CONCISE REPORT

Detection of Carriers of the Autosomal Form of Chronic Granulomatous Disease

By Arthur J. Verhoeven, Margriet L.J. van Schaik, Dirk Roos, and Ron S. Weening

The NADPH:O₂ oxidoreductase catalyzing the respiratory burst in activated phagocytes from healthy individuals is not operative in phagocytes from patients with chronic granulomatous disease (CGD). In a microscopic slide test using the dye nitroblue tetrazolium (NBT), carriers of X-linked CGD can be recognized by a mosaic pattern of NBT-positive and NBT-negative cells, governed by the expression of an unaffected or an affected X chromosome, respectively. Until now, it has not been possible to detect carriers of the autosomal form of CGD (other than by family studies) because all cells of these carriers stain positive in the NBT test. We have investigated whether neutrophils from carriers of autosomal CGD can be recognized by measurement of the rate of oxygen uptake upon stimulation of the cells. It was found that with the phorbol ester PMA as a stimulus, the respiratory burst is significantly lower in autosomal CGD carriers. With serum-treated zymosan as a stimulus, no difference between controls and carriers was observed. The addition of f-Met-Leu-Phe (1 μM) to PMA-activated neutrophils of control donors caused a transient increase in oxygen consumption of about 40%. Under these conditions, an increase of more than 100% was observed in neutrophils from carriers of autosomal CGD. These findings provide a simple method for the detection of carriers of the autosomal form of CGD.

MATERIALS AND METHODS

Patients. Neutrophils were isolated from heparinized blood from different parents or different sisters of four CGD patients on different occasions and from the blood of volunteer donors collected at the same time. The observations in the neutrophils of the sisters of the CGD patients were similar to those found in the neutrophils of their parents. The CGD patients were classified as autosomal CGDs on the basis of their clinical symptoms, failure of their neutrophils to respond with a respiratory burst to various stimuli, and 95% NBT-positive cells in the neutrophils from their mothers. The diagnosis of these patients was confirmed by positive complementation of NBT reductase activity in the patients' monocytes after fusion with monocytes from X-linked CGDs.

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Isolation and incubation of cells. After the blood was diluted with an equal volume of phosphate-buffered saline (PBS) containing 13 mmol/L trisodium citrate, it was centrifuged over Percoll with a specific gravity of 1.076 g/mL (20 minutes, 1,000 g, room temperature). Lysis of the erythrocytes in the pellet fraction was done by incubation in isotonic NH₄Cl at 4°C, as described previously. After two washings in PBS, the neutrophils were washed and resuspended to ~2 x 10⁶ cells/mL in an incubation medium (pH 7.4) containing 138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na₂HPO₄, 1.5 mmol/L KH₂PO₄, 0.6 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, 5.5 mmol/L glucose, and 0.5% (wt/vol) human albumin. Before measurement of the respiratory burst activity, the neutrophils were kept at room temperature and used as soon as possible.

Measurement of oxygen consumption. Oxygen consumption was measured at 37°C essentially as described before, with an oxygen electrode calibrated with air-saturated water. The cells (~2 x 10⁶ in 0.6 mL) were preincubated at 37°C for at least 15 minutes before any addition was made. For activators of the respiratory burst, we used serum-treated zymosan (STZ, 1 mg/mL), phorbol myristate acetate (PMA, 200 ng/mL) and formyl-methionyl-leucyl-phenylalanine (fMLP, 1 μmol/L). Rates of oxygen consumption given in the text are maximal values of the burst obtained with the different stimuli.

Results were expressed as mean ± SEM, with the number of observations in parentheses. Statistical significance was calculated with the Student’s t test.

RESULTS

After addition of STZ to activate the respiratory burst, no significant difference in the rate of oxygen uptake was observed between the neutrophils from carriers of the autosomal form of CGD and those from control donors (Table 1, line 1). When PMA was used as a nonphysiologic activator, however, the respiratory burst was lower in the neutrophils from the carriers than in those from control donors (Table 1, line 2). Consequently, a significant difference existed between carriers and controls when in each preparation of cells the PMA-induced burst was expressed relative to the STZ-induced oxygen consumption. In control neutrophils, the PMA-induced rate of oxygen uptake amounts to ~70% of the rate induced by STZ, whereas in neutrophils from autosomal CGD carriers this ratio is only ~0.4 (Table 1, line 3). This defect in the neutrophils of CGD carriers was not abolished by increasing the concentration of PMA to 1 μg/mL. Similar results were obtained when the respiratory burst was followed by measurement of lucigenin-enhanced chemiluminescence (results not shown).

Table 1. Comparison of Respiratory Burst Induced by STZ or PMA in Neutrophils From Control Donors and Carriers of Autosomal CGD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>CGD Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>STZ-induced burst</td>
<td>6.80 ± 1.19</td>
<td>6.65 ± 0.90</td>
</tr>
<tr>
<td>PMA-induced burst</td>
<td>2.93 ± 0.58†</td>
<td>6.14 ± 0.60</td>
</tr>
<tr>
<td>PMA/STZ ratio</td>
<td>0.43 ± 0.06†</td>
<td>0.72 ± 0.05</td>
</tr>
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</table>

Oxygen consumption was measured as described in the Materials and Methods section. The values are given in nmol O₂/10⁶ cells/min (mean ± SEM of six experiments with four different CGD carriers and six different control individuals).

*Not significantly different from control values.
†Significantly different from control values (P < .005).

Because both the interassay variation in the measurement of O₂ consumption and the individual variation within the group of control donors are ~10%, we decided to develop a procedure of carrier detection independent of these variabilities. For this purpose, the chemoattractant fMLP was added 2 to 4 minutes after addition of a saturating dose of PMA (Fig 1). In control neutrophils, the rate of oxygen uptake increased transiently by ~40% after the addition of fMLP (Table 2). In neutrophils from autosomal CGD carriers stimulated with PMA, however, the burst activity increased >100% after the addition of fMLP (Table 2, line 2).

DISCUSSION

We used different stimuli for the induction of a respiratory burst in neutrophils and developed a method to detect carriers of the autosomal form of CGD. This is a convenient method, because only ~5 x 10⁶ neutrophils (not necessarily pure) are required; the discriminative power is sufficient for reliable detection of carriers. The latter property is achieved.

Table 2. Comparison of Respiratory Burst Induced by PMA Before and After Addition of fMLP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>CGD Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA-induced burst</td>
<td>6.02 ± 0.60</td>
<td>3.01 ± 0.37†</td>
</tr>
<tr>
<td>Burst induced by fMLP</td>
<td>8.48 ± 0.55†</td>
<td>8.27 ± 0.76</td>
</tr>
<tr>
<td>PMA/fMLP + PMA ratio</td>
<td>0.38 ± 0.03*</td>
<td>0.72 ± 0.01</td>
</tr>
</tbody>
</table>

For experimental details see legend to Fig 1. Values of the respiratory burst are given in nmol O₂/10⁶ cells/min (mean ± SEM of nine different carriers and six different control individuals).

*Significantly different from control values (P < .005).
†Not significantly different from control values.
by measuring relative respiration rates obtained with different stimuli. The range of values for the rate of oxygen uptake in the presence of PMA relative to the rate obtained with PMA plus fMLP was 0.24 to 0.50 in the carriers and 0.68 to 0.75 in the control group. In the neutrophils of the mother of an X-linked CGD patient, this fraction was 0.64 (not shown), clearly different from the values obtained with carriers of autosomal CGD.

In general, a gene–dose effect of an enzyme activity on a metabolic flux can only be expected if that enzyme exerts a high control on the flux through that metabolic pathway. In neutrophils from carriers of autosomal CGD, this seems to be the case only when PMA is used to activate the respiratory burst (Table 1, line 2). This indicates that only with PMA as a stimulus does the enzyme affected in autosomal CGD exert a high control on the production rate of superoxide. At present, one can only speculate on the underlying mechanism of this phenomenon. Possibly, PMA activates the affected enzyme (the 47-kd protein?) to a lower extent than does STZ, resulting in a higher control of this enzyme on the respiratory burst. Alternatively, the extent of control might diminish by recruitment of additional 47-kd protein when a second signal is generated inside the cells, for instance by the addition of fMLP. With STZ as a stimulus, more than one signal may be generated, with the same consequences. Definite answers regarding these possibilities must await the elucidation of the role of the 47-kd protein in activation of the neutrophil respiratory burst.

REFERENCES

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