Effects of Oxygen Tension and pH on the Respiratory Burst of Human Neutrophils

By Theodore G. Gabig, Scott I. Bearman, and Bernard M. Babior

The respiratory burst of human neutrophils was measured under conditions of hypoxia and low pH. $O_2^-$ production by neutrophils activated with opsonized zymosan fell slowly as the oxygen concentration declined to 1%, then dropped more sharply, reaching negligible levels at oxygen concentrations less than 0.25%. Production was half maximal at an oxygen concentration of 0.35% (equivalent to \( \sim 10 \mu M \) dissolved oxygen). $O_2^-$ production by the cell-free $O_2^-$-forming system prepared from zymosan-activated neutrophils showed a similar dependence on oxygen concentration. A drop in pH caused decreases in both oxygen consumption and $O_2^-$ production by zymosan-treated neutrophils, values at pH 6.0 being 10%–20% of those observed at pH 7.5. Experiments with the cell-free $O_2^-$-forming system suggested that this decline in respiratory burst activity at low pH was due to inefficient activation of the $O_2^-$-forming enzyme under acidic conditions.

Among the mechanisms by which neutrophils kill bacteria are certain processes that involve the use of antimicrobial agents produced by the partial reduction of oxygen. These agents are generated during the "respiratory burst," a metabolic event initiated by the exposure of neutrophils to appropriate stimuli and characterized by large increases in oxygen uptake, $O_2^-$ and $H_2O_2$ production, and hexose monophosphate shunt activity. The basis for the respiratory burst appears to be activation of a membrane-bound flavoprotein, dormant in resting cells, that catalyzes the reduction of oxygen to $O_2^-$ using NADPH as the electron donor.

To date, measurements of the respiratory burst have almost always been made under room air at "physiologic" pH (\( \sim 7.4 \)). However, at infected sites where neutrophils act, the conditions may be considerably different from these. In particular, both oxygen tensions and pH levels are likely to be substantially lower than those usually employed experimentally. To obtain evidence regarding the capacity of neutrophils to use oxygen-dependent microbicidal mechanisms under these adverse conditions, we examined the effects of oxygen tension and pH on the activity of the respiratory burst in these cells.

MATERIALS AND METHODS

NADPH (preweighed vials), cytochrome c (type VI), catalase, superoxide dismutase (bovine erythrocyte), and zymosan were purchased from Sigma Chemical, St. Louis, Mo. Hanks’ balanced salt solution (HBSS) without phenol red was obtained from Grand Island Biological Co., Grand Island, N.Y. Gas mixtures were purchased from Med-Tech Gases, Medford, Mass. The gas mixtures were...
analyzed by the same company (analyses were accurate to 5 ppm). Oxygen-free gas (oxygen content < 4 ppm) was obtained by the use of a Deoxo catalytic unit (Fisher Chemical, Medford, Mass.) in conjunction with a 98% N₂-2% H₂ gas mixture. Citrate-phosphate buffer, used for determining the effect of pH on the respiratory burst, was prepared from glucose, salts, and distilled water according to the formulation used for HBSS without phenol red, except that the final concentration of NaH₂PO₄ was 10 mM, NaHCO₃ was replaced by citric acid (final concentration 10 mM), and the buffer was brought to the desired pH with 1-M NaOH before adjusting to the final concentration with distilled water.

Human neutrophils were prepared by dextran sedimentation and osmotic lysis as previously described and suspended in the desired buffer (HBSS or citrate-phosphate buffer, depending on the experiment). Zymosan was opsonized by incubation for 30 min at 37°C in autologous serum, washed once in the desired buffer, and then suspended in the same buffer. O₂⁻ production by neutrophils under defined atmospheres was measured by a previously described method that involves incubating the reaction mixtures in Warburg flasks that are completely submerged in a 37°C water bath. O₂⁻ production by neutrophils as a function of pH was determined by previously published techniques. Neutrophil oxygen uptake was measured manometrically by means of a submersible differential respirometer (Gilson Medical Electronics, Middleton, Wis.).

The particulate O₂⁻-forming system from zymosan-activated neutrophils was prepared as described elsewhere, except that for certain experiments activation was carried out in citrate-phosphate buffer instead of HBSS. In every case 1-mM NaN₃ was present in the activation mixture. The zymosan-activated neutrophils were homogenized in 0.34-M sucrose adjusted to pH 7.4 with 1-M NaHCO₃, and the particles were washed and suspended in the same solution (protein concentration in the final suspension 1 mg/ml by the Lowry method). O₂⁻ production by particles as a function of pH was measured by a previously described technique.

Zymosan uptake was determined by a modification of a previously described method. Neutrophils (1.5 × 10⁷) suspended in 1.8 ml of the desired buffer were placed in the main compartment of a Warburg flask. Opsonized zymosan (4 mg) in 0.2 ml of the same buffer was placed in the sidearm. The flasks were prepared as for incubation under defined atmospheres as described previously, gassing with nitrogen where indicated. The contents of the flasks were then mixed and incubated at 37°C for the times noted. The incubations were terminated by injecting 2 ml of ice-cold 2-mM N-ethylmaleimide through the syringe port used to stopper the mouth of the flask. A 0.2-ml portion of the assay mixture was then further processed and examined microscopically to determine zymosan uptake as described elsewhere.

Activity of Particulate O₂⁻-Forming System Incubated Under Defined Atmospheres

The activity of the particulate system under defined atmospheres was measured by the same method used for intact cells, with the following modifications: The main compartment of the Warburg flask contained 0.4 ml of particle suspension, 1.4 ml of 10-mM potassium phosphate buffer (pH 7.3), and 0.2 mg of cytochrome c, with or without 30 μg of superoxide dismutase. The sidearm contained 0.2 ml of 1-mM NADPH. The flask, its sidearm fitted with a venting plug and its mouth stoppered with a serum port vented with a needle as previously described, was placed in melting ice and gassed for 10 min. It was then submerged in a 30°C water bath (this temperature was used because the particles were found to lose their activity rapidly at 37°C), all the while maintaining the flow of gas, which was continued for an additional 3 min. The flask was then sealed; its contents were mixed, and the incubation was conducted for the times noted. The reaction was terminated by placing the flask in melting ice and injecting 30 μg of superoxide dismutase via a Hamilton syringe through the unopened serum port into each flask not initially containing dismutase. For each incubation a second flask (the zero-time control) was prepared and treated identically, except that it was placed in melting ice immediately after mixing the contents. The contents were then transferred to glass cuvettes (removal of particles by centrifugation was unnecessary), and cytochrome c reduction was determined spectrophotometrically, as previously described, using the incubated reaction mixture and its corresponding zero-time control as the sample and reference respectively. O₂⁻-dependent cytochrome c reduction was then calculated by subtracting the value for cytochrome c reduction in the dismutase-containing reaction mixture from the value obtained in the dismutase-free reaction mixture.
RESULTS

O$_2^-$ Production Under Decreased Oxygen Tension

O$_2^-$ production by neutrophils incubated under hypoxic conditions showed little decline until very low oxygen tensions were reached. Figure 1 shows that under 1% oxygen (a concentration less than one-twentieth that of room air) O$_2^-$ production over 25 min was more than 75% of the control value. Below that level, however, O$_2^-$ production fell off rapidly, reaching low to negligible values at oxygen concentrations of 0.25%. Similar results were obtained at 5 and 10 min of incubation.

Experiments with the cell-free O$_2^-$-forming system suggest that the effect of oxygen tension on O$_2^-$ production reflects an intrinsic property of this system. When particles containing the O$_2^-$-forming system were incubated under various oxygen tensions, O$_2^-$ production rates (expressed as a fraction of the rate obtained with room air) were similar to those observed with intact cells incubated under comparable oxygen tensions (Table 1). Thus the production of O$_2^-$ by both cells and particles is half maximal at approximately 10-µM oxygen, a property that permits the intact neutrophil to undergo a nearly normal respiratory burst under exceptionally hypoxic conditions.

Effect of pH on Respiratory Burst

Although the respiratory burst is relatively insensitive to hypoxia, it is rather sensitive to pH in the vicinity of the neutrophil. This is demonstrated by the results presented in Fig. 2. O$_2^-$-dependent cytochrome c reduction by neutrophils is seen...
here to decline substantially as the pH falls, values at pH 6.0 being only 11% of those at pH 7.5. Doubling the cytochrome c concentration at pH 6.0 was found not to affect the amount of cytochrome reduced during the incubation, thus indicating that the decrease in cytochrome c reduction at low pH was due to a true decline in \( \text{O}_2^- \) production, not to a decrease in \( \text{O}_2^- \) trapping, which theoretically might result because of the effect of pH on the rate of spontaneous dismutation of \( \text{O}_2^- \). Oxygen consumption fell similarly at low pH. Figure 3 shows that oxygen uptake by zymosan-treated neutrophils incubated at pH 6.0 was only 22% of that observed at pH 7.5. Thus by both of these measures respiratory burst activity is much lower under acidic conditions than at more physiologic pH levels.

The basis for this effect of pH on the respiratory burst appears to be a decline in the activation of the \( \text{O}_2^- \)-forming system at low pH. This was shown by measuring the activities of the particulate \( \text{O}_2^- \)-forming systems obtained from neutrophils treated with zymosan at pH 6.0 and 7.5. The results of these experiments are shown in Table 2. The specific activity of particles isolated from cells exposed to zymosan at pH 6.0 was much lower than that of particles from cells treated with zymosan at pH 7.5. In contrast, the pH at which the particles were assayed had relatively little effect on their activity, a result in accord with previous data. It thus appears that a

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Table 1. \( \text{O}_2^- \) Production by the Particulate \( \text{O}_2^- \)-Forming System as a Function of Oxygen Concentration.

<table>
<thead>
<tr>
<th>Oxygen Concentration (%)</th>
<th>( \text{O}_2^- ) Production (%) of value in air</th>
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<tbody>
<tr>
<td>20.9 (air)</td>
<td>(100)</td>
</tr>
<tr>
<td>0.34</td>
<td>45.4 ± 2.5</td>
</tr>
<tr>
<td>0</td>
<td>10.1 ± 2.2</td>
</tr>
</tbody>
</table>

\( \text{O}_2^- \) production was determined as described in the section Materials and Methods. For each experiment the assay was performed at the oxygen concentration shown. Results are expressed as percentages of the corresponding room air value and represent means ± 1 SE of three experiments. The table shows data obtained from 8-min incubations; similar results were obtained at 4 min. The actual control value was 22.5 ± 3.1 nmoles \( \text{O}_2^- \)/mg protein over the 8-min incubation.

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Fig. 2. \( \text{O}_2^- \) production by neutrophils as a function of pH. Reaction mixtures contained 10\(^6\) cells, 6 mg of opsonized zymosan, and 1.85 mg of cytochrome c, with or without 10 \( \mu \)g of superoxide dismutase, in 2.1 ml of citrate-phosphate buffer at the pH shown. Incubations were conducted for the times noted as described in the section Materials and Methods. Each point represents the mean ± 1 SE of four experiments, each using a different cell preparation.
Fig. 3. Oxygen uptake by zymosan-treated neutrophils at pH 6.0 and pH 7.5. The main compartment of the flask received $10^6$ cells in 1.8 ml of citrate-phosphate buffer, and the sidearm received either 6 mg of opsonized zymosan in 0.2 ml of citrate-phosphate buffer or 0.2 ml of buffer only. The flask was then attached to the differential respirometer and submerged in a 37°C bath. After 10 min the contents of the main compartment and the sidearm were mixed. Ten minutes later the manometer was zeroed, and readings were taken every 5 min thereafter for the period shown. Each experiment consisted of four incubations: with or without zymosan at pH 6.0 and at pH 7.5. The results shown are means ± SE for three experiments.

A drop in pH caused a reduction in the efficiency of activation of the $O_2^-$-forming system responsible for the respiratory burst, at least over the range of acidity used in these experiments.

**Phagocytosis**

One possible explanation for the effects observed here is that they may be secondary to differences in particle uptake under the various experimental conditions. This does not appear to be the case, however, since the rates of zymosan uptake by neutrophils incubated in air at pH 7.5, under nitrogen at pH 7.5, and in air at pH 6.0 were found to be essentially the same (Fig. 4).

<table>
<thead>
<tr>
<th>Conditions of Activation</th>
<th>$O_2^-$ Production (nmoles/min/mg protein)</th>
<th>Assayed at pH 6.0</th>
<th>Assayed at pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated at pH 6.0</td>
<td>2.9 ± 0.5</td>
<td>3.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Activated at pH 7.5</td>
<td>6.1 ± 1.3</td>
<td>7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Neutrophils were activated with opsonized zymosan in citrate-phosphate buffer at the pH shown. Particles were then prepared and assayed as described in the section Materials and Methods, except that the potassium phosphate buffer was replaced with citrate-phosphate buffer, the gassing procedure was omitted, and the incubations were carried out for 8 min at 22°C. The results shown are means ± 1 SE for three experiments, each carried out with particles from a different cell preparation.
Fig. 4. Effects of oxygen tension and pH on phagocytosis of opsonized zymosan. Phagocytosis was determined as described in the section Materials and Methods. Each panel shows the results of two separate experiments, each conducted with a different cell preparation, and each represented by a different symbol (circles and triangles). Top: Effect of oxygen tension. Incubations were conducted in air (open symbols) or under nitrogen (closed symbols) in HBSS at pH 7.3. Bottom: Effect of pH. Incubations were conducted under room air in citrate-phosphate buffer at pH 7.5 (open symbols) or pH 6.0 (closed symbols).

DISCUSSION

The mechanisms by which neutrophils kill bacteria can be divided into two classes: those that require oxygen and those that do not.2 Whereas many microorganisms are susceptible to both classes of killing mechanisms, some varieties, notably Staphylococcus aureus and the Enterobacteriaceae, are relatively resistant to the oxygen-independent microbicidal mechanisms of neutrophils.13 For these bacteria the respiratory burst is essential for efficient killing, and neutrophils that are unable to undergo the burst (e.g., neutrophils incubated under nitrogen or neutrophils from patients with chronic granulomatous disease14) have great difficulty in handling these organisms.

It has been shown that the oxygen tension at sites infected by organisms of this type is greatly reduced as compared with that of most uninfected tissues (PO2 ~ 25 mm, equivalent to 3% oxygen, according to Hays and Mandell6). For neutrophils to kill such organisms effectively at these sites, they must be able to employ their oxygen-dependent bactericidal mechanisms even at the low oxygen tensions prevailing in the infected regions. The present experiments show that neutrophils have the capacity to mount a nearly normal respiratory burst at these reduced oxygen tensions, strongly suggesting that the oxygen-dependent bactericidal mechanisms can operate under these conditions.

The diminution in the respiratory burst at low pH would appear to represent a disadvantage, in terms of host defense, since the pH at infected sites is usually lower than that of normal tissues.6 This disadvantage, however, is relatively minor.
Although decreased as compared with normal, the pH in the region of an infection seldom falls to less than 6.8–7.0, a level that permits substantial respiratory burst activity by neutrophils. Moreover, any impairment of killing due to the effect of low pH on the respiratory burst could be offset by its effect on myeloperoxidase. This enzyme, one of the components of a powerful oxygen-dependent bactericidal mechanism, is optimally active at acid pH. A fall in pH will augment the activity of myeloperoxidase at the same time it diminishes the respiratory burst, leading to what could well be a net increase in the potency of the oxygen-dependent bactericidal mechanisms of the neutrophil.

REFERENCES

7. GIBCO Catalog, 1972–73. Grand Island, NY, Grand Island Biological, p 96
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