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INFECTION AND IMMUNITY, Mar. 2002, p. 1367–1371 Vol. 70, No. 3
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.3.1367–1371.2002
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A Superoxide Dismutase C Mutant of *Haemophilus ducreyi* Is Virulent in Human Volunteers

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Received 3 October 2001/Returned for modification 13 November 2001/Accepted 10 December 2001

***Haemophilus ducreyi* produces a periplasmic copper-zinc superoxide dismutase (Cu-Zn SOD), which is thought to protect the organism from exogenous reactive oxygen species generated by neutrophils during an inflammatory response. We had previously identified the gene, *sodC*, responsible for the production and secretion of Cu-Zn SOD and constructed an isogenic *H. ducreyi* strain with a mutation in the *sodC* gene (35000HP-*sodC-cat*). Compared to the parent, the mutant does not survive in the presence of exogenous superoxide (L. R. San Mateo, M. Hobbs, and T. H. Kawula, Mol. Microbiol. 27:391–404, 1998) and is impaired in the swine model of *H. ducreyi* infection (L. R. San Mateo, K. L. Toffer, P. E. Orndorff, and T. H. Kawula, Infect. Immun. 67:5345–5351, 1999). To test whether Cu-Zn SOD is important for bacterial survival in vivo, six human volunteers were experimentally infected with 35000HP and 35000HP-*sodC-cat* and observed for papule and pustule formation. Papules developed at similar rates at sites inoculated with the mutant or parent. The pustule formation rates were 75% (95% confidence intervals [CI], 43 to 95%) at 12 parent-inoculated sites and 67% (95% CI, 41 to 88%) at 18 mutant-inoculated sites ($P = 0.47$). There was no significant difference in levels of *H. ducreyi* recovery from mutant- and parent-inoculated biopsy sites. These results suggest that expression of Cu-Zn SOD does not play a major role in the survival of this pathogen in the initial stages of experimental infection of humans.**

Haemophilus ducreyi is the etiologic agent of chancroid, a common genital ulcer disease found in many developing countries (7–9, 24, 29, 39). *H. ducreyi* is a strictly human pathogen that infects primarily genital and nongenital skin and mucous membranes. *H. ducreyi* infection enhances transmission of the human immunodeficiency virus (HIV) in populations where both forms of infection are present (17). Understanding the pathogenesis of chancroid may have implications for control of both *H. ducreyi* and HIV infections, especially in Africa and Southeast Asia (17).

To study *H. ducreyi* pathogenesis, we developed an experimental infection model with human volunteers (3, 34, 35). Throughout the papular and pustular stages of experimental infection, *H. ducreyi* is surrounded by professional phagocytes but remains extracellular (5, 6). In vitro, *H. ducreyi* adheres to the human macrophage-like cell line U-937 but resists phagocytosis (41). Neutrophils usually kill bacteria by ingesting them or by releasing toxic products such as superoxide radicals and antimicrobial proteins (10). *H. ducreyi* replicates during the initial stages of experimental infection in humans (3, 38), which suggests that the organism escapes both phagocytosis and killing by toxic products of neutrophils. How *H. ducreyi* evades phagocytic defenses is unclear.

Superoxide dismutases (SODs) are metalloenzymes which catalyze the conversion of superoxide radicals to oxygen and hydrogen peroxide. Theoretically, cytoplasmic SODs protect bacteria from superoxide and H_2O_2 toxicity generated during bacterial aerobic metabolism. SODs are highly metal specific, and cytoplasmic iron (Fe)- and manganese (Mn)-cofactored SODs are ubiquitous in bacteria (30). Once thought to be rare, periplasmic copper- and zinc-dependent SODs (Cu-Zn SODs) have now been described to occur in many bacterial species (11, 21, 23, 32, 37, 40). The functional significance of the Cu-Zn SODs in bacterial survival is not fully understood, but their conservation suggests that the ability to neutralize extracellular superoxide aids the survival of these organisms.

H. ducreyi produces a Cu-Zn SOD that localizes to the periplasm (21, 30, 36). In vitro, *H. ducreyi* Cu-Zn SOD does not complement the growth defect of an *Escherichia coli* strain that lacks cytoplasmic SODs, suggesting that the Cu-Zn SOD functions in a compartment different from that in which cytoplasmic SODs function (36). *H. ducreyi* Cu-Zn SOD expressed in *E. coli* confers resistance to phagocytic killing by murine macrophages (4). A Cu-Zn SOD-deficient *H. ducreyi* strain (35000HP-*sodC-cat*) was made by inserting a chloramphenicol acetyltransferase (*cat*) cassette into the *sodC* gene (30). Though the mutant and parent strains are equally resistant to induced cytoplasmic reactive oxygen species (ROS), the mutant is significantly more susceptible to killing by pyrogallol-generated extracellular superoxide (30). When the *sodC* mutation is complemented in the chromosome, resulting in a merodiploid (*sodC*$^{+}$*sodC*-mutant) strain, parent survival rates are restored (30).

Cu-Zn SOD may contribute in several ways to the survival of *H. ducreyi*. Earlier studies show that *H. ducreyi* is unable to synthesize heme and must be supplied with heme or heme-

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containing compounds, such as hemoglobin, for growth (13, 14, 18). Recent data suggest that Cu-Zn SOD binds heme and, under certain conditions, may protect *H. ducreyi* from toxic oxyradicals formed from the reaction of heme iron and oxygen (25). In addition, the histidine-rich N-terminal region of the Cu-Zn SOD complex has a high affinity for metals and is responsible for binding copper, which may be scarce in the periplasmic space (4). The ability to efficiently sequester copper for eventual delivery to the active site of the enzyme may enhance the function of the *H. ducreyi* Cu-Zn SOD (4).

In a swine model, the Cu-Zn SOD-deficient mutant of *H. ducreyi* 35000HP causes disease that is similar to that caused by its parent, as determined by histopathology (31). However, the mutant is recovered from significantly fewer inoculated sites and in lower numbers than the parent strain, implying that Cu-Zn SOD is a virulence factor for this bacterium in this animal model (31). When pigs are experimentally immunosuppressed by treatment with cyclophosphamide, the parent and mutant *H. ducreyi* strains survive equally well. These results suggest that suppression of neutrophil activity may decrease the generation of superoxide and allow the mutant to survive normally (31).

In this study, we tested the hypothesis that expression of the Cu-Zn SOD may be important for bacterial survival in humans. We compared the ability of a SOD C mutant (35000HP-*sodC-cat*) and its isogenic parent, 35000HP, to cause experimental infection in human volunteers.

MATERIALS AND METHODS

Bacteria. 35000HP is a human-passaged variant of 35000 (2, 3, 34). The 35000-*sodC cat* mutant, described previously (30), was constructed in 35000HP and has been renamed 35000HP-*sodC-cat* here.

OMP and LOS analysis. Lipooligosaccharides (LOS) and outer membrane proteins (OMP) were prepared from 35000HP and 35000HP-*sodC-cat* and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and silver staining as described previously (27, 42).

Human challenge protocol. Healthy adult male and female volunteers over 18 years of age were recruited for the study. Informed consent was obtained from the subjects for participation and for HIV serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University-Purdue University, Indianapolis. The experimental challenge protocol, preparation and inoculation of the bacteria, calculation of the estimated delivered dose (EDD), and clinical observations were done exactly as described previously (1–3, 34, 35). Each subject was inoculated with two doses (54 to 60 CFU) of live 35000HP and one dose of heat-killed bacteria on one arm and with three doses (ranging from 30 to 163 CFU) of live 35000HP-*sodC-cat* on the other arm. Subjects were observed until they reached a clinical end point, defined as either the development of a painful pustule 14 days after inoculation or the resolution of infection at all sites. When a clinical end point was achieved, the code was broken and up to two sites with active disease (one inoculated with the parent and one inoculated with the mutant), if present, were biopsied with punch forceps. The subjects were then treated with two doses of oral ciprofloxacin as described previously.

Surface cultures. For each subject, surface cultures were obtained from all inoculation sites at each follow-up visit, as described previously (19).

Biopsy specimens. For each subject, a mutant-inoculated site and a parent-inoculated site were biopsied with punch forceps of the same size. Each biopsy specimen was cut into halves with a razor blade. One half was fixed in formalin, and immunohistological studies were done as previously described (26, 34, 35). The other half was homogenized in 1 ml of freezing medium for 2 min on ice and cultured semiquantitatively as described previously (34, 35).

Phenotypes of recovered bacteria. Individual colonies from the inocula, surface cultures, and biopsy specimens were picked, suspended in freezing medium, and frozen in 96-well plates. The colonies were scored for susceptibility to chloramphenicol on chloramphenicol-containing chocolate agar plates. At least 30 individual colonies per specimen, if available, were analyzed. If the 30 colonies had the correct phenotype, then we were confident (95% probability) that, at most, only 11% of the colonies in a specimen could have had the incorrect phenotype.

TABLE 1. Response to inoculation of *H. ducreyi*

Subject[a]	No. of days of observation	Isolate	No. of initial papules	Final outcome after initial papule formation		
				No. of papules	No. of pustules	No. resolved
186	6	35000HP	2		2	
		35000HP-*sodC-cat*	1			1
187	9	35000HP	2		2	
		35000HP-*sodC-cat*	3		3	
188	9	35000HP	2		1	1
		35000HP-*sodC-cat*	3		1	2
190	10	35000HP	2		1	1
		35000HP-*sodC-cat*	3		2	1
191	7	35000HP	2	1	1	
		35000HP-*sodC-cat*	3		3	
192	7	35000HP	2		2	
		35000HP-*sodC-cat*	3		3	

[a] Each volunteer was inoculated at two sites with the parent (35000HP) and at three sites with the mutant (35000HP-*sodC-cat*). Volunteers 186, 187, and 188 were inoculated in the first iteration, and volunteers 190, 191, and 192 were inoculated in the second iteration.

RESULTS

Comparison of 35000HP-*sodC-cat* to 35000HP. The isogenic mutant 35000HP-*sodC-cat* was constructed in the 35000HP background in the laboratory of T. H. Kawula (30). We compared the OMP and LOS profiles and growth rates in the broth of the 35000HP stock used by members of the S. M. Spinola laboratory to infect human subjects with 35000HP-*sodC-cat*. The OMP and LOS profiles and the growth rates of the strains were identical (data not shown). Thus, we proceeded with the human challenge experiments.

Human inoculation experiments. Six healthy adults (four females, two males; age range, 24 to 51 years; mean age $\pm$ standard deviation, 35.8 $\pm$ 9.2 years) volunteered for the study. Three subjects (volunteers 186, 187, and 188) were challenged in the first iteration, and three subjects (volunteers 190, 191, and 192) were challenged in the second iteration (Table 1).

An escalating-dose response study was used to compare the levels of virulence of the mutant and the parent. We initially inoculated three subjects at six sites on both arms. One arm was inoculated at three sites with EDDs of 30, 60, and 119 CFU of 35000HP-*sodC-cat*. The other arm was inoculated at two sites with EDDs of 60 CFU of the parent and at a third site with 119 CFU of heat-killed 35000HP-*sodC-cat*. Papules developed at six of six sites inoculated with the parent and at seven of nine sites inoculated with the mutant. At end point, pustules developed at five of six sites inoculated with the parent and at four of nine sites inoculated with the mutant (Table 1).

Since the mutant was not impaired in its ability to cause pustules, we infected three more subjects to confirm these results. Our goal was to inoculate this group of subjects with

EDDs similar to those from the first iteration. Three sites were inoculated with EDDs of 41, 82, and 163 CFU of 35000HP-*sodC-cat*. Two sites were inoculated with 54 CFU of the parent, and a third site was inoculated with 163 CFU of heat-killed 35000HP-*sodC-cat*. Six of six parent-inoculated sites and nine of nine mutant-inoculated sites developed papules (Table 1). At clinical end point, pustules developed at four of six parent-inoculated sites and at eight of nine mutant-inoculated sites. Thus, 35000HP-*sodC-cat* was not impaired in its ability to form pustules.

The cumulative results for the two iterations showed that papules developed at 100% (exact-binomial 95% confidence intervals [CI], 74 to 100%) of 12 sites inoculated with 35000HP and at 89% (exact-binomial 95% CI, 65 to 99%) of 18 sites inoculated with 35000HP-*sodC-cat* (one-tailed Fisher's exact test, $P = 0.35$). Thus, the mutant was not impaired in its ability to cause disease in human volunteers. Pustules formed at 9 of 12 (75%; exact-binomial 95% CI, 43 to 95%) sites inoculated with 35000HP compared to 12 of 18 (67%; exact-binomial 95% CI, 41 to 88%) sites inoculated with 35000HP-*sodC-cat* (one-tailed Fisher's exact test, $P = 0.47$). Therefore, the mutant was not impaired in its ability to form pustules compared to the parent. For both iterations, small papules formed initially at three of six heat-killed control sites but resolved within 1 to 2 days, confirming that pustule formation was due to the inoculation of live bacteria.

Bacterial recovery and cellular infiltrate from mutant and parent lesions. Surface cultures were obtained by swabbing inoculation sites at each follow-up visit. No bacteria were recovered from sites inoculated with the heat-killed control. Of 78 cultures of parent-inoculated sites, three positive surface cultures with 42 colonies were recovered. Four surface cultures from a total of 117 cultures of mutant-inoculated sites were positive, and 32 colonies of 35000HP-*sodC-cat* were recovered.

In the swine model of infection, a *sodC*-deficient *H. ducreyi* strain was recovered from significantly fewer inoculated sites than the parent strain (31). By quantifying the number of bacteria present in halved biopsy specimens, the mutant was also less numerous in swine lesions than the parent (31). In this trial, *H. ducreyi* cells were recovered from five subjects who had parent- and mutant-inoculated sites that contained pustules at end point. *H. ducreyi* cells were recovered from all five parent biopsy samples (range, 6.2×10^2 to 2.7×10^6 CFU per g of tissue; mean ± standard deviation, $9.0 \times 10^5 \pm 1.1 \times 10^6$ CFU per g of tissue) and all five mutant biopsy samples (range, 8.8×10^3 to 1.2×10^6 CFU per g of tissue; mean ± standard deviation, $1.5 \times 10^5 \pm 2.4 \times 10^5$ CFU per g of tissue). In contrast to the swine model, there was no significant difference between the numbers of *H. ducreyi* cells recovered from parent and mutant biopsy specimens (paired t test after log transformation, $P = 0.72$).

In the swine model, the cellular infiltrates of mutant and parent biopsy samples were similar when they were analyzed by immunohistochemistry (31). Here, we also examined the cellular infiltrates in five parent and five mutant pustules. Micropustules with polymorphonuclear leukocytes (PMNs) were present in the epidermis of specimens from the subjects inoculated with the parent and mutant (data not shown). The dermis contained a perivascular infiltrate of mononuclear cells and some PMNs, and the venules were lined with reactive endothelial cells. The majority of mononuclear cells stained positively with a CD3 marker (data not shown). Thus, similar to what occurred in the swine model of infection, there were no notable differences between mutant and parent samples when they were analyzed by immunohistochemistry.

Confirmation of the phenotype of the recovered bacteria. To confirm that the inocula were correct and that no cross-contamination of sites had occurred during infection, individual colonies from each of the broth cultures used to prepare the inocula, from surface cultures and from biopsy specimens, were analyzed for chloramphenicol susceptibility. For the two parent and two mutant broth cultures used to prepare the inocula, all 96 parent colonies and all 96 mutant colonies tested were phenotypically correct (mutant, Cm^r; parent, Cm^s). All 42 colonies obtained from surface cultures of parent-inoculated sites and all 32 colonies from surface cultures of mutant-inoculated sites were phenotypically correct. All 108 parent colonies and 176 mutant colonies obtained from biopsy samples were phenotypically correct. Thus, all colonies tested from the inocula, surface cultures, and biopsy specimens had the expected antibiotic susceptibility.

DISCUSSION

In this study, we tested the ability of an isogenic *sodC*-deficient mutant (35000HP-*sodC-cat*) to infect human volunteers. In our volunteer group, the mutant formed papules at a rate similar to that of the parent. Pustules also formed at similar rates at mutant- and parent-inoculated sites, and halved biopsy specimens of mutant and parent pustules contained similar numbers of bacteria. Taken together, the data suggest that *sodC* activity is not a major factor in the ability of *H. ducreyi* to survive and cause disease during the initial stages of experimental infection of humans.

The bactericidal activities of neutrophils and macrophages are attributed in part to an induced oxidative burst upon exposure to bacteria (10). Activation of enzymes responsible for the respiratory burst depends on stimulation by a number of factors, including phagocytosis (10). In vitro, Cu-Zn SODs of a variety of organisms have been found to confer resistance to killing by detoxifying ROS generated by the oxidative burst (4, 28, 32, 33). In animal models, Cu-Zn SODs have a role in bacterial survival for some organisms (11, 15, 16, 31, 40) but not others (12, 23, 28, 33). Many of these data come from models which utilize animals that are not the natural hosts of the test organism. Thus, whether survival and/or virulence in animal models reflects altered host-bacterium interactions or the actual role of Cu-Zn SODs in virulence is unclear.

Previous studies that have evaluated Cu-Zn SOD mutants in models that utilize natural hosts include *Salmonella enterica* serovar Typhimurium and *Actinobacillus pleuropneumoniae* (11, 15, 16, 33). *S. enterica* serovar Typhimurium contains two distinct *sodC* genes (15). In a murine peritoneal infection model, the wild-type strain of *S. enterica* serovar Typhimurium causes a 50% mortality rate 6 to 7 days after inoculation (11, 15). A mutant that had both *sodC* genes inactivated was less lethal than the parent or than mutants having one intact *sodC* gene (15). In contrast, there is no difference in the abilities of wild-type and Cu-Zn SOD-deficient mutant strains of *A. pleuropneumoniae* to cause respiratory disease 42 h after inocula-

tion into the lungs of swine (33). The histopathology of the pulmonary lesions in pigs infected with the *sodC*-deficient mutant is the same as that of lesions caused by the parent strain (33).

The different contributions that Cu-Zn SODs make to *S.* serovar Typhimurium and *A. pleuropneumoniae* pathogenesis in their respective models may reflect an inherent difference in the way these two pathogens interact with phagocytic cells in their natural hosts. *S. enterica* serovar Typhimurium is a facultative intracellular organism. Macrophage killing of *Salmonella* spp. involves bacterial uptake and the combined effects of NADPH oxidase (the respiratory burst) and inducible nitric oxide (NO) synthase (11). These toxic radicals are active over short distances and need to be sequestered and/or concentrated in phagosomes to be effective in bacterial killing. After *S. enterica* serovar Typhimurium is phagocytosed, activated radicals produced by NADPH oxidase and NO synthase may induce cell damage by crossing the bacterial outer cell membrane and accumulating in the periplasmic space (11). DeGroote and colleagues proposed that Cu-Zn SOD protects *S. enterica* serovar Typhimurium by neutralizing these toxic products, thereby decreasing their local concentration within the periplasm (11). In contrast, *A. pleuropneumoniae* causes disease primarily as an extracellular pathogen (11, 33). SOD activity may be less critical to the survival of *A. pleuropneumoniae* because toxins released during the respiratory burst dissipate when they are released into the extracellular compartment. *A. pleuropneumoniae* cells probably survive host defenses primarily by evading phagocytosis and secreting cytolytic toxins (Apx I and II) that lyse neutrophils rather than by detoxifying ROS by Cu-Zn SOD activity (33).

Although Cu-Zn SOD has a role in the survival of *S. enterica* serovar Typhimurium in a short-term-infection model, its importance for other intracellular pathogens in the setting of chronic infection is not clear. For example, a Cu-Zn SOD-deficient mutant of *Mycobacterium tuberculosis* survives as well as the parent in a murine lung infection model 60 days after inoculation and in guinea pigs 35 days after subcutaneous injection (12, 28). Piddington and coworkers speculate that *sodA* may compensate for the loss of *sodC* during chronic infection (28). Alternatively, *M. tuberculosis* may not activate macrophages in vivo and may stimulate a vigorous respiratory burst (28), diminishing the potential role of Cu-Zn SOD for survival in these models.

To our knowledge, this study is the first evaluation of a Cu-Zn SOD mutant in humans. We did not detect differences between 35000HP and 35000HP-*sodC-cat* in terms of disease establishment, progression, and bacterial recovery after experimental infection. In vitro, *H. ducreyi* is phagocytosed by purified PMNs (22) but it resists phagocytosis by the macrophage cell line U-937 (41). Confocal microscopic studies show that *H. ducreyi* colocalizes with PMNs and macrophages but remains extracellular throughout experimental infection of human volunteers (5). Since *H. ducreyi* appears to resist engulfment, we speculate that the bacteria may not stimulate the respiratory burst in the human infection model. Alternatively, because *H. ducreyi* remains extracellular, any released toxins may not be concentrated enough to harm the bacteria. Our results parallel the findings from the swine model of acute *A. pleuropneumoniae* infection and are consistent with our observation that *H. ducreyi* causes disease primarily as an extracellular pathogen in the experimental infection of humans.

Analyses of mutant and parent biopsy specimens in our experiments did not show a difference in the numbers of mutant and parent bacteria recovered from pustules. Our results are in contrast to those noted from the swine model of *H. ducreyi* infection, in which the *sodC* mutant was recovered from fewer inoculated sites and in fewer numbers than the parent (31). The swine model and the human challenge model are similar in that Multi-Test applicators are used to deliver the bacteria to the skin of test subjects or animals. The histopathologies of experimental swine and human lesions are also similar (26, 33). However, the EDD used in the human model is on the order of 10^1 CFU, while the EDD used in the swine model is approximately 10^4 CFU. The larger EDD required for infection in the swine model suggests that *H. ducreyi* is a less efficient pathogen for pigs than for humans and that the absence of a candidate virulence determinant may have a more pronounced effect in swine than in humans. In the human model, *H. ducreyi* achieves a density of 10^5 CFU/lesion at the pustular stage, approximately 1 week after inoculation (38). In the swine model, the number of bacteria decreases from 1.7×10^4 CFU to 1.7×10^3 CFU 48 h after inoculation. Despite this initial decrease, *H. ducreyi* persists in swine lesions for weeks (20). Immunosuppression of swine with cyclophosphamide allows the Cu-Zn SOD mutant and the parent to achieve densities of 2.7×10^5 and 1.2×10^4, respectively, 48 h after inoculation (31). These findings suggest that swine neutrophils may be more efficient at clearing *H. ducreyi* than human neutrophils in the initial stages of infection.

In summary, our data show that expression of *sodC* is not required for the survival and virulence of *H. ducreyi* during experimental infection of human volunteers. Cu-Zn SOD may play a major role in bacterial survival for microbes that are phagocytosed and activate the respiratory burst. *H. ducreyi* appears to evade phagocytosis in the initial stages of experimental infection of humans, which may minimize the contribution of Cu-Zn SOD activity to pathogenesis in this model. For subject safety considerations, we did not allow experimental infection to progress beyond 14 days or to the ulcerative stage of disease. Whether *H. ducreyi* is engulfed by professional phagocytes during natural or later stages of infection is unknown, and we cannot test whether Cu-Zn SOD has a role in pathogenesis later in infection. Future studies should focus on examining whether the interactions between *H. ducreyi* and professional phagocytes change during the course of human infection and whether *H. ducreyi* interacts differently with swine neutrophils than with human neutrophils.

ACKNOWLEDGMENTS

This work was supported by grants AI27863, AI31494, and AI42824 from the National Institutes of Health (NIH) and by grant MO1RR00750 to the GCRC at Indiana University.

We thank Ferric Fang and Margaret Bauer for their thoughtful criticism of the manuscript.

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Editor: D. L. Burns