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Spore Membrane(s) as the Site of Damage Within Heated *Clostridium perfringens* Spores[1]

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Clostridium perfringens spores were injured by ultrahigh-temperature treatment at 105 C for 5 min. Injury was manifested as an increased sensitivity to polymyxin and neomycin. Since many of the survivors could not germinate normally the ultrahigh-temperature-treated spores were sensitized to and germinated by lysozyme. Polymyxin reportedly acts upon the cell membrane. Neomycin may inhibit protein synthesis and has surface-active properties. Injured spores were increasingly sensitive to known surface-active agents, sodium lauryl sulfate, sodium deoxycholate, and Roccal, a quaternary ammonium compound. Injured spores sensitive to polymyxin and neomycin also were osmotically fragile and died during outgrowth in a liquid medium unless the medium was supplemented with 20% sucrose, 10% dextran, or 10% polyvinylpyrrolidone. The results suggested that a spore structure destined to become cell membrane or cell wall was the site of injury. Repair of injury during outgrowth in the presence of protein, deoxyribonucleic acid, ribonucleic acid and cell wall synthesis inhibitors was consistent with this hypothesis.

Spores may suffer sublethal damage or injury during heating. Injury may be manifested as an increased sensitivity to inhibitors such as antibiotics (4), NaCl (23), nitrite (16, 24), nitrate (24), or fatty acids (31). Barach et al. (4) reported that *Clostridium perfringens* spores surviving ultrahigh-temperature (UHT) treatments were increasingly sensitive to certain antibiotics. The presence of polymyxin, neomycin, or kanamycin at concentrations that had little effect on the recovery of heat-activated spores severely decreased the recovery of UHT-treated spores. The possible action of the these antibiotics as surface-active agents suggested that the membrane system rather than an enzyme might be the site of injury within the spore.

Barach et al. (5) observed that these injured spores regained resistance to neomycin and polymyxin during outgrowth in the absence of these antibiotics. A study of the repair of injury could provide insight into the site of damage.

The objectives of this study were to examine the metabolic processes, if any, involved in repair and to ascertain the basis for this type of injury.

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MATERIALS AND METHODS

Test organism. The test organism was *C. perfringens*, type A, strain NCTC 8798 and was obtained from Charles L. Duncan, Department of Food and Nutrition, University of Wisconsin, Madison. Maintenance of the culture and preparation of spore suspensions have been described (2).

Media. The enumeration media were: (i) tryptone-sulfite-neomycin agar (TSN) of Marshal et al. (18); (ii) TSN plus lysozyme; (iii) TSN without antibiotics (BASE); (iv) BASE plus lysozyme; and (v) BASE plus various inhibitors. Repair media were: (i) repair broth (RB), prepared as BASE without agar; (ii) RB plus lysozyme; (iii) RB plus lysozyme plus osmotic stabilizers, dextran (10% wt/vol, Schwarz/Mann, Orangeburg, N.Y.; molecular weight, 17,700), polyvinylpyrrolidone (10% wt/vol, Schwarz/Mann), or sucrose (20% wt/vol, Fisher Scientific, Fair Lawn, N.J.); and (iv) RB plus metabolic inhibitors. Aqueous solutions of lysozyme and antibiotics were sterilized by filtration through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). Other inhibitors (sodium deoxycholate, sodium lauryl sulfate, Roccal) were autoclaved (121 C/15 min). Lysozyme, antibiotics, and other inhibitors were stored at 2 C and added to autoclaved (121 C/15 min) RB tempered to 35 C or to autoclaved enumeration media tempered to 47 C prior to use. Lysozyme was added to a final concentration of 5 μg/ml to plating media and 10 μg/ml to repair media. Media used in repair studies were prepared as prereduced media (13). These media contained resazurin (1 μg/ml) as an oxidation-reduction indicator

and cysteine hydrochloride (0.5 mg/ml) as a poising agent. All media were adjusted to pH 7.0 with 1 N NaOH prior to use.

Heat treatments. Portions of aqueous stock spore suspensions (10^8 to 10^9 spores/ml) were heat activated at 75 C for 20 min and UHT treated at 105 C for 5 min by the capillary tube method (2).

Alkali treatment. Spores suspended in prereduced RB or peptone water were adjusted to pH 13.0 with 1 N NaOH and held at 45 C for 15 min, and the pH was readjusted to pH 7.0 with 1 N HCl.

Germination of injured spores in peptone water. Alkali-treated spores were diluted into 0.1% peptone water plus 10 μg of lysozyme per ml and incubated at 35 C for 60 min. Since alkali-treated spores were incapable of germination in the absence of lysozyme (5, 8), the extent of germination was measured as the difference between colony counts on BASE and BASE plus lysozyme.

Measurement of injury and repair. Two types of injury were observed. Injury to the germination system was measured as a difference between colony counts of non-alkali-treated spores on BASE and BASE plus lysozyme. Injury to the spore outgrowth system was measured as a difference between colony counts on TSN or BASE plus inhibitors and BASE. Repair studies were done by diluting UHT alkali-treated spores, with or without germination treatment, into the repair medium and incubating at 35 C. Samples were periodically plated on BASE and TSN, both media with or without lysozyme. Dilutions were made in prereduced RB plus 10% dextran under an atmosphere of 90% N_2-10% CO_2 using the Virginia Polytechnic Institute anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.). Injured and uninjured spores were enumerated by the roll tube method (13). All tubes were incubated at 35 C for 48 h. Increasing colony counts on TSN during incubation while colony counts on BASE remained constant indicated repair.

RESULTS

Effect of germination in peptone water on repair of thermal injury. Barach et al. (5) found that injury was repaired after germination but prior to growth, suggesting that repair occurred during outgrowth. To distinguish germination from outgrowth, survivors were germinated in a medium unable to support outgrowth. UHT alkali-treated spores were germinated in 0.1% peptone water plus lysozyme, diluted into repair broth at 35 C, and periodically plated on BASE and TSN. Germination was complete within 60 min, as indicated by similar colony counts on BASE and BASE plus lysozyme (Fig. 1). No repair occurred during this time. Colony counts on TSN plus lysozyme decreased slightly. Colony counts on BASE plus lysozyme decreased by 83%. When the germinated spores were transferred to RB, repair occurred rapidly, as indicated by increasing colony counts on TSN.

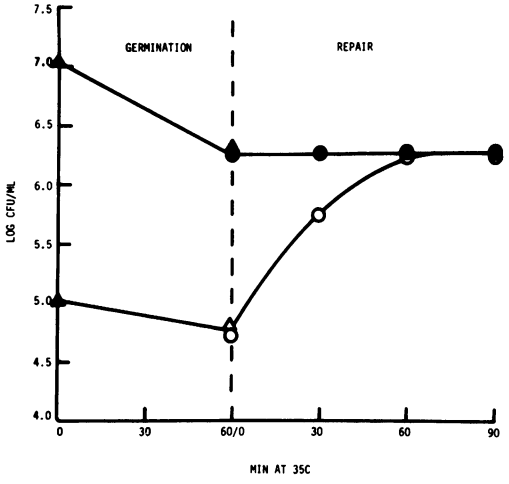

FIG. 1. *Effect of germination in peptone water on repair of thermal injury. UHT alkali-treated spores were plated on: BASE plus lysozyme, ▲; BASE, ●; TSN plus lysozyme, △; and TSN, ○.*

Repair of sensitivity to polymyxin and neomycin. TSN contained both polymyxin and neomycin and prevented the recovery of injured survivors. Earlier findings indicated that injured spores were sensitive to both antibiotics as well as kanamycin (4), but whether this resulted from only one type of damage was unknown. UHT alkali-treated spores were incubated in RB with lysozyme and 10% dextran. Periodically, the spores were plated on BASE plus lysozyme and BASE plus lysozyme with polymyxin, neomycin, or both. Resistance to both antibiotics was regained at the same time, as indicated by simultaneous increases in colony counts on all media containing antibiotics (Fig. 2). This indicated that the antibiotics were affecting the spores in a similar manner and that the increased sensitivity of injured spores was due to one type of damage.

Effect of inhibitors of macromolecular synthesis on repair of thermal injury. To gain further insight into the basis of thermal injury and its repair, the effects of metabolic inhibitors on repair were examined. UHT alkali-treated spores were germinated in peptone water and diluted into RB containing: inhibitors of protein synthesis, chloramphenicol (12) and tetracycline (1, 17); ribonucleic acid synthesis, actomycin D (15, 22) and rifampin (10, 30); deoxyribonucleic acid synthesis, hydroxyurea (25) and nalidixic acid (11); and cell wall mucopeptide synthesis, penicillin G (9, 28) and D-cycloserine (19). Concentrations used prevented the development of growth from uninjured spores (data not shown). The initial

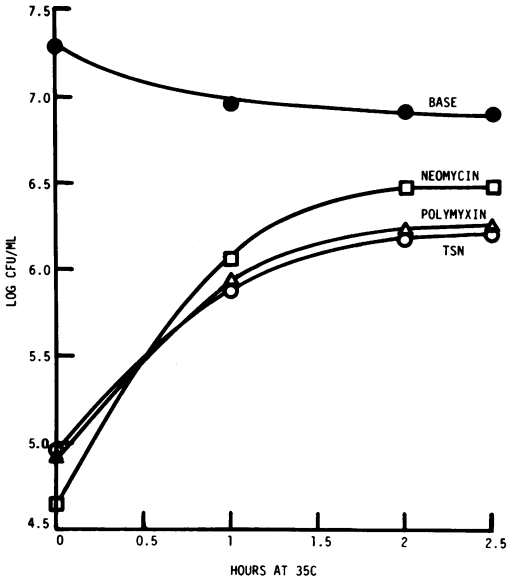

FIG. 2. *Repair of sensitivity to polymyxin and neomycin. UHT alkali-treated spores were incubated at 35 C in repair broth plus dextran and lysozyme and periodically plated on: BASE, ●; TSN, ○; BASE plus polymyxin, △; and BASE plus neomycin, □. Concentrations of antibiotics were the same as in TSN. All media contained lysozyme.*

percentage of uninjured survivors varied somewhat between experiments but was between 0.1 and 1.0% (Table 1). Repair occurred in the presence of each antibiotic. The percentage of uninjured survivors after 2 h for the controls of different experiments varied between 25 and 65%, with an average of 50%. The amount of repair in the presence of inhibitors after 2 h always was similar to that observed in the controls. After 4 h of incubation in repair media containing the inhibitors, the percentage of uninjured survivors ranged from 38 to 80%. Repair in the control after 4 h was masked by growth.

Osmotic fragility as a result of thermal injury. When UHT-treated spores were alkali treated and incubated in RB plus lysozyme, the number of viable spores decreased rapidly. Only approximately 5% of the initial population remained viable after 3 h at 35 C (Fig. 3). Heat-activated spores treated similarly exhibited no loss of viability. The addition of 10% dextran, an osmotic stabilizer, to the repair medium decreased the amount of death considerably (Fig. 3). Two other osmotic stabilizers, 10% polyvinylpyrrolidone and 20% sucrose, also protected the spores.

The relationship between osmotic fragility and sensitivity to polymyxin and neomycin is shown in Fig. 4. Spores which had been UHT and alkali treated were incubated in RB plus lysozyme with or without 10% dextran and RB

TABLE 1. *Effect of inhibitors of macromolecular synthesis on repair of thermal injury*

Inhibitors[a] (concn)	% Uninjured survivors[b]		
	0 h	2 h	4 h
Control[c]	0.1	50	
Chloramphenicol (1.5 μg/ml)	1.0	24	75
Tetracycline (1.0 μg/ml)	0.2	21	80
Rifampin (0.02 μg/ml)	0.1	43	50
Actinomycin D (10 μg/ml)	0.1	58	44
Penicillin G, 1,650 U/mg (0.15 mg/ml)	0.1	30	46
D-Cycloserine (1,750 μg/ml)	0.1	20	38
Hydroxyurea (1,500 μg/ml)	0.1	66	48
Nalidixic acid (40 μg/ml)	0.1	20	54

[a] Rifampin and penicillin G were from Mann Research Laboratory, Division of Becton-Dickinson, New York, N.Y.; tetracycline was from National Biochemicals Corp., Cleveland, Ohio; all other inhibitors were from Sigma Chemical Co., St. Louis, Mo.

[b] Colony-forming units on TSN divided by colony-forming units on BASE, times 100.

[c] RB less inhibitors.

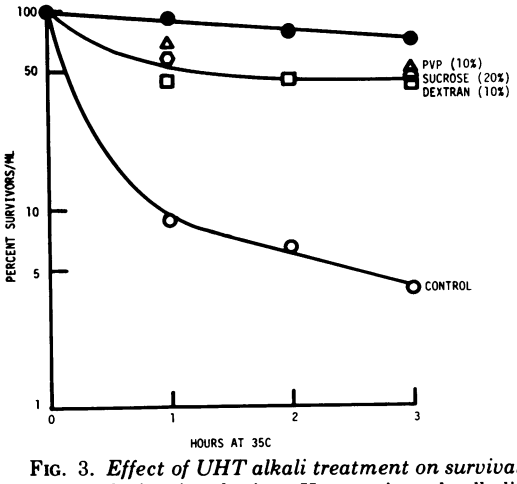

FIG. 3. *Effect of UHT alkali treatment on survival of spores during incubation. Heat-activated, alkali-treated spores were incubated in repair broth plus lysozyme (RB + L), ●. UHT alkali-treated spores were incubated in: RB + L, ○; RB + L with 10% dextran, □; RB + L with 10% polyvinyl pyrrolidone, △; RB + L with 20% sucrose, ◇. Enumeration was on BASE plus lysozyme.*

plus lysozyme with 10% dextran and neomycin or polymyxin. Heat-injured spores died rapidly in the absence of dextran, but little or no death was observed when the repair medium contained dextran (Fig. 4). However, when polymyxin or neomycin was present, death occurred even in the presence of dextran. Heat-activated spores treated similarly were not osmotically fragile (Fig. 3) and were able to grow in the presence of both polymyxin and neomycin, as indicated by similar colony counts on BASE and TSN (5).

Manifestation of injury in the presence of surface-active agents. Selective agents, polymyxin and neomycin, alone or in combination (TSN), in the recovery medium at concentrations that had little effect on the recovery of heat-activated spores reduced recovery of spores surviving UHT treatment by more than 99% (Table 2). Streptomycin had a similar effect. This same phenomenon also has been observed with kanamycin (4). Neomycin, streptomycin, and kanamycin are similar in structure and, although normally considered inhibitors of protein synthesis, may act as surface-active agents (14). Polymyxin is a cationic surface-active agent (20, 21, 26). Other known surface-active agents, sodium deoxycholate, sodium lauryl sulfate, and Roccal, a quaternary ammonium compound, also decreased recovery of UHT-treated spores at concentrations which were only slightly inhibitory to non-UHT-treated spores.

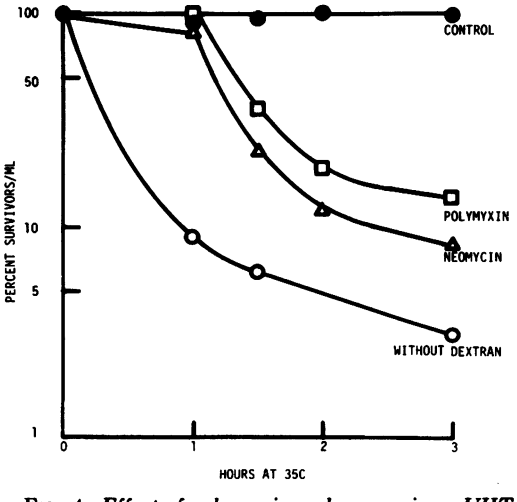

FIG. 4. *Effect of polymyxin and neomycin on UHT alkali-treated spores. UHT alkali-treated spores were incubated in: RB + L with 10% dextran (control), ●; RB + L without dextran, ○; RB + L with dextran and polymyxin, □; and RB + L with dextran and neomycin, △. Enumeration was on BASE with and without lysozyme.*

TABLE 2. *Effect of inhibitors on recovery of heat-activated and UHT-treated spores*

Medium (concn)	% Recovery[a]	
	Heat-activated	UHT
BASE	100	100
TSN	80	0.1
BASE plus neomycin (50 μg/ml)	78	0.2
BASE plus polymyxin (20 μg/ml)	80	0.7
BASE plus streptomycin (75 μg/ml)	75	0.3
BASE plus sodium deoxycholate (1 mg/ml)	88	10
BASE plus sodium lauryl sulfate (0.1 mg/ml)	70	6
BASE plus Roccal (20 μg/ml)	89	14

[a] Colony-forming units on BASE taken as 100%.

DISCUSSION

Heat treatments that do not completely inactivate spores may damage them, resulting in an increased sensitivity to certain inhibitors, including antibiotics (4), fatty acids (31), NaCl (23), KNO_3 (24), and $NaNO_2$ (16, 24). Barach et al. (4) reported that *C. perfringens* spores surviving UHT treatment were increasingly sensitive to kanamycin, neomycin, and polymyxin.

In a subsequent study, Barach et al. (5) reported that injured spores underwent repair in a medium lacking antibiotics. Repair was demonstrated for the whole population of injured survivors and appeared to occur during outgrowth (5). The findings reported here confirmed that repair occurred during outgrowth. Spores germinated by lysozyme in peptone water did not undergo repair until transferred to a medium that supported outgrowth. Likewise, repair occurred in the presence of concentrations of macromolecular synthesis inhibitors that prevented growth. The absence of repair during germination of survivors by lysozyme also indicated that injury, manifested as an increased sensitivity to selective agents, was unrelated to damage of the germination system.

TSN, used as the stress medium, contained both polymyxin and neomycin. Injured spores were sensitive to both antibiotics. During repair resistance was regained to both antibiotics stimultaneously, indicating one type of damage and a similar mechanism of action for the antibiotics. Injured spores were also inhibited by kanamycin (4) and streptomycin. Streptomycin, neomycin, and kanamycin inhibit protein synthesis, but they also can damage the cell membrane, resulting in leakage of essential

metabolites (14). Polymyxin, a cationic surface-active agent, affects the cell membrane, causing leakage of metabolites (19, 20, 26). Repair of injury in the presence of chloramphenicol and tetracycline indicated that the manifestation of injury in the presence of polymyxin, neomycin, kanamycin, and streptomycin was due to the surface-active properties of these antibiotics. This was confirmed by the increased sensitivity of injured spores to known surface-active agents, sodium lauryl sulfate, sodium deoxycholate, and Roccal, a quaternary ammonium compound. In the presence of polymyxin or neomycin injured spores died rapidly, even in the presence of osmotic stabilizers. Polymyxin and other surfactants reportedly combine with the cell membrane and become oriented between the lipid and protein components; the membrane can no longer function as an effective osmotic barrier (26). Defects in the membrane or wall of the outgrowing spore could allow increased penetration of surface-active agents (26), which would be manifested as an increased sensitivity to these agents.

Cell membrane or wall defects could be due to UHT damage of the plasma membrane or cortical membrane of the spore, which become the vegetative cell membrane and wall, respectively (27), or destruction of an enzyme or structural protein necessary for the normal development of the cell membrane or cell wall during outgrowth. However, inhibitors of ribonucleic acid and protein synthesis did not prevent repair, indicating that repair did not require the synthesis of structural proteins or enzymes. Therefore, the manifestation of injury is likely due to damage of the plasma or cortical membranes of the spore.

Heat-injured spores sensitive to polymyxin and neomycin also were osmotically fragile. In the absence of osmotic stabilizers, they died during incubation in repair broth or peptone water. Unheated spores treated similarly were not osmotically fragile or susceptible to destruction by polymyxin and neomycin. Likewise, survivors capable of forming colonies on TSN (uninjured survivors) suffered relatively little loss of viability during incubation in peptone water without osmotic stabilizers. Osmotic fragility of the injured survivors also indicated a defective cell membrane or wall.

The synthesis of new cell wall in outgrowing spores has been shown to occur in the presence of D-cycloserine but not penicillin (29). Repair occurred in the presence of both these antibiotics, indicating that cell wall synthesis was not required for repair and the cortical membrane was not the site of damage. Inhibitors of protein, ribonucleic acid, and deoxyribonucleic acid synthesis also did not prevent repair. These findings are consistent with the spore plasma membrane as the site of damage. Dawes and Halvorson (7) reported that early synthesis of cell membrane in outgrowing *Bacillus* spores occurred in the presence of macromolecular synthesis inhibitors.

It is possible that repair did not involve synthesis of new cellular material but merely reassembly of the damaged structure. Involvement of the cortical membrane, therefore, cannot be ruled out. Severe defects in the developing cell wall of an outgrowing spore would result in an increased sensitivity to surface-active agents by allowing greater access to the membrane and also would render the spore osmotically fragile.

The rate of damage to the spore membrane(s) (measured on TSN) was greater than the rate of damage of the germination system (measured on BASE) and the rate of death (measured on BASE plus lysozyme) (2-4). Thus, it appears that the more heat-sensitive locus within the spores is a spore membrane. The membrane damage resulted in osmotic fragility and increased sensitivity to polymyxin, neomycin, and other surface-active agents. Membrane damage also might explain the increased sensitivity of heated bacterial spores to NaCl (23), nitrite (16, 24), nitrate (24), and other inhibitors (e.g., fatty acids) (31). This is presently under investigation.

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