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Effect of Hydrogen Peroxide and Superoxide Radical on Viability of *Neisseria gonorrhoeae* and Related Bacteria[1] (39786)

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Phagocytosis by polymorphonuclear leukocytes (PMN) is accompanied by increased oxygen consumption and metabolic activities that can kill ingested microbes (for reviews see 1-3). While ingestion and killing of some bacteria may occur anaerobically (4), PMN bactericidal activity generally is enhanced in the presence of oxygen. The bactericidal activities of PMN are multiple and include both nonoxidative and oxidative processes. The former include lowering of intravacuolar pH and secretion into the phagosome of lysosomal hydrolytic enzymes and antimicrobial proteins. Oxidative bactericidal activities may be myeloperoxidase (MPO) dependent or MPO independent. The MPO-hydrogen peroxide-halide bactericidal system has been extensively investigated (1). Other bactericidal agents that result from PMN oxidative metabolism include: superoxide anion ($\cdot O^-_2$), hydrogen peroxide (H_2O_2), singlet excited oxygen ($^1O_2$), and hydroxyl radical ($\cdot OH$). The sensitivity of bacteria to these agents can be investigated *in vitro* by incubation of organisms with purine and xanthine oxidase (XO; 5, 6). The oxidation of purine by XO produces $\cdot O^-_2$ (Eq. [1]), which in turn can generate H_2O_2, $^1O_2$, and $\cdot OH$ by spontaneous reactions (Eqs. [2-4]; 7, 8). The purpose of this investigation was to determine whether *Neisseria* were killed when incubated with purine-XO and, if so, to determine the bactericidal agent.

$$\text{Purine} + H_2O + O_2 \xrightarrow{XO} \text{uric acid} + \cdot O^-_2 + 2H^+. \quad [1]$$

$$2 \cdot O^-_2 + 2H^+ \rightarrow H_2O_2 + O_2. \quad [2]$$

$$\text{(a) } 2 \cdot O^-_2 \rightarrow {}^1O_2 + O_2; \quad \text{or} \quad \text{(b) } \cdot O^-_2 + \cdot OH \rightarrow {}^1O_2 + OH^-. \quad [3]$$

$$\cdot O^-_2 + H_2O_2 \rightarrow \cdot OH + OH^- + O_2. \quad [4]$$

Materials and Methods. Chemicals. Xanthine oxidase, glucose oxidase, catalase, lactoperoxidase, purine, scopoletin, and horseradish peroxidase were from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase was from Truett Laboratories, Dallas, Tex.

Bacteria. N. gonorrhoeae, strain 2686, colonial types (T) 1 and 4 were provided by D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Ga. Colony type was maintained by selective subculture. Recent clinical isolates of *N. meningitidis, N. gonorrhoeae, N. perflava,* and *Branhamella catarrhalis* were provided by the Diagnostic Laboratories, University Hospital, Indiana University School of Medicine. Gonococci were grown on GCBIS, which was GC medium base (Difco, Detroit, Mich.) supplemented with 1% IsoVitaleX (Bioquest, Cockeysville, Md.), 1% V-C-N inhibitor (Bioquest), and 10% defined supplement (9). The other bacteria were grown on blood agar. After incubation for 18 hr in a candle-extinction jar at 36°, the bacteria were harvested (10) in LGCBIS, which was GCBIS less agar and V-C-N, and adjusted to a density of $10^{6.7}$ to 10^7 cells/ml as determined by count in a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, Pa.). Suspensions were discarded if significant clumping occurred. Percentage of viability (colony forming units per direct count) was determined by colony count on GCBIS. Viability of gonococci ranged from 55 to 70%. For one study, gonococci were cultured in 50 ml of LGCBIS plus 0.01% $NaHCO_3$ in 250-ml Erhlenmeyer flasks aerated by shaking on a reciprocal shaker at 36°. The bacteria were collected by centrifugation after 4 hr (expo-

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nential phase) or after 12 hr (stationary phase) and resuspended in LGCBIS as above.

Assay procedures. Bacterial killing by the products of the XO reaction was determined by a modification of the procedure of Babior *et al.* (5). The assay mixture was in a volume of 0.25 ml: 25 μl of an appropriate dilution of XO, 25 μl of the bacterial suspension, 25 μl of 0.05 *M* purine, and 0.075 *M* phosphate buffer, pH 7.3, containing 0.85% saline (PBS). The control contained XO that had been inactivated by boiling for 10 min. Where indicated, 50 μl of catalase or of superoxide dismutase (SOD) were added at final concentrations of 0.1 and 1.0 mg/ml, respectively. Inhibitors were added at the concentrations indicated.

Bacterial killing by products generated in the glucose oxidase (GO) reaction was examined in a similar assay mixture; GO replaced XO and 1.0 *M* glucose replaced purine. Controls contained heat-inactivated GO.

To test the effect of lactoperoxidase (LPO) and halide on bacterial killing in the XO reaction, 0.075 *M* phosphate buffer, pH 5.5, replaced PBS. LPO was at a final concentration of 60 units/ml, and KI was 0.1 m*M*.

Assays were initiated by the addition of XO or GO. After incubation at 37° for 10 min with XO or for 15 min with GO, the XO reaction was stopped by the addition of 0.25 ml of 0.6 m*M* allopurinol and the GO reaction was stopped by dilution. Then assay mixtures were diluted in LGCBIS and plated on GCBIS or on blood agar as appropriate. Percentage of bacterial survival was determined by colony count after incubation for 24 hr at 37°. The number of colonies from the heat-inactivated enzyme controls was assigned a value of 100% survival (0% killing), and other values were expressed as a percentage of that number that was killed.

The rates of H_2O_2 production in the XO and the GO systems were measured by a modification of the method of Root *et al.* (11). After incubation of enzyme and substrate for 5 min at 37°, 0.4 ml of 50 μM scopoletin in PBS was added. After 30 min of additional incubation, 0.4 ml of horseradish peroxidase, 1 mg/ml in PBS, was added, and the decrease in fluorescence was quantitated in a spectrophotofluorometer. The rate of H_2O_2 production was calculated by comparison to freshly prepared H_2O_2 standards using an extinction coefficient of 81 M^{-1} at 230 nm (12). With 0.2 units of XO and with 0.25 units of GO, production of H_2O_2 was 7 and 9.1 nmole/min/ml, respectively; these amounts of enzyme were selected after the results of the bactericidal assays above (see Results).

Results. The conditions required for killing of gonococci in the XO assay procedure are in Table I. Colony counts from tubes containing purine and heat-inactivated XO were not statistically different from controls in which gonococci were incubated in PBS in the absence of purine and of XO. When incubated with purine and active XO, gonococci were killed significantly; the percentage of killing in separate experiments showed good reproducibility, i.e., 53 ± 5 colonies per plate for heat-inactivated XO and 7 ± 2 colonies per plate for active XO (0.12 units). Killing required purine, and the purine concentration used was saturating. Addition of allopurinol, an inhibitor of the XO reaction (13), markedly decreased killing. Addition of azide, cyanide, or 3-amino-1,2,4-triazole, which inhibit the activity of various iron-containing enzymes including peroxidases (14), did not decrease killing; these results suggested that killing was not due to contaminating peroxidase activity in the XO preparation.

In gonococci, virulence relates to colonial morphology; T1 is virulent and T4 is aviru-

TABLE I. BACTERICIDAL ACTIVITY IN THE XO ASSAY.

Conditions[a]	Killing (%)
Heated XO	0
Less purine	10
1 m*M* Purine	95
5 m*M* Purine	94
1 m*M* Purine plus	
Allopurinol (0.6 m*M*)	2
Azide (1 m*M*)	84
Azide (2 m*M*)	86
Cyanide (2 m*M*)	92
Aminotriazole (2 m*M*)	88

[a] T4 gonococci were incubated for 10 min at 37° in PBS, pH 7.3, with XO (0.3 units/ml), and killing was determined as described in Methods.

lent in man (15). The relative sensitivity of *N. gonorrhoeae* 2686 T1 and T4 to products of the XO reaction is shown in Figs. 1A and B. Figure 1A relates killing in a 10-min period to XO concentration; at all XO concentrations tested, T1 and T4 were killed to the same extent. Figure 1B relates killing and the time of incubation; T1 and T4 were killed similarly.

To determine which of the products generated in the XO reaction was gonococcidal, we examined the effect of SOD and of catalase in the assay mixture. SOD catalyzes the conversion of $\cdot O^-_2$ to H_2O_2; this reaction occurs spontaneously (Eq. [2]) but is accelerated by SOD (8). SOD (i) should decrease the concentration of $\cdot O^-_2$ and, consequently, of $^1O_2$ and $\cdot OH$ (Eqs. [3] and [4]) but (ii) should increase the formation of H_2O_2. Catalase should destroy H_2O_2 generated from $\cdot O^-_2$. Both T1 and T4 were protected by exogenous catalase. Figure 2 shows that SOD slightly enhanced killing, whereas catalase markedly reduced killing of 2686 T4 in the XO reaction; similar results (not shown) were obtained with 2686 T1. Mannitol (0.1 *M*), a scavenger of $\cdot OH$ (16), did not alter killing. These results suggested that H_2O_2, and not $\cdot O^-_2$, $\cdot OH$, or uric acid, was the primary bactericidal agent generated in the XO reaction. Two recent gonococcal urethral isolates were similar to 2686 with respect to killing in the XO reaction, i.e., viable count declined to 13, 0.8, and 0.2% at 10, 20, and 30 min of incubation, respectively. These isolates also were protected by exogenous catalase but not by SOD. To determine the effects of the phase of growth on the sensitivity of gonococci to H_2O_2, organisms of exponential and stationary phase cultures of 2686 T1 in LGCBIS

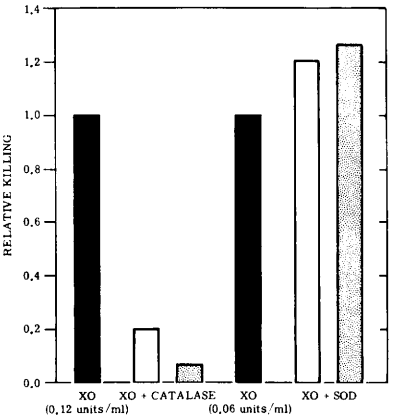

FIG. 2. Effect of catalase and SOD on killing of gonococci and meningococci in the XO assay. Killing of gonococci and of meningococci in the absence of catalase or SOD was normalized to 1 (solid bars). The enzyme concentrations were: XO (0.12 units/ml) +/− catalase (0.1 mg/ml) and XO (0.06 units/ml) +/− SOD (1.0 mg/ml). Open and stippled bars represent gonococci and meningococci, respectively.

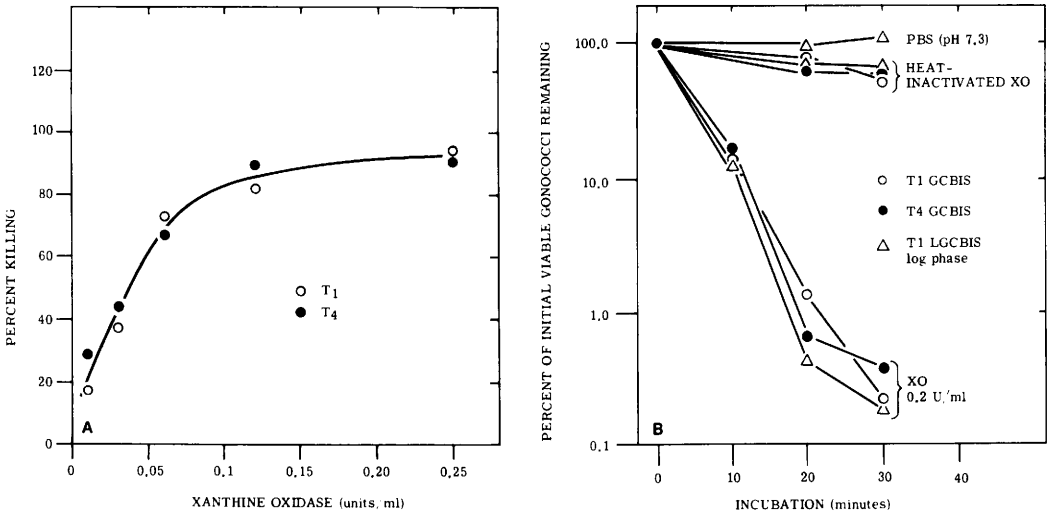

FIG. 1. Killing of gonococci in the XO assay. Killing is expressed relative to the heat-inactivated XO control which was assigned a value of 0% killing, i.e., 100% survival. (A) Killing in 10 min as a function of XO concentration. (B) Time course of killing at XO concentration of 0.2 units/ml.

plus 0.01% $NaHCO_3$ were compared for susceptibility to killing in the XO reaction. Both were killed at a similar rate (Fig. 1B), and both were protected by exogenous catalase.

Meningococci also were killed in the XO reaction. As with gonococci, exogenous catalase protected while SOD slightly enhanced killing (Fig. 2). Similar results were obtained with related commensal organisms, *N. perflava* and *B. catarrhalis*. The commensals were killed to the same extent, i.e., approximately 99.9% killing after 30 min of incubation (data not shown), and were protected by exogenous catalase but not by SOD.

To confirm the bactericidal activity of H_2O_2, we assayed killing of 2686 T4 in the glucose-GO reaction. This reaction generates H_2O_2 without the intermediate formation of $\cdot O^-_2$. Figures 3A and B show that killing increased with GO concentration and with time. Killing did not occur in the absence of glucose or in heat inactivated-GO controls. Catalase protected against killing in the GO system.

Figure 4 shows the effect of pH and of the addition of LPO and halide on killing of 2686 T4 gonococci in the XO reaction; similar results (not shown) were obtained with 2686 T1. For these experiments, 0.1 *M* phosphate buffer, pH 5.5, was used to provide the pH optimum for LPO; the number of bacteria was approximately 10^6 cells/ml and the amount of XO was 0.1 units/ml. At pH 5.5, killing of gonococci by the H_2O_2 generated in the XO reaction was only slightly enhanced, if at all, by the addition of

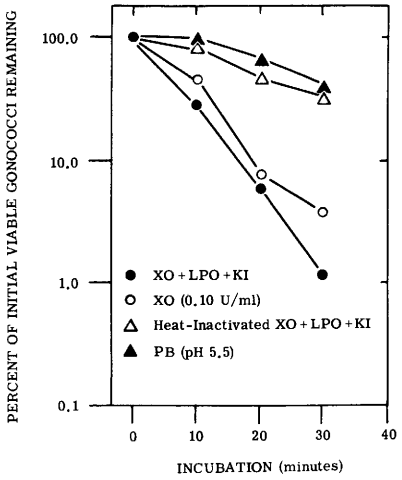

FIG. 4. Effect of lactoperoxidase (LPO) and KI on killing of T4 gonococci in the XO assay. XO concentration was 0.1 units/ml.

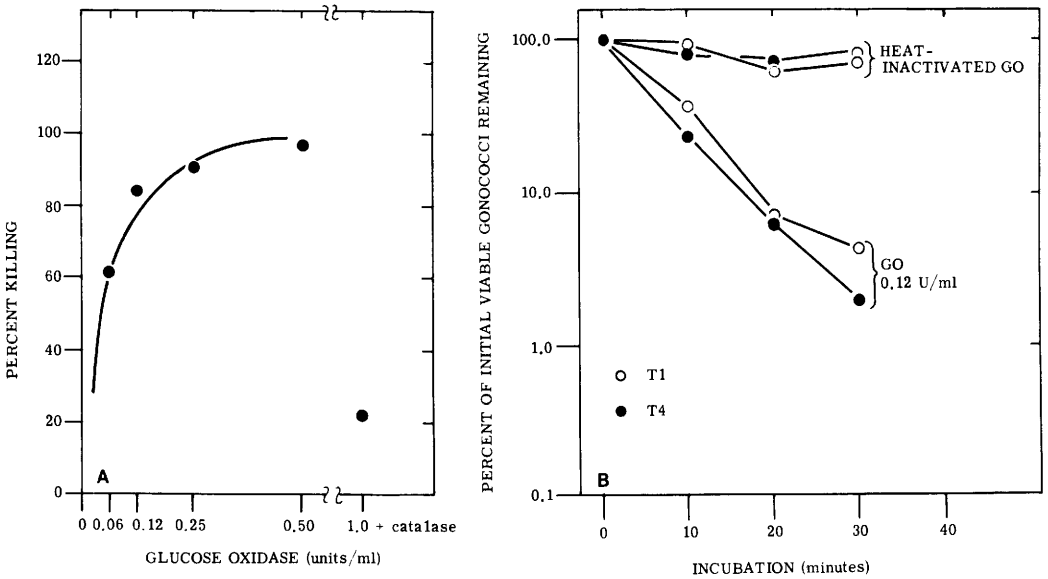

FIG. 3. Killing of T4 gonococci in the GO assay. Killing is expressed relative to the heat-inactivated GO control which was assigned a value of 0% killing, i.e., 100% survival. (A) Killing in 15 min as a function of GO concentration. (B) Time course of killing at a GO concentration of 0.12 units/ml. Catalase was added at 0.1 mg/ml.

KI and of LPO. The experiments were, however, complicated by slight loss of viability in the control tubes at this pH.

Discussion. The virulence of gonococci *in vivo* correlates with properties that can be measured *in vitro* including the greater resistance of the bacteria of virulent colonial types to ingestion by PMN (10, 17–20); some differ (21). Gonococci seem to behave as extracellular pathogens, and several studies indicate that many of both virulent T1 and avirulent T4 gonococci are killed after ingestion by PMN (10, 22–24). The present data support this view in that T1 and T4 gonococci were equally sensitive *in vitro* to the products generated in the XO assay.

In the XO assay, gonococci and meningococci appeared to be killed by H_2O_2 rather than by $\cdot O^-_2$, $^1O_2$, or $\cdot OH$. This conclusion is based on: (i) catalase, but neither SOD nor mannitol, protected against killing by the products of the XO system, and (ii) bacteria were killed in the GO system, which generates H_2O_2 directly. Bacteria differ in susceptibility to products generated in the XO reaction, e.g., *Escherichia coli* (5) appears to be killed by H_2O_2, whereas the killing of *Staphlococcus epidermidis* (5) and *Lactobacillus plantarium* (25) requires both H_2O_2 and $\cdot O^-_2$, presumably combining to form $\cdot OH$. Klebanoff (1, 6) and others (see reviews 1–3) demonstrated that MPO and halide (either Cl^- or I^-) markedly enhance killing of various microorganisms by H_2O_2. The MPO–halide system is thought to constitute a major bactericidal mechanism in human PMN, and activity is maximal at an acid pH that may be near that within the phagosome. Oxidative bactericidal processes that are MPO independent also exist in PMN; PMN that lack MPO due to a genetic deficiency or in which MPO activity has been inhibited by azide kill bacteria but at a slower rate (1). Further, in some species, e.g., chicken, MPO is absent (26). At pH 5.5, killing of gonococci by H_2O_2 generated in the XO reaction was not significantly enhanced by the addition of KI and LPO. LPO can substitute for MPO but only when the halide ion Cl^- is replaced by I^- (6). Azide and other inhibitors did not decrease killing in the purine–XO system. Therefore, the bactericidal activity of the XO system was not due to contaminating lactoperoxidase activity. We cannot exclude the possibility, however, that a peroxidase activity that is associated with the bacteria and is resistant to azide contributes to the bactericidal activity of H_2O_2.

Resistance of bacteria to $\cdot O^-_2$ and H_2O_2 has been related to SOD and catalase activities (27). Obligate anaerobes that are oxygen sensitive lack SOD (28). The resistance of gonococci to $\cdot O^-_2$ may relate to the conversion of $\cdot O^-_2$ to H_2O_2 by SOD. *E. coli* B has two enzymes that are differentially regulated and localized (29). Cytoplasmic SOD appears to protect against $\cdot O^-_2$ that is generated endogenously while SOD localized in the periplasmic space protects against exogenously generated $\cdot O^-_2$. SOD activity has not been studied in gonococci. In *S. aureus*, virulence correlates with catalase activity (30). Strains with high catalase activity are more resistant to exogenous H_2O_2 (31), are killed at a slower rate by PMN, and are more virulent in mice than strains with low catalase activity. In *E. coli* B (32), growth in iron-rich, as compared to iron-deficient, media results in bacteria that have increased ferri-SOD, peroxidase, and catalase activities and are less sensitive to killing both by exogenous H_2O_2 and by PMN. Gonococci are strongly catalase positive as evidenced by evolution of O_2 upon addition of H_2O_2, yet appear to be sensitive to H_2O_2. Since gonococci are obligate aerobes, one role for catalase may be to detoxify H_2O_2 generated during aerobic metabolism. The apparent ineffectiveness of the catalase of gonococci in protection against exogenous H_2O_2 has not been explained but may relate to several factors: (i) The concentration of H_2O_2 generated in the XO and GO assays may be too high to be detoxified rapidly and completely by the catalase present in the bacteria; (ii) the catalase may be localized in the cytosol and, thus, effective in detoxifying endogenously generated but not exogenous H_2O_2; (iii) H_2O_2 generated outside the cell may cause membrane damage which is irreversible, and this may not occur when H_2O_2 is generated endogenously; and (iv) other enzymes, e.g., peroxidases, may compete with catalase for H_2O_2 and, possibly, contribute to toxicity of H_2O_2.

Summary. H_2O_2, but not $\cdot O^-_2$ or $\cdot OH$

radical, appeared to be the bactericidal agent for *N. gonorrhoeae, N. meningitidis, N. perflava,* and *B. catarrhalis in vitro* in the purine–xanthine oxidase reaction, a system that generates products like those of the oxidative bactericidal system within PMN. Virulent T1 and avirulent T4 gonococci were killed equally. Both exponential and stationary phase gonococci were killed similarly. The addition of lactoperoxidase and iodide only slightly enhanced killing of gonococci by H_2O_2, if at all.

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