Effect of Nitric Oxide on Staphylococcal Killing and Interactive Effect with Superoxide

S. S. KAPLAN,[1,2]* J. R. LANCASTER, JR.,[2]† R. E. BASFORD,[3] AND R. L. SIMMONS[2]

*Departments of Pathology,[1] Surgery,[2] and Molecular Biology and Biochemistry,[3]
University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213*

The role of reactive nitrogen intermediates (RNI) such as nitric oxide (·NO) in host defense against pyogenic microorganisms is unclear, and the actual interactive effect of RNI and reactive oxidative intermediates (ROI) for microbial killing has not been determined. Since, in nature, ROI and RNI might be generated together within any local infection, we evaluated the separate and interactive effects of ·NO and O_2^- on staphylococcal survival by using a simplified system devoid of eukaryotic cells. These studies showed that prolonged exposure of staphylococci to ·NO does not result in early loss of viability but instead is associated with a dose-related delayed loss of viability. This effect is abrogated by the presence of hemoglobin, providing further evidence that the effect is RNI associated. Superoxide-mediated killing also is dose related, but in contrast to RNI-mediated killing, it is rapid and occurs within 2 h of exposure. We further show that the interaction of ·NO and O_2^- results in decreased O_2^--mediated staphylococcal killing at early time points. ·NO, however, appears to enhance or stabilize microbial killing over prolonged periods of incubation. This study did not produce evidence of early synergism of ROI and RNI, but it does suggest that ·NO may contribute to host defense, especially when ROI-mediated killing is compromised.

Nitric oxide (·NO) and other reactive nitrogen intermediates (RNI) derived from arginine have been shown to play a crucial role in diverse functions, including the regulation of blood pressure and flow (29, 45); neurotransmission, in which learning and memory are affected (15); and some of the effector functions of macrophages, such as cytotoxicity toward tumor cells and microorganisms (11, 22, 33, 34, 39, 44). Cells produce variable amounts of ·NO, and high-output ·NO-producing macrophages and liver cells can be induced to increase their production of ·NO by exposure to a synergistic mixture of the cytokines tumor necrosis factor alpha, interleukin-1, gamma interferon, and lipopolysaccharide (22, 53). The ·NO formed is uncharged, relatively hydrophobic, and short-lived. As a nonelectrolyte, it diffuses readily through lipid membranes and may act in both an autocrine and a paracrine fashion depending on the localized density of ·NO-producing cells (31, 59). While it is clear that cytokines induce nitric oxide synthase (NOS) in the rodent model system and that macrophage cytotoxicity against intracellular pathogens such as protozoa, viruses, fungi, and mycobacteria is up-regulated (an up-regulation inhibited by competitive inhibitors of NOS) (7, 19), the effectiveness of endogenous RNI against pyogenic organisms remains unclear and controversial. There is some evidence, however, to suggest that even pyogenic organisms such as staphylococci may be susceptible to RNI-mediated killing after phagocytosis (34). Further evidence in support of the antibacterial role of RNI includes the effectiveness of sodium nitrite (NO_2^-) (200 ppm) as an additive to cured meat (51). This usage was based upon studies performed by Tarr over 50 years ago which demonstrated the bacteriosta-

tic properties of nitrite at acidic pH (55). More recently, other groups examined the effect of NO_2^- on the viability of *Mycobacterium tuberculosis* and *Mycobacterium bovis* and demonstrated that at pH 5, but not at pH 7, all of the tested strains of *M. tuberculosis* were susceptible to being killed (9, 43). These observations are consistent with the well-known phenomenon that at pH 5 but not at pH 7 the NO_2^- anion is converted to the bactericidal product nitrous acid, which generates additional species of RNI, including ·NO. Although the molecular bases of the cytostatic and cytotoxic actions of RNI formation are not yet clearly delineated, important intracellular targets include iron-containing enzymes such as aconitase, which inhibits mitochondrial electron transfer and ribonucleotide reductase, resulting in inhibition of bacterial metabolic activity and therefore of bacterial growth (21, 32).

The activation of macrophages to produce RNI, however, often induces production of reactive oxygen intermediates (ROI) as well, creating the possibility of an interaction between these species. The interaction between ·NO and O_2^- produces peroxynitrite ($ONOO^-$), which upon protonation forms peroxynitrous acid (ONOOH) and rearranges to produce nitrate (NO_3^-) (3). The final effect of these reactions is likely to be mutual scavenging of ·NO and O_2^-. The intermediates $ONOO^-$ and ONOOH, however, also are highly reactive. It is interesting, therefore, that while many studies of the ·NO-O_2^- interaction demonstrate augmented cytotoxicity (3, 4, 20, 26, 30), others show diminished cytotoxicity (58, 59). The usual definitive experiment using competitive inhibitors of NOS will not necessarily differentiate between ·NO-mediated cytotoxicity per se and cytotoxicity via the interaction of RNI and ROI.

The cytotoxic role for ·NO in human cells is subject to substantial controversy. Several investigators have shown that human macrophages produce ·NO (12, 13, 37, 46, 61). Denis demonstrated that granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha induced an L-arginine-dependent cytotoxicity toward a virulent

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* Corresponding author. Mailing address: CLSI—Room 5930 CHP MT, Presbyterian University Hospital, 200 Lothrop St., Pittsburgh, PA 15213. Phone: (412) 647-3771. Fax: (412) 647-8567. Electronic mail address: KAPLAS@A1.ISD.UPMC.EDU.
† Present address: Departments of Physiology and Medicine, Louisiana State University Medical Center, New Orleans, LA 70112.

strain and an avirulent strain of *M. avium* even in patients with chronic granulomatous disease (12). Dumarey et al., however, found that significant amounts of ·NO were produced by human macrophages when they were infected with live, but not dead, *M. avium*, but the live *M. avium* organisms were not killed (13). Others, however, have failed to show ·NO-mediated killing by human macrophages (8, 27, 38, 50). Malawista et al., studying motile but non-ROI-producing neutrophil cytoplasts, also demonstrated a role for RNI in killing of staphylococci by showing that killing was inhibitable by N^G-monomethyl-L-arginine (NMA) (34). Indirect evidence that human neutrophils (5, 28) and monocytes (6) produce ·NO comes from studies showing inhibition of chemotaxis in the presence of the competitive inhibitor NMA and from studies showing L-arginine-dependent and NMA-inhibitable disaggregation of platelets (49). Yan et al., however, failed to find detectable NOS in human neutrophils (60). This absence of consensus suggests that at the very least, human mononuclear cells have lower levels of iNOS than rodent cells; however, studies with human hepatocytes have demonstrated ·NO production equal to that in rodent hepatocytes (41, 42).

While the predominant but still controversial evidence supports the theory that human neutrophils produce ·NO (17, 34, 52), the effectiveness of this radical in microbial killing is less certain. To probe this issue, we examined the effects of incubating staphylococci with ·NO on the survival of these organisms and also the effect of the simultaneous presence of ·NO and O_2^- derived from the action of xanthine oxidase on hypoxanthine. The effects of ·NO and O_2^- separately and together on staphylococcal viability were compared, and the kinetic patterns of bacterial killing under these three conditions were determined. We observed that O_2^- resulted in early bacterial killing, while ·NO-mediated killing was not manifested until about 5 h of exposure had occurred. The simultaneous presence of these mediators diminished the effectiveness of either mediator by itself.

MATERIALS AND METHODS

Materials. The nitric oxide donor *S*-nitroso-*N*-acetyl penicillamine (SNAP) was prepared as previously described and stored at $-20°C$ with protection from light (25). The powder was dissolved in dimethyl sulfoxide immediately before each use at a SNAP concentration of 500 mM. This mixture was diluted in Krebs Ringer phosphate buffer (KRPG; 16 mM Na_2HPO_4, 123 mM NaCl, 0.5 mM Mg SO_4, 5 mM KCl, 4.4 mM glucose [pH 7.4]) such that final concentrations of 0.01 to 1.0 mM SNAP were used. The parent compound, penicillamine, was used for comparison. Hypoxanthine (HX; 100 mM) and xanthine oxidase (XO; 10 U/ml; Sigma Chemical Co., St. Louis, Mo.) were prepared in water and stored at $-20°C$ and were diluted in KRPG before use each time. NMA was prepared as previously described, stored at $-70°C$ at a concentration of 200 mM, and diluted with KRPG before each experiment (25). Ninety-six-well flat-bottom culture plates were obtained from Becton Dickinson and Company, Lincoln Park, N.J. Ninety-six-well filter plates and a filtering apparatus were obtained from Milipore Corporation Lab Products, Bedford, Mass.

Cells. *Staphylococcus aureus* D_2C originally obtained from the American Type Culture Collection was grown overnight in Trypticase soy broth and then was quantitated and suspended in KRPG as previously described (16, 56).

Nitric oxide. The amount of ·NO released from 1 mM SNAP recoverable as NO_2^- was determined by the Griess reaction (18, 54) read at 550 nm in a Molecular Devices (Menlo Park, Calif.) plate reader. NO_3^- was measured according to the method of Kuhns et al. (30a). For this measurement, NO_3^- was reduced to NO_2^- with cadmium, which had been placed in the wells of Milipore filter plates. The reduction of NO_3^- to NO_2^- by cadmium also forms the basis of the more traditional high-performance liquid chromatographic method for detection of NO_3^-. In that method, the cadmium is copper coated to discourage oxidation of the cadmium. In the method described below, the cadmium must be freshly prepared immediately before each assay. To prepare the cadmium powder, the surface oxides were stripped with 0.1 M HCl followed by 10 washes with distilled water. The washed cadmium slurry (100 µl) was pipetted into the wells of the filter plates, and the suspending medium was drawn through by using a

Milipore vacuum extraction apparatus. Samples of 100 µl were pipetted onto the cadmium layer and incubated for 2 h at room temperature for detection of NO_3^-. The samples were removed by vacuum extraction to a plain 96-well plate placed beneath the cadmium-coated plate, and the cadmium layer was washed with 50 µl of ammonium chloride-borate buffer (1 M NH_4Cl plus 50 mM NaB_4O_7 [pH 8.5]). Griess reagent (150 µl) was then added to the filtrates. NO_2 content was determined by using identical filter plates but without the layer of cadmium. The value for NO_3^- plus NO_2^- was derived from the cadmium-prepared wells. The value for NO_2^- was obtained from untreated wells. Absorbances of standards and unknowns were determined as described above. The effects of reactants such as staphylococci (10^7/ml) and/or O_2^- in the buffer on relative amounts of NO_2^- and NO_3^- also were determined. Because the reduction of NO_3^- to NO_2^- by this procedure takes 2 h, the first NO_3^- measurement was at just past 2 h. Results are reported as micromolar NO_2^- or NO_3^- concentrations, where the concentration of NO_3^- equals $(NO_3^- + NO_2^-) - NO_2^-$.

Bacterial killing. The viability of *S. aureus* was determined by the previously published clonal culture technique (56). Studies were performed by using 25-ml Erlenmeyer flasks with incubations at 37°C in a rotary shaking water bath at 200 oscillations per min. Staphylococci (10^7/ml) were incubated in KRPG with 5% bovine serum at 37°C with and without SNAP at concentrations ranging from 0.001 to 1.0 mM. The interactive effect of ·NO and O_2^- on bacterial survival was evaluated by adding the O_2^--generating system HX plus XO to flasks containing 1 mM SNAP. HX at 0.1, 0.25, 0.5, and 1 mM in KRPG with XO at concentrations of 10, 25, 50, and 100 mU/ml, respectively, was added to the *S. aureus* in KRPG with 5% bovine serum. To determine whether removal of ·NO with a scavenging agent altered the observed effect of released ·NO on *S. aureus* survival, experiments with 1 mM SNAP also were performed with and without hemoglobin (1.5 mg/ml). Samples were removed at 0, 1, 2, 5, and 24 h to determine viability by placing the diluted samples on Trypticase soy agar plates and incubating the plates for 18 h at 37°C. The effect of the ·NO-O_2^- interaction also was evaluated by using 0.1 and 0.5 mM SNAP together with HX at 0.5 mM and XO at 50 mU/ml. These effects were compared with the microbicidal action of the above-mentioned levels of O_2^- in the absence of any SNAP.

In some experiments, the effect of authentic ·NO (50%) on *S. aureus* survival was evaluated. By using a gas-tight 15-ml test tube, 5 ml of KRPG was deoxygenated with argon, and then ·NO gas was bubbled into the solution for 30 min. This solution was diluted 1:1 with fresh deoxygenated KRPG to achieve a 50% ·NO solution. *S. aureus* organisms previously deoxygenated by gassing with argon then were incubated in this 50% ·NO solution for 1 h at 37°C under anaerobic conditions. At the end of this period, in order to prevent a drop in pH, the tube was centrifuged and the ·NO-saturated solution was removed and replaced by argon-deoxygenated KRPG before admission of room air. The pH was monitored by including 1% phenol red in the KRPG in some tubes.

Superoxide. The amount of superoxide was evaluated by the reduction of ferricytochrome *c* determined spectrophotometrically at 550 nm by using the micromolar extinction coefficient 0.021 (1). In some studies the kinetic assay described by Newberger et al. was used (40). Here, the superoxide-generating system at 37°C was placed in a 5-ml tube and vortexed vigorously before being transferred to a 1-ml cuvette in the carrier of a Beckman Du-62 spectrophotometer. Cytochrome *c* reduction was monitored for 5 min or until a plateau was reached.

When superoxide was generated by using HX and XO, the concentrations of HX were 0.1, 0.25, 0.5, and 1.0 mM and the corresponding concentrations of XO were 10, 25, 50, and 100 mU/ml. Assays were performed at time 0 and after 1 and 5 h. Aeration of the incubating samples was ensured by placement in a rotary shaking water bath (200 oscillations per min). When these measurements were conducted in the presence and absence of 1 mM SNAP, a considerable non-superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction ($1.36 \pm .05$ nmol/min) occurred in the presence of SNAP, and this was subtracted from the SOD-inhibitable reduction of cytochrome *c*.

Significance of differences was determined by the paired or grouped *t* test and in some cases by the Wilcoxon signed rank test with significance defined as $P < 0.05$.

RESULTS

Figure 1 shows that a fresh solution of 1 mM SNAP in KRPG at pH 7.4 resulted in the production of 30.1 ± 11.2 nmol of NO_2^- per ml during the first hour, and about half of the available ·NO was released in 6 h. The rate of NO_2^- formation decreased gradually during the 24 h of evaluation. Table 1 shows the effects of the presence of staphylococci or of superoxide on the quantities of NO_2^- and NO_3^- over 6 h of incubation. The total quantity of NO_2^- and NO_3^- was slightly greater when staphylococci were present, but this difference was not statistically significant. The simultaneous generation of superoxide by using 100 mU of XO, however, resulted in early release of NO_2^- plus NO_3^- at 2 and 3 h at levels far greater
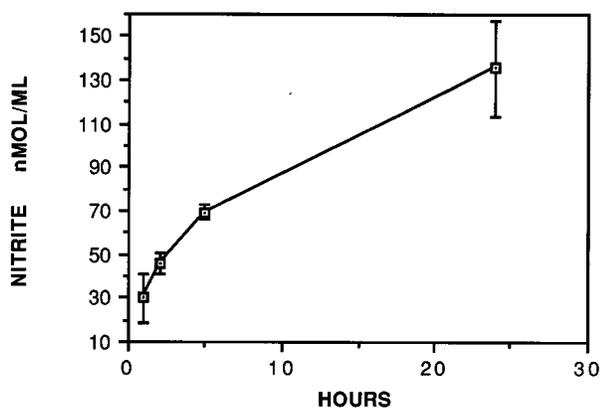
FIG. 1. The release of ·NO from SNAP was monitored as NO_2^- by using the Griess reaction.

($P = 0.033$ and 0.001, respectively) than those of the recoverable NO_2^- plus NO_3^- with SNAP alone. When O_2^- was present, the total amount of NO_2^- and NO_3^- detectable at 6 h was similar to the amount recoverable at 2 h. In addition, when HX and XO were present, the relative amounts of NO_2^- and NO_3^- favored NO_3^-.

Pattern of ·NO- and O_2^--mediated microbial killing. The effect of SNAP-generated ·NO on staphylococcal survival in the absence of neutrophils is shown in Fig. 2A. We showed that there was little or no decrease in bacterial survival at early time points (1 and 2 h), moderate reduction at 5 h, and a dose-related decrease in viability at 24 h. In the absence of SNAP, there was growth of the staphylococcal inoculum and the colony count was $237\% \pm 46\%$ of the initial count. When 1 mM SNAP was present, the percent viable staphylococci at 24 h was $0.42\% \pm 0.13\%$ of the starting inoculum ($P < 0.0002$ compared with the absence of SNAP). Figure 2B shows that when hemoglobin also was present, the staphylococcal viability was $32.1\% \pm 14.3\%$ of the starting inoculum ($P = 0.004$ compared with viability in the presence of SNAP when hemoglobin was not present). This small decrease in viability could have occurred if nitric oxide release continued after all available hemoglobin binding sites had been occupied. Table 2 shows that there was a similar effect

when staphylococci were exposed to 50% authentic ·NO for 1 h under anaerobic conditions and when 1 mM SNAP was used under anaerobic conditions. Killing of greater than one log once again was not evident until 24 h had elapsed. The parent compound, penicillamine, had no effect on staphylococcal survival. The bacteria instead were capable of growth in the anaerobic medium.

These effects of ·NO on staphylococcal survival were compared with the effect of O_2^- on survival by using the O_2^--generating system XO plus HX. Figure 3 shows that O_2^--mediated killing also is dose related but that in contrast to killing by ·NO, O_2^--dependent killing occurs during the first hour. At low concentrations of O_2^-, killing was incomplete and bacterial regrowth occurred within a period directly related to the O_2^- concentration. Only at the highest level of O_2^- generation did complete suppression of staphylococcal growth during this period occur. The amount of O_2^- initially produced when 1 mM HX and 100 mU of XO are used is similar to the amount of O_2^- produced by 10^6 human neutrophils per ml stimulated with opsonized zymosan (approximately 10 to 14 nmol/min). When 1 mM SNAP was present during the generation of O_2^- by HX and XO, however, the amount of measurable O_2^- was significantly smaller. The dose-related quantity of superoxide released by this system and the decrease in release of O_2^- during 5 h of evaluation are shown in Table 3. Table 3 also shows that after 1 h of coincubation, 1 mM SNAP obliterated measurable O_2^- when the amount of O_2^- generated was small and greatly decreased it when a larger amount of O_2^- (generated by using 1 mM HX and 100 mU of XO) was present.

Interactive effect of ·NO and O_2^- on staphylococcal survival. When SNAP-generated ·NO and variable amounts of O_2^- were present simultaneously, the effect on staphylococcal survival depended upon the concentration of O_2^-. These data are represented in Table 4. The data show that when small, sublethal amounts of O_2^- were generated, the presence of SNAP initially attenuated the killing effect of O_2^- and enhanced short-term staphylococcal survival. After a longer incubation, however, the killing effect of SNAP was asserted and a greater than 2-log decrease in the number of viable staphylococci was seen. When larger but still sublethal amounts of O_2^- were generated, SNAP did not adversely affect early killing and it slightly impaired killing at

TABLE 1. Effect of staphylococci or superoxide on NO_2^- and NO_3^- formation

Time (h)	SNAP only (set A)			SNAP + STAPH (set B)[a]			SNAP + 50 mU of XO (set C)			SNAP + 100 mU of XO (set D)		
	% of total[b]		Total (μM)[c]	% of total[b]		Total (μM)[c]	% of total[b]		Total (μM)[c]	% of total[b]		Total (μM)[c]
	NO_2^-	NO_3^-		NO_2^-	NO_3^-		NO_2^-	NO_3^-		NO_2^-	NO_3^-	
2	78 ± 8	22 ± 8	134 ± 39	70 ± 6	30 ± 6	155 ± 56[d]	41 ± 10	59 ± 10	319 ± 88[e]	37 ± 8	63 ± 8	392 ± 99[f]
3	71 ± 7	29 ± 7	188 ± 55	70 ± 9	30 ± 9	240 ± 76[g]	42 ± 1	58 ± 1	258 ± 43[h]	39 ± 2	61 ± 1	319 ± 58[i]
6	63 ± 9	37 ± 9	217 ± 76	63 ± 9	37 ± 9	269 ± 101[j]	33 ± 4	67 ± 4	294 ± 15[k]	30 ± 2	70 ± 2	321 ± 42[l]

[a] STAPH, staphylococci.
[b] Data are percentages (means ± standard errors of the means) of total NO_2 plus NO_3 measurements shown in Fig. 2.
[c] Data are total concentrations of NO_2 plus NO_3 (means ± standard errors of the means) shown in Fig. 2.
[d] $P = 0.258$ compared with set A.
[e] $P = 0.042$ compared with set A; $P = 0.026$ compared with set B.
[f] $P = 0.016$ compared with set C; $P = 0.033$ compared with set A; $P = 0.022$ compared with set B.
[g] $P = 0.171$ compared with set A.
[h] $P = 0.090$ compared with set A.
[i] $P = 0.096$ compared with set B; $P = 0.110$ compared with set C; $P = 0.001$ compared with set A.
[j] $P = 0.153$ compared with set A.
[k] $P = 0.367$ compared with set A.
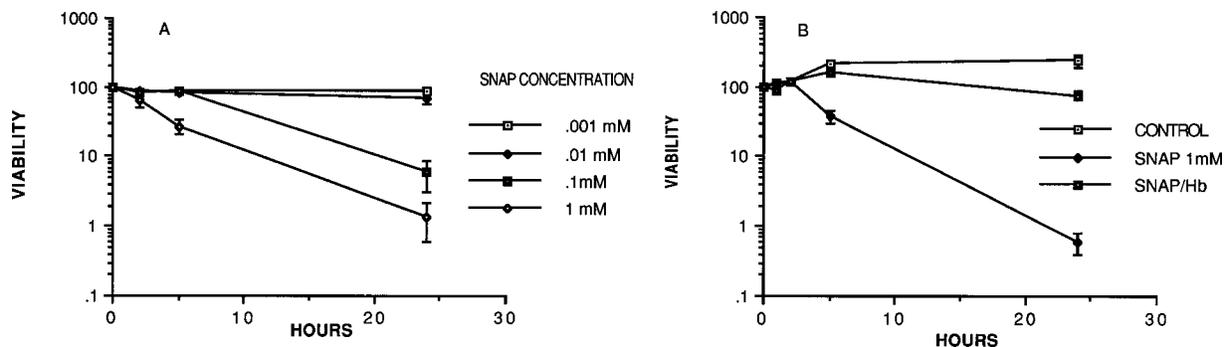[l] $P = 0.601$ compared with set C; $P = 0.108$ compared with set A.

FIG. 2. (A) Effect of ·NO quantity and time on staphylococcal survival. Staphylococcal killing in the presence of SNAP is dose related, and little killing was evident before 5 h had elapsed. Three logs of killing occurred at 24 h at the highest concentration of SNAP. (B) Effect of nitric oxide on staphylococcal viability. The microbicidal effect of SNAP is abrogated when hemoglobin is present.

5 h, but it enhanced staphylococcal killing at 24 h. At the highest level of O_2^-, SNAP had little effect on killing until 24 h had elapsed. After 24 h with fully lethal levels of O_2^-, the presence of SNAP impaired killing. Table 5 shows that when the amount of ·NO was decreased by using lower concentrations of SNAP (0.01 and 0.5 mM), the interactive effect was to slightly decrease the killing mediated by O_2^- alone at early time points (1, 2, and 5 h). These differences were not statistically significant. At 24 h, however, even with these lower concentrations of SNAP, there was significantly more bacterial killing than occurred in the absence of SNAP. The lowest concentration of SNAP killed relatively few staphylococci at 24 h (38% killing with 0.01 mM SNAP compared with approximately 99% with 0.5 and 1.0 mM SNAP). Here, the simultaneous presence of O_2^- significantly improved killing (64% killed). SNAP at 0.1, 0.5, and 1.0 mM killed most of the staphylococci at 24 h. With the 0.1 mM SNAP, however, the presence of O_2^- significantly decreased killing, while O_2^- did not significantly alter the ·NO-mediated killing at the higher concentrations of SNAP. The results of these studies suggest that ·NO derived from SNAP may have impaired O_2^--mediated staphylococcal killing at early time points because of a quenching effect of ·NO on O_2^-. A synergistic effect on killing was not observed under any of the conditions at 1, 2, and 5 h, and such an effect was observed only for the lowest concentration of SNAP at the 24-h time point.

DISCUSSION

On the basis of studies using rodent models of phagocyte-mediated cytotoxicity, it has been generally accepted that ·NO contributes importantly to host defense against hard-to-kill intracellular pathogens. When an intracellular organism such

as *M. avium* is considered, however, it should be taken into account that a variability of cytotoxic effect has been seen when the actions of mouse and human macrophages have been compared and that this variability could be related to the relative quantities of ·NO produced by these species. The role of ·NO in the control of pyogenic organisms has been even more uncertain. It was of some interest then to consider whether in a simpler system ·NO could kill a common pyogenic organism, *S. aureus*, and to determine the nature of the interactive effect with superoxide.

We asked the very simple question of whether ·NO kills staphylococci at all and demonstrated that when staphylococci were incubated with dissolved ·NO for 1 h their numbers did not appear to be reduced when viability was determined at 1 or 2 h after the cessation of ·NO exposure. However, when these bacteria were kept for 24 h and then cultured, we characteristically found a kill of greater than 1 log (up to 3 logs). We believe that this kill was due to the ·NO exposure because it could be abrogated by the presence of hemoglobin. Hemoglobin can react directly with ·NO and it also can react with nitrite, resulting in a scavenging effect on these molecules. Further support for the idea that ·NO exerts a delayed killing effect comes from our studies with the nitric oxide donor SNAP, which demonstrated dose-related, but delayed, staphylococcal killing. The release of ·NO from SNAP can occur by heterolytic cleavage, homolytic cleavage, or transnitrosation. Our data

TABLE 2. Effect of ·NO on staphylococcal survival
under anaerobic conditions

Time (h)	% of initial bacterial inoculum surviving (mean ± SEM) after exposure to:		
	50% authentic ·NO	1 mM SNAP	Penicillamine
0	100	100	100
2	112 ± 13	116 ± 5	124 ± 19
5	91 ± 13	38 ± 7	304 ± 61
24	3 ± 0.5[a]	0.6 ± 0.2[a]	451 ± 92[a]

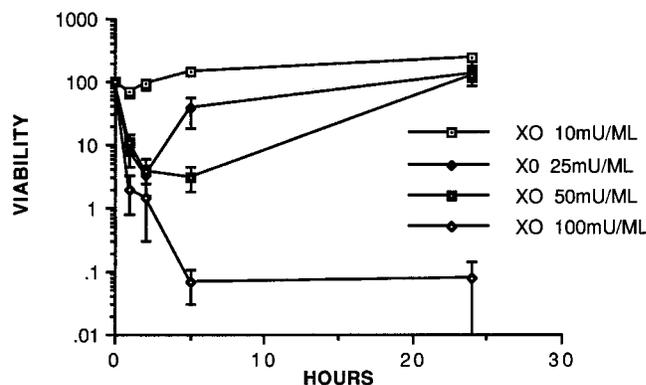[a] $P = 0.014$ compared with the starting inoculum.

FIG. 3. Effect of superoxide concentration and time on staphylococcal viability. The superoxide-generating mixture HX plus XO exerted a dose-related staphylococcal killing effect. Microbial killing was evident after 1 h of incubation, and at the highest concentration of reactants it was persistent. At lower levels of reactants bacterial regrowth occurred.

TABLE 3. Effect of SNAP on superoxide derived from HX-XO

Amt of XO (mU)	Rate of O_2^- production (nmol/min) as measured at[a]:							
	0 h		1 h		2 h		5 h	
	−	+	−	+	−	+	−	+
10	2.0 ± 1.0	0.5 ± 0.5	1.5 ± 0.7	0.3 ± 0.2	0.7 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0.02 ± 0.03
	$P = 0.023$		$P = 0.014$		$P = 0.086$		$P = 0.023$	
50	8.8 ± 4.5	3.1 ± 1.7	3.4 ± 1.9	0.8 ± 0.5	5.5 ± 2.8	0.7 ± 0.4	0.5 ± 0.1	0.3 ± 0.2
	$P = 0.014$		$P = 0.014$		$P = 0.014$		$P = 0.058$	
100	12.2 ± 5.0	4.3 ± 2.1	8.4 ± 3.6	2.0 ± 1.0	7.9 ± 3.6	0.7 ± 0.3	1.2 ± 0.3	0.3 ± 0.2
	$P = 0.014$		$P = 0.014$		$P = 0.014$		$P = 0.023$	

[a] Data are means ± standard errors of the means. −, no SNAP added; +, SNAP added.

showing that the amounts of end products of ·NO release (NO_2^- and NO_3^-) are increased when O_2^- also is released are consistent with the first alternative, heterolytic cleavage, which requires a reductant since O_2^- may serve both as a reductant and as an oxidant. We also showed that microbial killing can occur under both anaerobic and aerobic conditions, suggesting that the mechanisms of death might be similar under both conditions. In both cases, interaction with ·NO during the first few hours of the incubation is not irreversibly fatal. Removal of the ·NO-treated cells to an enriched culture medium such as Trypticase soy broth could resuscitate the bacteria (data not shown); leaving them in the ·NO-generating medium or even in a fresh serum-supplemented buffer was lethal. These results are consistent with the findings of others that ·NO interacts with enzymes essential for energy metabolism and that death occurs only when nutrient (e.g., glucose) is eliminated from the culture medium.

Nitric oxide is considered to be both a facilitator (3, 4, 20, 24, 26, 30) and a protector of free radical-mediated cytotoxicity due to ROI (57, 58). Superoxide and its derivative oxidants, peroxide and hypochlorous acid, are potent bactericidal products, and their production by human phagocytes is well known to be essential for effective host defense. This type of defense is effective against the so-called extracellular microbial infections (involving pyogenic organisms such as staphylococci) but also against fungal and viral infections. Our data, however, showed that the interactive effect of ROI generated by HX and XO and RNI generated by SNAP did not result in early synergistic killing of staphylococci but instead slightly decreased killing at the early time points when ROI-mediated killing normally occurs. At later time points, however, especially when there were in-

sufficient ROI to effect killing, the presence of RNI was associated with markedly decreased staphylococcal survival even when the amount of RNI by itself resulted in little killing. These data suggest that ·NO-mediated killing is not an early event but that ·NO might very well contribute to human neutrophil killing of staphylococci under conditions of compromised host defense. Certain organisms escape from the phagocytic vacuole and thereby evade ROI-mediated killing. These include mycobacteria, certain fungal pathogens, and parasites. These organisms are believed to be susceptible to RNI-mediated intracellular killing (20). However, virtually all of the studies showing that RNI-mediated host defense is important have been conducted with rodent models in which the macrophages possess a cytokine-inducible high-output NOS. Studies using human macrophages have shown that the induction of RNI is slower and occurs at a level much lower than that in rodent cells and that the conditions necessary for induction are not well defined. In addition, studies of cytocidal RNI-mediated function have yielded occasionally positive results (12, 37) but also negative results even with intracellular organisms (8, 27, 38, 50). In addition, the role of RNI in the killing of microorganisms such as salmonella, staphylococci, and pseudomonads has been somewhat controversial with rodent macrophages as well as with human macrophages (48). In part, this variability of effect may be due to the immuno-regulating role of ·NO with regard to the proliferation of lymphocytes (23). There is clear evidence, however, that ·NO plays a critical role in hepatocytes in control of plasmodia (41, 42, 47) and that RNI inhibit a number of enzyme systems, especially those mediated by iron-sulfur and heme-dependent enzymes (32). There is now a considerable body

TABLE 4. Interactive effects of ·NO and O_2^- on staphylococcal viability

XO concn (U/ml)	% of starting inoculum (mean ± SEM) surviving at[a]:							
	1 h		2 h		5 h		24 h	
	−	+	−	+	−	+	−	+
10	67 ± 12	91 ± 10[b]	90 ± 17	99 ± 21[c]	148 ± 18	66 ± 20[d]	236 ± 55	0.7 ± 0.7[d]
25	8 ± 4	32 ± 21[b]	3 ± 2	12 ± 4[b]	37 ± 19	80 ± 19[b]	138 ± 36	34 ± 5[d]
50	11 ± 4	10 ± 3[e]	4 ± 2	2 ± 1[e]	3 ± 1	22 ± 14[b]	121 ± 36	35 ± 18[d]
100	2 ± 1	3 ± 2[e]	1 ± 1	3 ± 2[e]	0.1 ± 0.04	3 ± 3[b]	0.1 ± 0.1	10 ± 12[b]

[a] −, no SNAP added; +, 1 mM SNAP added.
[b] SNAP attenuates O_2^- effect.
[c] No SNAP effect seen.
[d] ·NO-mediated kill is asserted.
[e] Too much O_2^- to have killing affected by SNAP.

TABLE 5. Separate and interactive effects of O_2^- and $\cdot NO$ on staphylococcal survival when the concentration of SNAP is lowered

SNAP concn (mM)	% Surviving staphylococci (mean ± SEM) at[a]:							
	1 h		2 h		5 h		24 h	
	−	+	−	+	−	+	−	+
0		26 ± 11^b		39 ± 13.4^b		42.3 ± 13.4		81 ± 16.3^b
0.01	ND^c	ND	89 ± 8	33 ± 7^d	92 ± 7	41 ± 6.5^d	$62 + 3.5$	$36 + 4^{d,e}$
0.1	103^f	44^f	94 ± 12	47 ± 8^d	93.3 ± 10.7	52.5 ± 6.5^d	7 ± 3	$31 \pm 14^{d,e}$
0.5	84 ± 7	45 ± 5^d	104.3 ± 3.2	66.3 ± 16.3^d	45 ± 5^g	68 ± 17	1.4 ± 1.0^g	1.0 ± 0.4^e
1.0	102 ± 5	32 ± 2.1^d	79 ± 6.7	52.2 ± 4.9^d	39.2 ± 16.4^g	38.8 ± 7.8	0.8 ± 0.4^g	11 ± 9.2^e

[a] n = 4 to 6 unless otherwise indicated. −, no HX-XO added; +, HX-XO added.
[b] HX and XO concentrations were 0.5 mM and 50 mU/ml, respectively (n = 9).
[c] ND, not done.
[d] $P < 0.05$ for the comparison of HX-XO alone and HX-XO plus SNAP.
[e] $P < 0.05$ for the comparison of HX-XO killing alone and HX-XO killing in the presence of SNAP.
[f] Single measurement.
[g] $P = 0.05$ for the comparison of the SNAP effect at 5 or 24 h with the effect at 1 or 2 h.

of evidence showing that RNI effects are reversible, producing cytostasis rather than cytolysis (2). Our data support such a role for authentic $\cdot NO$ under anaerobic conditions and for SNAP-generated $\cdot NO$ under aerobic conditions with *S. aureus*. Damage is done to the organism, but the damage is reversible if the $\cdot NO$ is removed and a replete medium is substituted. But if the medium is unchanged, microbial death ensues. Similar effects have been demonstrated to occur in eukaryotic cells. For example, evidence that the pathogenesis of Parkinson's disease and multiple sclerosis may be related to $\cdot NO$ which injures oligodendrocytes has recently been gathered (35).

The effect of $\cdot NO$ in human neutrophil-mediated host defense has been especially problematic, particularly because human neutrophils appear to produce very little of this mediator (17, 60). However, we and others have shown that NMA inhibits human polymorphonuclear leukocyte chemotaxis (5, 6, 28), and there is considerable evidence that neutrophil-derived $\cdot NO$ can inhibit platelet aggregation (49). Thus, while it seems likely that polymorphonuclear leukocytes produce $\cdot NO$, we have no evidence from the data presented that a more powerful microbicidal agent, peroxynitrite ($ONOO^-$), that might enhance microbial killing is formed. The conditions for formation of this molecule and for the chemical form that it takes, i.e., *cis*, leading to quenching of O_2^-, or *trans*, leading to cytocidal effector molecules, are poorly understood (2). Furthermore, others have shown that $\cdot NO$ inhibits NADPH oxidase and that if simultaneously formed, O_2^- effectiveness would be decreased both by inhibiting its production and by scavenging (10). Nevertheless, if $ONOO^-$ is produced, it might act on the polymorphonuclear leukocyte itself to impair phagocytic killing.

While our data support the idea that the simultaneous generation of O_2^- and $\cdot NO$ would reduce the effectiveness of O_2^-, a recent report by Fukahori et al. suggested that $\cdot NO$ may inhibit XO itself (14). Moilanen et al. have further shown that $\cdot NO$ donors inhibit neutrophil functions such as O_2^- release, degranulation, and chemotaxis because of the $\cdot NO$-induced formation of cGMP (36). The relevance of their finding to operational effects in vivo, however, may be limited, since the quantity of $\cdot NO$ used in their study was considerably larger than would be obtainable in human neutrophils. Wiedermann et al., however, demonstrated that L-arginine infusion reduced baseline and formyl methionyl leucyl phenylalanine (FMLP)-induced O_2^- production by human neutrophils and attributed this reduction to a direct effect of L-arginine on G-protein enhancement of $\cdot NO$ formation (57).

Our data add to this wealth of data from the literature to support the idea that the limited production of $\cdot NO$ by human phagocytes is of functional importance, because low-level $\cdot NO$ rather than high-level $\cdot NO$ protects both of the enzymes that make O_2^- and permits O_2^--derived ROI to carry out the functions of microbial killing in vivo (54). $\cdot NO$-mediated host defense also may be of functional importance when O_2^--mediated functions are poorly operative, such as in the relatively anaerobic environment of a wound. Here, however, an inducible NOS acting because of the same stimuli and in the same time frame as ROI would be biologically counterproductive and would tend to decrease levels of available superoxide even more. On the other hand, $\cdot NO$-producing cells, such as macrophages, which normally do not enter the wound until neutrophil infiltration has subsided, may serve as a backup system for host defense. Our observation that hemoglobin abrogates the effect of $\cdot NO$ on late staphylococcal killing is consistent with the well-known fact that blood in a wound is associated with a greater incidence of infectious complications of the wound, and it supports the notion that $\cdot NO$ participates in host defense. Thus, delayed formation of $\cdot NO$ or $\cdot NO$ formed over long periods of time under circumstances suggested above could benefit host defense.

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