SHORT COMMUNICATIONS

The Protective Antigenicity of Protoplasts and Sphaeroplasts of a Highly Protective Strain of *Clostridium chauvoei*

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INTRODUCTION

Clostridium chauvoei causes the fatal disease Blackleg of sheep and cattle. Properties of a highly protective vaccine strain of *C. chauvoei*, strain CH3, and a heat-labile, pH-sensitive, non-agglutinogenic protective antigen of this strain have been described (Chandler & Gulasekharam, 1970, 1974).

With strain CH3, difficulty in preparing cell walls or other subcellular fractions which were protectively antigenic was apparently due to the protective antigen being destroyed by the mechanical and chemical treatments involved in the disruption of the cells and the purification of their components. Therefore, to determine the role of the cell wall in the protective antigenicity of strain CH3, cells lacking cell walls (protoplasts), or having modified or incomplete cell walls (sphaeroplasts), have been grown.

We describe the production of protoplasts and sphaeroplasts of *C. chauvoei* strain CH3 using lysozyme and penicillin, respectively, and compare the protective antigenicity of these modified cell forms with that of unaltered control cells.

METHODS

Organisms. *Clostridium chauvoei* strains CH3 (a highly protective strain) and CH4 (a virulent strain used for challenging mice in active immunization protection tests), described by Chandler & Gulasekharam (1970, 1974), were used.

Culture medium and protoplasting agents. Digest broth contained 8% (w/v) polyethylene glycol (PEG), mol. wt 4000 (BDH; laboratory reagent) and 0.01 M-MgCl₂ (Brown, King & Sugiyama, 1970; McQuillen, 1960); glucose (0.5%, w/v) and cysteine hydrochloride (0.05%, w/v) were added immediately before inoculation with seed culture.

A stock solution of 100000 units of penicillin G/ml was prepared in isotonic saline from crystalline penicillin G (C.S.L., Melbourne, Australia). Lysozyme (3 times crystallized, A grade; Calbiochem) was dissolved in isotonic saline at 5 mg/ml and sterilized by membrane filtration.

Protoplasting procedure. Cultures of strain CH3 were activated by three serial transfers at 4 to 6 h intervals of organisms in the exponential phase of growth, using a 2 ml inoculum into 10 ml medium. A 2.5 ml inoculum of exponentially growing third transfer culture was then used for each 25 ml medium and (unless otherwise specified) the protoplasting agents were added after 2 to 3 h of incubation. These cultures were incubated at 37 °C for 12 h and the yields of protoplast- or sphaeroplast-like bodies assessed by total count in a Helber Glyn (Zeiss) counting chamber using a phase-contrast microscope.

Portions of each culture were then removed for protection-testing as vaccines. Sufficient penicillinase (Labpenase; C.S.L.) was added to the penicillin-containing cultures effectively to inactivate the penicillin, and all portions were killed by the addition of formalin (0.5%, v/v) and subsequent storage at 37 °C for 7 days.

Other portions were centrifuged at 7000 *g* for 20 min at 4 °C and the deposits resuspended in 8% (w/v) PEG solution in 0.85% (w/v) NaCl. Thin sections for electron microscopy were prepared by standard methods.

Vaccines. The total count (both cells and modified cells) for each formalinized culture was determined and standardized to 8×10^9 organisms/ml with isotonic saline. Further two-fold serial dilutions over the range 1/8 to 1/64 were prepared in 0.01% (w/v) Thiomersal saline for dosing of mice in the protection test.

Active-protection test in mice. Groups of ten white mice (C.S.L. strain), each weighing 18 to 21 g, were given four successive intraperitoneal doses (each 0.5 ml) of the various CH3 vaccine dilutions at 2 to 3 day intervals over 10 days. Four to five days after the final dose the mice were challenged intramuscularly with 0.25 ml of a CaCl_2 -activated spore suspension (50 lethal doses; 250 spores in 2.5% CaCl_2) of the virulent challenge strain, CH4.

Control unvaccinated mice (dosed with saline) were included in all protection tests and these always died within 36 h of challenge. Vaccinated mice surviving 5 days after challenge were considered to be fully protected.

RESULTS

Protoplasting procedure

Lysozyme at concentrations of 50 to 1000 $\mu\text{g/ml}$ was added to cultures in early exponential phase. After 12 h the cultures were examined under the phase-contrast microscope. Those containing concentrations of lysozyme greater than 400 $\mu\text{g/ml}$ were found to be composed almost entirely of large refractile spherical bodies, with some cell debris. No typical rod-shaped cells were apparent, whereas control cultures grown under the same conditions but without the added protoplasting agents consisted entirely of motile rods. Centrifuged deposits from these cultures were sectioned and studied under the electron microscope (Fig. 1*a, b*). In deposits from cultures containing greater than 400 μg lysozyme/ml, no cell walls were visible either adherent to the outside cell membrane or free in the surrounding medium (Fig. 1*b*).

Penicillin, at concentrations ranging from 1500 to 2500 units/ml, was added to cultures in early exponential growth. After 12 h, the cultures under phase contrast appeared similar to those grown in the presence of 500 to 1000 μg lysozyme/ml. The penicillin cultures consisted mainly of large, refractile, spherical cells, with some smaller spheres, distorted rods and cell debris. Very few intact rods were noted, whereas control cultures containing no penicillin consisted entirely of rod-shaped cells; electron microscopic sections of the deposits from these cultures are shown in Fig. 1(*c*) and (*d*). Many of the large spheres were found to be free of any recognizable cell wall, although pieces of cell wall-like material appeared to be present in the surrounding medium. The smaller spherical bodies and distorted rods were found to have discontinuous cell wall-like material partially adherent to, and partially free of the outer membrane.

Active-protection test in mice

The protective antigenicity of protoplasts and sphaeroplasts was assayed by this test. Vaccines prepared from six young exponential-phase cultures, grown in the presence of three different levels of lysozyme or penicillin, were compared with vaccines prepared from

Fig. 1. Ultrathin sections of *C. chauvoei*. (a) Control cells; (b) typical protoplast formed in the presence of 500 μ g lysozyme/ml; (c) and (d) altered cells grown in the presence of 2500 units penicillin/ml. Arrows indicate cell-wall material.

Table 1. *The effect of protoplasting agents on the protective antigenicity of Clostridium chauvoei strain CH3*

Protoplasting agent added to young exponential-phase culture	Amount of protoplasting agent/ml culture	Portion of culture tested for protective antigenicity	No. of surviving mice/total challenged*, amongst each vaccinated group			
			Dilution of culture in vaccine			
			1/8	1/16	1/32	1/64
Nil (control culture)	—	Whole culture	10/10	10/10	8/10	4/10
Lysozyme	100 µg	Whole culture	10/10	8/10	4/10	1/10
	500 µg	Whole culture	3/10	4/10	0/10	0/10
	1000 µg	Whole culture	4/10	1/10	0/10	0/10
Penicillin	1500 units	Whole culture	10/10	9/10	7/10	3/10
	2500 units	Whole culture	10/10	10/10	7/10	6/10
		Supernatant fluid	1/10	2/10	0/10	0/10
		Deposit	10/10	9/10	5/10	1/10
	3500 units	Whole culture	10/10	8/10	9/10	4/10

* Mice were given four intraperitoneal doses of 0.5 ml volumes of diluted vaccine, and challenged with 250 CaCl₂-activated spores of strain CH₄.

a control culture grown under identical conditions but without the addition of protoplasting agent (Table 1).

Cultures grown in the presence of lysozyme at concentrations of 500 to 1000 µg/ml were mainly composed of protoplasts, and were very poorly protective when compared with the control cultures of normal rod-shaped cells (Table 1). Lower concentrations of lysozyme resulted in a lower yield of protoplasts and proportionately more normal cells. At 100 µg lysozyme/ml, cultures consisted of approximately 60% normal cells.

The addition of penicillin to young exponential-phase culture resulted in the production of sphaeroplasts which, although appearing similar to the protoplasts formed in the presence of lysozyme, had incomplete cell-wall material either encircling the cells or free in the medium. The protective antigenicity of the sphaeroplasts produced at the three penicillin concentrations tested was similar to that of the control cells (Table 1).

The protective antigen of *C. chauvoei* strain CH₃ is normally cell associated (Chandler & Gulasekharan, 1974). To determine whether the protective antigen in the penicillin sphaeroplast culture was associated with particulate cellular components or released in soluble form into the medium, a portion of the sphaeroplast culture grown in the presence of 2500 units penicillin/ml was centrifuged at 27000 *g* for 30 min, and the deposit and supernatant fluid were separately prepared as vaccines. The protective antigenicity of these fractions was then compared with that of the culture from which they were derived (Table 1). Most of the protective antigen was associated with the particulate fraction of this sphaeroplast culture.

DISCUSSION

Lysozyme hydrolyses the β-1,4 glycosidic linkages between the *N*-acetyl muramic acid and *N*-acetyl glucosamine residues of cell wall peptidoglycan (Ghuysen, 1968). When the peptidoglycan of *C. chauvoei* strain CH₃ was destroyed by growing the cells in the presence of lysozyme, the protective antigenicity of the culture was greatly diminished. Penicillin blocks the cross-linking of linear peptidoglycan strands by inhibiting the terminal transpeptidation reaction (Strominger *et al.* 1967) and has been noted to cause an accumulation, at the cell-wall growing points, of a fibrous material thought to be peptidoglycan strands

without cross-links (Fitz-James & Hancock, 1965). The penicillin sphaeroplasts of strain CH3 had improperly-formed cell walls, partially enveloping and partially free of their plasma membranes. Such cultures remained highly immunogenic. These results indicate that the cell wall is the site of the protective antigen and since lysozyme destroys both the peptidoglycan and the protective antigen, it appears that peptidoglycan may have some function in antigenicity beyond merely support or location of the antigen.

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