Stimulation or Inhibition of the Respiratory Burst in Cultured Macrophages in a Mycobacterium Model: Initial Stimulation Is Followed by Inhibition after Phagocytosis

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Microorganisms cause varying degrees of stimulation of superoxide (O$_2^-$) production (respiratory burst [RB]) in macrophages but in some cases apparently inhibit the RB induced in the same monolayers by a conventional stimulator. We have explored these differences. A mycobacterium model, the slowly multiplying mouse mycobacterium Mycobacterium microti, induced a modest RB in resident macrophage monolayers, compared with the substantial RB induced by opsonized zymosan (Zy). However, if the 1-h M. microti pulse immediately preceded the Zy assay (instead of being concurrent), the RB was consistently less than that elicited by the Zy alone. Cytochalasin (an inhibitor of phagocytosis) enhanced Zy-induced RB, supporting the view that the burst is cell surface mediated, but this agent apparently eliminated the inhibition of the Zy-induced RB caused by prior M. microti exposure, suggesting that this inhibition may have an intracellular origin. The inhibition described extended not only to another mycobacterium (Mycobacterium bovis BCG) but also to a previous application of Zy itself. The general implications for macrophage functions of these observations on timing and sites of initiation are briefly discussed.

In general, microorganisms stimulate less superoxide anion (O$_2^-$) production (respiratory burst [RB]) in cultured macrophages (MΦ) than in neutrophils and less in resident MΦ than in elicited or activated cells. Microbial RB in MΦ is usually considerably less than that induced by stimulators such as zymosan (Zy), varying from substantial or moderate to little or none (10), the latter occurring with many pathogens, e.g., Toxoplasma gondii (17), Trypanosoma cruzi (13), Histoplasma capsulatum (7), Mycobacterium leprae (2, 14), Mycobacterium tuberculosis (15), and Mycobacterium leprae (9). Some authors report microbial inhibition of stimulators, e.g., by Leishmania spp. (3), Yersinia pestis (5), T. gondii (4), and H. capsulatum (18). In these cases, the inhibiting organisms were usually applied to the MΦ for a period prior to application of the stimulatory agent rather than concurrently. We suspected that a different mechanism might be involved in the difference of timing. We therefore examined the RB after both concurrent and two-stage applications. We selected mycobacteria because of their generally low RB and the mouse pathogen Mycobacterium microti because of its slow intracellular multiplication and its disturbance of the phagolysosome system (8), and we selected Zy as a conventional stimulatory agent.

Resident peritoneal MΦ were obtained from albino P strain mice, and M. microti OV254 were obtained from Tween-albumin cultures (8). After 1 or 2 days in monolayer culture (in high-concentration serum medium [8]), MΦ were overlaid with an M. microti suspension in balanced salt solution containing 3% mouse serum (to opsonize) at a concentration that gave 20 or more acid-fast bacilli per cell assessed in situ by Ziehl-Neelsen staining at the termination of the experiment; M. microti-free controls were also included. For concurrent exposure to Zy, this agent (preopsonized with mouse serum and usually at 0.25 mg/ml) was mixed with the M. microti inoculum (or with M. microti-free controls) plus glucose and cytochrome c, with and without superoxide dismutase (Zy package), and applied (in balanced salt solution) to the monolayers for 1 h of incubation at 37°C, followed by conventional spectrophotometric assay of reduced cytochrome c as a measure of O$_2^-$ release (11). Alternatively (and in most cases), the applications were in two stages, the 1-h pulse with M. microti being followed immediately by a wash and overlay of the Zy package. Incubation for a further 1 h. and analysis. Finally, by subtracting the reduction of cytochrome c in the presence of dismutase from the total reduction, the superoxide dismutase-inhibitable reduction of cytochrome c was used to calculate the O$_2^-$ release (expressed as nanomoles per hour per 10$^6$ cells).

M. microti, used alone, induced a modest RB (mean, 41 ± 12 nmol; n = 5), as did BCG (used for comparison), whereas opsonized Zy generated a substantial burst (mean, 134 ± 67 nmol; n = 5). Concurrent stimulation by M. microti and Zy showed inconsistent differences from Zy alone, but the uptake of acid-fast bacilli simultaneously with the Zy was so variable (presumably because of competition) that the concurrent approach was inconclusive and was abandoned.

When the two stimuli were applied at separate times (i.e., in two stages), uptake of Zy and acid-fast bacilli appeared unaffected by the experimental variations. Under these conditions, the RB induced by Zy was consistently and significantly less than that by parallel controls of Zy without M. microti (Table 1, normal culture; mean, −64%; also see footnote i). M. microti heated for 45 min at 100°C exhibited similar inhibition; mycobacterial superoxide dismutase is inactivated after 10 min at 80°C (6). Unstimulated monolayers treated similarly in the absence of M. microti and Zy gave only very small effects (mean, 9 ± 6 nmol; n = 7), which could be accounted for by the mouse serum.

Site of stimulation and inhibition. The view that stimulation of RB in MΦ, as in neutrophils, is substantially cell surface
TABLE 1. Superoxide anion production (RB) in normal and cytochalasin-treated Mφ preexposed for 1 h to M. microti and then stimulated by Zy

<table>
<thead>
<tr>
<th>Mφ culturea</th>
<th>Expt date (mo/day)</th>
<th>O₂⁻ release (nmol/h/10⁶ cells)b</th>
<th>Zy alone☆</th>
<th>Zy + M. microti</th>
<th>Difference☆ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.15.92</td>
<td>121</td>
<td>69.1</td>
<td>-43</td>
<td></td>
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<tr>
<td></td>
<td>4.06.92</td>
<td>160</td>
<td>32.4</td>
<td>-79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.22.92☆</td>
<td>118</td>
<td>43.6</td>
<td>-63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.11.92☆</td>
<td>210</td>
<td>74.3</td>
<td>-65</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>152</td>
<td>54.8</td>
<td>-64☆</td>
<td></td>
</tr>
<tr>
<td>Cytochalasin treated☆</td>
<td>7.14.92</td>
<td>293</td>
<td>220</td>
<td>-25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.23.92☆</td>
<td>324</td>
<td>393</td>
<td>+21</td>
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<tr>
<td></td>
<td>7.28.92☆</td>
<td>201</td>
<td>221</td>
<td>+10</td>
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</tr>
<tr>
<td></td>
<td>8.11.92☆</td>
<td>330</td>
<td>197</td>
<td>-40</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>287</td>
<td>258</td>
<td>-10☆</td>
<td></td>
</tr>
</tbody>
</table>

☆ 1 or 2 days old.
☆☆ Dimutase-inhibited reduction of cytochrome c
☆☆☆ Opsonized Zy at 0.125, 0.25, or 0.5 mg/ml. Difference of means for normal versus Cyto-treated samples, +88%. Two-sample t test: T = -3.67, with 5 df; increase in means significant, with P = 0.014.
☆☆☆☆ See the text.
☆☆☆☆☆ Percentage differences with Zy alone as the base.
☆☆☆☆☆☆ Dimethyl sulfoxide (6.5 mg/ml) present throughout.
☆☆☆☆☆☆☆ Same experiment.
☆☆☆☆☆☆☆☆ Same experiment.
☆☆☆☆☆☆☆☆☆ Paired t test: T = -4.82, with 3 df; decrease in means significant, with P = 0.017.
☆☆☆☆☆☆☆☆☆☆ Cyto (6.5 μg/ml) present throughout.
☆☆☆☆☆☆☆☆☆☆☆☆ Paired t test: T = -0.65, with 3 df; no difference in means (P = 0.56).

mediated (12, 16) was supported by the significantly increased O₂⁻ release (+88%) when Mφ were preexposed (for 1 h) to cytochalasin D (Cyto), a known inhibitor of phagocytosis by Mφ (1), prior to addition of the Zy package plus renewed Cyto (also for 1 h) (Table 1, Cyto-treated versus normal cultures; also see footnote c). On the other hand, when the two-stage procedure (M. microti pulse followed by Zy) was performed in the presence of Cyto, the inhibition of the Zy-induced RB (shown without Cyto) was apparently eliminated (Table 1, means of -64%, a significant decrease, with P = 0.017, and -10%, not significantly different from zero, with P = 0.56). These observations suggest that while the Zy-induced RB itself (enlarged in the presence of Cyto) has a surface origin, its inhibition by preexposure to M. microti may be intracellular in origin. Such a notion is supported by further observations that such inhibition was still considerable when the 1-h pulse of M. microti was followed by a 2- or 4-h bacterium-free chase to complete ingestion (data not shown). (At 24 h, the results became variable.) It must be emphasized that inhibition was also observed when M. bovis BCG, or even a preliminary application of Zy itself, was substituted for M. microti and similar procedures were followed (data not shown). Hence, this M. microti effect is not specific and should be regarded as a model for use in the timing variations studied. We suggest a different mechanism for the two-stage inhibition from that of the initial or single-stage stimulation of RB; a surface-mediated RB can be interfered with by changes originated by phagocytizable particles from a postphagocytic intracellular site.

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REFERENCES