

Lack of Antibacterial Activity After Intravenous Hydrogen Peroxide Infusion in Experimental *Escherichia coli* Sepsis

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Received 30 November 1984/Accepted 20 February 1985

The intravenous administration of hydrogen peroxide has been reported to benefit patients with pneumonia and to reduce *Plasmodium* parasitemia in experimentally infected mice. We assessed the antibacterial activity of intravenously infused hydrogen peroxide against hydrogen peroxide-susceptible *Escherichia coli* (MBC of hydrogen peroxide, 0.23 mM) in experimentally infected rabbits. No decrease in the level of bacteremia was detected at the maximum intravenous infusion rate of hydrogen peroxide physiologically tolerated by the rabbits (2.0 μ mol/h). Moreover, the addition ex vivo of greater amounts of hydrogen peroxide to human or murine blood containing *E. coli* resulted in no detectable antibacterial action. In contrast, ethyl hydrogen peroxide, which is not affected by catalase, was bactericidal when added ex vivo to human blood containing *E. coli*. These results suggest that extracellular hydrogen peroxide, whether of exogenous or endogenous origin, does not have antibacterial activity in the blood of animals having even low levels of catalase.

Hydrogen peroxide generated in mononuclear phagocytes and polymorphonuclear cells is of pivotal importance in the intracellular killing of several pathogens (2). Intracellular hydrogen peroxide may also mediate, in part, the antineoplastic activity of macrophages (13, 15). Recent reports have suggested that hydrogen peroxide released by mononuclear phagocytes and neutrophils may extend the antimicrobial, antitumor, and oxidant-injury activities of these cells to adjacent tissues (14, 16). These observations, together with reports of successful infusions of hydrogen peroxide into patients (12, 17), suggest that exogenous hydrogen peroxide might be effective in the therapy of selected infectious and neoplastic diseases.

Providing additional support for a potential therapeutic role for parenterally administered hydrogen peroxide, Dockrell and Playfair (6) reported that murine *Plasmodium yoelii* parasitemia was reduced after the intravenous administration of hydrogen peroxide. However, the fivefold lower level of catalase activity present in hemolysate prepared from mice compared with hemolysate from humans (11) and the use of intracellular parasites as markers of hydrogen peroxide activity limit the implications of this study. We therefore employed circulating hydrogen peroxide-susceptible *Escherichia coli* to assess the therapeutic potential of intravenously administered hydrogen peroxide in the rabbit, an animal whose levels of catalase activity in blood closely parallel those of humans.

MATERIALS AND METHODS

Bacteria. *E. coli* K1:O7 C94 (7, 18), kindly provided by Richard Moxon (Johns Hopkins University School of Medicine, Baltimore, Md.), was stored in skim milk at -70°C , recovered onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), and cultured in tryptic soy broth overnight at 37°C as previously described (20).

The susceptibility of this *E. coli* strain to hydrogen peroxide (Parke-Davis, Morris Plains, N.J.) was assessed with a modification of a previously described bactericidal assay (8). Briefly, *E. coli* K1 was grown in tryptic soy broth and

suspended in phosphate-buffered saline at a concentration of 800 CFU/ml. To 0.7 ml of this suspension was added 0.1 ml of phosphate-buffered saline containing various concentrations of hydrogen peroxide (serial twofold dilutions of a stock solution containing 1.25 M hydrogen peroxide). Immediately after the addition of hydrogen peroxide and again after incubation at 37°C for 45 min, 0.1-ml samples of this bacterial suspension were streaked onto a Trypticase (BBL) soy agar plate and incubated overnight at 37°C . The MBC of hydrogen peroxide was defined as the lowest concentration of hydrogen peroxide producing a $>90\%$ decrease in colony counts after the 45-min exposure interval.

Catalase activity. Whole blood catalase activity was measured by diluting heparinized whole blood in normal saline, adding 0.03 M hydrogen peroxide, and quantitating the rate of hydrogen peroxide decomposition by UV spectrophotometry as described by Aebi and Suter (1). Whole blood catalase activity was expressed as the velocity constant, k (1).

Animal model. Male New Zealand White rabbits weighing from 2.5 to 3.5 kg were obtained from a local rabbitry and received food and water free of antibiotics ad libitum. Paired rabbits were anesthetized by intramuscular injection of a solution containing ketamine, xylazine, and acepromazine, prepared with two indwelling femoral vessel catheters each, and maintained with a solution of 0.225% saline containing 5% glucose as previously described (20).

Mean arterial pressure and heart rate were monitored with a pressure transducer positioned at the level of the heart (Bentley, model 800), displayed continuously on a monitor (Siemens, model 404), and recorded on a four-channel strip-chart recorder (Western Graphtec). Arterial blood samples were collected periodically as described below in 1-ml glass syringes and analyzed on a calibrated, automated analysis system (Radiometer, model ABL3) for pO_2 , pCO_2 , pH, and calculated bicarbonate.

In preliminary experiments to determine the maximum rate at which hydrogen peroxide could be infused into rabbits without physiological disturbances, four rabbits received intravenous infusions of 0.225% saline with 5% glucose at 20 ml/h, to which hydrogen peroxide was added

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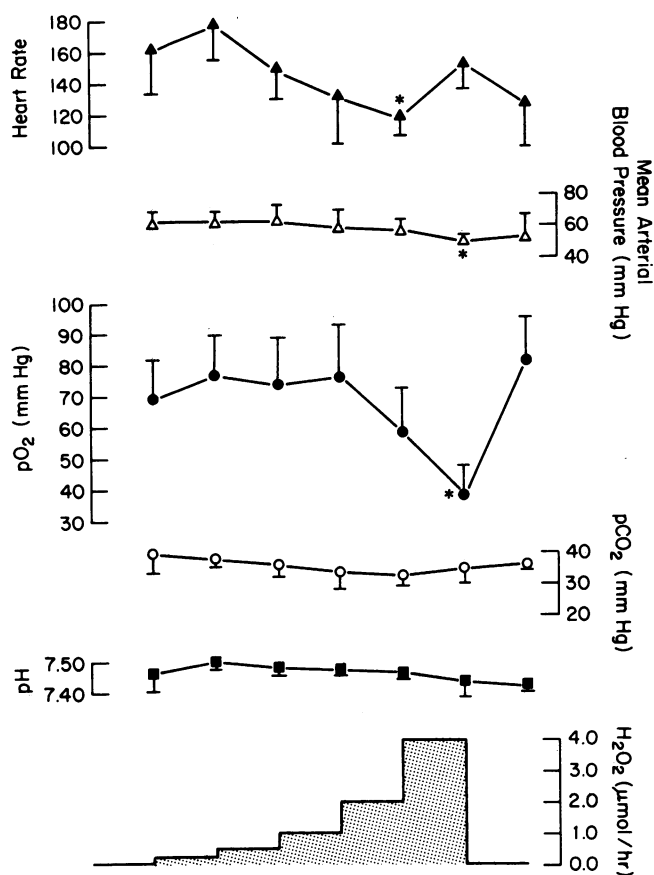


FIG. 1. Mean heart rate (▲), arterial blood pressure (Δ), pO₂ (●), pCO₂ (○), and pH (■) in four rabbits receiving incrementally increasing intravenous infusion rates of hydrogen peroxide. Bars represent one standard deviation. Measurements that differed significantly ($P < 0.05$) from the baseline measurement are denoted by an asterisk. Note that the hypoxemia and hypotension present after the intravenous infusion of 4.0 μmol of hydrogen peroxide per h was rapidly reversed after the infusion was discontinued.

with half-hourly twofold incremental increases in concentration starting at 0.0125 M hydrogen peroxide.

Porcine mucin (2 g; Sigma Chemical Co., St. Louis, Mo.) was added to 40 ml of the broth containing *E. coli* described above. Sepsis was induced in six paired rabbits by intraperitoneal administration of half of this suspension containing *E. coli* and mucin. The challenge dose of bacteria for different pairs of rabbits ranged from 5×10^9 to 1×10^{10} CFU of *E. coli*. Immediately after bacterial challenge, paired rabbits were intravenously infused with 0.10 M hydrogen peroxide in 0.225% saline with 5% glucose or with 0.225% saline with 5% glucose at 20 ml/h.

Blood samples (2 ml) for culture and endotoxin analysis were collected 5 min before bacterial challenge and hourly for 8 h after bacterial challenge. Blood samples were divided equally into two sterile, pyrogen-free Falcon tubes (Becton Dickinson and Co., Cockeysville, Md.), each containing 50 U of preservative-free heparin (Weddel Pharmaceuticals, Ltd., London, England), and stored on ice immediately.

Quantitation of bacteremia. Immediately after collection, serial 10-fold dilutions of a portion of each blood sample were prepared in phosphate-buffered saline (pH 7.4) containing 0.5% albumin. CFU were quantitated after overnight incubation at 37°C of 0.1-ml portions of each dilution cultured on Trypticase (BBL) soy agar.

Endotoxin assay. To assess whether bacterial lysis was occurring in the presence of hydrogen peroxide, a portion of each blood sample was taken and plasma-free endotoxin was separated from endotoxin bound to bacterial cells by centrifugation at $1,000 \times g$ for 10 min, followed by passage through a 0.45- μm -pore-size pyrogen-free filter (Millipore Corp., Bedford, Mass.) as previously described (20). Processed plasma samples were diluted 1:3 with pyrogen-free water and heated to 100°C for 10 min to inactivate inhibitors of the *Limulus* lysate assay (5).

Endotoxin levels in plasma were quantitated with the Pyrotell *Limulus* lysate assay (Associates of Cape Cod, Inc., Woods Hole, Mass.) according to the directions provided by the manufacturer. Samples were assayed in parallel with known concentrations of reference *E. coli* endotoxin (Associates of Cape Cod). Endotoxin concentrations were expressed as the amount of U.S. standard endotoxin (19) producing activity equivalent to that of the sample as assessed by the *Limulus* lysate assay. The sensitivity, specificity, relation of bound, free, and total endotoxin, and other characteristics of this assay relevant to this animal model have been previously studied and described (20).

Statistical analysis. Physiological parameters were compared by the comparison of two means *t* test program of an HP41-CV programmable calculator (Hewlett Packard, Corvallis, Oreg.). Geometric mean levels of bacteremia and plasma-free endotoxemia in paired control and hydrogen peroxide-treated animals were compared by the same *t* test.

RESULTS

Whole blood catalase activities. The mean catalase activities ($k \pm$ the standard deviation) in whole blood samples from three healthy adult volunteers, three New Zealand White rabbits, three CD1 mice, and three CBA/ca mice were $87 (\pm 21)$, $74 (\pm 12)$, $48 (\pm 9)$, and $38 (\pm 5) \text{ s}^{-1}$, respectively.

Physiological tolerance of rabbits for intravenous hydrogen peroxide infusion. Rabbits tolerated the intravenous infusion of 0.225% saline with 5% glucose containing 0.0125, 0.025, and 0.05 M hydrogen peroxide at 20 ml/h (0.25, 0.50, and 1.0 $\mu\text{mol/h}$, respectively) for 30 min with no significant change in arterial blood gases, heart rate, or mean arterial blood pressure (Fig. 1). Increasing the concentration of hydrogen peroxide in the infusion to 0.10 M (2.0 $\mu\text{mol/h}$) for 30 min significantly decreased the heart rate ($P < 0.05$) without significantly affecting the other monitored parameters (Fig. 1). After a 0.5-h infusion of 0.20 M hydrogen peroxide at 20 ml/h (4.0 $\mu\text{mol/h}$), the mean pO₂ decreased sharply ($P < 0.05$), the mean arterial blood pressure decreased ($P < 0.05$), and the heart rate increased toward the baseline rate. However, 0.5 h after discontinuing the 4.0 $\mu\text{mol/h}$ hydrogen peroxide infusion, the pO₂ and mean arterial blood pressure returned to baseline levels, indicating that the observed effects of hydrogen peroxide infusion were rapidly reversible.

Susceptibility of *E. coli* K1 to hydrogen peroxide in vitro. The MBC of hydrogen peroxide for the *E. coli* strain used was 0.23 mM hydrogen peroxide in phosphate buffered saline. At bactericidal concentrations of hydrogen peroxide, endotoxin bound to *E. coli* became liberated within 30 min.

Blood antimicrobial activity of intravenous hydrogen peroxide infusion in experimentally infected rabbits. The continuous intravenous infusion of 2.0 μmol of hydrogen peroxide per h for 8 h did not significantly decrease the geometric mean levels of bacteria in the blood of experimentally infected paired rabbits compared with control rabbits at any

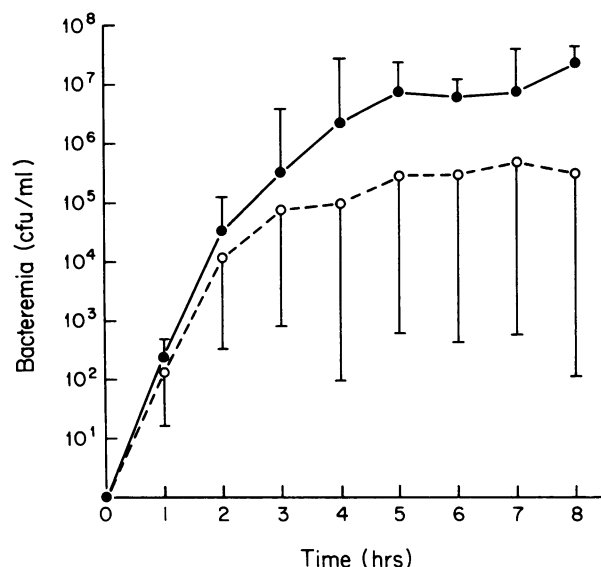


FIG. 2. Geometric mean levels of bacteremia in six paired, experimentally infected rabbits treated with either 0.10 M hydrogen peroxide in 0.225% saline with 5% glucose (●) at 20 ml/h (2.0 μ mol/h) or 0.225% saline with 5% glucose without hydrogen peroxide (○) at 20 ml/h. Bars represent one standard deviation. The mean levels of bacteremia were not statistically significantly different at any time. Not only was antimicrobial activity in rabbits treated with hydrogen peroxide not evident, but a trend toward lower levels of bacteremia in control rabbits was seen.

time (Fig. 2). To the contrary, a trend toward lower levels of bacteremia in control rabbits was apparent (Fig. 2).

The levels of free endotoxin in plasma were measured to determine whether bacterial lysis was occurring. The geometric mean levels of plasma-free endotoxin relative to the geometric mean levels of bacteremia (ng of free endotoxin per 10⁶ CFU) were not significantly different in hydrogen peroxide-treated rabbits versus control rabbits (Table 1).

Antimicrobial activity of hydrogen peroxide added ex vivo to human and murine blood containing *E. coli*. The addition of 0.125 or 1.25 M hydrogen peroxide in 0.225% saline with 5% glucose to equal volumes of fresh heparinized normal human whole blood containing 10⁷ CFU of *E. coli* per ml did not significantly alter the bacterial levels after 1 h at 37°C compared with parallel control samples of blood containing *E. coli* to which 0.225% saline with 5% glucose but without hydrogen peroxide were added. Similar experiments with murine blood (CD1 mice) also failed to demonstrate antibacterial activity after the ex vivo addition of hydrogen peroxide. In contrast, the ex vivo addition of ≥ 0.50 M ethyl hydrogen peroxide (Accurate Chemical Co., Westbury, N.Y.) to human blood containing *E. coli* sterilized the blood but immediately induced a change in hemoglobin color to chocolate brown, indicating the formation of methemoglobin.

DISCUSSION

In contrast to the promising results with parenteral hydrogen peroxide to treat experimental *Plasmodium* parasitemia in mice reported by Dockrell and Playfair (6), serial quantitative blood cultures in experimentally infected rabbits receiving intravenous hydrogen peroxide by continuous infusion failed to detect significant antibacterial activity (Fig. 2). The lack of endotoxin release observed in hydrogen peroxide-treated rabbits (Table 1) confirms the absence of hydro-

gen peroxide-associated antibacterial activity in blood, since endotoxin release is a sensitive gauge of bacterial lysis (20). Not only was there no antibacterial activity in rabbits receiving hydrogen peroxide, but there was a trend toward higher levels of bacteremia in these animals versus control animals, possibly because intravascular microbubbles resulting from the conversion of hydrogen peroxide to water and oxygen may have lodged in the lung (4), thereby interfering with bacterial clearance. In comparison, a single intravenous dose of a bactericidal antibiotic such as gentamicin or moxalactam administered 2 h after infection generally resulted in the complete clearance of bacteremia in similarly infected rabbits (20; J. L. Shenep, R. P. Barton, and K. A. Mogan, *J. Infect. Dis.*, in press). These observations in rabbits, combined with the lack of antibacterial activity after the ex vivo addition of hydrogen peroxide to human or murine blood containing *E. coli*, suggest that the reduced parasitemia observed by Dockrell and Playfair (6) after the parenteral treatment of mice with hydrogen peroxide may not have been a direct result of hydrogen peroxide antimicrobial activity, but rather may have resulted from hydrogen peroxide-induced lysis or clearance of infected erythrocytes. Alternatively, the observed differences in the effect of hydrogen peroxide administration on malarial parasites compared with the effect on *E. coli* may reflect species differences between these microorganisms, such as differences in endogenous catalase production.

Oliver and Murphy (17) first reported the therapeutic use of intravenously administered hydrogen peroxide in the management of critically ill patients with pneumonia complicated by hypoxemia. In their study, 10 delirious and 3 comatose patients reportedly had dramatic responses to the intravenous infusion of 300 ml of normal saline containing 0.25 M hydrogen peroxide, with lysis of fever and improved oxygenation, but without evidence of embolization. The results of our study fail to explain how hydrogen peroxide administration may have benefited these patients, since we found neither antimicrobial activity nor improved oxygenation.

The lack of antibacterial activity after the parenteral administration of hydrogen peroxide observed in our study is presumably a consequence of the rapid breakdown of hydrogen peroxide by catalase. One way to circumvent the effects of blood catalase is to use a hydrogen peroxide derivative, such as ethyl hydrogen peroxide, that is resistant to catalase. However, the minimal concentration of ethyl hydrogen peroxide required to achieve bactericidal activity

TABLE 1. Levels of free endotoxin in plasma relative to levels of bacteria in blood in septic rabbits infused with saline or hydrogen peroxide^a

H posttreatment	Geometric mean of level of free endotoxin in plasma relative to the level of bacteremia (ng/10 ⁶ CFU) ^b in septic rabbits infused with:	
	Saline	Hydrogen peroxide
2	6	2
4	5	1
6	2	6
8	2	2

^a In similar experiments in which gentamicin was administered to infected rabbits, the geometric mean of the levels of free endotoxin in plasma and the level of bacteremia exceeded 2×10^6 ng/10⁶ CFU (20).

^b None of the results were significantly different ($P > 0.05$).

in blood was sufficient to cause the apparent oxidation of hemoglobin iron, forming methemoglobin. Baehner and co-workers (3) circumvented the effects of blood catalase by binding glucose oxidase to latex spherules, thereby delivering hydrogen peroxide-generating particles to the granulocyte lysosome of patients with chronic granulomatous disease. The killing of non-hydrogen-peroxide-producing bacteria in the phagocytes of patients with chronic granulomatous disease was markedly enhanced on exposure of the phagocytes to these particles (10). Some therapeutic benefits of parenteral hydrogen peroxide administration may be possible despite the presence of catalase. Although attempts to lyse tumor cells in vivo by the parenteral administration of hydrogen peroxide have been unsuccessful for the most part (9), the intra-arterial regional administration of hydrogen peroxide increases the susceptibility of tumor cells to irradiation (12), presumably through oxidant injury.

Whereas endogenous intracellular hydrogen peroxide has an unquestionably important role in host defense, the role, if any, of extracellular hydrogen peroxide excreted by phagocytes has yet to be documented. Our failure to demonstrate antibacterial activity of hydrogen peroxide infused into rabbits at the maximum rate tolerated suggests that hydrogen peroxide released by mononuclear phagocytes and neutrophils has little, if any, effect on bacteria in the presence of catalase.

ACKNOWLEDGMENTS

We thank Dr. Edwin Thomas for suggestions and criticisms and Jamsheed Panahi, Paul W. Mackert, and Kathryn A. Mogan for technical assistance.

This work was supported by grant RR-00584-18 from the Division of Research Resources, National Institutes of Health, and by the American-Lebanese-Syrian Associated Charities.

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