Oxidative Metabolism in Cord Blood Monocytes and Monocyte-Derived Macrophages

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Little is known about phagocytosis-associated oxidative metabolism in mononuclear phagocytes from the human neonate. We investigated this phenomenon in monocytes from the cord blood of term newborn infants by measuring generation of superoxide anion (O$_{2}^{-}$) and hydroxyl radical (·OH) after stimulation with opsonized zymosan or phorbol myristate acetate. Production of these microbicidal oxygen metabolites by monocytes from neonates and healthy adult volunteers was equivalent. When cultured in the presence of the macrophage activator lipopolysaccharide or muramyl dipeptide, monocytes from neonates and adults differentiated into cells with the appearance of macrophages and with an enhanced capacity to release O$_{2}^{-}$ compared with cells cultured in the absence of an activator. Monocyte-derived macrophages from neonates produced only slightly less O$_{2}^{-}$ than did adult cells. Thus, unlike the cord blood neutrophil, which exhibits abnormalities in oxidative metabolism, the cord blood mononuclear phagocyte has a respiratory burst that is quantitatively comparable to that of the adult cell.

Monocytes play an essential role in host defense as circulating phagocytes, as well as precursors of macrophages. In an attempt to explain the enhanced susceptibility of neonates to systemic bacterial infections, certain aspects of monocyte function have been studied in cells from newborns (5, 6, 11–13, 15, 18, 22, 23, 26–28). Decreased chemotaxis was reported by a number of investigators. Phagocytosis and bactericidal activity were comparable with monocytes from healthy newborns and adult controls. However, activity of the phagocytosis-associated respiratory burst has not been thoroughly studied in monocytes from newborns. This activity consists of increased oxygen consumption and hexose monophosphate shunt activity and production of reactive oxygen metabolites, including superoxide anion (O$_{2}^{-}$) and hydrogen peroxide (H$_{2}$O$_{2}$) (9). These two metabolites may interact to produce hydroxyl radical (·OH), an extremely potent oxidant that appears to be important for the microbicidal activity of phagocytic cells (10). Recently, normal to increased O$_{2}^{-}$ production (10% to 127% of adult levels) but decreased ·OH generation (53 to 90% of adult levels) has been described in human neonatal neutrophils (1, 2). In this study we examined the production of oxygen metabolites by freshly isolated monocytes and cultured monocyte-derived macrophages during either phagocytosis of opsonized zymosan or perturbation of the cell membrane by phorbol myristate acetate (PMA; Consolidated Midland Corp., Brewster, N.Y.).

Umbilical cord venous blood was withdrawn from the placenta into heparin (1 to 20 U/ml) immediately after delivery of healthy, term newborns who were products of an uncomplicated pregnancy, normal labor, and vaginal delivery. General anesthesia was not used in any of the deliveries. The mean birth weight was 3,160 g; Apgar scores at 1 and 5 min were ≥8. Blood from healthy adult donors, similarly anticoagulated, was drawn immediately after procurement of cord blood. Informed consent was obtained from mothers and normal donors.

The blood was centrifuged at 200 × g for 20 min at room temperature, and the platelet-rich plasma was removed. Erythrocytes were sedimented in pyrogen-free dextran in saline (Cutter Biologicals, Berkeley, Calif.) (19), and granulocytes were removed by centrifugation through Ficoll-Hypaque (19). The resulting mononuclear cell preparation contained <1% contaminating granulocytes. The percentage of monocytes was determined by differential counting of 200 cells on smears stained with a nonspecific esterase stain for monocytes and macrophages (Technicon Instruments Corp., Tarrytown, N.Y.). These preparations, termed monocytes in this paper, contained 22.8 ± 2.6% (mean ± standard error of the mean) monocytes in the newborns and 24.2 ± 1.8% monocytes in the adult controls. The remainder of the cells were lymphocytes.

To obtain lymphocytes, 10 ml of a mononuclear cell preparation (as above) was plated on a 100-mm-diameter tissue culture dish and incubated at 37°C in a 5% CO$_{2}$–95% air incubator for 1 h to permit monocyte adherence. The nonadherent cells were washed and suspended in Krebs-Ringer phosphate buffer with dextrose; the incubations were repeated until monocytes composed <5% of the nonadherent mononuclear cells.

One milliliter of cell suspension (5 × 10$^{6}$ mononuclear cells in Krebs-Ringer phosphate buffer with dextrose) was added to each 35-mm-diameter tissue culture dish. After incubation for 2 h at 37°C in 5% CO$_{2}$–95% air, adherent cells were vigorously washed twice with Earle balanced salt solution. Some of the cells were immediately studied for release of O$_{2}^{-}$, and the remaining cells were placed in fresh lipopolysaccharide (LPS)-free medium (2 ml) without additive or with added LPS (19) or muramyl dipeptide (N-acetylglucosamine-β-d-glucopyranosyl(N-acetyl) muramyl-L-alanyl-d-isoglutamine [MDP]; Calbiochem-Behring, La Jolla, Calif.). The cells were then cultured for 3 days at 37°C in 5% CO$_{2}$. The LPS-free medium was prepared in our laboratory with a composition similar to that of medium 199 with Earle modified salts (19). Penicillin (50

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TABLE 1. Production of superoxide anion $O_2^-$ and hydroxyl radical (·OH) by monocytes from cord blood and adult controls

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Release of $O_2^-$ (nmol/2.5 × 10⁶ monocytes)</th>
<th>Production of ·OH (pmol/2.5 × 10⁶ monocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cord blood</td>
<td>Adult</td>
</tr>
<tr>
<td>None</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Opsonized zymosan</td>
<td>26.1 ± 1.0</td>
<td>24.5 ± 0.9</td>
</tr>
<tr>
<td>PMA</td>
<td>44.7 ± 1.2</td>
<td>46.4 ± 0.8</td>
</tr>
</tbody>
</table>

* Mean ± standard error of the mean of averages of duplicates of triplicates from each of 10 cord-adult pairs. Cells were in suspension.

$O_2^-$ was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c in a 60-min assay.

·OH was measured as ethylene production from α-keto-γ-methyl butyric acid, which was quantified by gas chromatography in a 60-min assay.

U/ml and streptomycin (50 μg/ml) were also added to the medium.

Release of $O_2^-$ by monocytes was determined as superoxide dismutase-inhibitable reduction of cytochrome c (20). Generation of ·OH was measured by interaction with α-keto-γ-methyl butyric acid to form ethylene quantitated by gas chromatography (3). The specificity of this system for ·OH has been previously demonstrated (3), and the majority of ethylene production was related to ·OH or a powerful oxidant with characteristics similar or identical to ·OH. For simplicity, we use the term ·OH for that oxidant in this paper. For freshly isolated monocytes, measurement of $O_2^-$ and ·OH was performed with cells in suspension in the absence of stimulants or after stimulation with opsonized zymosan (1 mg/2.5 × 10⁶ cells) or PMA (5 μg/ml). Cultured monocytes were stimulated with PMA for 60 min. Assays were performed in duplicate or triplicate. Statistical comparisons were made between cord and adult monocytes with the two-tailed t test.

The results of $O_2^-$ production and ethylene production by monocytes from adult donors and cord blood cells are summarized in Table 1. There was no significant difference in production of $O_2^-$ or ·OH by unstimulated or stimulated monocytes from the two populations. The scavengers of ·OH, benzoate and thiourea, inhibited ethylene production in monocytes from either source to the same extent. (For adult monocytes, inhibition was as follows: 50 mM benzoate, 56 to 65%; 1 mM thiourea, 83 to 87%; n = 3. For cord blood monocytes, inhibition was as follows: benzoate, 51 to 58%; thiourea, 83 to 92%; n = 3).

Because the monocyte preparations used in these studies contained a significant number of lymphocytes, studies were performed to determine the effects of these lymphocytes on generation of oxygen metabolites. Lymphocyte preparations did not release significant amounts of $O_2^-$ (<2 nmol/2.5 × 10⁶ cells, n = 3) or ·OH radical (<20 pmol of ethylene per 2.5 × 10⁶ cells, n = 3) after stimulation with either opsonized zymosan or PMA. Further addition of up to 10² purified lymphocytes to the monocyte preparations had no effect on the levels of $O_2^-\,$ or ·OH generated. Ethylene generation was dependent on monocyte number and concentration. When the monocyte concentration was kept at 10⁶ cells per ml, the amount of ethylene generated was a linear function of the total number of monocytes independent of contaminating lymphocytes.

Results with cultured monocytes are shown in Table 2. After a 2-h incubation on the day of isolation, cord blood monocytes generated as much $O_2^-$ in response to a soluble stimulus as did monocytes from adults. A decline in the ability to release $O_2^-$ in response to PMA after 3 days in culture in LPS-free medium without serum was noted in cells from both sources, as previously reported for adult monocytes (19). However, cells cultured in the presence of minute amounts of LPS (1 ng/ml) or MDP (10 ng/ml) were primed so that their $O_2^-$-generating capacity was equivalent to that of activated murine macrophages (9, 20); their size and pseudopod formation increased and they developed an appearance similar to that of macrophages (20). Release of $O_2^-$ by cord monocyte-derived macrophages (day 3 of culture) was significantly less than that by monocytes from adults in the absence of LPS or on treatment with LPS or MDP (P < 0.05 for each, unpaired t test), but differences were small.

Phagocytosis-associated oxidative metabolism has been systematically studied in neutrophils from newborns. Reduction of nitroblue tetrazolium (NBT) salts dye, which is partially dependent upon $O_2^-$ production (10), was reported to be greater in unstimulated and stimulated neutrophils from noninfected newborns than from adults (4, 7, 8, 21). These results correspond with the reported enhancement of oxygen consumption (21) and normal or elevated production of $O_2^-$ in neonatal neutrophils (1, 24) and in normal fetal granulocytes (17). However, when $O_2^-$ and ·OH production were studied simultaneously, neutrophils from healthy, term newborns, in comparison with those from adult controls, exhibited a significant defect in ·OH generation (1, 2). This disparity might be explained by the deficiency in cord neutrophils of lactoferrin (2), which can promote the formation of ·OH from the interaction of $O_2^-$ and $H_2O_2$ (3). Neonatal neutrophils have been reported to have a decrease in stimulated chemiluminescence (16, 25), a result of the interaction of oxygen metabolites with excitable substrates within the cell (14). This deficit might also be explained by decreased formation of ·OH or by variability in the content of excitable substrates.

In contrast to current knowledge of neutrophils from newborns, very little is known about oxidative metabolism in monocytes from the human neonate. We report here that both $O_2^-$ and ·OH were released to an equivalent extent by monocytes from healthy term newborns and adult controls. Thus, unlike results with neutrophils, ·OH production is not relatively decreased in monocytes from the newborn; oxidative metabolism appears perfectly normal in these cells. It is unlikely, then, that the increased susceptibility to

TABLE 2. Effect of preincubation with a macrophage-activating agent (LPS or MDP) on PMA-stimulated release of $O_2^-$ by cultured mononuclear phagocytes from cord blood and adults

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Release of $O_2^-$ (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cord blood</td>
</tr>
<tr>
<td>0</td>
<td>None (control)</td>
<td>1.247 ± 32</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>143 ± 9</td>
</tr>
<tr>
<td></td>
<td>LPS, 1 ng/ml</td>
<td>724 ± 36</td>
</tr>
<tr>
<td></td>
<td>MDP, 10 ng/ml</td>
<td>401 ± 17</td>
</tr>
</tbody>
</table>

* Blood mononuclear cells were incubated on tissue culture dishes for 2 h, washed vigorously, and either assayed immediately (day 0) or after culture for 3 days (day 3) in LPS-free medium (no treatment) or medium containing LPS or MDP. Numbers represent means ± standard errors of the means of averages of duplicates from each of three cord-adult pairs. The extent of $O_2^-$ release over 60 min is corrected for adherent cell protein, which was equivalent for the two populations.
infection exhibited by the human neonate can be related to an abnormality in the respiratory burst of the monocyte.

Monocytes or macrophages cultured in the presence of the bacterial product LPS or MDP develop an enhanced capacity to release O$_2^-$ when stimulated (19, 20). This enhanced (primed) state is a consistent and important characteristic of activated macrophages (9), which have increased microbicidal and tumoricidal capacity. In the studies described here, monocytes allowed to differentiate into macrophages in LPS-free medium had a rapid decline in their ability to produce O$_2^-$. Treatment with LPS or MDP enabled monocyte-derived macrophages from both populations to manifest the primed state with respect to release of O$_2^-$. The amount of O$_2^-$ release by primed macrophages was significantly decreased in newborns, but the difference was quantitatively modest. It seems unlikely that this difference would have clinical significance in regard to macrophage microbicidal activity. However, it is possible that it indicates a relatively diminished capacity of monocytes from newborns to undergo differentiation into activated macrophages. This important possibility remains to be proved.

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LITERATURE CITED