

## Leptospires Are Killed In Vitro by Both Oxygen-Dependent and -Independent Reactions

Rossella Murgia,<sup>1</sup> Rodolfo Garcia,<sup>2</sup> and Marina Cinco<sup>1\*</sup>

Department of Biomedical Sciences, University of Trieste, 34127 Trieste,<sup>1</sup> and International Centre for Genetic Engineering and Biotechnology, 34012 Trieste,<sup>2</sup> Italy

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**This study reports for the first time that leptospires are killed by H<sub>2</sub>O<sub>2</sub> and by low-molecular-weight primary granule components, which are agents normally released by neutrophils upon stimulation. Although both pathogenic and nonpathogenic strains were sensitive to H<sub>2</sub>O<sub>2</sub>-mediated killing, nonpathogenic organisms were found to be more susceptible. In addition, the killing of leptospires by H<sub>2</sub>O<sub>2</sub> was found to be independent of the presence of the neutrophil primary granule component myeloperoxidase and therefore not a consequence of halogenation reactions. We have also determined that leptospires are significantly sensitive only to primary granule components and, among those, to proteins and/or peptides of less than 30 kDa.**

Leptospires are the etiological agents of leptospirosis, a zoonanthroposis widespread throughout the world. The course of human leptospirosis ranges from mild to severe; some forms can be fatal, depending on the serovar of *Leptospira* involved and its virulence.

Although we are far from having a complete picture of the *Leptospira* pathogenic factors, it is clear that these organisms are invasive and that the success of their invasions is mainly due to their ability to survive and grow in tissues by escaping natural defense mechanisms such as phagocytic cells, the complement system, and pharmacologically active tissue peptides and mediators. In fact, pathogenic leptospires can survive in the nonimmune host by evading complement-mediated killing (6) and (probably) phagocytosis. Studies have shown that pathogenic leptospires bind nonopsonically to and are slowly internalized by guinea pig macrophages and human polymorphonuclear leucocytes (1, 5–7, 20). Although nonpathogenic spirochetes are rapidly engulfed and killed by human polymorphonuclear phagocytes, there is no clear evidence of the killing of pathogenic spirochetes other than that mediated by specific antibodies directed against some of their surface components (5). Moreover, live pathogenic leptospires can partially escape uptake by Kupffer cells from perfused rat liver (16). Overall, these data indicate that survival of leptospires is due primarily to scarce uptake by phagocytes. However, whether those leptospires that have been engulfed through nonopsonic phagocytosis are susceptible to intracellular killing is not known.

The bactericidal systems activated after uptake by phagocytes include the generation of oxygen metabolites (O<sub>2</sub>-dependent killing) and the release of granule components such as proteins and peptides with microbicidal activity (10, 15) into the phagocytic vacuole (O<sub>2</sub>-independent killing) and combinations of the two. Since it is not known which among the killing mechanisms of phagocytes are effective toward leptospires, we have investigated the in vitro sensitivity of pathogenic and

nonpathogenic strains of leptospires to the O<sub>2</sub>-dependent and -independent killing machinery of neutrophils.

The oxygen-dependent chlorinating system constitutes an important microbicidal strategy employed by neutrophils (13), and we tested its efficacy with respect to leptospires by means of a myeloperoxidase (MPO)-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> cell-free assay. As previously reported, the complete chlorinating system kills *Escherichia coli* K-12 (strain AB 1157) very efficiently (reference 11 and data not shown) whereas neither MPO nor H<sub>2</sub>O<sub>2</sub> is effective on its own. However, we found a pronounced killing effect with H<sub>2</sub>O<sub>2</sub> on both the pathogenic *Leptospira interrogans* strain Hardjoprajitno (serovar hardjo) and the nonpathogenic *Leptospira biflexa* strain Patoc1 (serovar patoc), regardless of the presence of MPO (Table 1). Both saprophytic and pathogenic leptospires were very sensitive to 0.33 mM H<sub>2</sub>O<sub>2</sub> in the presence or absence of MPO, with the decrease of viable leptospires being at least 2 logarithms. The leptospiricidal activity of H<sub>2</sub>O<sub>2</sub> alone was confirmed by using other pathogenic strains such as 142 (serovar icterohaemorrhagiae), Wijmberg (serovar copenhageni), and Ballico (serovar australis), with the microorganism survival level being at least 2 logarithms lower than that of the controls. We then tested the effect of lower H<sub>2</sub>O<sub>2</sub> concentrations on the Hardjoprajitno and Patoc1 strains, starting with 0.0018 mM (Fig. 1). The dose response curves showed that nonpathogenic Patoc1 was more sensitive than Hardjoprajitno to H<sub>2</sub>O<sub>2</sub>, with the level of killing 1 order of magnitude higher at 0.03 mM and 3 orders of magnitude higher at 0.18 mM, which is the concentration at which complete killing was achieved for Patoc1. The apparent absence of a contribution of MPO to the level of killing could have been due to too high a concentration of H<sub>2</sub>O<sub>2</sub>, to which these leptospires are particularly sensitive. Therefore, we investigated the possible contribution of MPO (0.1 guaiacol unit [1 guaiacol unit is the amount of enzyme which oxidizes 1 μmol of guaiacol per min]) to the level of killing at 0.033 mM H<sub>2</sub>O<sub>2</sub>, which is a concentration that results in only partial killing of the leptospires. As shown in Fig. 2, the presence of MPO did not increase the efficiency of H<sub>2</sub>O<sub>2</sub>-mediated killing after 10 or 30 min of incubation. As observed previously, Patoc1 was demonstrated to be the more susceptible of the two strains, with a

\* Corresponding author. Mailing address: Department of Biomedical Sciences, University of Trieste, 34127 Trieste, Italy. Phone: 39-040-5587178. Fax: 39-040-351668. E-mail: cinco@dsbmail.units.it.

TABLE 1. Oxygen-dependent killing of leptospires<sup>a</sup>

Treatment	Mean bacterial count (10 <sup>5</sup> MPN) ± SD for <sup>b</sup> :	
	Hardjoprajitno	Patoc1
None	4.4 ± 2.1	2.2 ± 2.9
MPO	3.7 ± 3.7	1.3 ± 1.8
H <sub>2</sub> O <sub>2</sub>	<0.01	<0.01
MPO + H <sub>2</sub> O <sub>2</sub>	<0.01	<0.01

<sup>a</sup> Leptospires (strain Hardjoprajitno or Patoc1) were incubated for 30 min at 37°C in MEM (containing 0.126 M chloride), with or without the addition of 0.1 or 1.0 guaiacol unit of MPO (kindly provided by G. Zabucchi) and with different amounts of H<sub>2</sub>O<sub>2</sub> as indicated. Incubation mixtures were tested for bacterial viability by using the microdilution test described previously for *Borrelia burgdorferi* (11), with the statistical elaboration described by Meynell and Meynell (17) for the calculations. As a positive control, *E. coli* bacteria were treated with MPO, H<sub>2</sub>O<sub>2</sub>, or MPO + H<sub>2</sub>O<sub>2</sub>, resulting in levels of viability of 100, 100, and 0%, respectively, as determined by measurements of the number of CFU. Viability counts were performed in triplicate in all cases.

<sup>b</sup> MPN, most probable number.

decrease of viable leptospires on the order of 2 log against a decrease of 1 log for Hardjoprajitno. Altogether, leptospires were found to be sensitive to H<sub>2</sub>O<sub>2</sub> independently of MPO, i.e., not as a consequence of halogenation reactions. This H<sub>2</sub>O<sub>2</sub> sensitivity can be postulated to be due to a scarce or absent production of catalase. In fact, no catalase activity has been detected in strain Patoc1, and strain Hardjoprajitno hydrolyzes only 16 μmol of H<sub>2</sub>O<sub>2</sub> per min per 10<sup>9</sup> cells (2). This is likely to be the reason that leptospires show a higher susceptibility to H<sub>2</sub>O<sub>2</sub> toxicity than other bacteria such as *Staphylococcus aureus* (21), *Legionella pneumophila* (12), *Listeria monocytogenes* (4), and *E. coli* (19). That H<sub>2</sub>O<sub>2</sub> is less reactive than other products of oxygen metabolism such as O<sub>2</sub><sup>-</sup>, OH, and hypohalous acid allows it to pass intact through cell membranes (8), diffuse within biological fluids containing little or no catalase, and act as an oxidizing agent (18). The lack of a role for MPO

in the killing of both pathogenic and nonpathogenic leptospires is in keeping with results previously obtained with *Borrelia* species (11). We have demonstrated here that MPO is not only not bactericidal but it is also unable to increase the microbicidal action of H<sub>2</sub>O<sub>2</sub>. MPO is a cationic protein and, as such, is generally thought to adhere to cell surfaces and result in cell injury (in the presence of H<sub>2</sub>O<sub>2</sub>) by increasing the local concentration of hypohalous acid at the target membrane (13). Therefore, it can be speculated that in the case of spirochetes in particular, MPO is not able to bind to the outer membranes and therefore chlorination reactions cannot take place. According to this hypothesis, the lack of such reactions excludes the possibility that the halogenation of *Leptospira* membrane proteins is the cause of functional damage leading to the killing of spirochetes.

The potential leptospiricidal contribution of oxygen-independent mechanisms was studied using different subcellular fractions from neutrophils isolated from the peripheral blood of healthy donors. Cytosolic, membrane (low and high density), and secondary and primary granule fractions were obtained by Percoll gradient fractionation of cells disrupted by nitrogen cavitation, as previously described (3, 11, 14). A very low level of cross-contamination between primary and secondary granules was demonstrated by using determinations of the ratios of MPO to lysozyme (11). Acid extracts from membranes and primary or secondary granules were prepared as previously described (11). We found that no leptospiricidal activity was found to be associated with cytosolic or membrane fractions. A very modest, not significant level of leptospiricidal activity was observed for both strains by using specific granule extracts, while a substantial level of killing of leptospires was found by using the same amounts of primary granule extracts (Table 2). A dose-response study (Fig. 3) confirmed that secondary granule extracts did not contribute in any significant proportion to levels of killing, while primary granule extracts

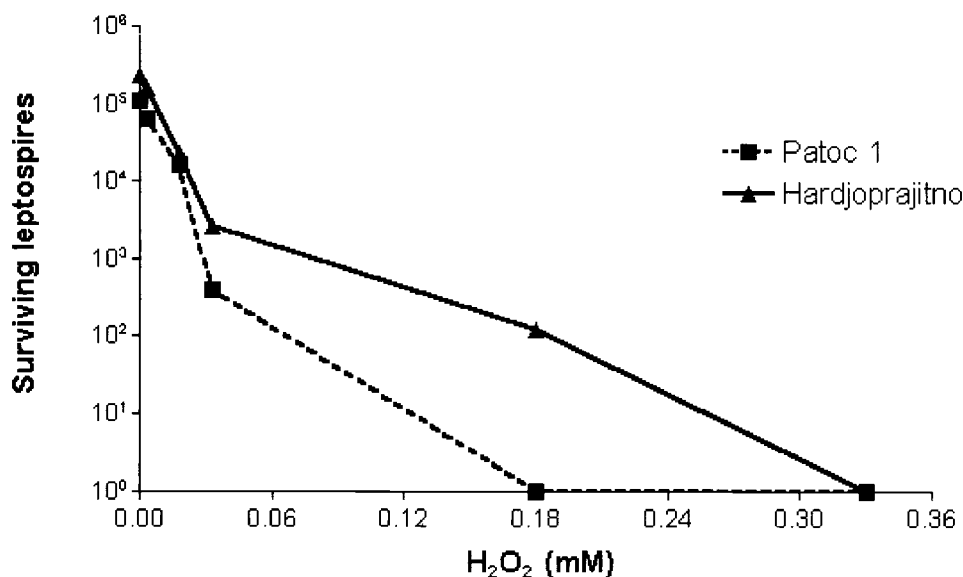


FIG. 1. Dose response of H<sub>2</sub>O<sub>2</sub> leptospiricidal activity. Leptospires (10<sup>5</sup>) were incubated at 37°C for 30 min with different concentrations of H<sub>2</sub>O<sub>2</sub> in a final volume of 100 μl of minimal essential medium (MEM). Bactericidal activity was evaluated as described in Table 1, footnote a. Values are the means of two representative experiments.

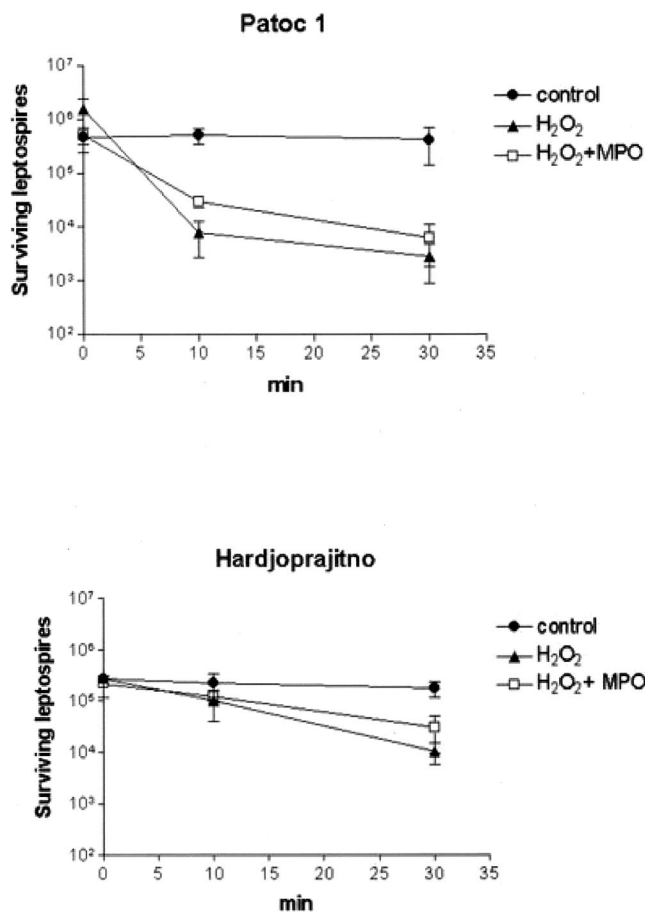


FIG. 2. Bactericidal effect of H<sub>2</sub>O<sub>2</sub>-MPO-Cl<sup>-</sup> on nonpathogenic and pathogenic leptospires. *Leptospira* strain Patoc1 (nonpathogenic) or Hardjoprajitno (pathogenic) was incubated at 37°C in the presence of 0.033 mM H<sub>2</sub>O<sub>2</sub>, with or without 0.1 guaiacol unit of MPO, in minimal essential medium. At different incubation times of up to 30 min, microbicidal activities were evaluated as described in Table 1; footnote *a*. Data shown are means ± standard deviations (*n* = 3).

were clearly effective. The relative difference between the effects of secondary and primary granules was found to be maximal at a dose of 40 μg.

Since MPO, a major primary granule component, had been

TABLE 2. Oxygen-independent killing of leptospires<sup>a</sup>

Subcellular fraction	Mean bacterial count (10 <sup>5</sup> MPN) ± SD ( <i>n</i> = 3) for <sup>b</sup> :	
	Patoc1	Hardjoprajitno
Control (buffer)	17.0 ± 2.5	18.5 ± 5.3
Cytosol	16.5 ± 2.6	25.2 ± 2.0
Low-density membranes	19.1 ± 3.0	26.4 ± 3.2
High-density membranes	18.2 ± 0.7	25.2 ± 1.4
Secondary granules	15.2 ± 0.7	14.5 ± 6.0
Primary granules	4.6 ± 1.5	5.5 ± 2.0

<sup>a</sup> *Leptospira* killing assays were performed by using 20 μg of protein from either cytosolic fractions or acid extracts from membrane and granule fractions. Control assays using the same amount of acid buffer were performed simultaneously. Viability was determined as described in Table 1, footnote *a*.

<sup>b</sup> MPN, most probable number.

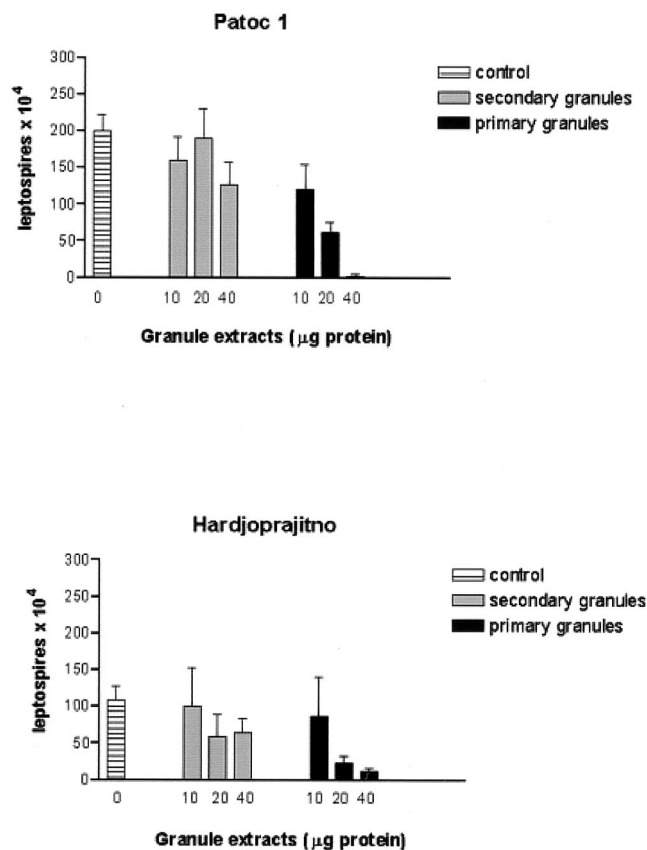


FIG. 3. Dose dependency of the microbicidal activity of primary or secondary neutrophil granule extracts toward leptospires. Nonpathogenic (Patoc1) and pathogenic (Hardjoprajitno) strains of leptospires were incubated at 37°C for 30 min in the presence of different amounts of granule extracts. Controls were incubated for 30 min with the same amount of acetate buffer (pH 4.2) as that present in the granule extracts. Microbicidal activities were evaluated as described in Table 1, footnote *a* and are expressed as means ± standard deviations (*n* = 3).

found not to be microbicidal towards leptospires (Table 1), the possibility that other granule constituents are responsible for the killing activity was examined. Acid extracts from primary granules were therefore subjected to gel filtration through a Sephadex G-75 column (11), and the eluted fractions were tested for leptospiricidal activity as well as by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein electrophoresis showed the presence of MPO in fractions 2 to 8, of serprocidins (9) in fractions 10 to 27, and of lysozyme in fractions 20 to 34. Leptospiricidal activity was found associated to fractions 25 to 32, with a peak from 26 to 29 (Fig. 4B). Figure 4A shows that after silver staining, the fractions with killing activity showed a protein doublet at 30 kDa (indicating the presence of serprocidins), a weak band at 20 kDa, a broad band at 15 kDa (lysozyme), and a doublet consisting of a minor and a broad band well below the 6.5-kDa molecular-mass marker, in a position corresponding to approximately 3 to 1 kDa. The latter, low-molecular-weight components are very likely to be defensins, the most abundant primary granule constituents, and/or cathelicidin-derived peptides. Both defensins and cathelicidins have been found to be leptospiricidal (22; V. Sambri, A. Marangoni, L. Giacani, R.

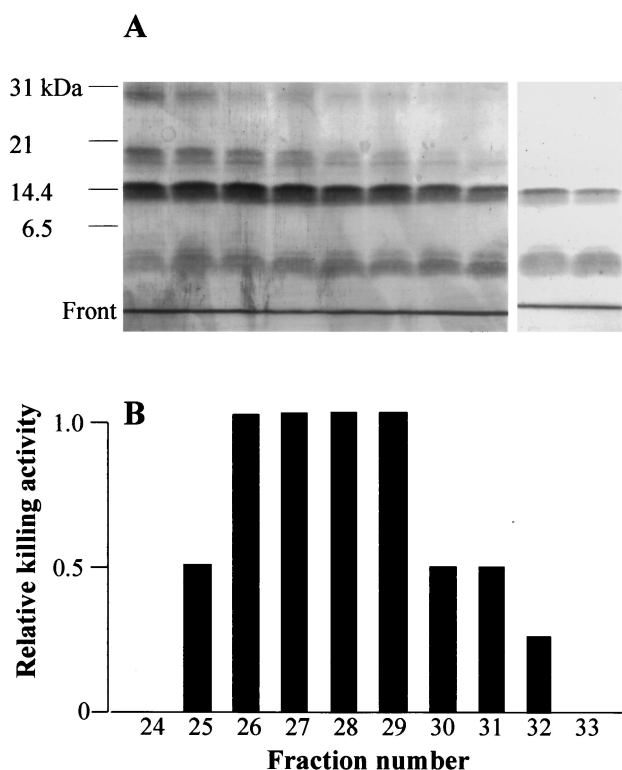


FIG. 4. *Leptospira* killing by neutrophil primary granule components. (A) SDS-14% PAGE and sequential Coomassie and silver nitrate staining of Sephadex G-75 fractions showing microbicidal activity. No bands were detected above 31 kDa. (B) Killing activity of the fractions whose SDS-PAGE profiles are shown in panel A, determined as described in Table 1, footnote *a*. A decrease of at least 1 log in the number of surviving bacteria is referred to as relative activity 1. No microbicidal effect was found before fraction 25 or after fraction 32.

Gennaro, R. Murgia, R. Cevenini, and M. Cinco, submitted for publication) when the purified molecules are used at high concentrations. However, we did not find any direct relationship between the concentration of the proteins detected in the G-75 fractions and the level of killing activity. For instance, fraction 24 shows no killing activity in spite of the fact that this fraction contains the same proteins as and in amounts similar to those in successive fractions, which are indeed microbicidal. This finding indicates that the leptospiricidal activity may be due to the presence of molecules not detected even after silver nitrate staining. Alternatively, killing may be the result of a more complex interaction of factors such as, for instance, a combination of killing enhancers and inhibitors whose concentration balance would determine the outcome of the microbicidal process or a synergy between peptides and microbicidal proteins, the latter being able to reach targets at the bacterial peptidoglycan or inner membrane level only after permeabilization by the peptides.

In brief, our results indicate that leptospires can be killed by both oxygen-dependent ( $H_2O_2$ ) and -independent (primary

granule components) mechanisms. Therefore, the poor in vivo clearance of phagocytosed, nonopsonized pathogenic leptospires is likely to be due to a modest uptake by neutrophils rather than to an intrinsic resistance of the microorganisms to killing.

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