The Biochemical Basis of Nitroblue Tetrazolium Reduction in Normal Human and Chronic Granulomatous Disease Polymorphonuclear Leukocytes

By Robert L. Baehner, Laurence A. Boxer, and Jacqueline Davis

Normal human polymorphonuclear leukocytes (PMN) placed in anaerobic chambers supports the idea that NBT reduction in reaching $P_{O_2}$ of less than 5 mm Hg fail to generate $O_2^-$, iodinate ingested particles, and stimulate glucose-$1^{-14}C$ oxidation through the hexose monophosphate shunt. The observation that anaerobic cells are incapable of generating $O_2^-$ or reducing nitroblue tetrazolium (NBT) to formazan supports the idea that NBT reduction in phagocytizing PMN is due exclusively to oxygen-dependent $O_2^-$ generating oxidase which is deficient in chronic granulomatous disease leukocytes, despite their hyperphagocytic capacity.

A series of redox reactions takes place in polymorphonuclear leukocytes (PMN) and monocytes during phagocytosis. These include the consumption of oxygen, which is insensitive to 1 mM potassium cyanide, stimulation of the hexose monophosphate shunt as determined by the evolution of $^{14}CO_2$ from glucose-$1^{-14}C$, and the iodination of protein particles and bacteria. Products of the reduction of oxygen include hydrogen peroxide ($H_2O_2$) and several highly reactive unstable intermediates of oxygen, i.e., superoxide anion ($O_2^-$), singlet oxygen ($^1O_2$), and hydroxyl radical (OH). Nitroblue tetrazolium (NBT) reduction has served as a useful marker of normal redox capabilities of the PMN and the failure of NBT reduction in the PMN of patients with chronic granulomatous disease (CGD) has corresponded to the lack of the other described oxidative reductive reactions in these cells.

Two possible biochemical bases for the reduction of NBT to insoluble formazan in phagocytizing PMN have been suggested: (1) a non-oxygen-dependent NADH or NADPH-NBT reductase; and (2) an oxygen-dependent oxidase generating $O_2^-$ and $H_2O_2$. In order to assess the contribution of oxygen to NBT reduction, normal phagocytizing PMN were exposed to air or kept in anaerobic chambers. Further metabolic comparisons were made between normal PMN placed either in air or in an aerobic environment and to CGD PMN in air.
MATERIALS AND METHODS

Collection of Human PMN

Human PMN were isolated from the peripheral blood of normal controls and laboratory volunteers, as well as two patients with CGD. Two hundred milliliters of heparinized blood were collected, and the white cells were isolated by a previously described technique. These cells and all solutions were then suspended in Krebs-Ringer phosphate buffer (KRP), pH 7.4, with 5 mM glucose, and placed in air or in an anaerobic chamber (Coy Manufacturing Co., Model AL 1213, Ann Arbor, Mich.) with the anaerobic gas mixture, LK 198095, containing 5% CO2 and 95% N2 (Union Carbide) for 5 hr prior to metabolic studies. The pO2 of the solutions was measured prior to the start of each experiment using a pH blood gas analyzer 313 (Instrumentation Laboratories, Inc.)

Metabolic Studies of Human PMN

The rate of iodination of opsonized zymosan particles was determined over a 60-min period by the method of Pincus and Klebanoff as modified by Hakim et al. The rate of hexose monophosphate shunt activity was determined by glucose-1-14C oxidation to 14CO2, as previously described. Quantitative O2− production was determined by measurement of the reduction of ferricytochrome C with and without the addition of superoxide dismutase (SOD) by the method of Babior et al. as modified by Johnston at al. The quantitative rate of NBT reduction was determined simultaneously with the rate of uptake of lipopolysaccharide-coated paraffin oil droplets (LPS) by a modification of the method of Stossel. Extractions were performed with pyridine instead of dioxane. The viability of cells was assessed by trypan blue exclusion before the assays were performed. NBT-NADH reductase was determined by the method of Baehner. Two x 10^7 PMN/ml were sonicated on ice with a Sonifier Cell Disruptor (Model W140, Heat Systems Ultrasonics, Inc., Plainview, N.Y.) at speed setting 3 for 1 min. With this procedure 90% of the cells were broken. The sonicates used for the anaerobic assay were initially prepared in air and were then placed in the anaerobic chamber for 5 hr prior to the assay.

RESULTS

Employing the anaerobic chamber, pO2 values of less than 5 mm Hg were achieved in cell suspensions following a 5-hr incubation period on ice with 95% cell viability. As noted in Table 1, O2− production by normal PMN in air was 38 ± 6 nmoles/10^7 PMN/10 min, and this was abolished in the anaerobic chamber. Similarly, CGD PMN failed to reduce cytochrome C in air, indicating that no superoxide anion was detected. The rate of iodination of zymosan particles in air by normal PMN was 12,836 ± 496 cpm/10^7 PMN/min, and this was reduced to 581 ± 152 cpm/10^7 PMN/min under anaerobic conditions. There was no iodination of particles by the PMN of two patients with CGD in air. Glucose-1-14C oxidation was 347 ± 59 cpm/10^7 PMN/30 min at rest in air and 1383 ± 383 cpm/10^7 PMN/30 min during phagocytosis. No glucose-1-14C oxidation occurred either at rest or in phagocytizing PMN under anaerobic conditions. A similar response was observed in CGD PMN in air. NBT reduction increased to 0.442 ± 0.042 μg formazan/10^7 PMN/min in air, and this was abolished in the anaerobic chamber. No reduction of NBT was observed at 0 time. Similarly, there was no detectable reduction of NBT by the CGD PMN. In order to assess whether H2O2 would directly reduce NBT, 10−6 M H2O2 was added to 0.2% NBT in the presence of 10−7 M NADH or NADPH. No reduction of NBT to formazan in air occurred.

Non-oxygen-dependent NADH-NBT reductase was quantitated in sonicates by observing the difference in formazan produced in 2 x 10^7 PMN exposed to air or to anaerobic chambers for 4 hr. Samples exposed to air reduced 0.205 ±
Table 1. Metabolic Reactions in Phagocytizing Human PMN

<table>
<thead>
<tr>
<th>Assay</th>
<th>Unit</th>
<th>Air</th>
<th>Anaerobic</th>
<th>CGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>nmoles/10^7 PMN/10 min</td>
<td>38 ± 6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Iodination</td>
<td>cpm/10^7 PMN/min</td>
<td>12,836 ± 496</td>
<td>581 ± 152</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-1-14C oxidation</td>
<td>cpm/10^7 PMN/30 min</td>
<td>1,036 ± 324</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NBT reduction</td>
<td>µg formazan/10^7 PMN/min</td>
<td>0.442 ± 0.042</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Uptake of particles</td>
<td>mg LPS/10^7 PMN/min</td>
<td>0.038 ± 0.010</td>
<td>0.057 ± 0.007</td>
<td>0.071</td>
</tr>
</tbody>
</table>

0.014 µg formazan/10^7 sonicated PMN/min, whereas samples in anaerobic chambers reduced 0.138 ± 0.004 µg formazan/10^7 PMN/min. Results were from three separate experiments, each done in triplicate. The addition of 1 mM KCN to cell sonicates in air in the presence of NADH and NBT did not alter the rate of reduction of NBT to formazan. This finding suggested that there was insufficient endogenous cyanide-sensitive SOD in the cytolytase to influence NADH-NBT reductase assay. Thus, although 67% of NBT reduction in PMN sonicates was independent of oxygen, intact phagocytizing PMN reduced NBT exclusively by generating O_2 from oxygen.

The uptake of LPS was increased in the anaerobic chambers to 0.057 ± 0.007 mg/10^7 PMN/min compared to 0.038 ± 0.010 mg LPS/10^7 PMN/min. Aerobically, CGD PMN from two patients also had an increased ingestion rate of 0.071 and 0.075 mg LPS/10^7 PMN/min when compared to normal PMN in an aerobic atmosphere.

DISCUSSION

The results of these studies clearly establish that oxygen is required for iodination, hexose monophosphate shunt activity, and NBT reduction in the intact PMN. Previous studies have indicated the necessity of an aerobic environment to kill efficiently many bacterial organisms. As Klebanoff has shown, the bactericidal activity of the PMN largely resides in its major product of oxygen reduction, H_2O_2. Recent studies of ours confirm that H_2O_2 is also required for iodination and hexose monophosphate shunt (HMPS) activity in PMN. Latex particles were coated with superoxide dismutase, an enzyme which partially depletes the PMN of O_2^- while increasing H_2O_2 generation, and then introduced into the PMN. The H_2O_2-dependent reactions, iodination of zymosan particles and the oxidation of glucose-1-14C, were enhanced by the dismutation of O_2^- to H_2O_2 and O_2. On the other hand, the reduction of NBT to formazan was diminished by 60% indicating that at least the majority of NBT reduction resulted from O_2^- generation.

\[
\text{Intact Phagocytizing PMN:} \quad O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2
\]

The failure of PMN to reduce NBT has proved a useful adjunct in establishing the diagnosis of CGD. It has been thought that the failure of NBT reduction has corresponded to an abnormality of either NADH or NADPH oxidase.
activity in the cell.\textsuperscript{25,26} Although oxidative metabolism in the CGD PMN is deficient, NBT reduction can occur in the CGD cells following their disruption or stimulation by methylene blue, suggesting a role for one or more NBT reductase enzymes.\textsuperscript{22} These studies indicate that approximately 67\% of the total reduction of NBT to formazan in sonicates of human PMN is catalyzed by an oxygen independent NADH or NADPH-NBT reductase. NBT reductase activity is mainly associated with the microsomal fraction of PMN homogenates.\textsuperscript{12}

\[
\text{Sonicate of PMN:} \\
\text{NAD(P)H}_2 + O_2 \xrightarrow{\text{oxidase}} O_2^- + O_2^- + \text{NBT} \rightarrow \text{NBTH}_2 (\text{formazan}) \quad (33\%) \\
\text{NAD(P)H}_2 + \text{NBT} \rightarrow \text{NBTH}_2 (\text{formazan}) \quad (67\%)
\]

On the other hand, the present findings now confirm that NBT reduction in phagocytizing PMN is due exclusively to oxygen-dependent oxidase activity in the form of excitable O\textsubscript{2} products and not to H\textsubscript{2}O\textsubscript{2} or to oxygen-independent NBT reductase. Furthermore, the failure of activation of this oxidase generating system in CGD PMN leads to a failure of the normal redox reactions, specifically, in their ability to reduce NBT to formazan. Previous studies of CGD and normal PMN homogenates exposed to SOD have shown similar inhibition of NBT reduction, suggesting that O\textsubscript{2}^- derived oxidase is not deficient in these cells.\textsuperscript{15} The recent observation that NADPH and NADH oxidase activity are not stimulated by 0.5 mM manganese chloride in CGD PMN 27,000 g pellets after phagocytosis supports the idea of a defect of enzyme activation rather than deficiency in CGD.\textsuperscript{26} The observation that anaerobic PMN are hyperphagocytic and the CGD PMN are also hyperphagocytic could be ascribed to the lack of free radical production and hydrogen peroxide production in these cells during phagocytosis.\textsuperscript{22} However, as pointed out by Stossel, PMN obtained from patients with infection are also hyperphagocytic, so that the real basis for this hyperphagocytosis in our CGD patients will require further evaluation.\textsuperscript{21}

**ACKNOWLEDGMENT**

We are indebted to Dr. James W. Smith, Department of Clinical Pathology, Indiana University Medical Center, for allowing us to use the Anaerobic Chamber and to Mrs. Elaine Carroll for typing and proofreading the manuscript.

**REFERENCES**

8. Allen RC, Stjernholm RL, Steele RH:
Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and in its participation in bactericidal activity. Biochem Biophys Res Commun 47:679, 1972


NITROBLUE TETRAZOLIUM REDUCTION
The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes

RL Baehner, LA Boxer and J Davis