Treponeme Outer Cell Envelope: Solubilization and Reaggregation

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The treponemal cell has a basic morphology consisting of an outer envelope (OE), axial filaments, and a cell wall-membrane complex enclosing the protoplasmic cylinder. The axial filaments lie between the OE and the cell wall-membrane complex and are attached to the latter structure (4, 8, 9). The OE, because of its peripheral location, probably plays an important role in the development of immunity to *Treponema pallidum* and other pathogenic treponemes. The importance of the OE as an immunogen has recently been demonstrated for the morphologically similar *Leptospira* (1).

A method for selectively removing the treponeme OE in quantities adequate for antigenic and chemical studies was investigated. Since the virulent *T. pallidum* cannot as yet be cultivated in vitro, the avirulent Kazan 5 treponeme was chosen as the initial test organism.

**MATERIALS AND METHODS**

**Culture and media.** Kazan 5 treponeme (*Treponema phagedenis* biotype Kazan 5) *T. scolidontum*, *T. denticola*, and *T. refringens* were the test organisms.

The prereduced culture medium used is a modification of that described by Moore (7) and consisted of (weight per liter) 20 g of Trypticase (BBL), 10 g of dextrose, 10 g of yeast extract, 0.5 g of (NH₄)₂SO₄, 0.9 g of cysteine, 0.5 g of NaHCO₃, 10 mg of MgSO₄, 10 mg of CaCl₂, 100 mg of NaCl, 5 mg of KH₂PO₄, 11.25 mg of K₂HPO₄, and 1 mg of resazurin. The pH was adjusted to 7.4, and the cysteine and NaHCO₃ were added after the medium was steam sterilized for 5 min and cooled under CO₂. The medium was dispensed and autoclaved under oxygen-free nitrogen. Rabbit serum (10%) and inoculum (10%) were added under oxygen-free nitrogen to the cooled medium, and incubation was at 35 C for 2 to 3 days.

**Solubilization of OE.** Cells from 1 liter of culture (approximately 250 mg [dry weight]) in the late log or early stationary growth phase were sedimented at 12,000 × g, washed once with distilled water, and suspended in 100 ml of 1.4 mM sodium dodecyl sulfate (SDS, Sigma Chemical Co., St. Louis, Mo.). After 15 min at room temperature, the cells were removed by centrifugation, and the supernatant fluid was passed through a 0.45-μm pore size membrane filter (Millipore Corp., Bedford, Mass.). Reaggregation of the solubilized OE present in the filtrate was accomplished by several procedures. The procedures were: (i) dialysis against distilled water (2 liters at 4 C for 72 hr with three changes), reduction of volume to approximately 10 ml with polyethylene glycol (compound 20-M, Union Carbide Corp., New York, N.Y.) and sedimentation of the reaggregated OE (36,000 g for 1 hr at 4 C); (ii) dialysis against 1.25, 2.5, 5, 10, 20, or 40 mM MgCl₂, and the aggregate was collected by centrifugation and washed twice with distilled water; (iii) sequential dialysis for 24-hr periods against distilled water, then 20 mM Mg, and finally distilled water; (iv) addition of Mg²⁺ (final concentration 80 mM) to the filtrate and harvest of the reaggregated OE by centrifugation. This OE preparation was then resolubilized with 30 mM ethylenediaminetetraacetic acid (EDTA), dialyzed against distilled water until visible reaggregation occurred (approximately 72 hr), and finally harvested by centrifugation.

**Electron microscopy.** All preparations were fixed with 1% OsO₄ (5), dehydrated, and embedded in Epon (6). Sections were cut with a diamond knife and mounted on parlodion-covered copper grids. The sections were examined with a Siemens Elmiskop I electron microscope after staining with 2% uranyl acetate.

**RESULTS**

The resuspension of harvested Kazan 5 cells in distilled water caused a cessation of motility, and many of the cells were converted to granular forms (3). Cells other than the grana-
lar forms had the typical treponeme morphology (Fig. 1). Exposure of the cells to 1.4 mM SDS for 15 min resulted in the solubilization of the OE while leaving the protoplasmic cylinder relatively intact (Fig. 2). Concomitant with the solubilization of the OE was the disappearance of the granular forms and a loss of brightness of the treponeme cells as viewed under dark-field illumination.

After the solubilized OE was separated from the protoplasmic cylinders by centrifugation and passage through a 0.45-μm pore size filter, its reaggregation was attempted by various treatments. In what follows the term OE will be used, for convenience, to designate the reaggregated material, although it is understood that the purity of the preparation is unknown at the present time. Dialysis of the OE preparation against distilled water resulted in reaggregated trilaminar structures characteristic of the original OE (Fig. 3). However, only a small amount of material was obtained by this treatment (0.4% of cell dry weight). Dialysis of the solubilized OE against increasing concentrations of MgCl₂ (1.25, 2.5, 5, 10, 20, and 40 mM) resulted in a concomitant improved yield of reaggregated OE. A maximal yield of OE (approximately 23% of cell dry weight) was obtained with Mg²⁺ concentrations of 20 mM or greater. The Mg²⁺ concentration had a pronounced effect on the structure of the reaggregated OE. As the Mg²⁺ concentration was increased to 5 mM there was a progressive loss of the triple-layered structure, and small amorphous aggregates became predominant (Fig. 4). At high Mg²⁺ concentrations (20 and 40 mM), the OE reaggregated largely as globular masses (Fig. 5). The removal of the SDS from the OE preparation prior to dialysis against 20 mM Mg²⁺ resulted in a modification in the form of the OE aggregate. As seen in Fig. 6, the size of the globular forms was markedly reduced. The solubilized treponeme OE was also reaggregated by the addition of 80 mM Mg²⁺ directly to the OE preparation. The aggregate formed was harvested by centrifugation and washed several times with distilled water. The structure of the OE prepared in this manner is seen in Fig. 7.

The capability of various cations to reaggregate the detergent-solubilized OE was investigated. After removal of the SDS by dialysis against water, reaggregation of the OE was attempted with monovalent, divalent, and

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**Fig. 1.** Normal Kazan 5 treponeme (section). OE, Outer envelope; WM, cell wall-membrane; AF, axial filaments. x96,000.
trivalent cations. Little or no OE aggregates were formed with the monovalent cations Na⁺, K⁺, and Li⁺. The divalent cations Mg²⁺, Mn²⁺, Ca²⁺, and Cu²⁺ as well as the trivalent cations Fe³⁺ and Al³⁺ were capable of OE reaggregation.

Exposure of the Mg²⁺-aggregated OE to the chelating agent EDTA (30 mM) brought about its resolubilization. When the EDTA was removed by dialysis against water a reaggregation of the OE occurred (Fig. 8). These aggregates closely resemble those seen in Fig. 6.

Solubilization and reaggregation of the OE of several other treponemes was attempted. The OE of Treponema denticola, T. scoliodontum, and T. refringens were solubilized with 1.4 mM SDS and reaggregated in the presence of Mg²⁺ under the same conditions used with the Kazan treponeme.

**DISCUSSION**

The structure and composition of Mycoplasma membranes have been extensively studied (10). These membranes are considered to be extremely sensitive to surface-active agents and are readily solubilized by 10 mM SDS. The action of this detergent results in a separation of the membrane lipid and protein components (2, 10). Reaggregation of the solubilized membrane into trilaminar structures resembling the original membrane requires the presence of cations (10, 11). These reaggregated membranous structures can be resolubilized with detergent but not with chelating agents (10).

The OE of the treponemes has a membrane-like structure. Jackson and Black (4) reported that exposure of the avirulent Nichols treponeme sequentially to -11C and to SDS resulted in the removal of the OE. We found this structure to be extremely sensitive to SDS. Exposure of the avirulent Kazan 5 treponeme to 1.4 mM SDS resulted in the solubilization of the OE while leaving the underlying protoplasmic cylinder intact. This “selective” solubilization of the OE is lost at higher SDS concentrations (10 mM), and both the OE and the cell wall-membrane complex of the protoplasmic cylinder are disaggregated. The OE of T. denticola, T. scoliodontum, and T. refringens as well as of the Leptospira (1) are similarly sensitive to low levels of SDS. These results suggest that the OE of most, if not all, spirochetes are

**Fig. 2.** Kazan 5 treponeme (section) after treatment with SDS. Note absence of OE on protoplasmic cylinder (PC). x96,000.
easily solubilized by surface-active agents.

The requirements for reaggregation of the treponeme OE resemble that of the mycoplasmal membrane in that cations appear to be required. At low Mg\(^{2+}\) concentrations (1.25 mM), the treponeme OE aggregates are primarily triple-layered, resembling the structure of the original OE. Aggregates formed with high Mg\(^{2+}\) concentration (20 to 80 mM) are largely globular with only a few triple-layered structures evident. The structures of the reaggregated *Mycoplasma* membranes are similarly affected by Mg\(^{2+}\) concentration (11). In contrast to the treponeme OE and the mycoplasmal membranes, the SDS-solubilized OE of the *Leptospira* does not require an exogenous source of cations for reaggregation (1).

Either divalent or trivalent cations are effec-
tive in reassembling the OE of the treponemes and the *Mycoplasma* membranes (10). The re-aggregated OE of the *Treponema* is resolubilized by EDTA, indicating that the cations are accessible to the chelating agent. This is in contrast to the reaggregated *Mycoplasma* membrane, which cannot be resolubilized with EDTA (10). Both of the above aggregates are soluble in SDS.

Although detailed studies of the treponeme ultrastructure have been made (4, 8, 9), little is known of the importance of the various structures in the development of immunity to the treponematoses. Recently, the OE of a parasitic leptospire was demonstrated to be an important immunogenic component of the cell. Hamsters vaccinated with the reaggregated OE were protected against a lethal challenge.
Fig. 5. Outer envelope reaggregated by dialysis against 20 mM Mg\(^{2+}\). ×96,000.
Fig. 6. Outer envelope reaggregated by sequential dialysis against distilled water; 20 mM Mg²⁺; distilled water. ×96,000.
FIG. 7. Outer envelope reaggregated by direct addition of 80 mM Mg²⁺ to SDS-solubilized OE. ×96,000.

(1). We have demonstrated in this report that the OE of an avirulent treponeme can readily be obtained in significant quantities. If techniques such as those reported here can be shown to be applicable to T. pallidum and if the purified OE of this spirochete can be shown to induce protective immunity, progress will have been made toward the development of a syphilis vaccine.

The treatment of the treponemes with SDS solubilizes other cellular components in addition to the OE. Whether these materials are present in the reaggregated OE is not known at this time. Antigenic and chemical analyses of the Kazan treponeme OE are presently being conducted in our laboratory.

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LITERATURE CITED


FIG. 8. Outer envelope reaggregated by direct addition of 80 mM Mg²⁺; resolubilization of aggregate with 30 mM EDTA; aggregation by dialysis against distilled water. ×96,000.


