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In Vitro Killing of *Mycobacterium ulcerans* by Acidified Nitrite

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***Mycobacterium ulcerans*, which causes Buruli ulcer, was exposed to acidified nitrite or to acid alone for 10 or 20 min. Killing was rapid, and viable counts were reduced below detectable limits within 10 min of exposure to 40 mM acidified nitrite. *M. ulcerans* is highly susceptible to acidified nitrite in vitro.**

Mycobacterium ulcerans disease (Buruli ulcer) is a serious ulcerative skin disease which is a major health problem in many tropical countries, particularly in West Africa (3, 11). It causes chronic, painless skin ulcers with undermined edges, usually on the limbs and predominantly in children (5). Treatment options for Buruli ulcer are surgery, antimycobacterial agents, and topical preparations. Surgery is curative for early nodules. However, patients often present late with large ulcers, which require wide surgical excision followed by skin grafting; the result is a long inpatient stay (4). The role of antimycobacterial drugs is being investigated under the auspices of the World Health Organization. Many different topical treatments have been tried (2, 15), but the only topical treatment shown to increase the rate of healing in a double-blind controlled trial is acidified nitrite creams, which generate nitric oxide and other oxides of nitrogen (16a).

In light of these encouraging clinical results, the present study was designed to investigate the in vitro susceptibility of *M. ulcerans* to nitrogen oxides. The antimicrobial activity of nitrogen oxides has been clarified to some extent in recent studies, although the exact molecular species responsible for killing is not known (9). Acidification of nitrite results in production of a complex mixture of nitrogen oxides including nitrous acid, dinitrogen trioxide, nitrogen dioxide, and nitric oxide, all good nitrosating agents (NO^+ donors) (20) which diffuse readily across membranes (8). They react rapidly with reduced thiols to form nitrosothiols, also thought to be important in microbial killing (7). Nitric oxide can inhibit respiratory chain enzymes through inactivation of iron-sulfur complexes (10) and can disrupt DNA replication by inhibiting ribonucleotide reductase (16).

A clinical isolate of *M. ulcerans* from Africa, *M. ulcerans* isolate 1, identified in our laboratory and maintained on Löwenstein-Jensen medium, was cultivated in Middlebrook 7H9 broth (pH 6.8; Difco Laboratories, Detroit, Mich.) supplemented with 10% ADC (albumin, dextrose, catalase; Difco) and incubated at 30°C. The concentration of bacteria was estimated by measuring the optical density in a spectrophotometer at a wavelength of 420 nm, where a reading of 0.15 is equivalent to 10^8 bacteria per ml (17).

Solutions of anhydrous sodium nitrite (Merck, Darmstadt, Germany) and citric acid monohydrate (BDH, Poole, England) were prepared in deionized water. Freshly prepared 0.4 M (3%), 0.9 M (6%), and 1.7 M (12%) sodium nitrite solutions and 0.2 M (4.5%), 0.4 M (9%), and 0.9 M (18%) citric acid monohydrate solutions were sterilized by passage through a 0.2-μm-pore-size sterile filter (Schleicher & Schuell, Dassel, Germany). Separate citric acid solutions were made, and the pH was adjusted with 1 M NaOH to that of each of the acidified nitrite solutions (0.2 M [pH 3.4], 0.4 M [pH 3.2], and 0.9 M [pH 3.0]).

Aliquots (0.2 ml) of the bacterial suspension prepared as described above were placed in sterile 2-ml screw-cap tubes (Sarstedt, Nümbrecht, Germany) to which 0.9 ml of nitrite solution and 0.9 ml of citric acid solution were added. Two sets of control tubes contained 0.2 ml of the bacterial suspension, 0.9 ml of sterile water, and 0.9 ml of pH-adjusted citric acid monohydrate solution. After exposures of 10 and 20 min, 0.2 ml of the contents was added to 1.8 ml of Middlebrook 7H9 broth enriched with ADC. Successive 10-fold serial dilutions of these bacterial suspensions were made, and 0.1-ml volumes of the broth mixture were then cultured in duplicate on Middlebrook 7H11 agar (pH 6.6) supplemented with oleic acid, albumin, dextrose, and catalase (OADC; Difco) to further neutralize the effect of the acidified nitrite solution. All cultures were incubated at 30°C in sealed bags, and the resulting CFU were counted after 28 days of incubation. Viable counts were expressed as $\log_{10}$ CFU per milliliter.

In human *M. ulcerans* lesions, the organisms grow in a high-protein environment. Therefore, the effect of acidified nitrite or citric acid on *M. ulcerans* viability was also tested in Middlebrook 7H9 medium with ADC in which the concentration of bovine serum albumin (BSA; Sigma, St. Louis, Mo.) was increased from 0.5 to 5% (wt/vol). We first determined the effect of exposure of *M. ulcerans* to acidified nitrite for 1 and 9 h, since we predicted that prolonged incubation in the presence of acidified nitrite would be necessary to kill *M. ulcerans*. However, complete killing was found after only 1 h. Table 1 shows the effect of acidified nitrite compared with that of pH-matched citric acid controls after 10- and 20-min incubations. Killing was again rapid, and viable counts were reduced to

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TABLE 1. Effect of exposure to acidified nitrite for 10 or 20 min on the viability of an *M. ulcerans* culture

Concn of acidified nitrite or citric acid alone[a]	pH of solution	Viable counts (log_{10}, CFU/ml)[b] after exposure for the indicated time in medium:					
		Without added protein			With protein added[c]		
		0 min	10 min	20 min	0 min	10 min	20 min
High							
Acidified nitrite	3.00	6.5	<2	<2	7.2	<2	<2
Acid alone	3.00	6.5	6.6	6.8	7.2	7.2	6.8
Medium							
Acidified nitrite	3.20	6.5	<2	<2	7.2	<2	<2
Acid alone	3.20	6.5	6.9	7.1	7.2	7.2	7.2
Low							
Acidified nitrite	3.41	6.5	<2	<2	7.2	<2	<2
Acid alone	3.41	6.5	6.9	6.8	7.2	7.0	7.3

[a] High concentrations, 1.7 M sodium nitrite and 0.9 M citric acid; medium concentrations, 0.9 M sodium nitrite and 0.4 M citric acid; low concentrations, 0.4 M sodium nitrite and 0.2 M citric acid.
[b] Mean results after 28 days of incubation. <2, no growth was observed on plates inoculated with 0.1 ml of the 10-times-diluted *M. ulcerans* suspension.
[c] Middlebrook 7H9 medium contained 5% BSA during exposure to acidified nitrite or citric acid.

below detectable limits after only a 10-min exposure to acidified nitrite. Controls showed no reduction in viable counts, suggesting that killing was due to the action of acidified nitrite and not simply to an acid environment. Also, sodium nitrite alone had no effect on the viable counts (data not shown). Increasing the protein content of the medium to 5% did not inhibit killing by acidified nitrite (Table 1). In similar experiments, the MIC of acidified nitrite (exposure time, 10 min) for *M. ulcerans* isolate 1 was determined (Table 2). The MIC of acidified nitrite for *M. ulcerans* with an exposure time of 10 min was below 40 mM sodium nitrite and 20 mM citric acid.

The concentrations of acidified nitrite chosen for this study were based on a recent clinical trial (16a), where a mixture of 6% (wt/wt) nitrite and 9% (wt/wt) citric acid was applied to ulcers caused by *M. ulcerans* infection. We have shown here that acidified nitrite, at the same concentrations, reduced the viable counts of a clinical isolate of *M. ulcerans* by more than 6 log_{10} units within 10 min and that this effect was not due to the low pH. This is the first study to demonstrate this effect in vitro. The duration of the exposure required to kill the organisms was short, and it is known that nitric oxide diffuses rapidly through human tissues, so these experiments are relevant to the treatment of human ulcers with topical nitrogen oxide-generating creams. The MIC of acidified nitrite for *M. ulcerans* with a 10-min exposure time was 22.5 times lower than the concentrations used in the clinical trial, suggesting that lower concentrations of this agent could be of benefit in vivo.

TABLE 2. Determination of the MIC of acidified nitrite with an exposure time of 10 min for *M. ulcerans* isolate 1

Concn (mM) of:		Viable counts (log_{10} CFU/ml)[a] after exposure for:	
Sodium nitrite	Citric acid	0 min	10 min
400	200	7.72	<2
40	20	7.72	<2
0	20	7.72	7.11
4	2	7.72	7.25
0	2	7.72	7.18

[a] Mean results after 28 days of incubation. <2, no growth was observed on plates inoculated with 0.1 ml of the 10-times-diluted *M. ulcerans* suspension.

In vitro, nitric oxide can kill *Escherichia coli* (12, 14), *Candida* spp. (6), *Leishmania* spp. (13, 18), and *M. leprae* (1), and it can inhibit *Staphylococcus aureus* and *Propionibacterium acnes* (19). *M. ulcerans* is an addition to the growing list of susceptible organisms. We have found that *Mycobacterium tuberculosis* is also susceptible to acidified nitrite at the same concentrations (unpublished data). These results help to explain the finding that Buruli ulcers caused by *M. ulcerans* heal more rapidly with topical treatment with acidified nitrite than without it. Further investigations of the actions of this treatment are desirable.

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