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# Counterprotective Effect of Erythrocytes in Experimental Bacterial Peritonitis Is Due to Scavenging of Nitric Oxide and Reactive Oxygen Intermediates

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**Erythrocytes (RBC) in the peritoneal cavity significantly increase the lethality of bacterial peritonitis. The lethality is known to be associated with, and perhaps due to, increased bacterial counts in the peritoneal cavity. The mechanism is unknown. In this study, we investigated the hypothesis that RBC scavenge reactive oxygen intermediates (ROI) and nitric oxide (NO), so that the counterprotective effect is due to a loss of the microbiostatic activity of both ROI and NO. To study this effect, rats were subjected to a peritoneal inoculation of live *Escherichia coli* without RBC (nonlethal dose) or with RBC (lethal dose). The adjuvant effect of RBC was not modified by $N^G$-monomethyl-L-arginine (NMA, an NO synthase inhibitor), superoxide dismutase, catalase, mannitol, or a combination of these agents. Furthermore, the increased number of bacteria in the peritoneal cavity in the presence of RBC was unaffected by these treatments. The administration of NMA with bacteria alone (no RBC) converted a nonlethal model into a lethal one associated with higher intraperitoneal bacterial counts. A similar effect was seen with superoxide dismutase and catalase but not with mannitol. During bacterial peritonitis in the absence of RBC, superoxide and NO formation (determined by the total nitrite plus nitrate formed) was detected in the ascites and inducible NO synthase mRNA expression was present in the peritoneal cells. In the absence of RBC, superoxide was detected and oxidation of dihydrorhodamine to rhodamine was observed, indicating that peroxynitrite was produced. Both were blocked by the inclusion of RBC. Preinjection with a low inoculum of killed bacteria protected the rats from a subsequent lethal peritoneal bacterial challenge; this effect was reversed by scavenging ROI and NO. The protective effect of killed bacterial pretreatment was lost when RBC were placed in the peritoneal cavity. In vitro bactericidal activity of NO- and ROI-generating macrophages was also inhibited by RBC or by inhibiting ROI and NO formation. Taken together, these data are consistent with the hypothesis that RBC can impair bacterial clearance by removing both NO and ROI, suggesting that NO in combination with superoxide may be important to the antimicrobial defenses of the peritoneal cavity.**

Phagocytes, both neutrophils and macrophages, provide the primary cellular defense against local and systemic bacterial infections in animals. Phagocytes, when exposed to microorganisms or a chemotactic stimulus, exhibit a burst in oxygen consumption coincident with the generation of reactive oxygen intermediates (ROI), such as superoxide ($O_2^{\cdot -}$) and $H_2O_2$, through the activation of NADPH oxidase. This is followed by the formation of even more-powerful oxidizing species such as the hydroxyl radical and HOCl. Such ROI play important roles both in antimicrobial host defenses and in oxidative tissue injury (42).

Phagocytes (particularly macrophages) activated by lipopolysaccharide (LPS) and/or cytokines also produce nitric oxide (NO) by the expression of inducible NO synthase (iNOS) (33) which catalyzes the conversion of one of the guanido groups in L-arginine to NO. NO is a diffusible and uncharged free radical capable of reacting with iron-containing proteins and thiol compounds. NO has a vast array of biological functions (32), including vasoregulation, neurotransmission, and immune regulation. NO produced by activated macrophages has been implicated in the inhibition or killing of tumor cells and various microorganisms through depletion of thiols and inhibition of ATP and DNA synthesis (34).

$O_2^{\cdot -}$ and NO react together at a diffusion-controlled rate (19) to yield peroxynitrite ($ONOO^-$), a potent oxidant with a half-life of about 1 s at neutral pH. Peroxynitrite inflicts cellular damage through oxidation of sulfhydryl groups, nitration of tyrosine residues in proteins (12), oxidation of α-tocopherol (17), DNA damage (44), and lipid peroxidation (35). It has also been implicated in inactivation of the Mn and Fe superoxide dismutase (SOD) (20) and aconitase (3). Peroxynitrite, therefore, could mediate NO-dependent microbe killing and cellular damage by phagocytic cells (2).

Erythrocytes (RBC) have several antioxidant enzymes, including SOD, catalase, and glutathione reductase, and are a well-known cellular antioxidant for ROI in biological systems (1). RBC and hemoglobin (Hb) have also been implicated in NO scavenging in vitro and in vivo (26, 36). The evidence suggests that RBC (or Hb) can inhibit the oxidative bactericidal mechanism in $O_2^{\cdot -}$-generating systems employing xanthine oxidase as well as phagocytes (13, 23). This is further supported by the finding that erythrophagocytosis can inhibit antibacterial function and oxidative bactericidal mechanisms in macrophages (14).

Although RBC and Hb have long been known to increase

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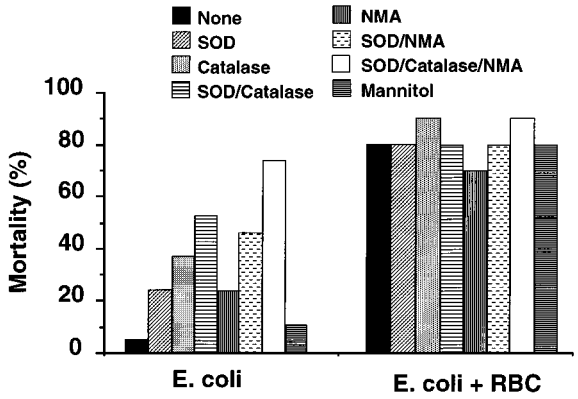


FIG. 1. Effects of putative radical scavengers on experimental bacterial peritonitis. Rats were injected i.p. with a nonlethal dose of *E. coli* ($4 \times 10^9$ CFU/kg) with or without RBC (12.4 μmol of monomeric Hb/kg). SOD (2,600 U/kg), catalase (11,000 U/kg), NMA (10 mg/kg), and mannitol (40 mg/kg) were injected i.p. at 0, 5, 10, and 15 h after bacterial administration. Mortality was determined at 24 h after administration. Each group consisted of 15 rats.

mortality in experimental bacterial peritonitis (6, 8, 15), all the mechanisms have not yet been clearly elucidated. Understanding how RBC increase mortality is important because peritonitis commonly occurs in situations associated with intraperitoneal hemorrhage such as trauma with bowel perforation, bowel perforation due to other forms of pathology, and following surgical procedures to the abdomen. In this study, we examined the hypothesis that the effect of RBC in peritonitis is due to the scavenging of both ROI and NO, thereby quenching the microbicidal properties of these species. Our study demonstrates that ROI and NO are both involved in inhibition of bacterial proliferation and bactericidal activity in vivo. Furthermore, our data directly demonstrate that the adjuvant effect of RBC on mortality in bacterial peritonitis is associated with their capacity to scavenge ROI and NO, thus inhibiting the formation of the antimicrobial agent $ONOO^-$.

## MATERIALS AND METHODS

**Materials.** Catalase (from bovine liver), SOD (from bovine erythrocytes), phorbal myristate acetate (PMA), cytochrome *c* (from horse heart), phosphate-buffered saline (PBS; pH 7.4), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and mannitol were obtained from Sigma (St. Louis, Mo.). DMPO was used after redistilling. RBC were prepared by collecting blood from rats; removing the plasma, leukocytes, and platelets after centrifugation at $2,000 \times g$ for 10 min; and washing four times with PBS. No detectable contamination with leukocytes was found in RBC samples by light microscopy. The Hb concentration was measured by spectrophotometry after hypotonic lysis in distilled water (45). Dihydrorhodamine 123 was obtained from Molecular Probes (Eugene, Oreg.).

**Preparation of bacterial solution.** *Escherichia coli* (ATCC 25922) was incubated on an agar plate at 37°C for 17 h, and a single colony was cultured in tryptic soy broth for an additional 18 h to obtain a suspension of approximately $10^9$ cells per ml. The bacteria were harvested by centrifugation, washed three times with saline, and resuspended in saline at $10^9$ bacteria per ml. The concentration of the bacteria was first estimated at 550 nm by spectrophotometry. The number of viable bacteria was directly determined by enumeration of developed colonies after serial dilution in saline, inoculation on agar plates, and incubation at 37°C for 18 h. Killed *E. coli* was prepared by treatment with formalin (7), and death was confirmed by plating on the agar plates.

**Animal mortality.** Male Sprague-Dawley rats (180 to 200 g) were given standard rat pellets and water ad libitum. Experimental peritonitis was induced in rats by intraperitoneal (i.p.) injection of *E. coli* ($4 \times 10^9$ CFU/kg of body weight) with or without RBC (12.4 μmol of monomeric Hb per kg). The injection mixtures were prepared immediately before injection and the injection volume was 5 ml/kg. Mortality was determined 24 h after administration. To determine the preinjection effect of killed *E. coli* on mortality, rats were preinjected i.p. with formalin-killed *E. coli* ($5 \times 10^7$/kg) 7 h before injection with either a lethal ($10^{10}$ CFU/kg) or nonlethal ($4 \times 10^9$ CFU/kg) dose of bacteria plus RBC (12.5 μmol/kg). Each group consisted of 12 or 15 rats.

**ESR and EPR spectra of DMPO spin adducts and Hb-NO.** For the determination of ROI generation, rats were injected i.p. with NMA ($N^G$-monomethyl-L-arginine; 20 mg/kg) at 3 and 5 h after bacterial injection ($4 \times 10^9$ CFU/kg). DMPO (2.5 ml of a 1 M solution per kg) was injected i.p. at 6 h. Rats were gently massaged for 30 s to ensure adequate mixing of peritoneal contents. After 20 min, peritoneal fluid (500 μl) was obtained after midline laparotomy and immediately transferred into an electron spin resonance (ESR) flat cell. ESR spectra were recorded with a Varian E-109 spectrometer at a microwave power of 20 mW, a modulation frequency of 100 kHz, a modulation amplitude of 1.0 G, a time constant of 0.25 s, and a receiver gain of $1.25 \times 10^4$. To measure Hb-NO, rats were injected i.p. with SOD (2,600 U/kg) twice, at 3 and 7 h after the i.p. injection of *E. coli* and RBC. The peritoneal ascites from rats treated with *E. coli* plus RBC were transferred into electron paramagnetic resonance (EPR) tubes and frozen in liquid nitrogen. EPR spectra were measured at 77 K at a power of 1 mW, a modulation amplitude of 8 G, and a receiver gain of $10^4$ (27).

**Oxidation of dihydrorhodamine.** After 7 h of treatment with saline, bacteria, or a combination of bacteria and RBC, rats were injected i.p. with diethylenetriaminepentaacetic acid (125 μmol/1.5 ml/kg) and then gently massaged for 30 s to ensure adequate mixing of the peritoneal contents. The rats were then injected i.p. 30 min later with dihydrorhodamine 123 (1 μmol/ml/kg) and gently massaged. After 20 min, peritoneal fluid was obtained and immediately centrifuged to remove peritoneal cells. The supernatant was filtered through a Centricon 10 (Amicon) filter. Oxidation of dihydrorhodamine to rhodamine was determined spectrophotometrically at 550 mm ($\varepsilon_{max}$ of 78,000 $M^{-1} \cdot cm^{-1}$) as described previously (11).

**Bacterial killing by macrophages.** Isolated peritoneal macrophages ($10^5$ cells per 200 μl per well) were plated on 96-well plates, incubated for 6 h, washed twice with Williams E medium, and cultured in the fresh medium for 12 h. After stimulation with LPS (10 μg/ml) plus gamma interferon (IFN-γ) (100 U/ml) for 10 h, the cells were incubated with *E. coli* ($10^6$ cells per 200 μl per well) in the presence or absence of PMA (0.5 μg/ml), SOD (400 U/ml), catalase (400 U/ml), and RBC (200 μM of Hb) for an additional 4.5 h. Bacterial killing was determined by counting colonies on agar plates after serial dilution of the culture solution and macrophage lysate (with 1% deoxycholate) in saline.

**Northern RNA blot analysis.** Peritoneal fluid was obtained from the rats 6 h after bacterial injection i.p. with or without RBC. Peritoneal cells were plated on 60-mm-diameter plates. The cells were allowed to adhere for 2 h, the plates were washed twice with PBS, and total RNA from adherent cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA (20 μg) was electrophoresed on a 1% agarose gel containing 1% formaldehyde, transferred to GeneScreen, hybridized with a murine iNOS $^{32}$P-labeled cDNA probe (gift from Charles Lowenstein, Johns Hopkins University), and exposed to autoradiography film as described previously (22).

**Assay of $NO_2^-$, total $NO_2^-$ plus $NO_3^-$, and $O_2^{\cdot -}$ production.** The combination of nitrite plus nitrate ($NO_2^-$ plus $NO_3^-$) in peritoneal fluid was determined by an automated high-performance liquid chromatography method with the use of a cadmium column after deproteinization (22). $NO_2^-$ only was assayed by the addition of 100 μl of Griess reagent to 100 μl of culture supernatant in flat-bottom 96-well plates (24). The $A_{550}$ of the reaction product was measured, and the $NO_2^-$ concentration was calculated from the standard curve of sodium nitrite. $O_2^{\cdot -}$ production was determined by SOD (100 U/ml)-inhibitable cytochrome *c* reduction, monitored at $A_{550}$ ($\Delta\varepsilon_{550}$, 21 $mM^{-1} \cdot cm^{-1}$ for reduced-oxidized cytochrome *c*) in PBS containing cytochrome *c* (100 μM), PMA (0.5 μg), and catalase (100 U/ml) (23).

**Statistical analysis.** The data are summarized as the means ± standard deviations unless indicated otherwise. The significance between the effects of SOD, catalase, and NMA on rat mortality was analyzed by the chi-square test. Significance between groups was determined by the Student unpaired *t* test.

## RESULTS

**Effect of antioxidant enzymes and NMA on mortality.** As shown in Fig. 1, the i.p. injection of live bacteria alone ($4 \times 10^9$ cells per kg) resulted in only a 6% mortality after 24 h, which is consistent with our previous findings (24). Under the same experimental conditions, coadministration of RBC increased mortality to 80% ($P < 0.01$). To determine if the increased lethality involved the generation of ROI and NO, we tested the effect of SOD, catalase, or NMA (an inhibitor of NOS) on mortality in the rat peritonitis model. In nonlethal bacterial peritonitis (no RBC), administration of SOD and catalase increased mortality to 27% ($P > 0.05$) and 38% ($P < 0.05$), respectively, while NMA increased mortality to 27% ($P > 0.05$). Combinations of these agents resulted in further increases in mortality: SOD and catalase, 53% ($P < 0.01$); SOD and NMA, 47% ($P < 0.02$). The combination of all three agents was associated with a mortality of 73% ($P < 0.01$). Mannitol, a scavenger of the hydroxyl radical, had no effect.

TABLE 1. Effect of ROI and NO scavengers on NOS and bacterial proliferation[a]

| Treatment | $NO_2^- + NO_3^-$ (nmol/rat) | Ratio of $NO_2^-/NO_3^-$ | No. of bacteria ($10^9$ cells/200 g of body wt)[b] |
|---|---|---|---|
| *E. coli* alone | 81 ± 32 | 0.54 ± 0.11 | 1.8 ± 0.4 |
| *E. coli* + RBC | 94 ± 20 | 0.08 ± 0.03 | 87.5 ± 8.7 |
| *E. coli* + SOD + catalase | 90 ± 35 | 0.47 ± 0.14 | 31.4 ± 1.5* |
| *E. coli* + NMA | 10 ± 5 | 0.57 ± 0.15 | 20.3 ± 0.8* |
| *E. coli* + SOD + catalase + NMA | 9 ± 3 | 0.49 ± 0.09 | 68.7 ± 7.5** |

[a] Data are presented as the means ± standard deviations for four animals.
[b] $P < 0.01$ versus RBC treatment (*) and $P > 0.05$ versus RBC treatment (**) by Student's unpaired $t$ test.

None of the combinations of SOD, catalase, and NMA or mannitol had any effect on the high rate of mortality for *E. coli* peritonitis when RBC were present (Fig. 1).

**Effects of antioxidant enzymes, NMA, and RBC on NO formation and bacterial proliferation.** Since there is evidence that ROI, NO, and their reaction breakdown products are involved in bacterial killing (2, 46), we examined the effects of the antioxidant enzymes and the NOS inhibitor on bacterial proliferation during bacterial peritonitis. As shown in Table 1, following bacterial injection ($4 \times 10^8$ cells per 200 g) the counts of viable bacteria at 14 h after inoculation were increased in the peritoneal cavity. Coinjection of RBC with the bacteria markedly increased bacterial proliferation in the peritoneal cavity. The administration of SOD and catalase or NMA resulted in a 10- to 15-fold increase in bacterial growth compared with that for injection of bacteria alone. Furthermore, the combination of the antioxidant enzymes with NMA further increased the bacterial counts. $NO_2^-$ plus $NO_3^-$ accumulated in the peritoneal cavity following bacterial injection in the presence or absence of RBC with or without SOD and catalase, while the accumulation was inhibited by coadministration with NMA. Equal amounts of $NO_2^-$ and $NO_3^-$ were detected following injection of bacteria only or bacteria with SOD and catalase, whereas coadministration with RBC resulted in the accumulation of $NO_3^-$ only.

**Detection of $O_2^{\cdot -}$ and NO formation and iNOS mRNA.** Detection of $O_2^{\cdot -}$ anion generation and NO formation in the peritoneal cavity during experimental peritonitis was accomplished by ESR and EPR spectrophotometric techniques, respectively. The DMPO-spin adduct of $O_2^{\cdot -}$ could be measured following bacterial injection but not after saline injection or coadministration of bacteria with RBC (Fig. 2A). EPR spectra of the Hb-NO complex could be detected following injection of bacteria plus RBC but not with saline injection or injection of bacteria alone (Fig. 2B). To determine if iNOS was expressed in the cells in the peritoneal cavity, Northern blot analysis was performed on the adherent cell population isolated from the peritoneal cavity (Fig. 3). An iNOS signal was detected in cells isolated from rats injected with bacteria, and administration of bacteria with RBC did not alter the iNOS mRNA level in the adherent cells. This signal was not detected in the cells from the sterile peritoneal cavities of control animals.

**Oxidation of dihydrorhodamine.** Dihydrorhodamine is known to be oxidized to rhodamine by $ONOO^-$ but not by NO, $O_2^{\cdot -}$, and $H_2O_2$ (11). To examine the formation of $ONOO^-$ in the bacterial peritonitis model, we determined the oxidation of dihydrorhodamine to rhodamine in the peritoneal cavity. As shown in Fig. 4, rhodamine was produced in the peritoneal cavities of the bacterium-treated rats (1.17 ± 0.27 μM rhodamine) but not in the peritoneal cavities of saline-treated rats (0.02 ± 0.02 μM). Coinjection of bacteria and RBC, however, abrogated the oxidation of dihydrorhodamine (0.23 ± 0.16 μM).

**Effect of pretreatment with killed bacteria on mortality.** It has long been known that pretreatment with a sublethal inoculum of *E. coli* renders the host less susceptible to subsequent bacterial infection (30). In this study, we examined the effect of preinjection of killed bacteria on the mortality of a higher lethal dose of live bacteria as well as the lethality of bacteria with RBC. As summarized in Table 2, in the absence of RBC, preinjection of killed bacteria reduced mortality induced by the subsequent injection of a high dose of bacteria. However, this protective effect was diminished when SOD plus catalase, NMA, or SOD plus catalase plus NMA was injected with the live bacteria. The inclusion of RBC in the challenging second inoculum also abrogated the protective effect of pretreatment with killed *E. coli*. However, pretreatment with killed bacteria

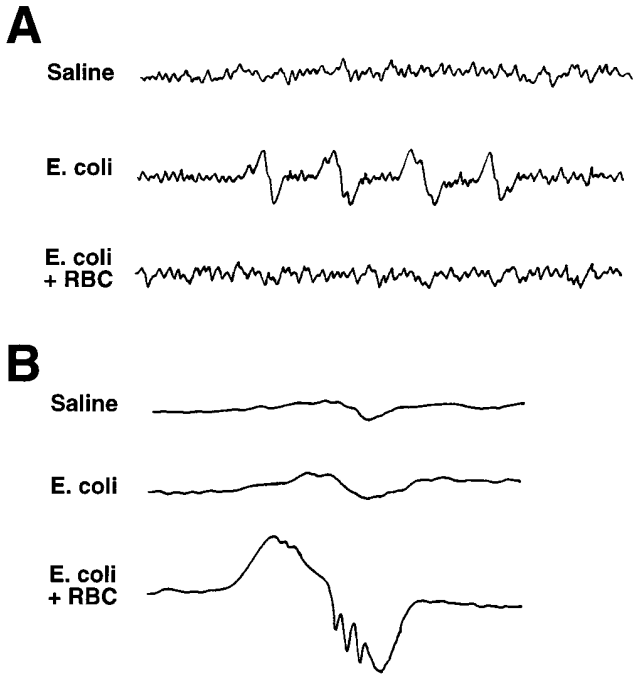


FIG. 2. Spectra of DMPO-spin adduct (A) and Hb-NO complex (B) formed in peritoneal cavities of rats with bacterial peritonitis. (A) NMA (20 mg/kg) was injected at 3 and 5 h after bacterial administration. DMPO (500 μl of a 1 M solution per rat) was injected at 6 h. After 20 min, peritoneal fluids were obtained for detection of spin adducts. (B) SOD (2,600 U/kg) was injected twice, at 3 and 7 h after administration of bacteria plus RBC. After 8 h, peritoneal fluids were obtained for determination of Hb-NO. The spin adducts were measured as described in Materials and Methods.

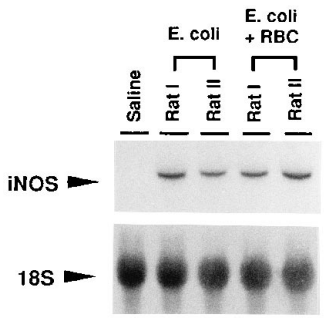


FIG. 3. Northern blot analysis of iNOS expression in adherent peritoneal cells during bacterial peritonitis. Peritoneal exudate cells obtained from rats with bacterial peritonitis were plated on 60-mm-diameter plates. After incubation for 2 h, the cells were washed twice with PBS, and total RNA was isolated. RNA (20 μg) was separated on an agarose gel, transferred to GeneScreen, hybridized with a $^{32}$P-iNOS probe, and exposed to X-ray film.

did not alter the adjuvant effect of RBC on mortality in the nonlethal bacterial peritonitis model. Also, the adjuvant effect of RBC was not abrogated by the addition of SOD, catalase, and NMA in the pretreatment model.

**Effect of ROI and NO on bacterial killing.** To determine if peritoneal macrophages exhibit a similar pattern of bacterial killing in vitro, cultured peritoneal macrophages were stimulated with LPS and IFN-γ (which normally induce iNOS in macrophages) and then incubated with bacteria in the presence or absence of PMA, SOD, catalase, NMA, or RBC. Macrophages exposed to LPS, IFN-γ, and PMA produced NO and $O_2^{\cdot-}$ at rates of 1.8 ± 0.4 and 1.6 ± 0.3 nmol/$10^5$ cells per h, respectively (data not shown). As shown in Fig. 5, the macrophages stimulated with LPS and IFN-γ exhibited a low level of bactericidal activity (12%; $P < 0.01$), which was inhibited by NMA, a combination of SOD and catalase, and RBC. The addition of NMA, SOD, catalase, or RBC to unstimulated macrophages had no effect on basal bactericidal activity. The addition of PMA increased bacterial killing by 35% in the absence of LPS and IFN-γ pretreatment and by 58% when the cells were pretreated with LPS plus IFN-γ. NMA, SOD, or a combination of NMA and SOD did not alter the killing with PMA alone but reduced the killing induced by PMA in LPS-plus-IFN-γ-pretreated cells by more than 20% ($P < 0.01$). Interestingly, catalase completely blocked bacterial killing by PMA in the nonstimulated system but only partially prevented the PMA-induced killing in the prestimulation system. Furthermore, the addition of catalase with SOD or NMA completely blocked bacterial killing by PMA in LPS-plus-IFN-γ-treated cells ($P < 0.01$). Finally, RBC completely blocked bacterial killing under all conditions.

## DISCUSSION

Blood in the peritoneal cavity is common after trauma or surgical procedures, and RBC are known to increase lethality in experimental peritonitis (5, 6). The most prominent characteristic reported so far for the adjuvant effect of RBC or Hb in experimental peritonitis is that these adjuvants seem to significantly increase the rate of bacterial growth in vivo (10, 15, 24) but not in vitro (39). RBC do not increase the lethality of dead *E. coli* or LPS, supporting a role for RBC in promoting bacterial growth. Previous experiments have shown that the influx of inflammatory cells into the peritoneal cavity is not diminished by RBC or Hb (21) but that neutrophils lose microbicidal activity in the presence of Hb (8). We have previously shown that lethality is related to the Fe component of the Hb molecule (28) and have postulated that Fenton and Habor-Weiss chemistry plays an important role in generating tissue toxic radicals in vivo (23). However, RBC are different from free Hb or iron-chelate complexes in their interaction with ROI because they have several types of antioxidant systems (see below).

Resident macrophages and exudated neutrophils in the peritoneal cavity play a role in the first line of defense against bacterial peritonitis. Phagocytes are known to produce ROI and NO, which are implicated in killing of bacteria (2) and tumor cells (29). As a result of this study, we hypothesize that RBC in the peritoneal cavity scavenge these reactive molecules and inhibit the antibactericidal mechanism(s) of these phagocytes. Our data presented here clearly show that ROI and NO are generated in the peritoneal cavity and that RBC scavenge both ROI and NO, allowing bacterial growth in the peritoneal cavity. Therefore, we conclude that ROI, NO, and their reaction products have important antimicrobial effects in the bacterial peritonitis model and that RBC prevent bacterial killing by scavenging these radicals. Our findings are consistent with the known effect of RBC on oxygen and nitrogen-free radicals (2, 11, 13, 22, 24, 27, 46).

Others have shown that RBC and an RBC lysate inhibit oxidative bactericidal mechanisms in a cell-free $O_2^{\cdot-}$-generating system through scavenging of ROI (13). Erythrophagocytosis by peritoneal and alveolar macrophages has also been known to inhibit bacterial killing (14). These effects on bacterial killing in macrophages and in the cell-free oxidative reactions by RBC or an RBC lysate could potentially be due to the antioxidant enzyme systems (SOD, catalase, and glutathione [GSH] peroxidase) or to interactions between RBC components (GSH and Hb) and reactive oxygen products. Kim et al. (24) have recently shown that free Hb inactivates bactericidal function of neutrophils stimulated by PMA. The cytotoxicity by purified Hb may be directly linked to the roles of pseudoperoxidase activity ($H_2O_2$ decomposition) and a Fenton-type reaction (production of a strong oxidant, ferryl Fe(IV) = O, which inactivates neutrophils but not bacteria) of Hb. However, the interaction of intact RBC with ROI is different from

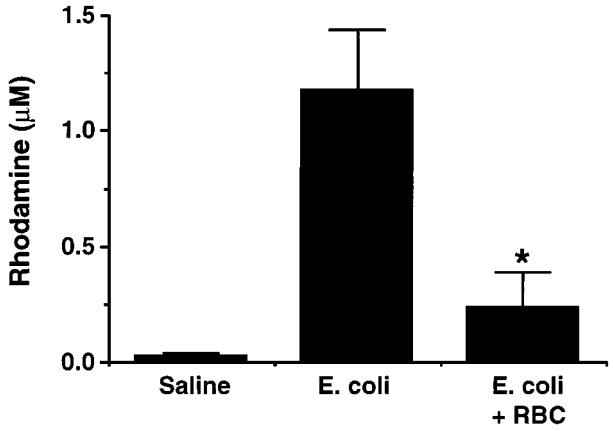


FIG. 4. Oxidation of dihydrorhodamine 123 in the peritoneal cavities of rats with bacterial peritonitis. Rats were injected i.p. with diethylenetriaminepentaacetic acid (125 μmol/1.5 ml/kg) 6 h after treatment with saline, bacteria, or a combination of bacteria and RBC and then injected i.p. 30 min later with dihydrorhodamine 123 (1 μmol/ml/kg). After 20 min, peritoneal fluid was obtained and centrifuged to remove peritoneal cells. The supernatant was filtered through Centricon 10 (Amicon) filters. Rhodamine was measured spectrophotometrically. Results are expressed as the means for three animals; error bars indicate standard deviations. *, $P < 0.01$ versus *E. coli* by Student's unpaired *t* test.

TABLE 2. Effect of preactivation of peritoneal macrophages by killed bacteria on mortality with bacterial peritonitis

| Pretreatment | Infective dose | Mortality (%) with[a]: | |
|---|---|---|---|
| | | Lethal dose | Nonlethal dose |
| Saline | *E. coli* | 75 | 0 |
| | *E. coli* + RBC | | 83 |
| Killed *E. coli* | *E. coli* | 17* | |
| | *E. coli* + RBC | | 75 |
| | *E. coli* + SOD/catalase | 42** | 83 |
| | *E. coli* + NMA | 33* | 75 |
| | *E. coli* + SOD + catalase + NMA | 67** | 75 |
| | *E. coli* + RBC | 67** | 75 |

[a] Each group consisted of 12 rats. Lethal dose of bacteria, $10^{10}$ CFU/kg; nonlethal dose of bacteria, $4 \times 10^9$ CFU/kg. By the chi-square test, $P < 0.01$ (*) and $P > 0.05$ (**) versus the saline-pretreated group.

that of purified Hb in many aspects, because RBC have several antioxidant systems in addition to high concentrations of Hb.

NO is a free radical and thus is highly reactive with iron-containing proteins. It is this biochemical characteristic that allows NO to mediate cell signalling through activation of guanylyl cyclase and tumoricidal activity through inhibition of cellular metabolism (32). OxyHb (oxyhemoglobin) also rapidly reacts with NO to neutralize cytotoxicity of NO (reactions 1 to 3). Although metHb (methemoglobin) formed by reaction with NO is detrimental in a biological system, RBC have a detoxifying system for metHb. Specifically, metHb reductase and NADPH generated by the hexose monophosphate pathway reduce metHb to oxyHb (reation 4). Thus, RBC have a large capacity to scavenge NO.

$$\mathrm{HbFe(II)O_2 + NO \rightarrow HbFe(III) + NO_3^-} \quad (1)$$

$$\mathrm{HbFe(II) + NO \rightarrow HbFe(II) - NO} \quad (2)$$

$$\mathrm{HbFe(II)NO + O_2 \rightarrow HbFe(III) + NO_3^-} \quad (3)$$

$$\mathrm{HbFe(III) + NADPH + H^+ \rightarrow HbFe(II) + NADP^+ + H_2O} \quad (4)$$

When stimulated with bacteria or other stimuli such as cytokines, macrophages and neutrophils produce $O_2^{\cdot -}$ and $H_2O_2$ by the activation of membrane-associated NADPH oxidase (4, 40). We show here that $O_2^{\cdot -}$ generation is detected by an ESR spin-trapping technique in the peritoneal cavity during bacterial peritonitis in the absence, but not in the presence, of RBC (Fig. 2). These reduced oxygen species have been known to be part of the antimicrobial defense system. Furthermore, in vitro bactericidal activity in the $O_2^{\cdot -}$-generating systems is almost completely inhibited by a combination of SOD and catalase (24). A role for $O_2^{\cdot -}$ and $H_2O_2$ is consistent with our in vivo data showing that administration of SOD and catalase increased the bacterial counts in the peritoneal cavity and caused high rates of mortality in the bacterial peritonitis model (Table 1 and Fig. 1).

Activated phagocytes produce not only $O_2^{\cdot -}$ but also NO. With the experimental conditions used in this study, we directly detected NO formation, as judged by the appearance of the Hb-NO complex as a reaction product of NO with adjuvant RBC in the peritoneal cavity (Fig. 2), by direct measurement of $NO_2^-$ and $NO_3^-$, and by the detection of iNOS mRNA from cells isolated from the peritoneal cavity (Fig. 3). Although $O_2^{\cdot -}$ and NO are individually not toxic against bacteria, their reaction product, $ONOO^-$, is a more powerful oxidant with bactericidal activity (2, 46). $ONOO^-$ can also produce many detrimental reactions in biological systems, including lipid peroxidation (35), inactivation of metal-containing enzymes (16), and inactivation of amiloride-sensitive sodium channels (18). Thus, this oxidant could be implicated in many pathological and antimicrobial defense processes. In this study, we showed that oxidation of dihydrorhodamine to rhodamine was measurable in the peritoneal cavity in the bacterial peritonitis rat. Although dihydrorhodamine can be oxidized by ROI generated from neutrophils (9), the method is sensitive and specific for $ONOO^-$ (11, 25). Furthermore, it is noteworthy that $O_2^{\cdot -}$ plus NO and the Fenton reaction system ($O_2^{\cdot -}$ plus $H_2O_2$ plus a transition metal) are known to oxidize dihydrorhodamine to rhodamine (37), indicating that $ONOO^-$ and a hydroxyl radical are responsible for dihydrorhodamine oxidation. We excluded the possibility of hydroxyl radical-mediated oxidation of dihydrorhodamine by preinjection of diethylenetriaminepentaacetic acid, an inhibitor of Fenton chemistry. Since $ONOO^-$ stoichiometrically produces 0.4 rhodamine from dihydrorhodamine (9), $ONOO^-$ was produced at about 3 μM/20 min (from 1.17 μM rhodamine/20 min) under the experimental conditions used in this study. Our data indicate that in bacterial peritonitis, $ONOO^-$ is essential for effective bacterial killing. It is also possible that metabolites of $ONOO^-$ participate in the bacterial killing. At physiological pH, 20% of $ONOO^-$ will be protonated to peroxynitrous acid. This protonated form isomerizes to a hydroxyl-like product, which is not identical to the chemical reactivity of the hydroxyl radical; typical scavengers of the hydroxyl radical do not attenuate the $ONOO^-$-mediated cytotoxicity. In our experiment, mannitol, a scavenger of the hydroxyl radical, did not show any effect on bacterial growth or mortality, suggesting that this radical was not involved in our observed effects.

Since activated phagocytes usually produce ROI rapidly and then generate NO hours later, infected bacteria may be exposed sequentially. Although NO itself is not toxic to bacteria, bacteria preexposed to ROI exhibit increased sensitivity to the aerobic products of NO and $ONOO^-$ (2). This increased sensitivity may be due to a decrease in intracellular antioxidant

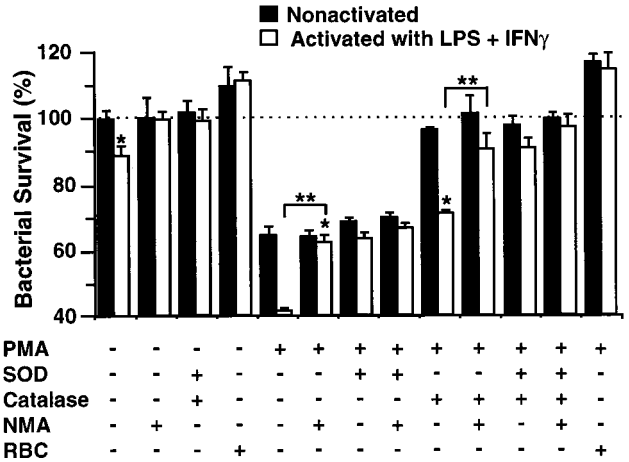


FIG. 5. In vitro bacterial killing by NO- and ROI-generating macrophages. Peritoneal macrophages were stimulated in culture with LPS plus IFN-γ for 10 h. The cells ($10^5$ cells per well in 96-well plates) were then incubated with *E. coli* ($10^6$ cells per 200 μl per well) in the presence or absence of PMA (0.5 μg/ml), SOD (400 U/ml), catalase (400 U/ml), and RBC (200 μM of Hb) for 4.5 h. Bacterial killing was determined by enumeration of developed colonies after serial dilution in saline, plating on agar plates, and incubation at 37°C for 18 h. $P < 0.01$ versus nonactivated group (*) and between indicated two groups (**) by Student's unpaired *t* test.

systems, including reduced glutathione, which is an excellent scavenger of ROI, NO, and $ONOO^-$. The greatest increase in bacterial proliferation in vivo and bacterial killing in vitro was seen when both ROI and NO were removed from the system. The effect of RBC was identical to removing ROI and NO, strongly suggesting that RBC increase bacterial proliferation and mortality by scavenging both ROI and NO.

Substances (zymosan, muramyl depeptide, and glucan) known to activate phagocytes and induce neutrophil influx have been shown to increase resistance of animals to i.p. infections (21, 25, 37). Under the conditions studied here, preinjection of killed bacteria into the peritoneal cavity decreased lethality of a subsequent exposure to a lethal inoculum of bacteria. This protective effect was diminished by combinations of antioxidant enzymes and NMA (Table 1). The capacity of RBC to act as an adjuvant was also not reduced by pretreatment. Thus, bacterial killing in a previously unstimulated animal is linked not only to ROI and NO but also to the heightened response due to prestimulation with killed bacteria. Although not determined in this study, the more effective protection may be due to prestimulated production of ROI, NO, or both, resulting in the immediate exposure of live bacteria to ongoing ROI and NO production. The fact that RBC continued to promote mortality suggests that the amounts of ROI and NO produced were not sufficient to overcome the enormous scavenging capacity of the RBC. It is important that a peritoneal injection of an agent such as NMA may involve a systemic effect which could influence outcome. However, we found that systemic injection of NMA into rats treated with a nonlethal dose of bacteria plus RBC did not significantly increase the final mortality rates (data not shown). Therefore, we believe that we have provided clear evidence for an important local effect of NMA on NO production.

In summary, RBC are effective biological scavengers not only of ROI (31, 43) but also of NO and $ONOO^-$ (2, 26). It should be noted that $O_2^{\cdot -}$ enters through RBC anion channels, while $H_2O_2$, NO, and $ONOO^-$, which are amphipathic, readily diffuse into RBC. RBC are highly enriched with SOD, catalase, GSH, GSH reductase, and Hb. These cellular components would be expected to inactivate extracellular toxic chemicals, acting as a metabolic sink. Thus, RBC protect the other cells and tissues from cytotoxicity or cellular injury caused by ROI and NO. In contrast, contamination of RBC at the site of infection can protect bacteria from oxidative bactericidal activity of the host defense system by scavenging $O_2^{\cdot -}$ and NO in the peritoneal cavity. We suggest that our observations provide the mechanistic basis for the well-known adjuvant effect of RBC in bacterial peritonitis (7, 8, 15).

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## REFERENCES

1. **Agar, N. S., S. M. H. Sadrzadeh, P. E. Hallaway, and J. W. Eaton.** 1986. Erythrocyte catalase: a somatic oxidant defense? J. Clin. Invest. **77:**319–321.
2. **Brunelli, L., J. P. Crow, and J. S. Beckman.** 1995. The comparative toxicity of nitric oxide and peroxynitrite to Escherichia coli. Arch. Biochem. Biophys. **316:**327–334.
3. **Castro, L., M. Rodriguez, and R. Radi.** 1994. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J. Biol. Chem. **269:** 29409–29415.
4. **Cummings, N. P., M. J. Pabst, and R. B. Johnston, Jr.** 1980. Activation of macrophages for enhanced release of superoxide anion and greater killing of Candida albicans by injection of muramyl dipeptide. J. Exp. Med. **152:**1659–1669.
5. **Davies, J. H., and A. B. Yull.** 1964. A toxic factor in abdominal injury. II. The role of red cell component. J. Trauma **4:**84–90.
6. **Davis, J. H., and A. B. Yull.** 1962. A possible toxic factor in abdominal injury. J. Trauma **2:**291–300.
7. **Dunn, D. L., R. A. Barke, J. T. Lee, Jr., R. M. Condie, E. W. Humphery, and R. L. Simmons.** 1983. Mechanism of the adjuvant effect of hemoglobin in experimental peritonitis. VII. Hemoglobin does not inhibit clearance of Escherichia coli from the peritoneal cavity. Surgery **94:**487–493.
8. **Dunn, D. L., R. D. Nelson, R. M. Condie, and R. L. Simmons.** 1983. Mechanisms of the adjuvant effect of hemoglobin in experimental peritonitis. VI. Effects of stroma-free hemoglobin and red blood cell stroma on mortality and neutrophil function. Surgery **93:**653–659.
9. **Emmendorffer, A., M. Hecht, M. L. Lohmann-Matthew, and J. Roesler.** 1990. A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. J. Immunol. Methods **131:**269–275.
10. **Filler, R. M., and H. K. Sleeman.** 1966. Pathogenesis of peritonitis. I. The effect of Escherichia coli and hemoglobin on peritoneal absorption. Surgery **61:**385–392.
11. **Haddad, I. Y., J. P. Crow, P. Hu, Y. Ye, J. Beckman, and S. Matalon.** 1994. Concurrent generation of nitric oxide and superoxide damages surfactant protein A. Am. J. Physiol. **267:**L242–L249.
12. **Haddad, I. Y., G. Pataki, P. Hu, C. Galliani, J. S. Beckman, and S. Matalon.** 1994. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. J. Clin. Invest. **94:**2407–2413.
13. **Hand, W. L.** 1984. Inhibition of cell-free oxidative bactericidal activity by erythrocytes and hemoglobin. Infect. Immun. **44:**465–468.
14. **Hand, W. L., and N. L. King-Thompson.** 1983. Effect of erythrocyte ingestion on macrophage antibacterial function. Infect. Immun. **40:**917–923.
15. **Hau, T., R. Hoffman, and R. L. Simmons.** 1978. Mechanism of the adjuvant effect of hemoglobin in experimental peritonitis. I. In vivo inhibition of peritoneal leukocytosis. Surgery **83:**223–229.
16. **Hausladen, A., and I. Fridovich.** 1994. Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. J. Biol. Chem. **269:**29405–29408.
17. **Hogg, N., J. Joseph, and B. Kalyanaraman.** 1994. The oxidation of alpha-tocopherol and trolox by peroxynitrite. Arch. Biochem. Biophys. **314:**153–158.
18. **Hu, P., H. Ischiropoulos, J. S. Beckman, and S. Matalon.** 1994. Peroxynitrite inhibition of oxygen consumption and sodium transport in alveolar type II cells. Am. J. Physiol. **266:**L628–L634.
19. **Huie, R. E., and S. Padmaja.** 1993. The reaction of NO with superoxide. Free Radical Res. Commun. **18:**195–199.
20. **Ischiropoulos, H., L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith, and J. S. Beckman.** 1992. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch. Biochem. Biophys. **298:**431–437.
21. **Joyce, L. D., T. Hau, R. Hoffman, R. L. Simmons, and R. C. Lillehei.** 1978. Evaluation of the mechanism of zymosan-induced resistance to experimental peritonitis. Surgery **83:**717–725.
22. **Kim, Y. M., H. A. Bergonia, C. Muller, B. R. Pitt, W. D. Watkins, and J. R. Lancaster, Jr.** 1995. Loss and degradation of enzyme-bound heme induced by cellular nitric oxide synthesis. J. Biol. Chem. **270:**5710–5713.
23. **Kim, Y. M., I. Yamazaki, and L. H. Piette.** 1994. The effect of hemoglobin, hematin, and iron on neutrophil inactivation in superoxide generating systems. Arch. Biochem. Biophys. **309:**308–314.
24. **Kim, Y. M., I. Yamazaki, and L. H. Piette.** Involvement of reactive oxygen species in adjuvant effect of hemoglobin on rat experimental bacterial peritonitis. Submitted for publication.
25. **Kooy, N. W., J. A. Royall, H. Ischiropoloulos, and J. S. Beckman.** 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radical Biol. Med. **16:**149–156.
26. **Lancaster, J. R., Jr.** 1994. Simulation of the diffusion and reaction of endogenously produced nitric oxide. Proc. Natl. Acad. Sci. USA **91:**8137–8141.
27. **Lancaster, J. R., Jr., J. M. Langrehr, H. A. Bergonia, N. Murase, R. L. Simmons, and R. A. Hoffman.** 1992. EPR detection of heme and nonheme iron-containing protein nitrosylation by nitric oxide during rejection of rat heart allograft. J. Biol. Chem. **267:**10994–10998.
28. **Lee, T. J., D. H. Ahrenholz, R. D. Nelson, and R. L. Simmons.** 1979. Mechanisms of the adjuvant effect of hemoglobin in experimental peritonitis. V. The significance of the coordinated iron component. Surgery **86:**41–48.
29. **Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell.** 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. J. Biol. Chem. **268:**1908–1913.
30. **Maddaus, M. A., D. Ahrenholz, and R. L. Simmons.** 1988. The biology of peritonitis and implications for treatment. Surg. Clin. N. Am. **68:**431–443.
31. **Matsumoto, K., T. Otani, T. Une, Y. Osada, H. Ogawa, and I. Azuma.** 1983. Stimulation of nonspecific resistance to infection induced by muramyl dipeptide analogs substituted in the γ-carboxyl group an evaluation of *N*α-muramyl dipeptide-*N*ε-strearoyllysine. Infect. Immun. **39:**1029–1040.
32. **Moncada, S., R. M. Palmer, and E. A. Higgs.** 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43:**109–142.
33. **Nathan, C., and Q. W. Xie.** 1994. Regulation of biosynthesis of nitric oxide. J. Biol. Chem. **269:**13725–13728.

34. **Nathan, C. F., and J. B. Hibbs, Jr.** 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. **3:**65–70.
35. **Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman.** 1991. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. **288:**481–487.
36. **Rioux, F., E. Petitclerc, R. Audet, G. Drapeau, R. M. Fielding, and F. Marceau.** 1994. Recombinant human hemoglobin inhibits both constitutive and cytokine-induced nitric oxide-mediated relaxation of rabbit isolated aortic rings. J. Cardiovasc. Pharmacol. **24:**229–237.
37. **Royall, J. A., and H. Ishiropoulos.** 1993. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular $H_2O_2$ in cultured endothelial cells. Arch. Biochem. Biophys. **302:**348–355.
38. **Scott, M. D., B. H. Lubin, L. Zuo, and F. A. Kuypers.** 1991. Erythrocytes defense against hydrogen peroxide: preeminent importance of catalase. J. Lab. Clin. Med. **118:**7–16.
39. **Simmons, R. L., J. W. Diggs, and H. K. Sleeman.** 1968. Pathogenesis of peritonitis. III. Local adjuvant effect of hemoglobin in experimental E. coli peritonitis. Surgery **63:**810–815.
40. **Tanner, W. G., M. B. Welborn, and V. L. Shepherd.** 1992. Tumor necrosis factor-alpha and interleukin-1 alpha synergistically enhance phorbol myristate acetate-induced superoxide production by rat bone marrow-derived macrophages. Am. J. Respir. Cell Mol. Biol. **7:**379–384.
41. **Tsan, M. F., and J. E. White.** 1988. Red blood cells protect endothelial cells against $H_2O_2$-mediated but not hypoxia-induced damage. Proc. Soc. Exp. Biol. Med. **188:**323–327.
42. **Ward, P. A., J. S. Warren, and K. J. Johnson.** 1988. Oxygen radicals, inflammation, and tissue injury. Free Radical Biol. Med. **5:**403–408.
43. **Williams, D. L., I. W. Browder, and N. R. DiLuzio.** 1983. Immunotherapeutic modification of Escherichia coli-induced experimental peritonitis and bacteremia by glucan. Surgery **93:**448–454.
44. **Wink, D. A., K. S. Kasprzak, C. M. Maragos, R. K. Elespuru, M. Misra, T. M. Dunams, T. A. Cebula, W. H. Koch, A. W. Andrews, J. S. Allen, and L. K. Keefer.** 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science **254:**1001–1003.
45. **Winterbourn, C. C.** 1985. Reactions of superoxide with hemoglobin, p. 137–141. *In* R. A. Greenwald (ed.), CRC handbook of methods for oxygen radical research. CRC Press, Inc., Boca Raton, Fla.
46. **Zhu, L., C. Hunn, and J. S. Beckman.** 1992. Bactericidal activity of peroxinitrite. Arch. Biochem. Biophys. **298:**452–457.

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