A STUDY OF METABOLIC FACTORS INVOLVED IN THE INTRACELLULAR GERMINATION OF *CLOSTRIDIUM BOTULINUM* SPORES AFTER PHAGOCYTOSIS

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THE chemical environment within the metabolically active leucocyte during the phagocytic act is conducive to intracellular spore germination. This leucocyte-induced germination of *Clostridium botulinum* type A spores has been studied in various in-vitro systems after Booth, Suzuki and Grecz (1971a) reported that heat-resistant spores of this organism are converted to heat-sensitive germinated spores and vegetative cells in the peritoneal cavity after they have been injected intraperitoneally (IP) into mice.

These workers used new staining techniques (Booth, Suzuki and Grecz, 1971b) to demonstrate phagocytosis by leucocytes *in vivo* and *in vitro* and to show subsequent germination of the spores within the leucocyte. Furthermore, Suzuki, Booth and Grecz (1970) recently found that *Cl. botulinum* spores must be actively phagocytosed to release toxin. The production of an equivalent ionic environment or exposure of the spores to leucocyte cytoplasmic extracts did not, by themselves, induce spore germination or toxin release. Studies involving the implantation of Millipore diffusion chambers containing spores alone or spores plus leucocytes into the peritoneal cavity of guinea-pigs further supported this concept (Suzuki, Booth, Benedik and Grecz, 1971). Ten per cent. of the animals given chambers containing spores alone died with typical symptoms of botulism, whereas chambers containing spores plus leucocytes killed 90 per cent. of the test guinea-pigs. Passively immunised guinea-pigs were able to survive the implantation of chambers containing *Cl. botulinum* spores plus leucocytes. Thus, it has been established that polymorphonuclear (PMN) leucocytes can provoke a pathogenic state when interacting with *Cl. botulinum* spores.

During phagocytic engulfment, lactic acid accumulates as a result of glycolysis and causes a rapid decrease in pH of the phagocytic vacuole (Cohn, 1968) to values as low as pH 3.0–4.5 (Rous, 1925; Rowley, 1962). The pentose phosphate pathway present in peripheral leucocytes (Bona, Macovschi and Gheordunescu, 1969; Morton, Moran and Stjernholm, 1969) generates its metabolic by-product H₂O₂ and brings the reaction of the phagocytic vacuole to near neutrality. Concurrently, lysosome degranulation proceeds and brings into the proximity of the phagocytic vacuole a multitude of enzymes that include lysozyme and myeloperoxidase; the latter forms an effective bactericidal complex with H₂O₂ (Klebanoff, 1968, 1970; Paul *et al.*, 1970).

The purpose of the present study was to determine the relationship of metabolic processes within the PMN leucocyte to the induction of intracellular *Cl. botulinum* spore germination and subsequent toxin release.

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MATERIALS AND METHODS

Culture methods. A culture of *Clostridium botulinum* type A, strain 33A was grown at 30°C in 5 per cent. trypticase (Baltimore Biological Labs, Baltimore, Md), 0.5 per cent. peptone (Difco, Detroit, Mich.), 0.1 per cent. sodium thioglycollate and 20 µCi per litre of ⁴⁵Ca (International Chemical and Nuclear Corp., Irvine, Calif.). After 5–6 days, the radioactively labelled spores were harvested by centrifugation in a Sorvall RC-2 continuous flow centrifuge, and vegetative contaminants cleaned with trypsin and lysozyme as previously described (Grecz, Anellis and Schneider, 1962). This treatment does not affect spore resistance. ⁴⁵Ca-labelled spores were stored in 0.85 per cent. NaCl in water at 2°–4°C.

Germination of bacterial spores was ascertained by observing the release of Ca (Powell and Strange, 1953) in the form of ⁴⁵Ca as described by Suzuki, Booth and Grecz (1971b).

Leucocytes. Venous blood samples from three healthy male donors and two patients with chronic granulomatous disease (CGD) were separately held in 30×115 mm Falcon plastic centrifuge tubes (Falcon Plastics, Oxnard, Calif.) containing heparin 10 units per ml in 20 per cent. clinical dextran in saline solution (Travenol, Morton Grove, Ill.) and allowed to stand at room temperature for 45 min. Dextran promotes sedimentation of erythrocytes by increasing rouleau formation.

The supernatant plasma containing leucocytes, 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 leucocyte pellet washed with heparinised (1 unit per ml) Hanks' balanced salt solution at pH 7.4 (HBSS, Grand Island Biol. Co., Grand Island, N.Y.) and resuspended in a known volume. PMN leucocytes and monocytes were counted in a haemocytometer, but small lymphocytes were omitted from the count; thus, cell counts refer to actual numbers of phagocytic cells. Lymphocytes were present but usually represented less than 20 per cent. of total numbers of cells. After another centrifugation, the cells were resuspended to a final concentration of 2×10⁷ cells per ml in calcium-free HBSS with 30 per cent. fresh human adult serum.

Phagocytic index. Phagocytic indices were determined by the radioisotope method of Suzuki *et al.* (1971a) and expressed as percentages of total labelled spores engulfed.

Phagocytosis systems with metabolic inhibitors

All in-vitro incubations were carried out at 37°C in a shaking waterbath. Siliconised, sterile, Falcon plastic centrifuge tubes were used. Human PMN leucocytes and ⁴⁵Ca-labelled spores of *Cl. botulinum* type A were added to HBSS with 30 per cent. human adult serum (Animal Blood Center, Syracuse, N.Y.) to give final concentrations of leucocytes at 10⁷ per ml and spores at 10⁸ per ml. Control suspensions were then compared with leucocyte-spore suspension to which the following inhibitors were separately added: Glyceraldehyde (final concentration of 8 mM) was added to the first test-tube concurrent with the addition of ⁴⁵Ca-labelled spores to inhibit leucocytic glycolysis and, ultimately, phagocytosis (Selvaraj and Sbarra, 1966). Colchicine (0.04 mM) was introduced to a second leucocyte-spore suspension. This drug does not impair engulfment processes, but it interferes with leucocyte lysosomal degranulation during phagocytosis (Malawista and Bodel, 1967). The anti-thyroid agent, methimazole (1-methyl-2-mercapto imidazole), which inhibits thyroidal iodination, was found to inhibit the iodination reaction in leucocytes (Klebanoff and White, 1969). Accordingly, methimazole at a final concentration of 0.5 mM was added to a third leucocyte-spore suspension and incubated at 37°C. At the concentration employed, methimazole had no effect on the phagocytosis of micro-organisms as determined by enumeration of intracellular bacteria in stained smears (Klebanoff and White). Azide also has no effect on phagocytosis or microbicidal activity at 0.1 mM (Klebanoff, 1970), and this was tested in a fourth leucocyte-spore suspension. Azide forms complexes with the iron of haem-containing enzymes such as myeloperoxidase, and therefore inhibits these enzymes. To a fifth in-vitro phagocytic system, methimazole and azide were added simultaneously at the concentrations

employed above, thereby effecting a metabolic block at both the iodination and myeloperoxidase sites. A sixth leucocyte system was exposed to hydrocortisone (0.21 mM). Hydrocortisone selectively inhibits NADPH oxidase and therefore causes a lack of H_2O_2 production (Mandell, Ruben and Hook, 1970). The site of action of these metabolic inhibitors is summarised in the figure.

FIGURE.—Known metabolic pathways and physiological processes in the leucocyte during phagocytosis.

The numbers refer to the following metabolic inhibitors or genetic blocks that interfere with normal phagocyte function at the sites indicated: (1) glyceraldehyde, (2) chronic granulomatous disease, (3) colchicine, (4) hydrocortisone, (5) methimazole, (6) azide.

At specific time intervals (0, 1, 2, 4, 6, 8, 12, 24 and 48 hr), 1.2-ml volumes of each test mixture were filtered through a Millipore Swinney device equipped with a $0.22 \mu\text{m}$ filter. A 0.2-ml sample of this filtrate was placed in a glass scintillation vial with 10 ml Bray's solution (Bray, 1960) and radioactivity was measured in a Beckman LS-200 Liquid Scintillation Counter at 14°C for 10 min. The remainder of the filtrate (1 ml) was injected IP into two mice to assay for botulinal toxin; one of the two mice passively immunised with type-A botulinal antitoxin served as a control for this assay.

Phagocytosis system with chronic granulomatous disease cells

One ml of prepared leucocytes (2×10^7 cells per ml) derived from patients with CGD and 1 ml of ^{45}Ca -labelled *Cl. botulinum* spores (2×10^8 per ml) were mixed in siliconised test-tubes

and placed in a shaking waterbath at 37°C. Control mixtures contained prepared normal leucocytes at the same concentration. At 0, 1, 2, 4, 8, 12, 18, 24, 36 and 48 hr, 0.2-ml vols were filtered through a Millipore Swinney device equipped with a 0.22 μ m filter. The filtrate was placed in a glass scintillation vial, and radioactivity was determined as above.

TABLE I

Release of free ^{45}Ca from labelled spores of Clostridium botulinum type 33A in leucocyte cultures as an index of intracellular spore germination in the presence of various leucocyte inhibitors at 37°C

Inhibitor added	Relative amount of free ^{45}Ca released* (counts per min.) from unit volumes of leucocyte-spore mixtures at the stated time (hr) of incubation								
	0	2	4	8	12	18	24	36	48
Control (no inhibitor added)	81	84	89	203	430	1253	1509	1537	1626
Glyceraldehyde	83	85	91	91	103	110	132	183	210
Colchicine	78	82	90	103	142	194	281	314	371
Methimazole	69	73	83	191	403	1201	1489	1530	1588
Azide	71	74	90	188	381	1222	1461	1529	1597
Azide+methimazole	79	79	91	192	392	1241	1496	1526	1584
Hydrocortisone	84	89	94	207	419	1243	1519	1530	1629
Cell-free leucocyte extract with spores	80	81	83	84	87	94	98	103	143

* Free ^{45}Ca determined by counting 0.22 μ m Millipore filtrate of in-vitro leucocyte-*Cl. botulinum* spore systems. Average of three experiments ($P = 0.05$). Background count has been subtracted in each case.

RESULTS

In the control suspension of ^{45}Ca -labelled *Cl. botulinum* spores and leucocytes incubated *in vitro*, ^{45}Ca release representing spore germination occurred at 6–8 hr (table I). ^{45}Ca release correlated with microscopic observations of spore phase-darkening.

The data given in table I indicate that, in the presence of glyceraldehyde or colchicine, ^{45}Ca release was substantially inhibited. Spore germination was depressed to a greater extent with glyceraldehyde than with colchicine. This observation suggests that glycolysis-dependent leucocyte engulfment is the predominant factor in this type of spore germination. However, neither methimazole (0.5 mM) nor azide (0.1 mM), both known inhibitors of the iodine-myeloperoxidase bactericidal system of Klebanoff (1968), significantly affected leucocyte-induced spore germination. Even in combination, azide+methimazole had negligible effects. Phagocytic indices of all leucocyte-spore in-vitro systems containing colchicine, methimazole, azide or hydrocortisone at the stated concentrations were not depressed; this indicates that there was no interference with engulfment. Glyceraldehyde depressed the phagocytic index by 53 per cent.; since glyceraldehyde blocks glycolysis, the metabolic

pathway responsible for phagocytic engulfment, the observed depression is consistent with the findings of Selvaraj and Sbarra. Hydrocortisone, which selectively inhibits NADPH oxidase (Mandell *et al.*, 1970)—the enzyme responsible for generation of H_2O_2 in the leucocyte—was found to have no effect on intraleucocytic *Cl. botulinum* spore germination.

Germination of ^{45}Ca -labelled spores of the test strain *Cl. botulinum* 33A in trypticase-peptone broth was studied by determining the release of free ^{45}Ca . Spores in the bacteriological culture medium to which glyceraldehyde,

TABLE II

Release of toxin from Cl. botulinum spores ingested by polymorphonuclear leucocytes and the influence of various inhibitors on toxin release during incubation at 37°C

Inhibitor added	Number of mice killed* by challenge with filtrate taken during the stated incubation period	
	0-4 hr	6-48 hr
Control (no inhibitor added)	0	5
Glyceraldehyde	0	0
Colchicine	0	0
Methimazole	0	5
Azide	0	5
Methimazole + azide	0	5
Hydrocortisone	0	5

* Total of five mice challenged per experiment at each of the following time intervals: 0, 2, 4, 6, 8, 12, 24 and 48 hr. A dose of 1 ml of filtrate was injected intraperitoneally. Control mice immunised with botulin type A antitoxin survived.

colchicine, methimazole, azide or methimazole plus azide was added at the test concentration showed normal germination patterns compared with control suspensions in the medium alone.

The results summarised in table II suggest that phagocytosis was required for release of *Cl. botulinum* toxin from spores. When *Cl. botulinum* spores are incubated with leucocytes without chemical inhibitors, botulin toxin began to be demonstrable in filtrates at 6 hr. However, no toxin was demonstrable when glyceraldehyde or colchicine was present in the test mixtures. Neither methimazole nor azide inhibited release of toxin; when the two inhibitors were used simultaneously, there was still little effect on release of free ^{45}Ca or botulin toxin.

At 24 hr, trypan blue dye exclusion staining indicated that only 25 per cent. leucocyte viability was lost when treated with metabolic inhibitors and the loss of viability in control non-treated leucocytes averaged 20 per cent.

In table III the capacity to initiate germination by leucocytes derived from a normal patient is compared with that of leucocytes from two different CGD patients (T. W. and M. S.). Although CGD leucocytes can induce some germination, they are clearly defective in this regard. This defect is evident at

8 hr and pronounced by 12–18 hr. The maximum release of ^{45}Ca was less than half that of normal cells in both instances. The results for a patient's cells are compared with those obtained with normal cells tested on the same day.

DISCUSSION

The results of the present study concur with previous findings that spores of *Clostridium botulinum* are capable of germination after phagocytosis by polymorphonuclear (PMN) leucocytes (Suzuki, Booth and Grecz, 1970; Booth,

TABLE III

*Release of free ^{45}Ca from labelled spores of *Cl. botulinum* 33A ingested by normal and chronic granulomatous disease polymorphonuclear leucocytes as an index of spore germination*

Test material	Relative amount of free ^{45}Ca released* (counts per minute) from unit volumes of leucocyte-spore mixtures or leucocyte extract-spore mixtures at the stated time (hr) of incubation								
	0	2	4	8	12	18	24	36	48
Normal leucocytes	94	96	100	108	268	1198	1412	1483	1496
CGD†—T. W.‡	97	99	101	103	124	589	590	691	760
leucocytes									
CGD—M. S.‡	96	96	99	104	133	604	710	753	773
leucocytes									
Cell-free leucocyte extract	80	81	83	84	87	94	98	103	143

* Free ^{45}Ca determined by counting 0.22 μm Millipore filtrates from in-vitro leucocyte-spore systems. Average of three experiments ($P = 0.05$). Background count has been subtracted in each case.

† Chronic granulomatous disease.

‡ T. W. and M. S. are the initials of the CGD patients.

Suzuki and Grecz, 1971a). The inhibitors used in the present study did not prevent spore germination *per se*. This was not unexpected since many of the metabolic inhibitors normally used in leucocyte function tests are not inhibitory to spore germination (Wynne, 1957; Murrell, 1961). Spores of *Cl. botulinum* strain 33A did not proceed to outgrowth when held in Krebs-Ringer phosphate medium + leucocyte extracts + serum at pH 7.4 (Suzuki, Booth and Grecz, 1970).

The data clearly demonstrate that normal physiological functions within the leucocyte, particularly glycolysis and the pentose-phosphate pathway, are involved in triggering the germination of engulfed spores. It is interesting that methimazole and azide were found not to affect leucocyte-induced germination or release of toxin from *Cl. botulinum* spores. Methimazole and azide are known to be selective inhibitors respectively of iodination (Klebanoff and White, 1969) and myeloperoxidase (Klebanoff, 1970), two important bactericidal mechanisms of the leucocyte. These apparently paradoxical results may be explained by the observation that the act of cell killing, i.e., bactericidal

action, which is influenced by methimazole and azide, is immaterial with respect to spore germination because: (i) mature spores are extremely resistant to direct chemical attack such as that found in the leucocytes; (ii) spore germination is not necessarily linked to spore viability (spores killed by radiation are still phase-bright and refractile to stains); and (iii) after germination within the leucocyte, the germinated spores are destroyed by lytic processes that are independent of iodination and myeloperoxidase.

Hydrocortisone blocks the enzyme NADPH oxidase requisite for H_2O_2 production and lysosome degranulation. Evidently H_2O_2 does not play a major role in leucocyte-induced spore germination or toxin release since hydrocortisone did not significantly inhibit spore germination.

The relationship of leucocyte metabolism to changes in the ingested *Cl. botulinum* spore was further evaluated in our studies with the genetically defective leucocytes found in patients with chronic granulomatous disease of childhood (see Good *et al.*, 1968). CGD cells engulf normally, but intracellular events are defective: there is a profound depression of the pentose pathway and, in addition, the burst of oxygen consumption and production of H_2O_2 that normally occurs in association with phagocytic uptake is greatly reduced (Holmes, Page and Good, 1967).

Phagocytic leucocytes from two male patients with chronic granulomatous disease were found to be greatly reduced in their capacity to induce *Cl. botulinum* type A spore germination. It is postulated that this reflects the basic genetic defect in CGD leucocytes and that optimum intracellular germination of *Cl. botulinum* spores in normal leucocytes requires metabolic events or substances that are defective or lacking in CGD cells. Evidently CGD leucocytes engulf normally. Phagocytic engulfment has been correlated with a normal glycolytic pathway in such cells (Holmes *et al.*, 1967). Thus, even though germination of *Cl. botulinum* spores was decreased in normal leucocytes in the presence of inhibitors of glycolysis, it is reasonable to state that glycolysis alone does not induce germination. The role of the glycolytic pathway is to provide metabolism necessary for phagocytic uptake; intracellular action upon the engulfed material depends on other processes, some of which may be defective in CGD cells (i.e., H_2O_2 production, lysosome degranulation).

Gould and Hitchins (1963) presented evidence that H_2O_2 could cause changes in spores of *Bacillus* and *Clostridium* species with resultant 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 disulphide bonds. Similar pretreatment was required to make such spores susceptible to lysozyme. Hydrogen peroxide, on the other hand, seemed to cause progressive spore lysis in addition to the germinative changes. The authors postulated that disulphide bond rupture in spore coats permitted the action of lysozyme or H_2O_2 on previously protected substrates with consequent germination.

Exposure of spores to acidic pH within phagocytic vacuoles could possibly alter protein disulphide bonds enough to allow lytic enzyme molecules, such as lysozyme, to pass through to the underlying susceptible layers. Toxin release

could possibly then be effected by continual action of lysozyme on the germinated spore or the bacterial cell wall of the outgrowing cell.

The depressed spore germination in the CGD leucocyte system may be attributed to a defect in the H_2O_2 -generating system of CGD cells. However, it must be noted that the CGD leucocytes contain normal amounts of lysozyme (Quie *et al.*, 1967) and that hydrocortisone-treatment of leucocytes whereby H_2O_2 production is blocked, did not inhibit germination of the ingested spores.

It is possible then that, in regard to phagocytic activity in the CGD leucocyte, lysosomes may not completely degranulate or fuse with phagocytic vacuoles, thereby providing decreased amounts of lysozyme in the phagocytic vacuole. This would explain a depressed but not totally inhibited induction of spore germination. Although Quie *et al.* contended that lysosomal degranulation is completely absent in CGD cells, others (Holmes *et al.*, 1967; Kauder *et al.*, 1968; Baehner, Karnovsky and Karnovsky, 1969) have demonstrated that this may not be the case. With a CGD defect in lysosomal degranulation, spore germination would be depressed—provided that the lysosomal enzyme, lysozyme, is required for leucocyte-induced spore germination. If lysozyme is responsible for induction of germination, as suggested by the present results, small amounts of the enzyme must be available to engulfed spores enclosed in the phagocytic vacuole. Partial degranulation of lysosomes would account for the observed, although decreased, liberation of ^{45}Ca from labelled spores.

The present results suggest that digestive processes occurring during normal phagocytosis, i.e., glycolysis resulting in acidic pH and the action of lysosomal enzymes (e.g., lysozyme), seem to create conditions conducive to spore germination. It is possible that this action is a general one for many types of bacterial spores and that elucidation of this process may shed further light on some host-parasite interactions.

SUMMARY

The environment within the metabolically active polymorphonuclear leucocyte during phagocytosis is conducive to the germination of phagocytically ingested spores of *Clostridium botulinum* type A. Specific metabolic inhibitors of leucocyte function—glyceraldehyde, hydrocortisone, colchicine, methimazole and azide—were used to study metabolic pathways during phagocytosis that may be responsible for inducing spore germination within the leucocyte.

Leakage of ^{45}Ca from labelled *Cl. botulinum* spores was taken as evidence of spore germination. Release of free ^{45}Ca from phagocytosed spores in spore-leucocyte mixtures in in-vitro systems was normal in the presence of hydrocortisone, methimazole, azide and methimazole+azide. These findings indicate that H_2O_2 production, iodination and myeloperoxidase activities during phagocytosis are not required for germination. In the presence of glyceraldehyde or colchicine, release of ^{45}Ca from spore-leucocyte mixtures was profoundly inhibited and this indicates that glycolysis and lysosome degranulation are essential for intraleucocytic spore germination.

In leucocytes from patients with a genetic abnormality of phagocytosis, i.e., chronic granulomatous disease of childhood (CGD), germination of spores was significantly depressed. Since normal mechanisms of H_2O_2 production and possibly of lysosomal degranulation are blocked in CGD leucocytes, the results suggest that H_2O_2 and lysosomal enzymes are involved in triggering spore germination in this model. Nevertheless, a limited release of ^{45}Ca was observed and this seems to indicate that small amounts of lysosomal enzymes are available for reaction with engulfed spores in CGD leucocytes.

The results of these studies with metabolic inhibitors and genetic blocks lead to the view that germination of spores of *Cl. botulinum* within leucocytes is triggered by the combined action of lowered pH resulting from glycolysis together with lytic enzymes resulting from degranulation of lysosomes.

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