Reduced Germination of *Clostridium botulinum* Type A Spores In Vitro by Polymorphonuclear Leukocytes from Chronic Granulomatous Disease

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Leukocytes from two patients with chronic granulomatous disease of childhood (CGD) and from one female carrier were examined for their capacity to induce germination of *Clostridium botulinum* type A spores in vitro. Normal human leukocytes induced germination of *C. botulinum* spores to the same extent as guinea pig neutrophils. Germination was depressed by more than half when cells from CGD patients were used. A noticeable, but less severe, abnormality was present in leukocytes from a female carrier for this X-linked trait. CGD leukocytes are defective in cellular production of H₂O₂, and the latter is known to be effective in inducing germination of clostridial spores. Lysozyme is also known to be effective in spore germination and is present in CGD leukocytes in normal amounts. The possibility that *C. botulinum* spores require a mechanism which sensitizes them to the action of H₂O₂ and lysozyme is raised, and it is suggested that the methodology used in this work measures a defect of such a mechanism in CGD leukocytes.

Germination of *Clostridium botulinum* type A spores has been examined in various animal systems upon intramuscular (13) and intraperitoneal challenges (9, 10). When *C. botulinum* type A spores are recovered from the peritoneal cavity of mice, a loss of heat resistance is noted, indicating conversion to germinated spores or vegetative cells (2), and special staining techniques suggest that intraperitoneal spores germinate within the leukocytes (3).

Isolated guinea pig leukocytes can induce spore germination as measured by conversion of heat-resistant spores to heat-sensitive forms by 6 to 8 hr with concomitant release of botulinic toxin (22). Spore germination within leukocytes in vitro can also be observed by measuring release of ⁴⁰Ca from labeled spores (23). Both germination and botulinic toxin release from engulfed spores in the in vitro leukocyte system can be blocked by inhibition of glycolysis in the pentose phosphate pathway (Suzuki, Benedik, and Grecz, unpublished data).

Since addition of metabolic inhibitors directly affects spore metabolism, these findings were further evaluated utilizing leukocytes which are genetically defective in limited aspects of metabolism. Such leukocytes are found in patients with chronic granulomatous disease of childhood (CGD; reference 7). CGD cells engulf normally, but intracellular events are defective. This is associated with a profound depression of the pentose pathway as measured by the utilization of the first carbon of glucose. In addition, the burst of oxygen consumption and production of H₂O₂ which normally occurs in association with phagocytic uptake is greatly reduced (12). Thus, such cells offer a unique opportunity to examine the effects of selected aspects of leukocyte metabolism on such living systems as bacterial spores.

**MATERIALS AND METHODS**

**Patients.** Two male children with CGD were available for study. Both had been shown to have defective leukocytes by bactericidal assay, nitro-blue tetrazolium reduction, and metabolic assays including glucose-¹⁴C utilization and formate oxidation (Katz et al., unpublished data). Both boys clearly had the X-linked form of the disease in that one (M.S.) was the proband in the S-P family reported in the original genetic study (26). The other (T.W.) patient's mother had been shown to be a carrier by the leukocyte bactericidal assay utilizing *Staphylococcus aureus*. In addition, when her leukocytes were incubated with tetrazolium dyes, there appeared to be some normal cells as well as some with a defect similar to that of her son's cells (25, 27). This mother's cells were also examined with the *C. botulinum* germination assay described below.

**Culture methods.** Spores of *C. botulinum* type A strain 33A were grown at 30 °C in 5% Trypticase,
0.5% peptone, 0.1% sodium thioglycolate broth (TP broth), containing 10 μCi of 45Ca (International Chemical and Nuclear Corp., Irvine, Calif.). After 6 days, radioactive spores were harvested by centrifugation at 200 × g for 40 min and cleaned with trypsin and lysozyme as previously described (8).

Preparation of human leukocytes. Venous blood from CGD patients and from suitable controls was placed in plastic centrifuge tubes (30 by 115 mm; Falcon Plastics, Oxnard, Calif.) containing 10 units of heparin per ml in 20% clinical dextran in saline solution and allowed to stand at room temperature for 45 min. Dextran effects sedimentation of erythrocytes by increasing rouleaux formation.

The supernatant plasma containing leukocytes, platelets, and some erythrocytes was withdrawn with a Pasteur pipette and centrifuged at 50 × g for 10 min. The cell-free plasma was decanted and the leukocyte pellet was washed with heparinized (1 unit/ml) Hanks balanced salt solution and resuspended in a known volume. Polymorphonuclear leukocytes (PMN) and monocytes were counted in a hemocytometer, but small lymphocytes were omitted from the count; thus, cell counts refer to actual numbers of PMN plus monocytes. Lymphocytes were present but usually represented less than 20% of total numbers of cells. After another centrifugation, the cells were resuspended in calcium-free Krebs-Ringer phosphate buffer (pH 7.4) and 30% fresh human adult sera to a final concentration of 2 × 106 cells/ml and used immediately.

Phagocytosis system. One milliliter of 2 × 106/ml of prepared CGD or normal leukocytes and 1 ml of 2 × 107 45Ca-labeled C. botulinum spores per ml were mixed in siliconized test tubes and placed in a shaking water bath at 37 C. At 0, 1, 2, 4, 8, 12, 24, and 48 hr, 0.2-ml samples were filtered through a swinney equipped with a 0.22-μm filter (Millipore Corp., Bedford, Mass.). The filtrate was placed in a glass scintillation vial, to which 10 ml of Bray’s solution (4) was added, and counted in a Beckman LS-200 liquid scintillation counter with a P-32 window for 10 min at 14 C.

RESULTS

The appearance of free 45Ca in 0.22-μm membrane filtrates (Millipore) when C. botulinum type A spores are incubated in vitro with normal human peripheral leukocytes is shown in Fig. 1. This is compared with incubation of the spores in TP broth and in the medium containing human serum which is used for the leukocyte cultures. No germination of C. botulinum spores is observed in the cell-free culture medium as indicated by no release of 45Ca. Leukocyte-induced germination is somewhat less than that of TP broth, as expected, since the latter is an ideal microbiological growth medium for these spores. In both growth systems, germination was clearly present at 8 hr. Maximum release of 45Ca was observed by 24 hr.

Fig. 1. Free 45Ca appearing in 0.22-μm membrane filtrates from in vitro incubation of 10⁷ labeled C. botulinum type A spores per ml and 10⁴ normal (I) human PMN leukocytes per ml. Average of three experiments. Background has been subtracted. Trypticase-peptone broth, TP. Leukocyte cell-free extracts in Hanks balanced salt solution plus serum.

Figures 2 and 3 compare the capacity of leukocytes from three different normal adults (I, II, and III) and leukocytes from two different CGD patients (T.W. and M.S.) to initiate germination. CGD leukocytes, although they can induce some germination, are clearly defective in this regard. This defect is evident at 8 hr and pronounced by 12 to 18 hr. The maximum release of 45Ca was less than half that of normal cells in both instances. The results for patients’ cells are compared with normal cells tested on the same day.

Figure 4 illustrates the findings when cells from the mother of T.W. were examined with this method, and Table 1 compares actual counts per minute, at 24 hr, of control cells, CGD cells, and carrier female cells. The data suggest that carrier cells are also abnormal but not as severely deficient as the patients’ cells in this assay.

In wet mounts and stained smears, the process of engulfment by CGD leukocytes appeared to be normal. By using microscopic evaluation at 15 min, approximately 5 to 7 spores were engulfed per leukocyte in all cultures.
DISCUSSION

In this study, germination of *C. botulinum* spores by phagocytic leukocytes from normal human venous blood was similar to that induced by guinea pig peritoneal PMN leukocytes (24). Under identical in vitro conditions and cell ratios, phagocytic leukocytes from two male patients with CGD were found to be greatly reduced in their capacity to induce *C. botulinum* type A spore germination. It is postulated that this reflects the basic genetic defect in CGD leukocytes and that optimum intracellular germination of *C. botulinum* spores in normal leukocytes requires metabolic events or substances which are defective in CGD cells.

In agreement with previous reports (1, 11, 12), the CGD leukocytes engulfed normally. This feature has been correlated with a normal glycolytic pathway in such cells (11). Thus, even though germination of *C. botulinum* spores was decreased in normal leukocyte mixtures in the presence of inhibitors of glycolysis, it is reasonable to state that glycolysis alone does not induce germination. Rather, it would appear that in this system, as in others (17, 21), the role of the glycolytic pathway is to provide metabolism necessary for phagocytic uptake and that intracellular action upon the engulfed material depends on other processes, some of which are defective in CGD cells.

CGD leukocytes do have a profound defect in the burst of oxygen utilization, glucose-1-14C utilization, and H2O2 production (measured by formate oxidation) which normally occurs during phagocytic uptake (11).

Gould and Hitchins (7) presented evidence that H2O2 could cause changes in spores of *Bacillus* and *Clostridium* which resulted in phase darkening and loss of dipicolinic acid typical of normal germination. However, this occurred only if the spores were previously treated in a manner known to rupture protein disulfide bonds. Similar pretreatment was required to make such spores susceptible to lysozyme. This latter agent could be used in the system in such a way as to induce the changes characteristic of germination but with no detrimental effect on spore viability. H2O2, on the other hand, seemed to cause progressive spore lysis in addition to the germinative changes. The authors postulated that disulfide bond rupture in spore coats permitted the action of lysozyme or H2O2 on previously protected substrates with consequent germination.

**FIG. 2.** Free 45Ca appearing in 0.22-μm membrane filtrates from in vitro incubation of 10⁶ labeled *C. botulinum* type A spores per ml and 10⁵ PMN leukocytes from a patient, TW, with chronic granulomatous disease per ml. Average of three experiments. Background has been subtracted. Normal cells (II and III) were examined on the same day.

**FIG. 3.** Free 45Ca appearing in 0.22-μm membrane filtrates from in vitro incubation of 10⁶ labeled *C. botulinum* type A spores per ml and 10⁵ PMN leukocytes from a patient, M.S., with chronic granulomatous disease (CGD) per ml. Average of three experiments for normal cells and two experiments for CGD (M.S.). Background has been subtracted.
Although the defective \( \mathrm{H}_2\mathrm{O}_2 \) system in CGD cells may be critical for explaining our observations, it must be noted that the CGD leukocytes contain normal amounts of lysozyme (19). Hence, if the findings of Gould and Hitchin apply in this system it might be expected that germination could proceed in CGD leukocytes in a slowed but ultimately fully manifest manner since, as an enzyme, lysozyme could continue to act as long as substrate were available. Instead, total amounts of \( ^{45}\mathrm{Ca} \) liberated are markedly reduced. It is possible, of course, that in regard to spore germination in the leukocytes the role of lysozyme may be minor, with \( \mathrm{H}_2\mathrm{O}_2 \) being more important for triggering the germination events.

On the other hand, spore germination may reflect somewhat different aspects of leukocyte action than does bacterial kill. In this regard, it seems reasonable to expect that leukocytes could affect changes in spore coats similar to those caused by the pretreatment used by Gould and Hitchins to make the spores susceptible to lysozyme and \( \mathrm{H}_2\mathrm{O}_2 \) action. If this hypothetical leukocyte capacity were linked metabolically to the generation of \( \mathrm{H}_2\mathrm{O}_2 \), a defect at that point could then result in defects of both systems. As an approach to examining this question, the concept of reduction of spore protein disulfide bonds discussed by Gould and Hitchins might be considered. Thus, examination for leukocyte protein disulfide reductase activity would be appropriate.

The possibility that male CGD leukocytes are manifesting a defect in handling of bacteria which is not limited to the failure to produce \( \mathrm{H}_2\mathrm{O}_2 \) is quite important. Several other genetic defects in leukocyte function have been described (28), including several instances of decrease in leukocyte \( \mathrm{H}_2\mathrm{O}_2 \) production, in which the clinical disease is much less severe than in these boys. The non-X-linked female patients with CGD are a prime

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<th>Table 1. Counts per minute of ( ^{45}\mathrm{Ca} ) released in membrane filtrates of leukocytes from normal and chronic granulomatous disease patients.</th>
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<td>Patient (^{b} )</td>
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<td>Normal</td>
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<td>CGD carrier</td>
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<td>CGD (MS)</td>
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<td>CGD (TW)</td>
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\(^{b} \) Membrane filter: 0.22-\( \mu \)m, Millipore Corp., Bedford, Mass. Leukocytes incubated 24 hr in vitro with labeled Clostridium botulinum type A spores.

\(^{b} \) CGD, chronic granulomatous disease.
example, in that their cells are, in nearly every measurable way, like those of the males with CGD. The women with lipochrome histiocytosis described by Ford et al. (5) have been shown by Rodey et al. (20) to have the leukocytes which are similarly defective. However, the female CGD patients and Ford’s patients seem to live longer and have fewer life-threatening infections than the male CGD patients, suggesting that the X-linked form has more profound abnormalities which are, as yet, unmeasured. Additionally, a rare genetic absence of neutrophil and monocyte myeloperoxidase has been reported in one family (18). This defect causes a severe depression of the leukocyte peroxidase-H2O2-halide bactericidal system, yet one affected adult is quite healthy and the other is an adult male whose chronic candidiasis is far from the lethal disease seen in X-linked CGD.

Even if these considerations should prove invalid, the work reported in this paper demonstrates that leukocyte action on the spores of *C. botulinum* involves selective aspects of leukocyte metabolism. It is possible that this action is a general one for many types of bacterial spores and that understanding this process may shed further light on host-parasite interactions.

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**LITERATURE CITED**


