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QUANTITATION OF SUPEROXIDE PRODUCTION IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES FROM NORMALS AND 3 TYPES OF CHRONIC GRANULOMATOUS DISEASE

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SUMMARY
Conditions for quantitating the superoxide producing capacity of polymorphonuclear leukocytes are given. It is shown that the production is partly dependent on glucose but independent on Mg^{2+}, Ca^{2+} and extracellular pH in the range 6.6-8.2. The capacity of normal cells to produce superoxide is estimated to 13.4±0.13 SEM (n=9) femtomoles per min per cell, whereas superoxide production is absent in all cases of chronic granulomatous disease, irrespective of the type. It is suggested that at least 3 enzymes cooperate intimately in the superoxide producing system of the cell. This supports the theory of an electron transport chain of significance for oxygen consumption.

In the phagocytic process neutrophilic polymorphonuclear leukocytes consume oxygen for the production of the superoxide anion, O_2^-, which again is converted to H_2O_2 and hydroxyl radicals. These oxygen derivatives are all believed to be essential in the intracellular killing of catalase positive aerobic bacteria by the neutrophils (1-4), and are not formed in patients with chronic granulomatous disease (1, 5, 6). It is the current concept that the oxygen consumed in the phagocytic process is reduced to O_2^- in an oxidase system that may be linked to NAD(P)H oxidation (7, 8) and/or may be part of an electron transport chain as seen in bacteria (9). From experiments with cytochalasin B (4), the oxidase system has been allocated to the plasmamembrane. In order to quantitate the capacity of the oxygen consuming and superoxide producing system, we decided to use the soluble phagocytosis imitator phorbol myristate acetate as stimulus, since it was thought to give a more reproducible effect than IgG-coated latex particles or opsonised bacteria. The activity of the system was followed by measuring the liberation of the product O_2^- rather than the disappearance of the substrate O_2.

METHODS

The neutrophils were prepared as described (10), except that the cells were resuspended in Hanks balanced salt solution, pH 7.3, containing 5.5 mM glucose or as indicated. The preparations were more than 97% pure as judged by differential cell counting. Spectroscopic recordings were carried out at 37°C on a Beckman 24 double-beam spectrometer. 600 µl cell suspension plus 200 µl 0.8 mM cytochrome c in buffer were added to the sample and reference cuvettes. 5 µl phorbol myristate acetate (0.8 mg/ml in 0.9% NaCl) prepared as described (11) was then added to the sample cuvette. The cuvettes were vigorously shaken and the difference in absorbance at 550 nm was taken as the phorbol myristate acetate induced superoxide production, which was calculated using the absorbance coefficient of 21.1 mM^{-1} cm^{-1} for reduced cytochrome c (12). The increase in absorbance at 550 nm was totally inhibited by

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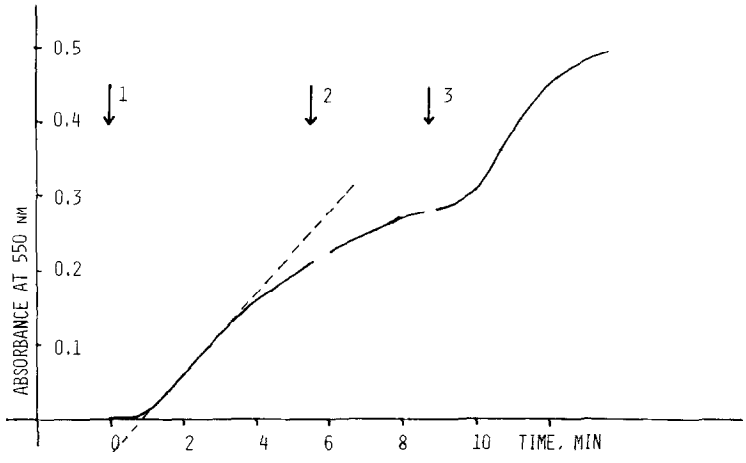

Figure 1
Increase in absorbance at 550 nm when phorbol myristate acetate, 5 µg/ml, is added to 2 x 10^5 neutrophils per ml in Hanks balanced salt solution with 5.5 mM glucose and 0.2 mM cytochrome c. Arrow 1 indicates addition of phorbol myristate acetate, arrow 2 that the sample cuvette is vigorously shaken for 30 sec, and arrow 3 the further addition of 50 µl cell suspension (3 x 10^6 cells per ml) to the sample cuvette. The stipled line is the inflexion from which maximal O_2^- production rate is calculated.

including superoxide dismutase (300 µg/ml) in the sample cuvette, showing that the reduction of cytochrome c was due to superoxide.

The diagnosis of chronic granulomatous disease and the mode of inheritance was determined by measuring intracellular killing, ^{14}C-1-glucose oxidation and chemiluminiscense as in ref. (10). The activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, glutathione peroxidase, and myeloperoxidase were determined as in (10) and found normal.

For measurements of cytochrome b, the procedure given in ref. 10 was followed. Cytochrome b was identified by the absorption bands at 428 and 559 nm (9). The spectra did not show any absorption bands at 552 nm, indicating that there was no contamination by eosinophils (13, 14).

RESULTS

The reduction of cytochrome c is nonlinear with time (Fig. 1). After a lag period of about 30 sec, the rate of reduction increases until a well defined maximum is reached in 2-4 min as indicated by the inflexion. The subsequent decrease in the rate of cytochrome c reduction is not due to inadequate oxygen diffusion, since vigorous shaking of the cuvettes does not result in increased reduction (Fig. 1, arrow 2). Furthermore, reduction of cytochrome c is resumed by readdition of cells (Fig. 1, arrow 3), indicating that the decrease in reduction rate is not due to lack of oxygen or oxidised cytochrome c. Increasing the cytochrome c or phorbol myristate acetate concentration or both did not change the rate of cytochrome c reduction, indicating that the

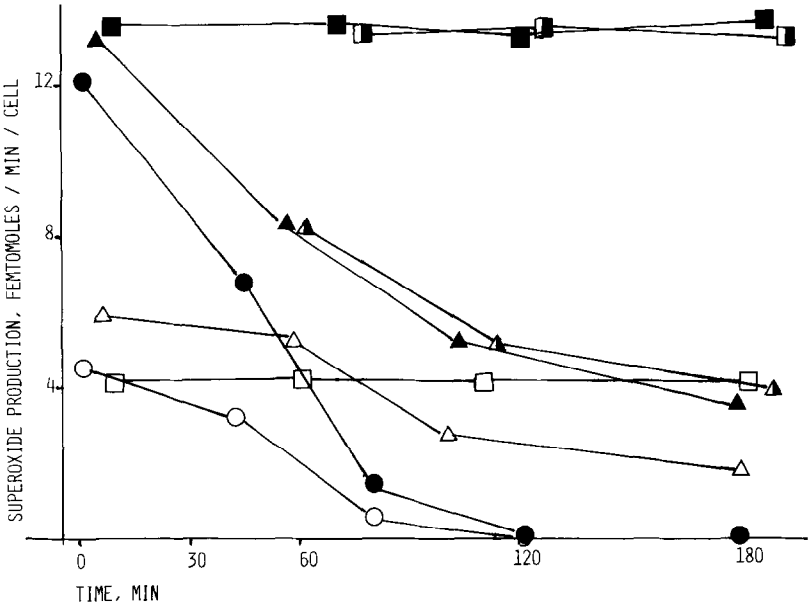

Figure 2
Neutrophils ($1o^7$/ml) were preincubated at 37°C with shaking in buffer as indicated by symbols. Samples were withdrawn, diluted to a cell concentration of $1o^6$ cells per ml with the incubation buffer and superoxide production measured by addition of phorbol myristate acetate and cytochrome c, as described in Methods. Open symbols indicate that glucose is absent from the medium. Closed symbols that glucose is present in 5.5 mM concentration. Half closed symbols indicate that glucose is absent during the preincubation, but present when superoxide production is determined. ■ □ ◨ Hanks balanced salt solution pH 7.3, ▲ △ ◭ 1o mM phosphate, 145 mM NaCl, pH 7.3 ● ○ 1o mM Tris, 145 mM NaCl, pH 7.3.

concentration of phorbol myristate acetate (5 μg/ml) stimulates the cells maximally and that o.2 mM cytochrome c traps the superoxide produced effectively. This is in accordance with other reports (15, 16). The maximal rate of cytochrome c reduction was a linear function of cell concentration below 3 x $1o^6$ cells per ml (data not shown). Routinely 7.5 x $1o^5$ cells per ml were used. 5.5 mM glucose has traditionally been included in the buffer in phagocytosis experiments and is also sufficient for maximal superoxide production after phorbol myristate acetate stimulation (Fig. 2). Although omission of Mg^{2+}, Ca^{2+} and P_i from the buffer were without effect on superoxide production by freshly isolated cells, the presence of these ions were necessary to maintain the ability of the cells to produce superoxide (Fig. 2). The decrease in superoxide production in phosphate-buffered saline and tris-saline was not prevented by the presence of glucose or reversed by subsequent incubation in Hanks balanced salt solution for 15 min (data not shown). The

Table I

Donor	O_2^- production (femtomoles/min/cell)	cytochrome b in neutrophils
Normals	13.4±0.013 SEM (n=9)	present
Patient 1, male	0	absent
Mother of 1	2.6	present
Patient 2, male	0	present
Mother of 2	5.4	present
Patient 3, female	0	present

The superoxide production was determined in Hanks balanced salt solution containing 5.5 mM glucose.

superoxide production was independent on variations in extracellular pH in the range 6.6-8.2 when measured in Hanks balanced salt solution (data not shown). Table I gives the figures for maximal superoxide production of neutrophils from 9 healthy unrelated individuals and from 3 patients suffering from chronic granulomatous disease, one girl having the autosomal recessive form and two boys the X-linked form. As described elsewhere (lo, 14) cytochrome b is present in the membrane of the neutrophils from the girl and one of the boys, whereas the other boy lacks cytochrome b. In all three cases of chronic granulomatous disease superoxide was not formed, whereas the production was heavily reduced in the mothers of the X-linked cases. Unfortunately, it was not feasible to obtain blood from the mother of the girl. The variable reduction of maximal superoxide production seen in the mothers, as compared to the very narrow range observed in normals, is in accordance with the Lyon hypothesis showing different degrees of inactivation of the unaffected chromosome in the mothers.

DISCUSSION

Our result show that maximal superoxide production of neutrophils is linear with cell concentration. Others (15) have found that superoxide production decreases with increasing cell concentration. However, in those studies, superoxide was measured by adding cytochrome c after the production had been stopped. Since superoxide is known to undergo spontaneous dismutation ($O_2^- + O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$), a reaction that is second order, we believe that an effective scavenger should be included in the incubation mixture in order to prevent spontaneous dismutation of superoxide. The production rate given by us is considerably higher than reported previously (15). We believe this is due to phorbol myristate acetate being more effective in stimulating superoxide production than

IgG-coated latex particles (17) and to the inclusion of cytochrome c in the incubation medium.

It is known that the increase in oxygen consumption induced by phagocytosis is tightly linked to an increase in hexose-monophosphate shunt activity, being reduced both in patients suffering from chronic granulomatous disease and in a patient with total glucose-6-phosphate dehydrogenase deficiency (18). Our finding that glucose causes a considerable increase in phorbol myristate acetate induced superoxide production suggests that maximal superoxide production by cells suspended in a glucose-free medium is limited by the activity of the hexose monophosphate shunt, which in turn is limited by the intracellular level of glucose-6-phosphate that can be provided by glycogen breakdown through the action of glycogen phosphorylase, known to be activated during phagocytosis (19). Inclusion of glucose, which is known to be freely permeable to the cell membrane of leukocytes (2o), raise the intracellular glucose-6-phosphate concentration.

Recently Segal et al. (9) discovered cytochrome b in the plasmamembrane of neutrophils and suggested that it was active in an electron transport chain functioning as an oxidase, since it was found lacking in patients suffering from chronic granulomatous disease. We have found, however, that cytochrome b is present in some and absent in other cases of chronic granulomatous disease (1o, 14). It was found present in all 3 patients with the autosomal recessive inherited form and present in 2, but absent in 3 of 5 patients with X-linked chronic granulomatous disease investigated by us. Thus, absence of cytochrome b cannot account for all types of chronic granulomatous disease. Two other enzymes must exist, one absent in the autosomal recessive form of chronic granulomatous disease and the other absent in that form of X-linked chronic granulomatous disease in which cytochrome b is present. Since the superoxide production was nil in all three types of chronic granulomatous disease, as shown in table I, an intimate functional relation must exist between cytochrome b and the two other enzymes not yet identified. An electron transport chain as suggested by Segal et al. (9) with at least three components which consumes oxygen and liberates superoxide would account for these findings.

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