The Effect of an NADH Oxidase Inhibitor (Hydrocortisone) on Polymorphonuclear Leukocyte Bactericidal Activity

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ABSTRACT Polymorphonuclear neutrophils (PMN) from patients with chronic granulomatous disease of childhood have impaired bactericidal activity and are deficient in diphosphopyridine nucleotide, reduced form of (NADH) oxidase. Since hydrocortisone had been shown to inhibit NADH oxidation, experiments were undertaken to determine the effect of hydrocortisone on several parameters of human PMN function.

The phagocytic and bactericidal capacity of PMN with or without hydrocortisone (2.1 mM) was determined by quantitation of cell-free, cell-associated, and total bacteria. Phagocytosis of Staphylococcus aureus and several gram-negative rods was unimpaired by the presence of hydrocortisone in the media. In contrast, killing of bacteria was markedly impaired by hydrocortisone. After 30 min of incubation, there were 20-400 times as many bacteria surviving in hydrocortisone-treated PMN as in simultaneously run controls without hydrocortisone.

The defect of intracellular killing noted in the presence of hydrocortisone was not related to impaired degranulation. Quantitative kinetic studies of degranulation revealed no difference in the release of granule associated acid phosphatase in hydrocortisone-treated and control PMN after phagocytosis. Electron microscopy of PMN also indicated that the presence of hydrocortisone had no effect on the extent of degranulation after phagocytosis. These observations were confirmed by studies using histochemical techniques to detect lysosomal enzymes.

After phagocytosis, hydrocortisone-treated PMN demonstrated less NADH oxidase activity, oxygen consumption, and hydrogen peroxide production than postphagocytic control PMN. In addition, Nitro blue tetrazolium dye reduction was diminished in hydrocortisone-treated PMN.

Thus, impairment of NADH oxidase activity in normal human PMN by hydrocortisone results in reduced intracellular killing of bacteria, diminished postphagocytic oxygen consumption, decreased ability to reduce Nitro blue tetrazolium, and decreased hydrogen peroxide production. These abnormalities are similar to those seen in the PMN of patients with chronic granulomatous disease of childhood.

INTRODUCTION

Normal polymorphonuclear neutrophils (PMN) rapidly kill most phagocytized microorganisms (1). The factors responsible for intracellular killing are incompletely understood but probably include an acid intracellular pH (2), antibacterial cationic proteins (3), and hydrogen peroxide (4, 5).

New insights into leukocyte function have been obtained from the study of neutrophils from patients with chronic granulomatous disease (CGD) of childhood. Patients with CGD suffer from severe and recurrent bacterial infections caused by Staphylococcus aureus and gram-negative rods, and PMN from these patients have impaired bactericidal activity for those organisms (6). Leukocytes from these patients are unable to kill ingested organisms normally and recently have been found to be deficient in diphosphopyridine nucleotide, reduced form of, (NADH) oxidase (7). NADH oxidase may be essential for the production of intracellular hydrogen peroxide by the leukocyte. Since hydrogen peroxide in combination with myeloperoxidase and an appropriate oxidizable substance such as iodide has been shown to be a potent bactericidal system in PMN (4, 5), a deficiency of hydrogen peroxide may be the reason for impaired bactericidal activity of CGD PMN.

In order to obtain more information about the role of NADH oxidase in leukocyte bactericidal function, we inhibited NADH oxidation in normal neutrophils with hydrocortisone and compared the resultant abnormalities.
to those seen in the NADH oxidase-deficient neutrophils of patients with CGD. In these studies, hydrocortisone-21-succinate (a potent inhibitor of NADH oxidase in several biological systems) (8) was used at a concentration that impaired leukocyte respiration but not phagocytosis (2.1 mM).

METHODS

Phagocytosis

10 ml of heparinized venous blood (heparin sodium, Connaught Medical Research Laboratories, Toronto, Canada, 10 U/ml of blood) from normal volunteers were sedimented for 60 min with equal volumes of 3% dextran (6% dextran in saline, Abbott Laboratories, North Chicago, Ill., diluted to 3% with normal saline solution). The supernatant containing plasma, leukocytes, and platelets was divided into two aliquots and centrifuged at 280 g for 12 min. The white blood cell (WBC) buttons were resuspended in Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.) with 10% autologous serum to give a concentration of about 5 x 10^6 leukocytes/ml. 1 mg/ml of hydrocortisone-21-succinate (HC) (Sigma) was added to one of the cell suspensions. The suspensions were then rotated end over end at 12 rpm at 37°C, with or without staphylococci or candida. Slides were prepared by allowing the cells to settle on coverslips in a humid 5% CO₂ in air incubator at 37°C for 10 min. The coverslips were then rapidly dried with compressed air and stained with Wright's stain. 100 PMN were examined from preparations that had been incubated with bacteria for 15, 30, and 60 min and the numbers of intracellular bacteria were recorded.

Phagocytosis and bactericidal capacity

The bacteria used in these studies included S. aureus (strain 502A), Serratia marcescens (nonpigmented), Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli (strain 011B4), Pneumococcus (Type 19), group A streptococcus, Staphylococcus albus, Enterococcus, and Salmonella typhimurium. The organisms were grown for 18 hr in trypticase soy broth, washed twice in sterile saline, and then diluted 100 times original volume. A WBC button was prepared from 10 ml of heparinized peripheral blood as described above. The cells were then washed with heparinized saline (25 U of heparin in 100 ml of saline), divided into two aliquots, and resuspended in 3.6 ml of Hanks' solution and 0.4 ml of freshly obtained autologous serum. HC was then added to one aliquot to give a final concentration of 2.1 mM. The cell suspensions were placed in screw top siliconized tubes (15 x 75 mm) with 0.2 ml of bacterial suspension. This usually gave a ratio of one bacterial cell per PMN. The suspensions were immediately inverted by inversion and 0.6 ml was removed and placed in an ice bath. This was the "0 time" specimen. The rest of the mixture was placed in a 12 rpm/rotator in an incubator at 37°C. At 15, 30, and 60 min 0.6 ml of the mixture was removed; and total, supernatant, and sediment bacterial counts were performed on each specimen to determine the fate of bacteria in the mixture (9).

Degranulation

Histochemistry. Slides of leukocytes were prepared as described above, both before and after phagocytosis of heat-killed staphylococci or candida. Leukocytes were then stained for peroxidase (10), an enzyme which is primarily granule-associated. Acid phosphatase. The influence of HC on the distribution of acid phosphatase activity in leukocytes before and after phagocytosis of staphylococci was studied. A modification of the methods of Malawista and Bodel (11) was used. The WBC were separated from heparinized peripheral blood by dextran sedimentation; erythrocytes remaining in the supernatant were lysed with distilled water for 10 sec. Approximately 20 x 10^6 WBC were suspended in 3 ml of Hanks' balanced salt solution with 10% autologous serum and were tumbled end over end with or without 10 g of boiled S. aureus for 15, 30, or 60 min. Identical suspension were prepared with 2.1 mM HC. The leukocytes were then washed, lysed in 0.34 M sucrose, and homogenized with a Teflon pestle for 5 min in order to disrupt the leukocytes but not the granules. The mixture was spun at 8200 g for 20 min to sediment the granules. After lysis of intact granules with saponin, acid phosphatase activity in the granule fraction was measured by dephosphorylation of n-nitrophenol phosphate (Sigma). The acid phosphatase activity of the incubation media and the supernatant material obtained after the granules were spun down was also determined.

Leukocyte viability was evaluated by examining preparations incubated with equal volumes of 1% trypan blue. Exclusion of dye from the nucleus was used as an indication of viability (12).

Electron microscopy

Leukocyte suspensions were prepared from the blood of normal donors as described above and incubated at 37°C in the presence or absence of 2.1 mM HC and with S. aureus (502 A) in ratios of 1 staphylococcus:1 PMN, or 10 staphylococci:1 PMN. Incubations were stopped after 15, 30, and 60 min by ic, and the tubes were centrifuged at 200 g for 8 min.

The pellets were fixed for 24 hr at 4°C in 2.5% glutaraldehyde buffered at pH 7.4 with 0.1 M phosphate. They were postfixed in osmic acid in the same buffer at 4°C for 3 hr, dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812 (13). Thin sections were cut with an ultramicrotome, placed on copper grids, stained with uranyl acetate (14) and/or lead citrate (15), and examined and photographed with a Philips EM300 electron microscope at original magnifications of 2800-50,000 X. Leukocytes incubated in the presence of hydrocortisone were compared with their corresponding controls from each time interval.

Leukocyte oxygen consumption

Leukocytes were separated from peripheral blood by dextran sedimentation of erythrocytes. The erythrocytes remaining in the supernatant with the leukocytes were lysed with icc distilled water for 10 sec (11). Suspensions of 1-3 x 10^6 leukocytes in Hanks' solution with 10% autologous serum plus 5 x 10^9 boiled staphylococci and varying concentrations of HC were placed in the chambers of a polarograph oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, Model 53) (16).

Nitro blue tetrazolium dye reduction

The Nitro blue tetrazolium (NBT) test was done by the method of Bachner and Nathan (17) as modified by Windhorst, Holmes, and Good (18). Cells were incubated with the dye and latex particles with and without 2.1 mM HC.
and smears were examined to determine the extent of cellular reduction of NBT.

Quantitative Nitro blue tetrazolium dye reduction

The methods of Baehner and Nathan were modified (19). 15 ml of blood was allowed to sediment for 60 min with 15 ml of 3% dextran and 150 U of heparin. The supernatant was removed and centrifuged at 200 g for 10 min. 3 ml of iced distilled water was added to the resulting button and the suspension was mixed for 20 sec to lyse erythrocytes. 1 ml of 3.6% heparinized saline was added to restore isotonicity and the suspension centrifuged at 200 g for 15 min. The cell button was then washed in 5 ml of heparinized Hanks' balanced salt solution. A leukocyte count and chamber differential was performed at this time. Four 15-ml siliconized glass tubes were prepared with 1 mM KCN, 0.4 mg of Nitro blue tetrazolium, and 0.1 ml of the previously prepared cell suspension in a total volume of 1 ml of Hanks' solution. Approximately 10^6 boiled staphylococci were added to two of the tubes and 1 mg/ml of HC was added to two of the tubes so as to provide the following mixtures: (a) WBC alone, (b) WBC + 2.1 mM HC, (c) WBC + Staph, and (d) WBC + Staph + 2.1 mM HC. The tubes were incubated in a Dubnoff shaker at 37°C for 15 min at which time 10 ml of iced normal saline was added to each tube and the tubes were centrifuged in the cold at 200 g for 15 min to sediment cells and cell-associated reduced NBT. 10 ml of 0.5 M hydrochloric acid was added to the buttons and extractions, and a spectrophotometric determination of reduced NBT was done as described by Baehner and Nathan (19).

NADH oxidation by WBC extracts

The methods of Cagan and Karnovsky (20) were modified as follows. 50 ml of blood was sedimented with 50 ml of 3% dextran and 500 U of heparin. A leukocyte button was prepared by centrifugation of the supernatant at 200 g for 15 min. The erythrocytes were twice lysed with distilled water as described above and the cell suspension was added and resuspended in 3.6 ml of Hanks' balanced salt solution. To this suspension was added 0.4 ml of autologous serum and 10^6 boiled S. aureus. The cell-bacterial mixture was tumbled for 15 min at 37°C and then centrifuged in the cold at 200 g for 15 min. 2 ml of isotonic alkaline KCl was added to the button and the resulting suspension was homogenized with a Teflon pestle in ice for 5 min. The homogenate was spun down at 8200 g for 20 min in a cold Spinco ultracentrifuge. The oxidation of NADH to NAD was measured at 340 nm on a Beckman DU spectrophotometer. The standard was distilled water and each cuvette contained (a) 0.8 ml of supernatant from the ultracentrifugation, (b) 0.8 ml of pH 7.0 potassium phosphate buffer with 3 mM KCN, and (c) NADH (Sigma Chemical Co., St. Louis, Mo.) in 0.8 ml of pH 7.0 potassium phosphate buffer to give a final concentration of 1.2 X 10^{-4} M NADH in the cuvette. HC was added to one cuvette to give a final concentration of 2.1 mM. The protein content of the cell homogenate was determined by the Folin-Lowry method (21), and the results were expressed as change in OD416/mg of protein per 20 min.

Hydrogen peroxide production by leukocytes

50 ml of blood from normal donors was sedimented with 50 ml of 3% dextran for 60 min. The leukocyte rich supernatant fluid was centrifuged at 200 g for 5 min and the resulting pellet was subjected to three 20-sec rinses of distilled water (as described above) in order to eliminate most of the erythrocytes. Preparations were incubated with or without 2.1 mM hydrocortisone and heat-killed S. aureus at a ratio of 20 bacteria per PMN. After 30 min of incubation, the suspensions were iced and centrifuged at 200 g for 5 min. The hydrogen peroxide content of the pellet was determined by spectrophotometric determination of the oxidation of titanium tetrachloride (22).

Effect of hydrocortisone on peroxide production by pneumococci

Plates containing benzidine and lysed erythrocytes were prepared as described by Kraus, Nickerson, Perry, and Walker (23). Peroxide-producing organisms caused the media to blacken. Pneumococci were streaked over the plate and 0.5 ml of 2.1 mM HC in Hanks' balanced salt solution was put in one metal well, and Hanks' solution alone was put in another well. The plates were incubated and examined after 18 hr.

RESULTS

Phagocytosis. Phagocytosis was similar in normal PMN and PMN incubated with 2.1 mM HC at several

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multiplicities of bacteria to leukocytes. At 15 min, 100 control PMN contained 506 ±151 (SEM) staphylococci, and 100 HC-treated PMN contained 471 ±130 staphylococci (P > 0.4 for four paired experiments). The 30-min values were 614 ±245 staphylococci found in 100 control PMN and 568 ±203 staphylococci found in 100 HC-treated PMN (P > 0.2 for four paired experiments). After 60 min, 707 ±199 staphylococci were found in 100 control PMN and 720 ±169 staphylococci were found in 100 HC-treated PMN (P > 0.5 for three paired experiments).

Phagocytosis and bactericidal capacity. Phagocytosis also appeared similar when studied by differential centrifugation tests (Fig. 1) but intracellular killing, as measured by quantitation of viable cell-associated bacteria, was markedly impaired in HC-treated cells (Ta-
ble 1). This observation was consistent with all organisms tested. When concentrations of HC of 0.21 mM or less were used, no consistent effect on intracellular killing was noted. 98–100% of PMN incubated with or without 2.1 mM HC for 60 min were viable as determined by nuclear exclusion of trypan blue.

Degranulation. Both control and HC-treated polymorphonuclear neutrophils showed abundant blue-staining, peroxidase-positive granules. After phagocytosis of boiled staphylococci or candida, the number of granules staining positively for this enzyme decreased markedly in both HC-treated cells and control cells. The enzyme stain could be seen localized in the phagosomes indicating transfer of lysosomal contents to the phagocytic vacuole.

The distribution of the granule-associated enzyme, acid phosphatase, before and after phagocytosis was used as a measure of degranulation. Total leukocyte acid phosphatase activity decreased in both HC-treated and control leukocytes after 15, 30, and 60 min of incubation. The decrease was maximal at 15 min and did not change with increasing time with the 20:1 bacteria:PMN multiplicity used. When smaller multiplicities were used, no consistent criteria for degranulation could be ascertained. There was no influence of HC on either total, granule-associated, or supernatant acid phosphatase values (Table II). A marked decrease in the total amount of acid phosphatase activity recoverable from phagocytizing WBC was noted in these studies and in similar experiments by Malawista and Bodel (11) and Holmes, Sater, Rodey, Park, and Good (24).

Electron microscope studies. The HC-treated leukocytes exhibited no morphologic differences from the corresponding control leukocytes harvested after the same period of incubation. HC-treated leukocytes indicated many bacteria and seemed to degranulate as extensively as did their untreated controls (Fig. 2).

Leukocyte oxygen consumption. The normal post-phagocytic increase in oxygen consumption was diminished in HC-treated PMN (Fig. 3).

Nitro blue tetrazolium dye reduction. After phagocytosis, 60–80% of PMN contained tiny particles of blue dye in both HC-treated and untreated preparations. The total amount of blue precipitate seemed greater in the PMN without HC. In order to quantitate this difference, the amount of reduced NBT was measured spectrophotometrically as described below.

Quantitative Nitro blue tetrazolium dye reduction. Since hydrocortisone itself can reduce NBT (25), only reduced dye actually in the cell buttons was determined. Fig. 4 shows that HC-treated PMN reduce less NBT after phagocytosis than do untreated PMN, confirming the impressions of the qualitative test.

NADH oxidation by WBC extracts. Oxidation of NADH to NAD by WBC extracts was inhibited in the presence of HC (Table III). When WBC were incubated with HC and then washed and the assay was performed without HC in the cuvette, there was no diminution of NADH oxidation (Table IV).

Hydrogen peroxide production by leukocytes. Phagocytizing leukocytes that were treated with hydrocortisone produced less hydrogen peroxide than control preparations (Table V). Other experiments showed that 2.1 mM HC did not cause the breakdown of H$_2$O$_2$.

Hydrogen peroxide production by pneumococci. 2.1 mM HC in Hanks’ solution placed in a well caused inhibition of hydrogen peroxide formation by pneumococci. Control wells containing Hanks’ solution without HC did not affect pneumococcal peroxide production.

![Figure 3](image-url) **Figure 3** The influence of HC on the oxygen consumption of phagocytizing leukocytes. Boiled staphylococci were added to leukocytes incubated with varying concentrations of HC in a polarographic oxygen monitor.

![Figure 4](image-url) **Figure 4** The amount of cell-associated reduced NBT (expressed as OD$_{515}$) increased after incubation of both control and HC-treated leukocytes with boiled staphylococci (staph) for 15 min. However, HC-treated phagocytizing leukocytes reduced less of the dye than control phagocytizing leukocytes ($P < 0.01$ for paired data).
The study leukocyte bactericidal activity. After phagocytosis these abnormal CGD cells seem to degranulate normally (9, 26, 27) but are deficient in postphagocytic hexose-monophosphate shunt activity, oxygen consumption, and hydrogen peroxide production (16). Recently Baehner and Karnovsky reported that these cells are deficient in NADH oxidase (7).

The oxidation of NADH in the presence of NADH oxidase may be necessary for the production of hydrogen peroxide, and recent evidence suggests that hydrogen peroxide stimulates the direct oxidation of glucose by means of the hexose-monophosphate shunt (28).

Our studies indicate the importance of NADH oxidation and hydrogen peroxide production to normal antibacterial activity by polymorphonuclear neutrophils and shed some light on the sequence of postphagocytic metabolic events.

In order to create a model of chronic granulomatous disease leukocytes, NADH oxidation was inhibited with hydrocortisone-21-succinate. Hydrocortisone has been shown to be a potent inhibitor of NADH oxidase in several other biological systems (8). Large doses were required to inhibit this enzyme in intact leukocytes. The concentration of 2.1 mM is about 100 times greater than that achieved in the blood of an adult after 500 mg of hydrocortisone-21-succinate intravenously (29).

Although some studies have reported that leukocyte migration (30) and phagocytosis (31) is diminished by hydrocortisone, our data indicate that phagocytosis was not impaired by this drug. Since we assayed phagocytosis in a system where active tumbling of white cells

Table II

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<th>Acid Phosphatase Activity of Leukocyte Fractions (Sigma units/10^7 WBC)</th>
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<td>Total activity</td>
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Numbers of experiments performed are shown in parentheses. The SEM is indicated. There was no difference noted when HC-treated cells were compared with control cells (P > 0.5). When WBC alone were compared with WBC + staph or WBC + HC + staph, total activity and granule-associated activity were significantly different (P < 0.01). Supernatant activity was similar (P > 0.5) but the per cent supernatant activity was significantly different (P < 0.05) (see text for details).

Table III

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<th>NADH Oxidation by Leukocyte Extracts</th>
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<td>△OD/20 min per mg of protein</td>
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A greater change in OD indicates more NADH converted to NAD. Thus, HC diminished the oxidation of NADH to NAD by leukocyte extracts. 2.1 mM HC alone did not reduce NADH.

Table IV

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Leukocytes were incubated with 2.1 mM HC and staphylococci. The cells were thoroughly washed and extracts prepared as described in the text. Incubation of extracts from WBC pretreated with HC (but with no HC in the cuvette) oxidized NADH to the same extent as did control leukocytes.
and bacteria takes place, the steps of chemotaxis and leukocyte locomotion are bypassed.

The defect in bactericidal activity noted with HC-treated PMN is similar to that seen in leukocytes of patients with CGD, with the exception that leukocytes from patients with CGD are able to kill certain hydrogen peroxide-forming organisms normally while HC-treated PMN are not. This is probably because hydrogen peroxide formed by the bacteria compensates for the lack of peroxide formed by the CGD cell (32 33). In essence, the bacterium produces the hydrogen peroxide that is instrumental in its own destruction. Pneumococci, which produce hydrogen peroxide, probably do so by means of an enzyme system utilizing NADH oxidase (34). Hydrocortisone inhibits this enzyme system in the bacterium as it does in the leukocyte. Thus, these microorganisms are unable to produce hydrogen peroxide in the HC-treated cell and do not aid in their own destruction.

Weissmann has shown that hydrocortisone stabilized liver lysosomes (35), and initially we considered that the mechanism of action of hydrocortisone might be through this route. However, morphological, histochemical, and electron microscopic studies, plus quantitative enzyme analyses, all showed that hydrocortisone did not prevent normal degranulation of PMN.

Degranulation follows phagocytosis and probably follows other metabolic changes noted in the leukocyte (36). This study suggests that degranulation is not dependent on either the postphagocytic oxygen burst or postphagocytic hydrogen peroxide production.

Thus, by inhibiting NADH oxidation in normal neutrophils with HC, abnormalities similar to those found in neutrophils from patients with chronic granulomatous disease are seen. Inhibition of NADH oxidation leads to a lesser than normal postphagocytic stimulation of cyanide-insensitive oxygen consumption, and in turn, to a diminution of the normal postphagocytic hydrogen peroxide formation. Since hydrogen peroxide is essential for normal intracellular killing by the neutrophil, the bactericidal capabilities of neutrophils are strikingly impaired by HC inhibition of NADH oxidase.

ACKNOWLEDGMENTS

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