NOTES

Toxic Effect of the Peroxidase-Hydrogen Peroxide-Halide Antimicrobial System on *Mycobacterium lepraes*

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*Mycobacterium lepraes* are killed by myeloperoxidase (or eosinophil peroxidase), H$_2$O$_2$, and a halide, thus suggesting a mechanism for their destruction by peroxidase-containing phagocytes.

Among the antimicrobial systems of phagocytes is one that consists of peroxidase, H$_2$O$_2$, and a halide (7, 8). The neutrophil peroxidase (myeloperoxidase [MPO]) is released from cytoplasmic granules into the phagosome where it reacts with both H$_2$O$_2$ formed by the phagocytosis-induced respiratory burst and a halide (chloride, bromide, iodide) to form agents toxic to the ingested organism. A different peroxidase is present in eosinophil granules (eosinophil peroxidase [EPO]); it can be released either into a phagosome (3, 9) or extracellularly onto the surface of an adjacent target (14). EPO and, to a lesser degree, MPO are strongly basic proteins which bind strongly to the negatively charged surfaces of microorganisms and other targets; organisms with EPO on their surface become exquisitely sensitive to the toxic effect of added H$_2$O$_2$ and a halide (12, 13, 16, 18). Monocytes contain a peroxidase that is identical to that of the neutrophil. However, as the monocyte matures into a macrophage this granule peroxidase is lost; mature macrophages thus do not have this mechanism for the amplification of the toxic activity of the H$_2$O$_2$ formed during the respiratory burst. Macrophages can acquire peroxidase by the ingestion of organisms with EPO bound to their surface (13, 16, 18). The peroxidase taken up in this way potentiates the cidal activity of macrophages.

Studies of the susceptibility of *M. leprae* to the antimicrobial systems of phagocytes are complicated by limitations of the assays now available. The organisms currently cannot be grown in culture and thus must be obtained after growth in vivo (22). The mouse footpad, armadillo liver or spleen, or a biopsy specimen of an untreated lepromatous patient may be used as a source of viable organisms. We have chosen to use the mouse footpad as it is a convenient source of the numbers of organisms needed for our studies. After in vitro incubation of the organisms with an antimicrobial system, the loss of viability must be measured. Of the several procedures developed for this purpose, most have low sensitivity and allow the measurement of only about a 1 log$_{10}$ loss of "viability." These include morphologic index or solid staining (15, 19), fluorescein-ethidium bromide staining (5, 11), incorporation of radioactive precursors in bacteriologic medium (2) or in macrophages (17), and adenosine triphosphate content (4). Moreover, a loss in viability may precede the observed change in *M. leprae* by several months (24). A titration of viable *M. leprae* in mouse footpads is tedious, and because the organism grows slowly, the results are not available for 8 to 12 months. However, the method is sensitive and accurate, and the results are unambiguous. We have used it here in a study of the susceptibility of *M. leprae* to the peroxidase-H$_2$O$_2$-halide system.

Canine MPO, prepared as described by Agner (1), and horse EPO, prepared as described by Jörg et al. (6), were assayed by guaiacol oxidation (10). *M. leprae* (B2409 strain) were obtained from the footpads of female CFW mice inoculated ca. 6 months earlier (21). Briefly, each infected footpad was removed, minced with a scissors, and agitated in Hanks balanced salt solution with 3-mm glass beads in a Mickel homogenizer. The supernatant fluid was incubated with trypsin (0.125% trypsin [1:250]; Difco Laboratories, Detroit, Mich.) for 5 min at 37°C with stirring. The organisms were washed, suspended in Hanks balanced salt solution containing 0.1% bovine serum albumin, counted as acid-fast bacilli (25), and stored overnight at 4°C. The organisms were collected by centrifugation and suspended in 0.1 M sodium phosphate buffer (pH 7.0) at a concentration of 2 x $10^9$ acid-fast bacilli per ml. Where indicated, 5 x $10^8$ organisms in 0.5 ml of sodium phosphate buffer were preincubated with 0.02 ml of EPO (ca. 5 U) for 15 min at 37°C. The organisms were washed once and suspended in 0.5 ml of sodium phosphate buffer.

*M. leprae* were incubated with the other components of the reaction mixture (see the legends to Tables 1 and 2) for 30 min at 37°C. The reaction was stopped by the addition of 0.5 ml of Hanks balanced salt solution containing 0.2% bovine serum albumin, and the tubes were placed on ice. Smears were prepared for acid-fast staining, and the remaining suspension was diluted in Hanks balanced salt solution–0.1% bovine serum albumin for inoculation into mouse footpads. Groups of 10 mice each were injected with serial dilutions containing (in controls) $10^4$ to $10^{-2}$ acid-fast bacteria per mouse, and after 8 to 12 months 5 mice per group were killed for counts of acid-fast bacteria in suspensions of footpad tissues (25). Counts of greater than $10^5$ acid-fast bacteria per mouse were scored as positive. The positive counts averaged ca. $10^6$ and did not vary significantly with the dilution of the inoculum. The results are expressed as the number of acid-fast bacilli required to produce infection in 50% of animals (ID$_{50}$) and are analyzed statistically by the Spearman-Kärber method (23).

The cidal activity of the peroxidase-H$_2$O$_2$-halide system on *M. leprae* is shown in Table 1. When *M. leprae* were
incubated in sodium phosphate buffer alone, the ID$_{50}$ was 32 organisms. However, when MPO, H$_2$O$_2$, and chloride were added, the ID$_{50}$ was greater than 32,000 organisms. Clearly, the great majority of the acid-fast bacilli had been killed by the peroxidase system under these conditions, as defined by their inability to replicate in the footpads. The bactericidal activity was lost upon deletion of each component of the MPO-H$_2$O$_2$-chloride system. Chloride (0.1 M) could be replaced by 10$^{-4}$ M iodide with comparable results; however, 10$^{-4}$ M bromide was ineffective under our conditions. When MPO was replaced by EPO, no bactericidal effect was observed with chloride as the halide; however, the EPO-H$_2$O$_2$-iodide system was strongly bactericidal, whereas a lesser but significant effect was observed with bromide as the halide. These findings suggest that the presence of MPO in neutrophils and monocytes and of EPO in eosinophils provides these cells with a mechanism for the destruction of *M. leprae*.

*M. leprae*, ingested by mature macrophages, can survive and replicate (20). Macrophages lack a granule peroxidase; however, EPO bound to the surface of certain organisms increases their sensitivity to destruction by the H$_2$O$_2$ formed by macrophages (13, 16, 18). When *M. leprae* were preincubated with EPO and then washed to remove excess enzyme, a portion of the EPO remained bound to the organisms, as indicated by the bactericidal effect of added H$_2$O$_2$ and iodide (Table 2). However, *M. leprae* coated with EPO and then injected directly into the footpads of mice were not killed in situ more effectively than uncoated organisms. The basis for the resistance of *M. leprae* to the antimicrobial systems of macrophages is currently under study.

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**LITERATURE CITED**


**TABLE 1. Bactericidal effect of the peroxidase-H$_2$O$_2$-halide system**

<table>
<thead>
<tr>
<th>Supplements</th>
<th>ID$_{50}^a$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>MPO + H$_2$O$_2$ + chloride</td>
<td>&gt;32,000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chloride omitted</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$ omitted</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>MPO omitted</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>MPO + H$_2$O$_2$ + iodide</td>
<td>&gt;32,000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MPO + H$_2$O$_2$ + bromide</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>EPO + H$_2$O$_2$ + chloride</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>EPO + H$_2$O$_2$ + iodide</td>
<td>&gt;32,000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EPO + H$_2$O$_2$ + bromide</td>
<td>501</td>
<td>&lt;0.001</td>
</tr>
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*a* The reaction mixture contained 0.02 M sodium phosphate buffer (pH 7.0), 0.067 M sodium sulfate, and 10$^{-6}$ M *M. leprae*, 10$^{-5}$ M *M. leprae* with bound EPO, 10$^{-4}$ M H$_2$O$_2$, 10$^{-6}$ M sodium iodide. Final volume, 0.5 ml. Probability values are shown for the difference from uncoated *M. leprae* where significant (P < 0.05).

**TABLE 2. Effect of EPO bound to the organism surface**

<table>
<thead>
<tr>
<th>Supplements</th>
<th>ID$_{50}^a$</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td><em>M. leprae</em></td>
<td>316</td>
<td></td>
</tr>
<tr>
<td><em>M. leprae</em> with bound EPO + H$_2$O$_2$ + iodide</td>
<td>&gt;20,000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>M. leprae</em> with bound EPO</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

*a* The reaction mixture contained 0.02 M sodium phosphate buffer (pH 7.0), 0.067 M sodium sulfate, and where indicated: 10$^{-6}$ M *M. leprae*, 10$^{-5}$ M *M. leprae* with bound EPO, 10$^{-4}$ M H$_2$O$_2$, 10$^{-6}$ M sodium iodide. Final volume, 0.5 ml. Probability values are shown for the difference from uncoated *M. leprae* where significant (P < 0.05).

*b* Results are expressed as the estimated number of bacilli, based on microscopic counts, which was required to produce infection in 50% of inoculated animals.


