The Outer Membrane of *Treponema pallidum*: Biological Significance and Biochemical Properties

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Rabbits infected intravenously with *Treponema pallidum* were not markedly febrile, and the pyrogenicity of treponeme preparations administered intravenously to rabbits was negligible. The antibiotic polymyxin B did not induce any ultrastructural changes on the treponemal surface and was not lethal (immobilizing) for *T. pallidum*, which was, however, highly susceptible to detergents such as SDS. Extraction of treponemes with Triton X-100 removed the outer membrane (despite the presence of Mg²⁺) as shown by electron microscopy, and solubilized a limited number of proteins detectable by SDS-PAGE, including a dominant antigen (47 kDa) demonstrated by immunoblotting. None of the proteins were heat-modifiable. Periodic acid–silver staining of polyacrylamide gels for carbohydrate together with protease K digestion did not demonstrate major carbohydrate components in whole treponemes, or in the Triton-soluble fraction. Surface iodination of intact treponemes revealed very little surface exposure of treponemal proteins, although a protein which co-migrated with host albumin was labelled and appeared to be associated with the treponemal surface. Many treponemal proteins were, however, labelled when iodination was done in the presence of Triton. These observations indicate that the outer membrane of *T. pallidum* differs significantly from those of many Gram-negative pathogens.

INTRODUCTION

Syphilis is essentially a chronic infection and the causative organism, *Treponema pallidum* does not elicit host reactions in many stages of the disease. Clearly the treponemes are capable at these times either of sequestration in sites where host reactions cannot be activated, or of some form of ‘disguise’ so that they are not recognised by the host as foreign substances, or of active suppression of host defensive responses.

Treponemes may be found intracellularly in human and rabbit infections; this possibly protects them from host defence mechanisms and favours their persistence in the face of an immune response (Sykes & Miller, 1971; Sykes et al., 1974; Turk, 1979). However, in early lesions treponemes are predominantly extracellular, often near blood vessels, and clearly accessible to host defences (Drusin et al., 1969; Hasegawa, 1969; Penn, 1981; Penn & Clay, 1982). Thus, while intracellular survival may promote long term persistence of small numbers of organisms, it cannot explain the initial extracellular multiplication of treponemes without excitation of host defences.

The ‘disguise’ of pathogens to avoid immunological recognition may comprise surface acquisition of host substances, or similarity between surface constituents of the pathogen and those of the host. The former has been suggested for *T. pallidum* by the finding of host proteins, including albumin and immunoglobulins, associated with the surface of organisms taken from infected rabbits (Alderete & Baseman, 1979). The significance of this in pathogenicity is yet to be determined, however: other pathogens growing in vivo may bind host proteins (Finn et al., 1982), but these would not necessarily disguise the foreign antigens of the organism and alter its mode of pathogenicity. Similarity between the surfaces of *T. pallidum* and the host might result...
from a mucopolysaccharide capsule, possibly responsible for the mucoid exudate found in syphilis lesions (Fitzgerald, 1981). The treponemal origin of this material is disputed, however, and recent work by Strugnell et al. (1984) indicates that the material is synthesized by the host. T. pallidum exhibited enhanced reactivity with antibody after treatment with Triton X-100, concomitant with the removal of the outer membrane (Penn & Rhodes, 1982; Penn & Lichfield, 1982). Hence the Triton-soluble outer membrane and associated layer(s) appeared crucial to the antigenic crypticity of intact treponemes. The nature and composition of the intact treponemal surface and the Triton-soluble fraction are now described further.

METHODS

T. pallidum strain. The Nichols strain of T. pallidum was maintained and propagated in rabbits, and harvested for study in vitro as described previously (Penn, 1981; 1983). Organisms suspended in extraction medium were killed by addition of 0·1% (w/v) sodium azide and stored at 4°C for at least 24 h. They were used within 48–72 h.

Pyrogenicity testing. Treponemes were concentrated and washed by centrifugation at 10000 g for 20 min and re-suspension in phosphate buffered saline pH 7·2 (PBS: 7·2 mM-Na2HPO4, 2·8 mM-NaH2PO4, 0·15 mM-NaCl) to a density of 1 × 10⁶ ml⁻¹ determined by total visual counts on the original suspensions in a counting chamber. They were then fragmented by ultrasonication at 20 kHz for 5 min (Penn & Rhodes, 1982). For comparison, a suspension of gonococci (strain BS4; Penn et al., 1976) grown on complex agar medium containing peptone, yeast extract and serum was treated similarly. Bacterial suspensions (0·1 or 1·0 ml), or diluent alone, were injected intravenously into healthy adult male Californian rabbits which had shown stable (±0·2°C), normal rectal temperatures for at least 1 beforehand. Rectal temperatures were monitored at 1 h intervals for 5–6 h.

Reactions of organisms with polymyxin B. Bacteria were incubated with polymyxin B (Sigma) as described by Wiegel & Quandt (1982), with the addition to the buffer of 0·15 mM-NaCl when the organisms were to be fixed and sectioned, to stabilize them osmotically. Organisms were centrifuged at 10000 g for 20 min and suspended in polymyxin B at a density of 1 × 10⁸ ml⁻¹ for treatment. Salmonella typhimurium strain 395 MS (obtained from Dr E. Kihlstrom, Gothenburg) was similarly treated as a positive control organism known to contain lipopolysaccharide (LPS). Processing for electron microscopy, including post-fixation with uranyl acetate, was as described by Penn & Lichfield (1982).

Effects of polymyxin B and SDS on treponemal motility. Viable treponeme suspensions, in the medium in which they had been extracted from rabbit testes, were treated immediately by the addition of 10% (v/v) of solutions of polymyxin B or SDS in PBS to give final concentrations of 1000 U polymyxin B ml⁻¹ or 1·0 or 0·01% (w/v) SDS in the treponemal suspension. Treponemal motility in test and control (addition of PBS alone) samples was assessed after incubation at 37°C for 1 h.

Treatment with Triton X-100. Azide-killed treponemes (batches of 1·5 × 10¹⁰) were centrifuged at 10000 g for 20 min, washed by suspending in 10 ml PBS and re-centrifuged. The sedimented treponemes were then resuspended in PBS containing 10 mM-MgCl₂ and 0·2% (v/v) Triton X-100 to a density of 2 × 10¹⁰ ml⁻¹. After 30 min incubation at 37°C, treponemes were centrifuged as above and both the supernate and the pellet (resuspended in the same volume of PBS) were retained for polyacrylamide gel electrophoresis. Some organisms from the pellet were also suspended in 2% (w/v) potassium phosphotungstate, pH 7·2, in water, for the preparation of negatively stained samples for electron microscopy.

Electrophoretic analysis. SDS-PAGE was done as described by Laemmli (1970). Molecular weight standards were obtained from BioRad. Samples (25 μl) of Triton-treated treponemes, soluble fraction (supernate) or resuspended pellet were applied to 10 mm wide slots in 1 mm thick slab gels. In some experiments the same volume of a suspension of whole treponemes (2 × 10¹⁰ ml⁻¹) was also run. Periodic acid–silver staining for carbohydrate and digestion of protein with protease K (Boehringer) were done by the method of Hitchcock & Brown (1983). LPS (from Escherichia coli, type O111) was obtained from Sigma and samples of 10 μg were run for positive control staining.

Immunoblotting was done by the method of Towbin et al. (1979) with an electroblot apparatus (BioRad), and antigenic components were detected by reaction of blots with pooled hyperimmune antiserum from rabbits which had been boosted by immunization with sonicated treponemes (Penn & Rhodes, 1982). The visualization of immuno-reactive components was done with alkaline phosphatase conjugated to goat anti-rabbit serum (Miles) by the method of O’Connor & Ashman (1982) with ASMX phosphate and Fast Red (Sigma).

Surface iodination. Two 1·5 ml portions of freshly harvested, live treponemes, extracted in a minimal volume of medium to obtain counts of at least 5 × 10⁶ ml⁻¹, were centrifuged at 200 g for 5 min to remove host cells, and were then centrifuged for 5 min at 11600 g on a microcentrifuge to sediment the treponemes. These were then resuspended in 0·1 ml testis extraction medium (Hanks’ solution with 20 mM-HEPES and 2 mM-dithiothreitol, pH 7·4) with or without 0·2% (v/v) Triton X-100. To this was added 10 μl [approx. 100 μCi (3·7 MBq)] Na¹²⁵I.
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(Amersham) in 0-01 m-NaOH, and 1 Iodobead (Pierce & Warriner). Iodination was allowed to proceed for 15 min at room temperature with occasional gentle agitation, and was terminated by removal of the Iodobead. The suspension was centrifuged at 11600 g. Material iodinated in the presence of Triton was retained as supernate and pellet (resuspended in the same volume of medium). The reaction supernate of the material iodinated without Triton was retained, and the pellet resuspended in medium with Triton. After 15 min at room temperature, this was again centrifuged to provide supernate and pellet, and the latter was resuspended as above. All samples were analysed by SDS-PAGE followed by autoradiography of the dried gels.

**RESULTS**

*Fever in T. pallidum infection and pyrogenicity of the organism*

Rectal temperatures of a group of acutely orchitic rabbits infected with *T. pallidum*, and uninfected animals housed in the same conditions, are shown in Fig. 1. The mean temperature of the normal rabbits was 39.15 °C, SD 0.16 °C, and in infected rabbits 39.5 °C, SD 0.51 °C. Thus there was no statistically significant difference in temperatures between the two groups. However, some infected rabbits undoubtedly had elevated temperatures, although they were seldom severely pyretic; other acutely orchitic rabbits had normal temperatures.

Intravenous injection of ultrasonically disintegrated *T. pallidum* produced only a slight fever in comparison with an equivalent dose of *Neisseria gonorrhoeae* (Fig. 2). A tenfold increase in the number of *T. pallidum* injected led to some increase in the degree of fever, but the kinetics of this response did not accord with the typical dual peaks at 1 and 3 h characteristic of bacterial LPS (Milner & Finkelstein, 1966), and seen here after injection of *N. gonorrhoeae*.

*Effect of polymyxin B on treponemal ultrastructure*

*T. pallidum* treated with polymyxin B, at either 200 or 1000 U ml⁻¹, were macroscopically agglutinated but light microscopy did not reveal any other change in their appearance. Control *S. typhimurium* cells were similarly affected. Ultrastructurally, negative staining showed that characteristic surface structures, often annular in appearance, were prominent on the surface of *S. typhimurium* but were not seen on *T. pallidum* (Fig. 3). In thin section, the surface of *T. pallidum* appeared unaffected, but *S. typhimurium* showed the surface structures visible in negatively stained preparations (Fig. 4); in section these appeared to be vesicular projections of the bacterial surface, adjacent to the outer membrane. The membrane itself did not appear to be substantially disrupted.

*Effect of polymyxin B and SDS on treponemal motility*

*T. pallidum* was not immobilized by exposure to polymyxin B (1000 U ml⁻¹) for 1 h. Tests with SDS showed immobilization within a few minutes at concentrations as low as 0-01% (w/v).

*Solubilization of the outer membrane of T. pallidum by Triton X-100*

Treponemes treated with Triton X-100 in the presence of Mg²⁺ underwent the morphological changes which were previously shown to accompany removal of the outer membrane (Penn & Lichfield, 1982). These are shown in Fig. 5.

*Electrophoretic analysis of the Triton-soluble fraction*

SDS-PAGE of the Triton-soluble fraction showed that a minority of the total treponemal polypeptides were significantly extracted into this fraction, notably those of approximately 60, 47 and 40 kDal (Fig. 6a). Of these, only the 47 kDal polypeptide, and two or more minor components, including one of about 33 kDal and another of about 16 kDal near the dye front, which was poorly resolved under these conditions, appeared to be extracted almost completely, suggesting their location in the outer membrane. The position of the largest major polypeptide on this gel at 70 kDal (starred) differed from its position (80 kDal) when organisms not treated with Triton were examined (unpublished observation). The reason for this is not known.

Periodic acid-silver staining for carbohydrate components stained many bands in the various untreated treponemal fractions (Fig. 6b). Those which were stained strongly with Coomassie blue did not generally stain well with the silver stain, and indeed clear unstained areas were often
Fig. 1. Rectal temperatures of identically housed normal rabbits and rabbits acutely infected intratesticularly with *T. pallidum*.

Fig. 2. Fever curves of groups of 4-5 rabbits injected intravenously with $1 \times 10^8$ (□) or $1 \times 10^9$ (●) sonicated *T. pallidum* or $1 \times 10^8$ (○) sonicated *N. gonorrhoeae*. Error bars indicate sd. Mean temperatures of two control rabbits which received diluent alone (■) are also shown.

Fig. 3. Negatively stained untreated (a) and polymyxin B-treated (b) *S. typhimurium*, and polymyxin B-treated *T. pallidum* (c). The arrow indicates the annular appearance of surface vesicle on *S. typhimurium* after reaction with polymyxin B. Bars represent 0.2 μm.

present in these positions. A sample of 10 μg LPS from *E. coli* was stained well by this method. After treatment of treponemal components with protease K, no silver-stainable bands remained, except at the dye front.

Western blotting (Fig. 6c) of whole treponemes and the Triton-soluble fraction showed that an immunodominant antigen was extracted by Triton treatment. Examination of a section of the blot stained for protein with amido black, and of the original polypeptide profiles in polyacrylamide gel, showed this immunodominant polypeptide to be the major, 47 kDal polypeptide of the Triton-soluble fraction.
Fig. 4. Ultrathin section of mixed *T. pallidum* and *S. typhimurium* after treatment with polymyxin B. Note vesicles on the surface of *S. typhimurium* (1), and unaffected outer membrane of *T. pallidum* (2). Bar represents 0.2 μm.

Fig. 5. Negatively stained *T. pallidum*, intact *(a)*, or treated with Triton in the presence of Mg²⁺ *(b)*. The arrow indicates cytoplasmic filaments, made more visible by Triton treatment. Bars represent 0.2 μm.
Fig. 6. (a) SDS-PAGE analysis of the Triton-soluble fraction (1), cell residue after Triton extraction (2), Triton-treated but unseparated treponeme suspension (3) and molecular mass markers (4). The arrows indicate polypeptides substantially solubilized by Triton with their molecular weights. (b) Periodic acid–silver stained SDS-PAGE gel, with 10 µg E. coli LPS (1), whole treponemes (2, 5), Triton-extracted residue (3, 6) and Triton-soluble fraction (4, 7). Material in lanes 2–4 was untreated; material in lanes 5–7 was digested with protease K. (c) Western blot of whole treponemal cells (1) and Triton-soluble fraction (2), reacted with hyperimmune rabbit serum. The arrow indicates the major 47 kDa antigenic polypeptide, extractable into the Triton-soluble fraction.
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Surface labelling

Surface iodination of intact treponemes, viable until addition of the iodination reagents, led to incorporation of significant amounts of label into only one polypeptide as analysed by SDS-PAGE (Fig. 7, lanes 1 and 2). This polypeptide was shown in other experiments to co-migrate with bovine serum albumin, and its absence from the washed treponeme preparations analysed as shown in Fig. 6(a) suggests its loose association with the surface of the unwashed organisms used for surface labelling. No significant presence of label in the cell pellet remaining after Triton extraction was seen (lane 3). After iodination in the presence of Triton, considerable incorporation of label into a variety of treponemal proteins was observed (Fig. 7, lanes 4 and 5). Both the Triton-soluble fraction (lane 4) and the insoluble residue (lane 5) had incorporated much label. The apparently greater incorporation into the albumin-like protein in the presence of Triton (lane 1 compared with lane 4) did not appear to be due merely to the presence of Triton since control iodination of solutions of bovine serum albumin in the presence and absence of Triton showed no difference in incorporation.

DISCUSSION

T. pallidum establishes foci of infection but not acute inflammatory responses resulting in pus formation. Thus reactive components such as LPS may be absent from T. pallidum. The limited availability of T. pallidum precludes the use of many standard analyses of cell wall composition. Therefore biological and ultrastructural investigations are important in the elucidation of the surface structure.

Fever is not prominent in syphilis. Since the pyrogenicity of Gram-negative bacteria results partly from their LPS content, fever in T. pallidum infection in rabbits and the pyrogenicity of treponemes might indicate presence of LPS. The fever observed in some acutely orchitic rabbits was less than 1 °C, and depression of body temperature occurred in some animals. The low pyrogenicity of treponemes in comparison with gonococci suggests the absence of typical Gram-
negative LPS from *T. pallidum*. This conclusion was supported by the absence of surface vesicles from *T. pallidum* following treatment with polymyxin B. Vesicle formation, as seen on *S. typhimurium* treated with polymyxin B, is characteristic of Gram-negative bacteria (Wiegel & Quandt, 1982), and LPS appears to be the target for action of polymyxin B (Perkins, 1983). The resistance of *T. pallidum* to killing (immobilization) by polymyxin B reinforces the morphological evidence for the lack of effect of this antibiotic. The SDS susceptibility of *T. pallidum* also suggests the absence of at least a smooth type of LPS, since the relative resistance of many wild-type Gram-negative enteric bacteria to detergents is attributed to LPS of the smooth type which presents a hydrophilic exterior of the bacterium (Wright & Tipper, 1979). Additional evidence for the absence of LPS activity in *T. pallidum* has recently been reported by Hardy & Levin (1983), who failed to detect any Limulus amoebocyte lysate gelation activity associated with this organism. In other studies (Bailey *et al.*, 1985) we have demonstrated by immunoblotting a ladder profile of protease-resistant antigenic material characteristic of LPS in the non-pathogenic Reiter treponeme, but no protease-resistant antigens were detected in *T. pallidum*.

The removal of the outer membrane of *T. pallidum* by Triton in Tris/HCl buffer has been described previously (Penn & Lichfield, 1982). In Gram-negative enteric bacteria, the outer membrane is often resistant to solubilization by such detergents unless divalent cations are removed (Schnaitman, 1971). The susceptibility of the outer membrane of *T. pallidum* to solubilization by Triton X-100 even in the presence of Mg$^{2+}$ again suggests the absence of LPS.

Analysis by SDS-PAGE of Triton-soluble material from the surface of *T. pallidum* showed that only three polypeptides appeared to be completely solubilized. These are likely to be outer membrane components, since this layer appeared to be completely removed ultrastructurally. None of the polypeptides detected showed heat modification as determined by a comparison of the molecular mass on SDS-PAGE of material solubilized by boiling with the molecular mass of material held at 37°C for 30 min. In this respect *T. pallidum* differs from many pathogenic Gram-negative bacteria. Lack of reactivity with the periodic acid–silver stain after protease digestion indicated that carbohydrates are not prominent components of this organism, but staining reactions alone cannot show that LPS or other carbohydrates were completely absent. The many bands silver-stained before protease digestion could have been glycoproteins or proteins (Hitchcock & Brown, 1983).

The almost total absence of iodination of proteins in intact treponemes, compared with the promotion by Triton X-100 of the iodination of a large number of protein species, indicated that the intact outer membrane shields the majority of treponemal proteins from surface exposure. This conflicts with other reports of surface iodination of a number of treponemal proteins (Alderete & Baseman, 1980; Jones *et al.*, 1984), a discrepancy possibly resulting from variations in treatment of treponemes before iodination. In our hands, long exposures during autoradiography demonstrated low levels of iodination of other polypeptides, but this was insignificant in comparison with the level of incorporation of label in the presence of Triton. Neither the Triton-extractable 40 kDal polypeptide, which resembled a 39 kDal polypeptide shown by Norris & Sell (1984) to be labelled after surface-iodination of purified, washed treponemes, nor the 47 kDal Triton-extractable polypeptide, appeared to be accessible for surface iodination in the intact, unwashed organism. The labelling of presumed albumin associated with the treponemal surface agrees with other observations of host protein binding by *T. pallidum* (Alderete & Baseman, 1979), but whether this protects against host defences remains unclear. Enhanced iodination of the albumin-like protein in the presence of Triton suggests that binding of albumin to the treponemal surface may be sufficiently intimate to afford some protection against its iodination in the native configuration.

These observations indicate many differences in outer membrane properties of *T. pallidum* from those of other Gram-negative pathogens, which may contribute to the low reactivity towards the host of this pathogen and its adaptation to long term survival in chronic infection despite host immune responses to internal antigens. The latter may become accessible to the host immune system after damage to or death of some, but presumably not all treponemes in the course of infection.
REFERENCES


