.01 to 0.02 (~10^6 to 10^7 CFU/ml). Triplicate 100-μl aliquots were incubated with either GSNO or H_2O_2 at the indicated final concentrations in 96-well plates at 37°C under 5% CO_2. A 10-μl sample of the bacterial mixture from each reaction condition was taken out at 2, 4, and 6 h and put into 100 μl of LB-AlamarBlue in new 96-well plates. The plates could be kept at 4°C overnight without any effect on the fluorescence curves generated at 37°C the following day (data not shown). The number of surviving bacteria at each incubation time point was then deduced from the growth of the bacteria in LB-AlamarBlue as described above.

Bactericidal assays with macrophages. Mice lacking iNOS and their wild-type littermates (C57BL/6 × 129/SvEv) were as described previously (10); mice lacking the $gp91^{phox}$ subunit of the NADPH oxidase flavocytochrome *b* (C57BL/6) were raised from breeders generously provided by Mary Dinauer (23). The mice were given intraperitoneal injections of 1 ml of 5 mM sodium periodate in PBS, prepared immediately before use and filtered through a 0.22-μm-pore-size Millex filter (Millipore Corp.). Sodium periodate, a lymphocyte mitogen, stimulates cytokine production and macrophage activation (18). Four days later, the mice were sacrificed by CO_2 inhalation and peritoneal cells were harvested by lavage with 10 ml of ice-cold, sterile RPMI. The cells were centrifuged at 250 × *g* and 4°C for 10 min, and the pellet was resuspended in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 1% L-glutamine (complete medium) plus 10 μg of gentamicin per ml. Trypan blue-excluding cells were counted with a hemocytometer, and the proportion of macrophages (≥60%) was determined by differential counting of Diff-Quik-stained cytocentrifuge preparations. The cells were plated in triplicate in 96-well plates (one plate per time point) and incubated at 37°C under 5% CO_2 for 48 h prior to infection. At 24 h prior to infection, the plates were washed twice with sterile PBS by flicking over a basin, and 100 μl of fresh complete medium without antibiotics was added to each well. The manner of emptying the wells during washing is emphasized; neither inversion nor aspiration completely removed the medium (data not shown). For opsonization, an overnight culture of *S. typhimurium* was diluted to an optical density at 670 nm of 0.02 (~2.5×10^7 bacteria/ml) in sterile PBS and opsonized in 10% normal mouse serum at 37°C for 30 min, with end-over-end rotation. Immediately prior to infection, the plates were washed twice with sterile PBS by flicking over a basin, and 100 μl of fresh complete medium without antibiotics was added to each well. Then, 10 μl of opsonized bacterial suspension (~10^6 bacteria) was added to each well and the plates were centrifuged at 250 × *g* and 25°C for 5 min. Plates were transferred to a 37°C 5% CO_2 incubator for 10 min for additional phagocytosis and then washed three times with sterile PBS as described above. All the wells in one plate were immediately lysed by the addition of 100 μl of sterile 0.1% sodium deoxycholate in PBS per well. Neither deoxycholate up to 0.5% nor phagocyte lysate interfered with the AlamarBlue assay (data not shown). The well contents were mixed by vigorous pipetting to ensure disruption of macrophages. From each well, 10 μl of lysate was transferred via a multichannel pipette into 100 μl of LB-AlamarBlue in a readout plate. The readout plate could be wrapped in foil and stored at 4°C overnight. The remaining cell culture plates were replenished with 100 μl of complete medium plus 10 μg of gentamicin per ml to kill extracellular bacteria, and incubation was resumed at 37°C under 5% CO_2. At each indicated time point, another cell culture plate was lysed and aliquoted to a readout plate as described above. Following the last time point, all the readout plates were transferred from 4 to 37°C and shaken at 75 rpm. Their fluorescence was determined every 30 min until it was maximal. Comparison of $T_{1,500}$ data to the standard curve yielded CFU equivalents for each well. The percent survival was determined by dividing the CFU at each time point and condition by the starting CFU for that condition.

Bactericidal assays with neutrophils. Human PMN were isolated from heparinized blood of normal donors by a one-step, modified Ficoll-Hypaque gradient separation (neutrophil isolation medium; Cardinal Associates, Santa Fe, N.Mex.) as described previously (14). Erythrocytes were lysed by hypotonic shock, and PMN were resuspended in ice-cold KRPG. A 96-well plate was

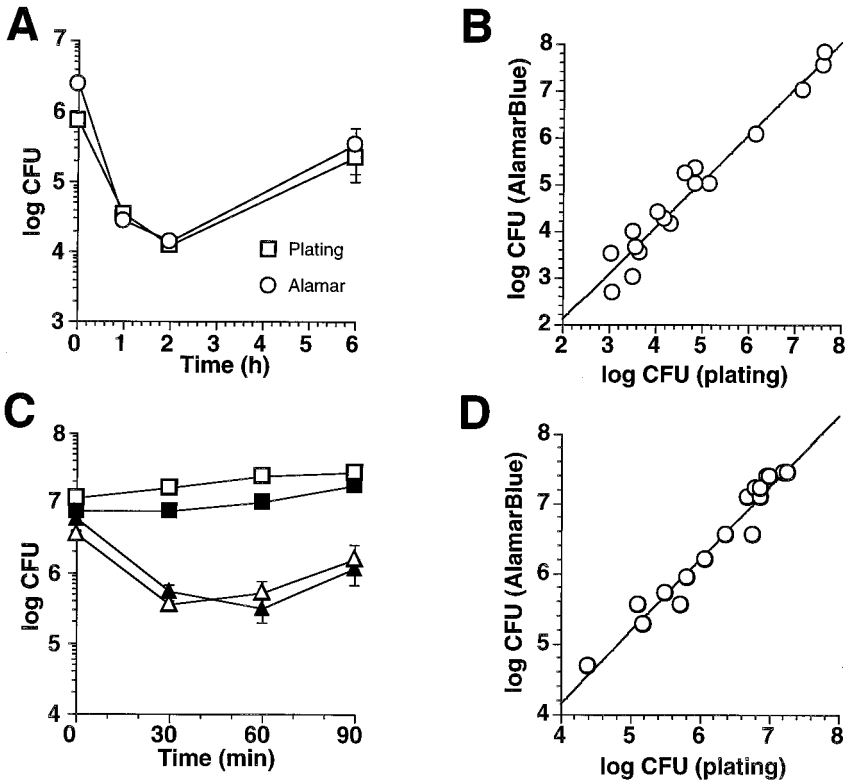

FIG. 2. Comparison of unknown bacterial concentrations determined by dilutional plating versus AlamarBlue. (A) Murine peritoneal cells, ~5×10^5/well, were infected with *S. typhimurium* as described in the text. The wells were lysed at various time points, and the number of CFU/well was determined either by plating or by the AlamarBlue assay. (B) Correlation of log CFU determined by plating versus log CFU determined by AlamarBlue from four independent experiments like that in panel A. The line drawn to fit the data was calculated by linear regression and represents $y = 0.99x + 0.12$ ($r^2 = 0.96$). (C) *S. typhimurium* was added to 100 μl of either KRPG (squares) or human neutrophils (5×10^5/well) suspended in KRPG (triangles). The plate was centrifuged for 5 min to enhance and synchronize infection and subsequently incubated at 37°C. The appropriate wells were lysed at 0, 30, 60, and 90 min by the addition of 10 μl of 1% sodium deoxycholate, and 12 μl of lysate was transferred to the readout plate. CFU was determined either by plating (solid symbols) or AlamarBlue (open symbols). One of three similar experiments is shown. (D) Correlation of log CFU determined by plating versus log CFU determined by AlamarBlue from three independent experiments like that in panel C. The line drawn to fit the data was calculated by linear regression and represents $y = 1.0x + 0.03$ ($r^2 = 0.96$).

coated at 37°C under 5% CO_2 for 1 h with 50 μl of fetal bovine serum (Hyclone) per well to prevent nonspecific activation (16); this was followed by three washes with sterile 0.9% saline. *S. typhimurium* cells were opsonized as described above but with human serum instead of mouse serum. PMN were plated in triplicate at 5×10^5/well (100 μl) in parallel with cell-free control wells, followed immediately by the addition of 10 μl of serum-coated *S. typhimurium* at ~2.5×10^7/ml (~2.5×10^5/well). The plate was centrifuged at 250 × *g* and 25°C for 5 min to reduce the time required for bacteria to reach the PMN. The plate was incubated at 37°C, and the PMN were lysed by the addition of 10 μl of 1% sodium deoxycholate at 0, 30, 60, or 90 min. The number of bacteria in PMN-containing wells and cell-free control wells was determined by transferring 12 μl (from a total of 120 μl) of the lysates to 100 μl of LB-AlamarBlue in a readout plate and determining $T_{1,500}$ for each well. Comparison of $T_{1,500}$ data to the standard curve yielded CFU equivalents for each well.

Statistical analyses. Where indicated, the data were analyzed by analysis of variance followed by Student's two-tailed unpaired *t*-test with StatView 4.5 (Abacus Concepts, Inc., Berkeley, Calif.) statistical software.

RESULTS

Comparison of AlamarBlue and CFU assays. Three species of bacteria were diluted serially in LB-AlamarBlue, and fluorescence was measured at 15- to 30-min intervals (Fig. 1A). Curves of fluorescence versus time shifted to the right as the starting concentration of bacteria decreased. For each dilution, $T_{1,500}$ (see Materials and Methods) was calculated by regression analysis of the linear portion of the fluorescence curve. $T_{1,500}$ was plotted against log CFU/ml (ascertained by counting colonies on plates; see Materials and Methods). $T_{1,500}$ was a linear function of log CFU/ml over the range 10^9 to 10^2 bacteria/ml for *S. typhimurium* (Fig. 1B) and *E. coli* HB101 (Fig. 1C) and over the range 10^8 to 10^3 bacteria/ml for *L. monocytogenes* (Fig. 1D) (correlation coefficients [r^2] ≥ 0.99 for all three). Fluorescence was maximal for *E. coli* and *S. typhimurium* by 10 h and for *L. monocytogenes* by 12 h. Repeated experiments on different days showed negligible variation in standard curves. Hence, at least three independently determined standard curves for each organism were averaged for use in subsequent experiments.

The ability of wild-type activated macrophages to kill *S. typhimurium* was quantitatively concordant when assessed by the AlamarBlue assay and by determination of the CFU on the plates (Fig. 2A). Pooling results from 4 such experiments with macrophages, close correlation was evident ($r^2 = 0.96$) over a wide range of surviving organisms (Fig. 2B).

Concordance extended also to the killing of *S. typhimurium* by human PMN (Fig. 2C and D). PMN reduced absolute numbers of *S. typhimurium* by ~1.0 $\log_{10}$ unit after 60 min compared to time zero and by ~1.5 $\log_{10}$ units compared to cell-free controls at 60 min, consistent with previous reports (19). The consistently observed difference at time zero between starting concentrations in cell-free and PMN-containing wells may reflect bacterial killing by PMN during the 5-min centrifugation prior to the start of the experiment.

Susceptibility of bacteria to individual products of phagocytes in chemical form. *E. coli* was inhibited in a time-depen-

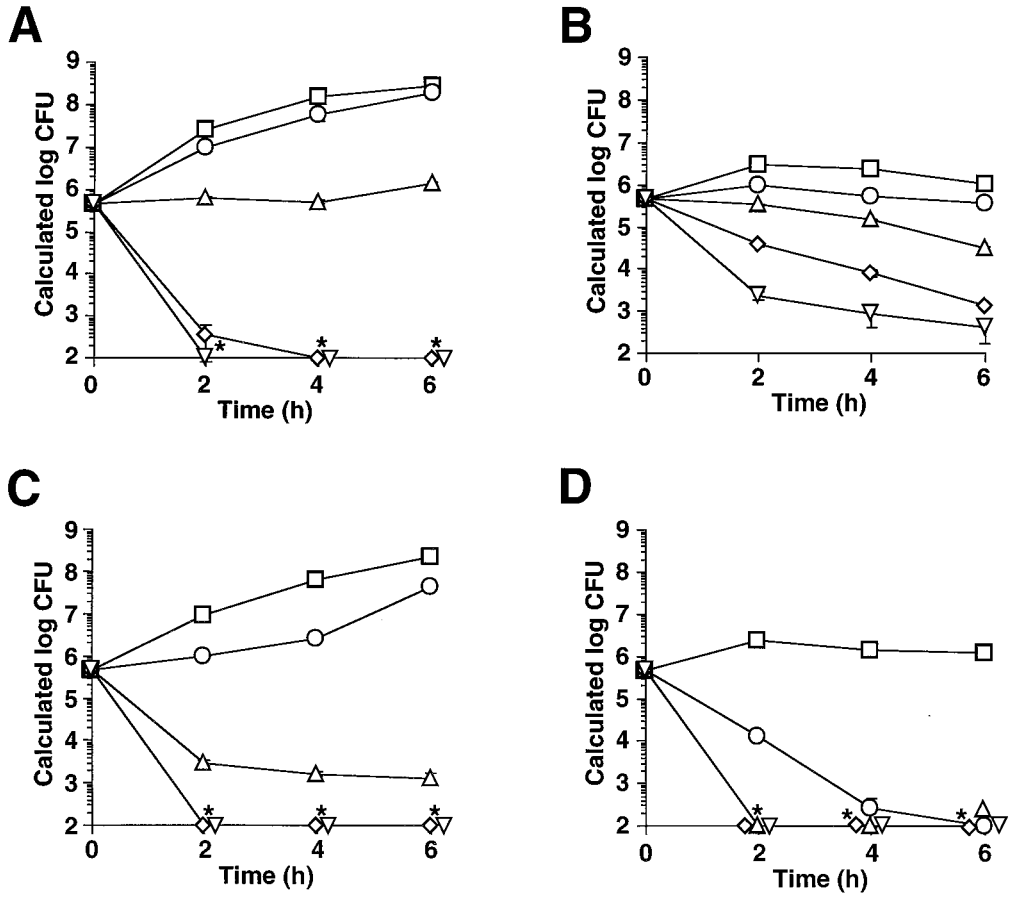

FIG. 3. Survival of *E. coli* HB101 in GSNO and H_2O_2. An overnight culture of *E. coli* HB101 was diluted to ca. 10^6 CFU/ml in either LB broth (A) or M9 (B), and 100 μl was exposed to 0 (□), 2 (○), 5 (△), 7.5 (◇), or 10 (▽) mM GSNO for 2, 4, or 6 h. For the response to H_2O_2, bacteria were diluted as above in LB broth (C) or M9 (D) and 100 μl was exposed to 0 (□), 0.5 (○), 1 (△), 2 (◇), or 5 (▽) mM H_2O_2 for 2, 4, or 6 h. At each time point, 10 μl was removed from each well and transferred to 100 μl of LB-AlamarBlue for CFU quantitation. Asterisks indicate concentrations below the limit of sensitivity of the assay. One of two similar experiments is shown.

dent manner by both GSNO and H_2O_2. However, GSNO was markedly more bactericidal in LB broth (Fig. 3A) than in M9 (Fig. 3B). In contrast, H_2O_2 was more bactericidal in M9 (Fig. 3D) than in LB broth (Fig. 3C). At high concentrations of each agent, >4 $\log_{10}$ units of killing drove the assay below the lower limit of sensitivity. Only at GSNO or H_2O_2 concentrations of >1 mM in the LB-AlamarBlue solution did these compounds begin to render AlamarBlue fluorescent in the absence of bacteria (data not shown). Since the readout plate represented a 1:10 dilution of the culture plate, the maximum concentrations of GSNO and H_2O_2 in the readout plate were 1 and 0.5 mM, respectively. Thus, the fluorescence changes observed were attributable to bacteria, not to GSNO or H_2O_2.

Impaired killing of *S. typhimurium* by macrophages from gp91^{phox}-deficient mice. In vivo-activated mouse peritoneal macrophages were infected with opsonized *S. typhimurium*. Wild-type and iNOS-deficient macrophages effected rapid bacterial killing (Fig. 4). However, gp91^{phox}-deficient macrophages killed *Salmonella* much less efficiently than either wild-type or iNOS-deficient macrophages did (Fig. 4).

DISCUSSION

As bacteria grow, their environment becomes more reducing (7), converting AlamarBlue from its nonfluorescent oxidized form to its fluorescent reduced form. Previous investigators have used AlamarBlue to determine the MICs of test agents against a fixed number of bacteria (1, 17, 30) and fungi (21, 22). Resistance to a given concentration of the agent tested results in growth of the bacteria in the well concurrent with reduction of the dye to its red, fluorescent form. Susceptibility, manifest

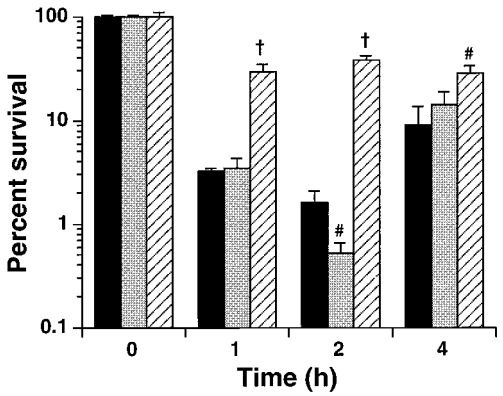

FIG. 4. Survival of *S. typhimurium* in an in vitro macrophage bactericidal assay. Periodate-elicited peritoneal cells (~4 × 10^5/well) from C57BL/6 × 129/SvEv (black bars), iNOS$^{-/-}$ (grey bars), or gp91^{phox}-deficient (striped bars) mice were infected with *S. typhimurium*. At the indicated times, macrophages were lysed and surviving bacterial CFU were determined. Values are means ± standard error for three independent experiments, each in triplicate. †, $P \leq 0.0001$ versus wild-type macrophages; #, $P < 0.05$ versus wild-type macrophages.

as absence of bacterial growth, maintains the dye in its oxidized, blue, nonfluorescent form. Thus, a dose-response curve can be generated with a binary readout: red/blue or fluorescent/nonfluorescent. The present approach renders such assays more versatile by exploiting the strict inverse relationship between the input number of bacteria and the time required to reach a given fluorescence.

Compared to some conventional assays, the dye reduction method is inexpensive and its reagents are few and nonhazardous. Provided that a plate-reading microfluorimeter and computer are available, the labor required is minimal. For example, the experiment illustrated in Fig. 4 consumed four 96-well plates and required ~4 h for readout. To obtain the same information from conventional dilution and plating would have required more than 250 LB agar plates and ~20 h for readout. Moreover, with the present assay, bacterial numbers can be calculated in a single well of a 96-well plate over a wide range—10^2 to 10^9 per ml for *S. typhimurium* and *E. coli* and 10^3 to 10^8 per ml for *L. monocytogenes*. To cover the same range by dilution and plating would require four plates per sample for *S. typhimurium* and *E. coli* and three plates per sample for *L. monocytogenes*. The accuracy of the dye reduction assay is reflected in the excellent correlation ($r^2 = 0.96$ to 0.99) between the number of bacteria inferred from fluorescence and the number of CFU documented by dilutional plating. The precision of the assay is manifest in the negligible standard errors for replicates. Finally, the assay allows simultaneous testing of multiple variables in a single experiment. This is helpful for testing dose-response curves, combinations of agents, different assay media, various bacterial strains or species, and different populations of phagocytes.

A potential drawback of this assay is the possibility that a test agent will interfere with the ability of the bacteria to lower the redox potential and thereby reduce the dye without affecting the replicative potential of the bacteria, leading to a false interpretation of diminished viability. However, impaired ability to generate reducing equivalents is unlikely to occur in the absence of an effect on colony formation. Nonetheless, with each new test agent or organism, it would be prudent to compare the dye reduction assay with classical plating.

Colicidal activity required higher concentrations of GSNO than of H_2O_2. It is difficult to extrapolate these results to the intraphagosomal milieu because of the marked effect of the composition of the medium in which the experiment was conducted. This may reflect variable formation of NO in the two media due to different concentrations of transition metals such as Cu^{2+} or Fe^{2+}, which catalyze the decomposition of nitrosothiols (11), and/or different contents of proteins that could serve as targets for extracellular oxidants or reductants.

The dye reduction assay demonstrated that macrophages from mice with an induced form of chronic granulomatous disease were markedly impaired in their bactericidal activity toward *S. typhimurium*, an organism that frequently infects patients with chronic granulomatous disease (13). In contrast, iNOS appeared to be dispensable for activated mouse peritoneal macrophages to kill *S. typhimurium* in vitro, although De Groote et al. showed that mice treated with aminoguanidine, a NOS inhibitor, were more susceptible to *S. typhimurium* (5).

Given that the dye reduction assay also demonstrated microbicidal activity in human PMN, it may prove clinically useful by allowing the rapid identification of bactericidal defects in patients' leukocytes and assessing responses to treatments (6, 25). Finally, the assay will be useful in comparing the response of phagocytes to pathogens with altered genomes.

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