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Involvement of Cytochrome b_{-245} in the Respiratory Burst of Human Neutrophils

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The cytochrome b_{-245} of human neutrophils was reduced when the neutrophils under anaerobic conditions were stimulated by serum-treated zymosan, immune complexes, or phorbol myristate acetate. Reintroduction of oxygen rapidly reoxidized the cytochrome; azide was without effect on any of these reactions. This indicates that cytochrome b_{-245} participates in the respiratory burst of neutrophils.

Ingestion of particles by neutrophils elicits a dramatic increase in oxygen consumption, known as the respiratory burst of phagocytosis (2). This response may be triggered not only by interaction of opsonized particles with C3b and Fc receptors but also by certain soluble stimuli, such as the tumor promotor phorbol myristate acetate (PMA) (3). Oxygen consumed in the respiratory burst is reduced to superoxide anions or hydrogen peroxide in an azide-insensitive process by electrons ultimately originating from oxidation of glucose in the hexose monophosphate shunt (6, 10). This reduction of oxygen takes place in the plasma membrane, which is incorporated into the developing phagocytic vacuole (10) where the H_2O_2 formed interacts with myeloperoxidase and a halide to form a highly bactericidal system (7). Genetic defects causing complete absence of the neutrophil respiratory burst and a consequent impairment of bactericidal and fungicidal functions are exhibited by patients with chronic granulomatous disease (CGD) (6).

The exact biochemical events which result in the respiratory burst remain to be resolved. There is considerable support for the involvement of an NADPH-oxidase, possibly containing a flavin (1). A *b* cytochrome which has a midpoint potential of −245 mV (5) and which binds carbon monoxide is present in the plasma membrane of human neutrophils and becomes incorporated into the phagocytic vacuole (12). The finding that this *b* cytochrome is not detectable in neutrophils from patients with classical X-linked CGD (13) points to its possible involvement in an electron transport chain necessary for the expression of the respiratory burst. This concept has gained further support from the observation that this cytochrome is reduced when intact neutrophils are stimulated by PMA under anaerobic conditions (13) and that this does not happen in those CGD cells in which the cytochrome is present (autosomal recessive CGD) (13). However, since PMA is an unphysiological stimulus with unknown mechanisms of action, data obtained solely with this agent must be interpreted cautiously. It is therefore critical for the understanding of the role of this *b* cytochrome in the respiratory burst to demonstrate that activation of neutrophils by other more physiological stimuli (e.g., immune complexes or C3b-coated particles) also induces reduction of the *b* cytochrome.

Blood was withdrawn from healthy adult donors who had given informed consent to the procedure. Neutrophils were isolated by dextran sedimentation of erythrocytes, followed by centrifugation through Ficoll-Hypaque as previously described (4). Residual erythrocytes were lysed two to three times; the first lysis was done in distilled water for 45 s and the second and third in NaCl (2 g/liter) for 45 s. Isotonicity was restored by adding hypertonic NaCl. After being washed, the neutrophils were suspended in Hanks balanced salt solution containing 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.05) (Hank-HEPES). The final cell concentration was 2×10^7/ml. The cells were deoxygenated at room temperature in stoppered, siliconized cuvettes by blowing N_2 over the surface while the cell suspension was stirred. Anaerobiosis was obtained after 30 min as assessed by the inability of the cells to reduce ferricytochrome *c* (0.08 mM) after stimulation with PMA. The ability of the cells to reduce cytochrome *c* was completely normalized when oxygen was reintroduced. Difference spectra of stimulated ver-

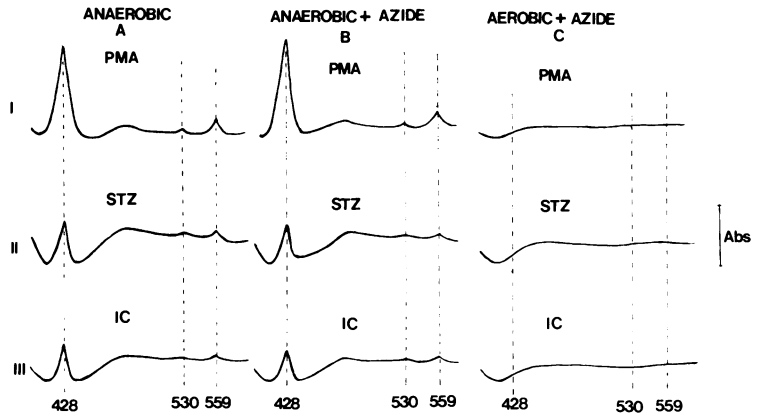

FIG. 1. Difference spectra from 400 to 600 nm of human neutrophils (2×10^7/ml) in Hank-HEPES buffer. The cells in the sample cuvette were stimulated by (I) PMA, 5 μg/ml; (II) serum-treated zymosan, 1 mg/ml; (III) immune complexes, 0.2 mg/ml. The stimuli were added to anaerobic cells while being stirred at 37°C. Scans were made 5 min after stimulation. (A) Stimulated anaerobic cells versus unstimulated anaerobic cells. (B) Stimulated anaerobic cells to which 1 mM azide was added 30 min before stimulation versus unstimulated anaerobic cells. (C) Cells from experiment B 10 min after oxygen was reintroduced to the sample cuvette (by removing the stopper and blowing atmospheric air over the surface during stirring of the cell suspension) versus unstimulated anaerobic cells. Results of typical experiments are shown. Abs, 0.01 absorbance.

sus unstimulated cells were recorded on a Perkin-Elmer 576 spectrophotometer. Zymosan was obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. Serum-treated zymosan was prepared as previously described from pooled human sera (3). Immune complexes were prepared by Deiren Mark, Boston University; rabbit immunoglobulin G anti-bovine serum albumin (Cappel Laboratories, Downingtown, Pa.) was mixed 5:1 (wt/wt) (14) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). After being washed, the complexes were suspended in phosphate-buffered saline (pH 7.4) at a concentration of 2.2 mg/ml as determined by the Lowry analysis (9). PMA was obtained from Sigma and dissolved at 10 mg/ml in dimethyl sulfoxide and kept frozen until use.

Stimulation of neutrophils via receptors for C3b (serum-treated zymosan) or Fc (immune complexes) as well as by PMA triggered an event in the cells which led within minutes to reduction of the cytochrome b_{-245} (Fig. 1). The possibility that the *b* cytochrome is active in oxidative phosphorylation, which, although known to be of very little significance in neutrophils (2a, 11), might be enhanced by the stimulation of the cells with consequent reduction of the cytochrome under anaerobic conditions, is ruled out by several observations. Incubation of neutrophils under anaerobic conditions for as long as 4 h at 37°C did not result in reduction of the cytochrome in the absence of a stimulus (data not shown); addition of azide, which blocks cytochrome oxidase (8), did not result in reduction of the *b* cytochrome (data not shown), indicating that it did not participate in electron transport associated with the generation of ATP; and reduction of the *b* cytochrome in stimulated cells was carried out equally well in the presence and absence of azide, and the cytochrome was reoxidized by reintroduction of oxygen in the presence of azide. Thus, the electron transport chain in which cytochrome b_{-245} participates is, like the respiratory burst itself (11), insensitive to azide.

The results presented here add further evidence for the concept that the cytochrome b_{-245} is part of an electron transport chain which participates in the reduction of oxygen during the respiratory burst in human neutrophils. The other components of such a system and the mechanism of their activation remain to be determined.

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