Improvement of Leukocyte Bactericidal Activity in Chronic Granulomatous Disease

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Chronic Granulomatous Disease (CGD) is a genetic disorder characterized by chronic and recurrent pyogenic infections, with abscess and granuloma formation throughout the reticuloendothelial system. Patients' leukocytes fail to demonstrate the increase in oxygen consumption, hexose monophosphate shunt activity, nitroblue tetrazolium reduction, and hydrogen peroxide formation which normally occur during phagocytosis.1,2 These oxidative defects have been ascribed to a deficiency of leukocyte DPNH (NADH) oxidase activity.1,3

Phagocytes from patients with CGD can ingest bacteria normally, but can kill only those bacteria which effectively produce hydrogen peroxide, such as streptococci and lactobacilli.4,5 Bacteria which are not effective peroxide producers, such as staphylococci and serratiae, are not killed by these phagocytes.6 Klebanoff has shown that hydrogen peroxide combined with halides and myeloperoxidase constitute a potent bactericidal system,7 and that this system is lacking in CGD leukocytes.5 We have previously reported marked improvement in intracellular formate oxidation and hexose monophosphate shunt activity by CGD leukocytes following the introduction of a hydrogen peroxide-producing system into these cells.8 This report describes the improved killing of ingested Staphylococcus aureus and Serratia marcescens achieved by this technique.

Materials and Methods

Binding of Glucose Oxidase to Latex Spherules. Glucose oxidase (type V, 880 units per ml., Sigma Chemical Co.) was diluted 1:10 with distilled water to a final volume of 2 ml. Latex spherules, 0.81-μm diameter (Difco Laboratories) were dialyzed exhaustively against deionized water and resuspended to original volume. One milliliter of these spherules and 2 ml of the glucose oxidase solution were incubated together for 15 minutes at 25°C. The mixture was centrifuged at 18,000 g. for 15 minutes, and the spherules with bound enzyme were resuspended in distilled water to 1 ml.

Determination of Glucose Oxidase Activity on Latex Spherules. Glucose oxidase activity was determined by a modification of the Glucostat method for determination of glucose.
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(Worthington Biochemical Corp.). The incubation mixture included 1.8 ml. Chromogen solution (Worthington, made by addition of 150 ml. of 0.01 M potassium phosphate buffer, pH 7, to the contents of one vial), 0.2 ml. of a 2 mM glucose solution, and 0.1 ml. latex coated with glucose oxidase. The reaction was started by the addition of 0.01 ml. horseradish peroxidase (type I, Sigma) at a concentration of 600 units/ml. in distilled water, incubated at 25°C, and stopped after exactly six minutes by the addition of one drop 4N HC1. The reaction was linear for at least 10 minutes and proportional to glucose oxidase concentrations of 0.4 to 1.8 units per total reaction volume.

Preparation of Phagocytes. Venous blood was obtained from controls and two patients, and processed as previously described,9 except that the final cell washing was made in Krebs Ringer phosphate (KRP) buffer, pH 7.35, without glucose. Phagocytes (monocytes and PMNs) were suspended in KRP buffer with one per cent human serum albumin to a concentration of $2.5 \times 10^6$ cells per 0.5 ml. For experiments with S. aureus, this buffer also contained 0.2 per cent d-mannitol (Sigma); for experiments with S. marcescens, it contained 0.2 per cent maltose hydrate (grade II, Sigma).

Preparation of Bacteria. Staphylococcus aureus was grown overnight in nutrient broth (Difco Laboratories) containing 0.2 per cent mannitol. Serratia marcescens was grown overnight in broth consisting of 2 per cent Bacto-Peptone (Difco), 0.5 per cent NaCl, 0.2 per cent maltose, 0.25 per cent Na9HPO4, and HC1 to a final pH of 7.35. Cultures were transplanted to fresh medium in the morning, and after a five-hour incubation, 2 ml. of the broth culture was centrifuged at 8000 g. for 15 minutes. The bacteria were resuspended in a 17-mm.-diameter tube with KRP buffer (containing albumin, and either mannitol or maltose) to 95 per cent transmission at 620 mjs in a Coleman Junior spectrophotometer.

Phagocytic Bactericidal Assay. The ability of peripheral blood leukocytes to kill S. aureus and S. marcescens was studied using a modification of the method of Quie.6 Normal adult serum was separated from freshly clotted blood, frozen in small aliquots at −65°C and thawed immediately before use. One-tenth ml. serum, 0.1 ml. latex spherules, either with or without bound glucose oxidase, and 0.3 ml. bacteria were added to 12 × 75 ml. sterile capped plastic tubes (Falcon Plastics Co.) in an ice bath. At five-minute intervals, $2.5 \times 10^6$ phagocytes in 0.5 ml. buffer were added to begin the reaction. This was calculated to provide four or five bacteria per phagocyte in a reaction volume of 1 ml. A control tube contained bacteria, 0.1 ml. serum, 0.1 ml. latex spherules with bound glucose oxidase, and 0.5 ml. buffer in place of the suspension of leukocytes. The contents of the tube were immediately mixed, and 0.05 ml. of the mixture was transferred by sterile disposable micropipette (Drummond Scientific Co.) to 4.8 ml. sterile water in a 100 × 17 mm. capped plastic tube (Falcon). After vigorous shaking of the tube for 30 seconds, a 0.5-ml. aliquot was transferred to a second tube containing 4.8 ml. water. After this tube was shaken vigorously for 30 seconds, a 0.5-ml. aliquot was removed to make a pour plate with 12 ml. of agar made from the broth appropriate for that bacterium. The original reaction tube was then placed on a revolving wheel at 37°C, and additional samples were obtained for colony counting at 30-minute intervals for 90 minutes. Colonies were counted the following day and expressed as the number of surviving bacteria per ml. of incubation mixture.

RESULTS

Binding of Glucose Oxidase to Latex Spherules. Approximately five per cent of the glucose oxidase in the incubation mixture was bound to latex spherules after a 15-minute incubation at 25°C, eight units of glucose oxidase activity being detected per 1 ml. latex spherules. This activity remained on the latex after repeated washings in distilled water. However, progressive loss of activity was noted after several washings in 0.85 per cent NaCl solution.

Histologic Study of the Phagocytic System. The latex spherules bound with glucose oxidase were phagocytized to an equal extent by normal and CGD leukocytes as demonstrated by light microscopy of Wright's-stained smears. All normal and CGD phagocytes contained spherules. The number of spherules
ingested after 30 minutes’ incubation averaged approximately 55 per phagocyte. Using Gram’s-stained smears, we noted that 87 per cent of phagocytes contained staphylococci and 84 per cent of phagocytes contained serratiae, after 30 minutes’ incubation in their respective phagocytic systems. Very few extracellular bacteria were noted. It was not possible to determine with light microscopy whether or not bacteria and latex particles were present within the same phagocytic vacuole.

Improvement of Bactericidal Activity by Introduction of Glucose Oxidase Activity into CGD Leukocytes. Improved bactericidal activity of the CGD leukocytes was consistently noted following the simultaneous ingestion of bacteria and latex spherules bound with glucose oxidase. As noted in Fig. 1, minimal killing of *Staphylococcus aureus* by CGD leukocytes occurred during ingestion of plain latex spherules, but ingestion of latex spherules bound with glucose oxidase definitely improved the extent of killing. Glucose oxidase bound to latex had no bactericidal effect in a leukocyte-free medium.

There was also consistent improvement in the rate and extent of killing of *Serratia marcescens* by CGD leukocytes which ingested latex coated with glucose oxidase along with bacteria (Fig. 2). When leukocytes were omitted, glucose oxidase and the remainder of the incubation mixture was not bactericidal for *Serratia*. When serum was also deleted from the reaction mixture, the number of bacteria almost doubled during the 90-minute incubation period (data not shown).

Although there was some variation in the extent to which bactericidal activity was enhanced, definite improvement was regularly noted by 30 minutes and was persistent throughout the 90-minute period of incubation. However,
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Fig. 2.—Effect of glucose oxidase on killing of Serratia marcescens by CGD leukocytes. Two representative experiments shown. Effect of latex bound with glucose oxidase in absence of phagocytes shown only for first experiment.

The improved bactericidal activity of the CGD leukocyte never equaled the bactericidal activity of normal leukocytes under the same conditions. The addition of latex particles, with or without bound glucose oxidase, impaired the rate and extent of bacterial killing by normal leukocytes (Fig. 1). Moreover, when we tried to introduce more glucose oxidase-bound latex than the optimal 0.1 ml. (Figs. 1, 2), we achieved less bacterial killing (data not shown).

DISCUSSION

The clinical manifestations of chronic granulomatous disease (CGD) can be ascribed to an inability of patients’ phagocytes to kill ingested bacteria. Several potential mechanisms for the killing of bacteria reside in the normal peripheral blood phagocyte. These include: lysosomal bactericidal agents, such as cationic proteins, lysozyme, and hydrolytic enzymes; lactic acid generated by increased glycolysis during phagocytosis; and the hydrogen peroxide–myeloperoxidase iodination system. Quie and co-workers have shown that the CGD leukocyte has a normal content of cationic proteins (phagocytin) and lysozyme. Normal activities of various hydrolases and of peroxidase are also present. The release of these enzymes from the CGD granule during phagocytosis appears to be normal, although controversy exists over this point. Lactic acid production during ingestion of particles has also been found normal in these patients’ leukocytes.

However, Holmes and her coworkers have demonstrated that hydrogen
peroxide formation does not occur in CGD leukocytes during phagocytosis. Moreover, Klebanoff has shown that normal leukocytes utilize peroxide and granule peroxidase to iodinate and kill phagocytosed bacteria. In contrast, CGD leukocytes fail to kill most bacteria, but can iodinate and kill peroxide-forming bacteria, such as *Lactobacillus casei* and streptococci.

Thus, the immediate cause for the inability of CGD leukocytes to kill ingested bacteria appears to be related to impaired production of hydrogen peroxide and, consequently, failure to complete the iodination reaction. The deficiency of DPNH oxidase demonstrated to exist in the CGD leukocyte could account for the failure of hydrogen peroxide production during phagocytosis. DPNH oxidase is apparently the most active respiratory enzyme in the human leukocyte during phagocytosis, and Cagan and Karnovsky have shown that this enzyme catalyzes the reaction between DPNH and oxygen to yield DPN and hydrogen peroxide. Glucose oxidase catalyzes the reaction between glucose and oxygen to yield gluconic acid and hydrogen peroxide. We have reported previously that phagocytosis of latex spherules bound with glucose oxidase introduces a hydrogen peroxide-generating system into the CGD leukocyte as measured by peroxide-dependent formate oxidation. (These studies will be presented fully in a subsequent publication.) Since leukocytes contain 18 per cent by dry weight of glycogen, ample intracellular substrate for phagocytosed glucose oxidase is available. This permits the incubation medium to be glucose-free, thus preventing the formation of extracellular hydrogen peroxide which might decrease bacterial survival regardless of phagocytosis. Indeed, no significant bacterial killing occurred when latex bound with glucose oxidase was incubated with bacteria.

The presence of latex spherules in the phagocytic mixture diminished bacterial killing by normal leukocytes (Fig. 1), presumably because the latex and bacteria were ingested competitively. The improvement in bacterial killing by CGD leukocytes in the presence of latex particles occurred in spite of this competition.

The importance of oxygen in the killing of bacteria during phagocytosis was shown by MCRipley and Sbarra. These investigators noted much greater intracellular killing of several bacteria when phagocytosis was carried out in oxygen compared to a nitrogen atmosphere. The hydrogen peroxide deficiency of the CGD leukocyte would seem to offer a genetic analogy to the anaerobic conditions of the above experiment. Correction of this defect in peroxide production may provide a rational basis for treatment of patients with CGD.

**SUMMARY**

Chronic granulomatous disease (CGD) is characterized by an inability of patients’ leukocytes to generate hydrogen peroxide and to kill non-peroxide-forming bacteria, such as staphylococci and serratia. We have introduced glucose oxidase into CGD leukocytes in order to generate peroxide and thereby kill *S. aureus* and *S. marcescens*. These results support the concept that a leukocyte oxidative deficiency is primary to the pathogenesis of CGD.
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