The Preparation of Immunogenic Cell Walls from a Highly Protective Strain of *Clostridium chauvoei*

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**SUMMARY**

Conventional methods for the preparation of cell walls of a highly protective strain of *Clostridium chauvoei* destroy the protective antigen. Bacteria were therefore lysed by the enzyme pronase instead of by the mechanical disintegration methods commonly employed. Final purification and separation of cell walls and membranes was achieved by equilibrium density-gradient centrifugation with sodium iodide in a zonal rotor. The resultant cell walls had a two-layered structure when seen in ultra-thin section and were highly immunogenic when used to immunize mice against challenge with *C. chauvoei*. Rabbit antisera raised against the cell walls provided passive protection against challenge in mice and the level of protection was not diminished by the absorption of all agglutinins from the sera. These results confirm previous observations that the protective antigen is a heat-labile cell wall antigen which stimulates the production of non-agglutinating protective antibody.

**INTRODUCTION**

*Clostridium chauvoei* causes the important disease Blackleg of sheep and cattle. Previously we have described the properties of the protective antigen of a highly protective strain of *C. chauvoei*, strain CH3 (Chandler & Gulasekharam, 1970, 1974; Chandler & Hamilton, 1975). Chandler & Hamilton (1975) present evidence which suggests that the cell wall was the site of the main protective antigen of strain CH3.

The terms 'immunogenic' and 'immunogenicity' are used throughout to denote the capacity to stimulate active immunity in mice against experimental infection with a virulent challenge strain of *C. chauvoei*.

Although methods for the preparation of pure bacterial cell wall suspensions are well established (Salton, 1964; Work, 1971), application of either grinding or pressure disruption (X-Press, AB-Biox, Sweden) techniques to strain CH3 gave cell walls that were not immunogenic (unpublished results). We report a method by which pure and highly immunogenic cell walls may be prepared from cells of strain CH3.

**METHODS**

*Bacterial strains.* The following strains of *C. chauvoei*, previously described by Chandler & Gulasekharam (1970, 1974), were used: strain CH2 – a poorly protective strain used in one of the agglutination tests; strain CH3 – a strain possessing a highly immunogenic protective antigen; strain CH4 – a virulent strain used for challenging mice in protection tests.

*Culture of bacteria.* Bacteria were grown as previously described (Chandler & Gulasekharam, 1974).

*Deflagellation.* In mid-exponential phase, before pleomorphism or sporing became apparent, the bacteria were centrifuged (23000 g, 20 min, 4 °C) and resuspended in ethanol.
for 10 min at 37 °C. The resulting deflagellated bacteria were collected by centrifuging and washed with distilled water.

**Lysis.** The deflagellated bacteria were again centrifuged and resuspended in 200 ml sodium phosphate buffer (0.02 M, pH 7.4) containing 0.2 mg pronase/ml (A. F. Halcyon Proteins, Melbourne, Australia). After incubation for 30 min at 37 °C, the bacteria were examined at 10 min intervals by phase-contrast microscopy. When nearly all of the bacteria were observed to have lysed they were centrifuged and washed twice with distilled water.

**Trypsin-ribonuclease digestion.** The lysed bacteria were suspended in a sodium phosphate buffer (0.1 M, pH 7.6) containing (per ml): 1 mg trypsin (BDH; laboratory reagent) and 0.5 mg ribonuclease (Boehringer, Mannheim; analytical reagent). Digestion continued at 37 °C for 1 h. After centrifuging (27000 g, 20 min, 4 °C), the bacterial deposit was washed sequentially with physiological saline, sodium phosphate buffer (0.1 M, pH 7), and distilled water.

**Equilibrium density-gradient centrifugation.** The separation of cell walls from cytoplasmic membranes and other cell debris was accomplished by equilibrium density-gradient centrifugation, initially with caesium chloride gradients in a swinging bucket rotor and in later experiments with the much less expensive sodium iodide in a zonal rotor.

With the former method, the bacterial deposit was mixed with CsCl to give a density of 1.3 g/cm³. After centrifuging at 40000 g for 20 h in a preparative ultracentrifuge the cell walls were recovered from the lower region of the centrifuge tubes, the cytoplasmic membranes and lighter cell material having separated upwards. The cell walls were then freed from heavier material by centrifuging (40000 g, 20 h) in CsCl with a density of 1.4 g/cm³. This time the cell walls were recovered from the top layer in the centrifuge tube. For complete purification more than one centrifugation at each density was required.

More rapid and effective purification was achieved by ultracentrifugation through a pre-formed sodium iodide gradient in a zonal rotor. A NaI solution of density 1.85 g/cm³, and containing 0.025 M-Na₂SO₄·5H₂O, was diluted to give solutions with densities of 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 1.7 g/cm³. These solutions were then used to form a density gradient of 1.1 to 1.85 g/cm³ in a zonal rotor (Christ Omega 2, B XIV rotor; Herabus-Christ, Ostbrode, West Germany). The cell deposit was ultracentrifuged through this gradient and complete separation of cell walls from cytoplasmic membranes achieved after 5 h at 125000 g. The average densities of the NaI-containing cell walls and cytoplasmic membranes were 1.38 and 1.26 g/cm³, respectively.

The CsCl or NaI was removed from the cell walls by ultrafiltration (Diaflo XM50 membrane, Amicon Corp., U.S.A.) and a suspension containing 1.5 mg dry wt cell walls/ml prepared in distilled water. The cell wall suspension was stored at -20 °C.

**Electron microscopy.** (i) Thin sections. This method has been described by Chandler & Hamilton (1975).

(ii) Negatively stained samples. A small drop of cell wall suspension was placed on a Formvar-coated grid, the excess fluid removed with filter paper, and the grid inverted on to a drop of 2 % phosphotungstic acid which had been neutralized to pH 7.0 with potassium hydroxide. After 30 s the grid was removed and the excess stain absorbed by filter paper. The grid was then air-dried and examined immediately with the electron microscope.

(iii) Shadowed samples. A drop of cell wall suspension in distilled water was placed on a Formvar-coated copper grid. When dry, the grid was placed in a vacuum evaporator and shadowed with platinum–carbon (Ladd Research Industries, Vermont, U.S.A.). The grid was then examined in the electron microscope.

**Active-protection test in mice.** This test has been described by Chandler & Hamilton (1975).
Cell walls of Clostridium chauvoei

Vaccines containing a selected range of dry weights of whole culture or cell walls were prepared in isotonic saline containing thiomersal (0.01%, w/v) and used to immunize groups of white mice. Protection was calculated as the dry weight of the smallest quantity of cell walls or whole culture that would protect 50% of the mice against challenge (ED₅₀).

Cell wall antiserum production in rabbits. Three female English albino rabbits (1.2 to 1.8 kg) were used for immunization. Two of the rabbits were each dosed with a total of 5 mg of cell walls in Freund's (1951) complete adjuvant. The other rabbit was the source of control antiserum and was dosed with Freund's complete adjuvant only. Two 2 ml doses were given three weeks apart, each dose being injected subcutaneously at three separate sites. The rabbits were bled by cardiac puncture 10 days after the second dose.

Passive-protection test in mice. White mice were given intraperitoneal doses of serum over the dose range 0.05 to 0.5 ml, using 4 mice/dose. After 6 h the mice were challenged with spores of strain CH₄ prepared as for the active-protection-test challenge.

Agglutination tests. Details of the O and H agglutination tests are described by Chandler & Gulasekhararam (1970). As before, H antigen was prepared from formalinized cells, O antigen from boiled cells.

Absorption of sera. A portion of the cell wall antiserum was absorbed with living cells from mid-exponential growth phase culture of strain CH₃ until no trace of agglutinating activity (O or H) was detectable in the serum. Another portion was absorbed with boiled cells (O antigen) until all O agglutinins were removed. In each case the serum and absorbing antigen were mixed, 0.01% (w/v) thiomersal was added and the mixture incubated at 37°C for 4 h. The cells were then centrifuged at 10000 g for 20 min and the serum concentrated to its original volume through an ultrafiltration membrane (Diaflo PM₁₀, Amicon Corp.). As a control, a portion of serum was mixed with physiological saline in lieu of the absorbing antigen and also reconcentrated by ultrafiltration.

RESULTS AND DISCUSSION

Ghosh & Murray (1967) noted that pancreatic lipase partially digested the cell wall of Listeria monocytogenes, forming protoplasts and cell wall 'ghosts'. With C. chauvoei, pancreatic lipase caused limited cell wall digestion and lysis of the cells. The pancreatic lipase used (Steapsin; Nutritional Biochemicals Corp., U.S.A.) had both lipolytic and proteolytic activity. To test which of these activities was responsible for cell lysis, the effect of the proteolytic enzyme pronase was investigated. Pronase caused rapid lysis of the cells and was thereafter used for this purpose. Approximately 30 min incubation with pronase caused sufficient digestion of the cell walls to effect lysis of nearly all the bacteria, leaving cell walls which had generally lost one or both polar caps and which contained burst and collapsed cytoplasmic membranes trapped inside (Fig. 1). Whether the cell lysis was due to the proteolytic activity of the enzymes or whether the latter also possessed muralytic activity has not been determined.

Differential centrifugation was ineffective for the removal and separation of the trapped cytoplasmic membranes. However, equilibrium density-gradient centrifugation in caesium chloride or sodium iodide yielded cell walls which were free of any visible contaminants when examined under the electron microscope.

Typical cell walls produced by the method described are shown in Figs. 2 and 3. Cell walls generally lacked polar caps (Figs. 1a, 2 and 3) and many complete and apparently undamaged caps were free of the main cell-wall bodies (Fig. 3).
Fig. 1. Cell walls before equilibrium density-gradient centrifugation. Note cytoplasmic membranes trapped inside. (a) Platinum–carbon shadowed. (b) Thin section.
Cell walls of *Clostridium chauvoei*

Fig. 2. Negatively stained cell wall.

Fig. 3. Negatively stained cell wall with polar caps.
The surface of the negatively stained cell walls appeared to be covered with indentations or perforated by many small holes. However, in ultra-thin section most of the cell walls had a distinct two-layered structure which showed little sign of perforation or indentation (Fig. 4).

The rigid two-layered structure of these cell walls contrasted with the fragile, one-layered structure of the cell walls produced in early experiments (Fig. 5). The latter, produced by either enzymic or mechanical cell-disruption methods, were always poorly immunogenic when used to immunize mice in active protection tests (unpublished results).

The immunogenicity of purified cell walls

The immunogenicity of the cell walls was assessed by preparing vaccines containing known dry weights of cell wall, and using these to immunize mice in an active-protection test. The immunogenicity of the cell walls was compared with that of the original culture from which they were derived, and the heat-stability of the cell wall protective antigen was determined by including vaccines prepared from a portion of the cell wall suspension that had been boiled for 2 h (Table 1). The cell walls possessed a heat-labile protective antigen which was highly immunogenic; the stimulation of equivalent protective immunity in the mice required a cell wall dry weight of less than 1% of the comparable dry weight of whole culture.

The protective activity of cell wall antiserum

Rabbits were immunized with cell walls, and portions of the resultant cell wall antiserum absorbed with either boiled cells (O antigen) or live cells of strain CH3. The agglutinating activities of the absorbed and unabsorbed sera were then tested against the antigens of the challenge strain CH4, and their protective activities against this strain measured using the
Cell walls of Clostridium chauvoei

Fig. 5. One-layered cell walls produced in early experiments. (a) Negatively stained. (b) Thin section.
Table 1. Immunogenicity and heat stability of the cell wall protective antigen

<table>
<thead>
<tr>
<th>Source of vaccine</th>
<th>Total dry weight of vaccine per mouse (µg)</th>
<th>No. of surviving mice/total challenged*</th>
<th>ED₅₀ (µg)</th>
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<tr>
<td>Whole culture</td>
<td>1300</td>
<td>4/10</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>2600</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5200</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>Cell walls</td>
<td>5</td>
<td>2/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6/10</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>Boiled cell walls</td>
<td>20</td>
<td>0/10</td>
<td>&gt; 80</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2/10</td>
<td></td>
</tr>
</tbody>
</table>

* Mice given four intraperitoneal doses of 0.5 ml of vaccine. Challenged with CaCl₂-activated spores of strain CH₄.

Table 2. Protective and agglutinating properties of cell wall rabbit antiserum

<table>
<thead>
<tr>
<th>Antiserum against:</th>
<th>Absorption of serum</th>
<th>Agglutinating activity* (titre)</th>
<th>Mouse passive protection ED₅₀ (ml)</th>
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<tbody>
<tr>
<td>Cell wall</td>
<td>Unabsorbed</td>
<td>O 640 H 1</td>
<td>0.1</td>
</tr>
<tr>
<td>Cell wall</td>
<td>Absorbed with boiled cells of strain CH₃ (O antigen)</td>
<td>-- --</td>
<td>0.15</td>
</tr>
<tr>
<td>Cell wall</td>
<td>Absorbed with live cells of strain CH₃</td>
<td>-- --</td>
<td>No detectable protection</td>
</tr>
<tr>
<td>Control†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Against antigens of challenge strain CH₄.
† Rabbits dosed with adjuvant only.

mouse passive-protection test (Table 2). The cell wall antiserum conferred a considerable level of passive protection on mice, a level of protection not substantially reduced by the absorption of all agglutinating antibodies from the serum.

To confirm the absence of agglutinins in the live-cell absorbed antiserum, it was also tested in agglutination tests with living cells and O and H antigens of strains CH₃ and CH₂. In no case was any trace of agglutination detected.

These results confirm previous findings (Chandler & Gulasekharam, 1970; Chandler & Hamilton, 1975) which indicated that the main protective antigen of C. chauvoei strain CH₃ is a heat-labile cell wall antigen which stimulates the production of protective antibody that is incapable of agglutinating the O or H antigens of the organism. Cell walls prepared by the present method are currently under further investigation.

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REFERENCES


