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Identification and Localization of Integral Membrane Proteins of Virulent *Treponema pallidum* subsp. *pallidum* by Phase Partitioning with the Nonionic Detergent Triton X-114

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Integral membrane proteins of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) were identified by phase partitioning with the nonionic detergent Triton X-114; antigens with apparent molecular masses of 47, 38, 36, 34, 32, 17, and 15 kilodaltons (kDa) were identified in the detergent phase. Immunoblotting with murine monoclonal antibodies directed against pathogen-specific 47- and 34-kDa *T. pallidum* antigens confirmed their presence in the detergent phase. Endoflagellar proteins of *T. pallidum* were not detected in immunoblots of detergent-phase proteins when monospecific antisera directed against endoflagella of the nonpathogenic *T. phagedenis* biotype Reiter were used. At detergent concentrations (0.02 and 0.1%) which appeared to solubilize selectively the outer membranes of treponemes radiolabeled with ^{35}S in vitro, limited amounts of detergent-phase proteins were immunoprecipitated. Greater amounts of detergent-phase proteins were extracted at higher detergent concentrations (0.5 and 2.0%) which resulted in both outer membrane solubilization and ultrastructural derangements of the residual cytoplasmic bodies. Furthermore, Triton X-114 extraction of both intact treponemes and organisms without outer membranes yielded detergent phases with similar protein profiles. The results of these experiments indicate that the hydrophobic proteins identified by Triton X-114 are not located exclusively in the *T. pallidum* outer membrane. The results are also consistent with the hypothesis that the *T. pallidum* outer membrane is a protein-deficient lipid bilayer.

Treponema pallidum subsp. *pallidum* (*T. pallidum*), the etiologic agent of venereal syphilis, morphologically consists of an outer membrane that surrounds the endoflagella, cytoplasmic membrane, and protoplasmic cylinder of the organism (13, 14). It has been presumed that molecules important to the immunopathogenesis of syphilis are located in the outer membrane, although the structure has not been subjected to rigorous molecular characterization. Isolation of the outer membrane has been complicated by its physical lability (26, 29, 32) and by the need to propagate *T. pallidum* via intratesticular inoculation of rabbits. Expression of treponemal polypeptides in *Escherichia coli*, recently accomplished by several laboratories (4, 21, 22, 33, 34, 37, 38), has the potential to circumvent these problems and provide unlimited quantities of individual antigens for pathogenesis investigations. However, the lack of a priori knowledge of the protein constituents of the outer membrane has hindered selection from genomic libraries of clones expressing recombinant outer membrane proteins.

Selective detergent solubilization has been used to isolate outer membranes from cultivatable spirochetes, such as the nonpathogenic, host-indigenous *T. phagedenis* biotype Kazan (15) and the pathogenic *Leptospira interrogans* (2). More recently, Stamm et al. (32) used low concentrations of sodium dodecyl sulfate (SDS) to isolate putative outer membranes from viable *T. pallidum*. In the present study, we have extended these methods by using Triton X-114, a nonionic detergent with polyoxyethylene head groups similar to Triton X-100 but possessing a relatively low cloud point (20°C) (3). Solutions of Triton X-114 warmed above the cloud point can be separated by centrifugation into distinct aqueous and detergent phases; hydrophobic proteins, such as integral membrane proteins, segregate into the heavier detergent phase (3). Phase partitioning with this detergent has been used to identify integral membrane proteins in both eucaryotic and procaryotic membranes (5, 6, 30). Identification of *T. pallidum* membrane proteins by phase partitioning with Triton X-114 was particularly attractive because of the recognized limitations of conventional techniques, such as extrinsic radiolabeling with ^{125}I and immunoelectron microscopy, for identification of *T. pallidum* outer membrane proteins (7, 24, 26, 27, 29).

Initially, we used relatively high Triton X-114 concentrations to identify and characterize integral membrane proteins from whole *T. pallidum* organisms without regard to their location in either the outer or cytoplasmic membranes. Proteins which were specific to the outer membrane were then sought by extracting organisms radiolabeled in vitro with ^{35}S at different detergent-to-protein ratios and by correlating the immunoprecipitates with electron microscopic examination of the extracted organisms. Our results provide support for the hypothesis that the *T. pallidum* outer membrane is a protein-deficient lipid bilayer with properties that differ greatly from the outer membranes of typical gram-negative bacteria.

MATERIALS AND METHODS

Source of treponemes. The virulent Nichols strain of *T. pallidum* was propagated by intratesticular passage in male New Zealand White rabbits without the use of cortisone acetate injections. Ten days after inoculation, rabbits were sacrificed by intravenous injection of 1 ml of T-61 Euthanasia solution (American Hoescht Corp., Somerville, N.J.),

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and the testes were aseptically removed. Treponemes were extracted on a rotary shaker in either phosphate-buffered saline (PBS [pH 7.4]) or an enriched medium used for in vitro radiolabeling (see below). Organisms were purified by Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation (9).

Protein assay. Protein content of freshly extracted and Percoll-purified *T. pallidum* cells was determined by the BCA Protein Assay micromethod (Pierce Chemical, Rockford, Ill.).

Immunologic reagents. Murine monoclonal antibodies 11E3 (immunoglobulin G2a) and 3B5 (immunoglobulin G1), directed against *T. pallidum* antigens with apparent molecular masses of 47 and 34 kilodaltons (kDa), respectively, have been described previously (16, 19, 34). (Anti-*Treponema* monoclonal antibodies are protected under U.S. patent 4,515,498, April 1985.) Human syphilitic sera were obtained from patients with classic stigmata of secondary syphilis and reactive nontreponemal and treponemal serodiagnostic tests. Rabbit antiserum to purified endoflagella of *T. phagedenis* biotype Reiter was generously provided by David Blanco, Michael Lovett, and James N. Miller (University of California, Los Angeles Medical Center, Los Angeles).

SDS-PAGE, two-dimensional electrophoresis, and immunoblotting. Samples for SDS-polyacrylamide gel electrophoresis (PAGE) were boiled for 10 min in final sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, and 2% SDS and then separated by electrophoresis on 2.5% stacking and 12.5% separating gels, both with 2.6% bisacrylamide cross-linking, by the discontinuous buffer system of Laemmli (17). After electrophoresis, gels were either stained with Coomassie brilliant blue or silver (39) or transferred to nitrocellulose for immunoblotting.

Two-dimensional electrophoresis (2DE) was performed as described by O'Farrell (25) by using the modifications introduced by Norris et al. (23). Samples contained either 7 μl of washed, detergent-phase proteins (approximately 15 μg of total protein) or 2×10^8 to 3×10^8 Percoll-purified, sonically disrupted *T. pallidum*. Isoelectric focusing was performed for 7,200 V · h in tube gels (0.25 by 11 cm) containing 3.2% (pH 5 to 7) and 0.8% (pH 3.5 to 10) Ampholines (LKB, Bromma, Sweden). The second dimension consisted of SDS-PAGE on 8 to 20% linear gradient polyacrylamide gels (1.5 mm thick by 10 cm long). Molecular size standards or 2×10^8 Percoll-purified *T. pallidum* were run adjacent to the tube gels for standardization.

Specimens separated by SDS-PAGE and 2DE were transferred for immunoblotting to nitrocellulose sheets (0.2-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) at 250 mA for 3 h in a TransBlot Cell (Bio-Rad Laboratories, Richmond, Calif.). Nonspecific binding sites were blocked by incubation for at least 30 min in PBS containing either 5% nonfat dry milk or 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). Transfers were incubated with 1:100 (vol/vol) or 1:250 (vol/vol) dilutions, respectively, of human syphilitic serum or rabbit antiserum directed against *T. phagedenis* biotype Reiter endoflagella, with 20 μg of purified monoclonal antibody 11E3 or 5 ml of fresh culture supernatant containing monoclonal antibody 3B5. Immunoblots were probed with either ^{125}I-staphylococcal protein A, horseradish peroxidase-conjugated staphylococcal protein A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), or, in the cases of 2DE immunoblots reacted with monoclonal antibodies, with affinity-purified goat anti-mouse and rabbit anti-goat horseradish peroxidase conjugates (Cappel Laboratories, Malvern, Pa.). Autoradiographs were exposed on XAR-5 film (Eastman Kodak Company, Rochester, N.Y.) at −70°C. Horseradish peroxidase conjugates were diluted 1:1,000 in PBS-Tween 20 or PBS-milk and developed in 0.06% 4-chloronaphthol (Sigma) with 20% methanol and 0.02% hydrogen peroxide.

In vitro radiolabeling of *T. pallidum* polypeptides. Polypeptides of virulent *T. pallidum* were radiolabeled by using slight modifications of the protocol described by Stamm and Bassford (31). Testes were extracted in the medium described by these investigators except that both cysteine and methionine were deleted (31). Organisms were incubated at 34°C for 20 h at a final concentration of 5×10^8 cells per ml. Radiolabeling was performed with 0.2 mCi per 10^9 treponemes in an approximate 80:20 mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans ^{35}S-label; ICN Radiochemicals, Irvine, Calif.). Portions (approximately 200 μl) containing 10^8 radiolabeled organisms were immediately extracted at 0°C in varying concentrations of Triton X-114 in PBS as described below.

Extraction and phase partitioning of treponemal polypeptides with Triton X-114. Extraction and phase separation of treponemal polypeptides was performed as described by Bordier (3) except that precondensation was omitted in favor of extensive washing of the separated aqueous and detergent phases (which produced identical results). Sucrose cushions also were omitted inasmuch as their use did not, in our hands, improve separation between detergent and aqueous phases.

Percoll-purified *T. pallidum*, in portions of 5×10^9 organisms (representing approximately 1 mg of total protein), were added to 1 ml of ice-cold 1% (vol/vol) Triton X-114 in either 50 mM Tris hydrochloride, 0.1 M KCl, or PBS (detergent-to-protein ratio, approximately 10:1). After a 20-min incubation at 0°C with frequent gentle agitation, the insoluble material was separated by centrifugation at 4°C for 30 min at 20,000 × *g*. The supernatant, containing the detergent-soluble material, was removed and either stored at −70°C or immediately phase separated. Phase separation was performed by warming the supernatant for 10 min in a 37°C water bath, followed by centrifugation for 10 min at 13,000 × *g*. In the presence of residual Percoll, the detergent phase developed above, rather than below, the aqueous phase. The separated detergent and aqueous phases were then washed at least four times in the following manner. The detergent phase (approximately 50 μl) was diluted to 1 ml in the original buffer at 0°C, rewarmed, and spun in a microcentrifuge as described before. The aqueous phase was cleansed by the repeated addition of fresh 10% Triton X-114 to a final concentration of 2% and phase separated as described above.

Virulent, radiolabeled organisms were extracted at different detergent-to-protein ratios by adding portions of 10^8 live cells in 200 μl of incubation medium to 800 μl of ice-cold Triton X-114 at concentrations of 0.25, 0.12, 0.6, and 2.4% (vol/vol) in PBS (final detergent concentrations [vol/vol] of 0.02, 0.1, 0.5, and 2%, respectively). After 20 min of incubation on ice with gentle agitation, insoluble material was removed by centrifugation as described above. Supernatants containing Triton X-114 at concentrations of less than 1% were brought to a detergent concentration of 2% by the addition of appropriate volumes from a 10% Triton X-114 stock solution in PBS before phase separation.

Immunoprecipitation and fluorography of Triton X-114-extracted and phase-partitioned treponemal polypeptides. The presence of large amounts of nonspecific rabbit immunoglobulins in the incubation medium precluded immunoprecipita-

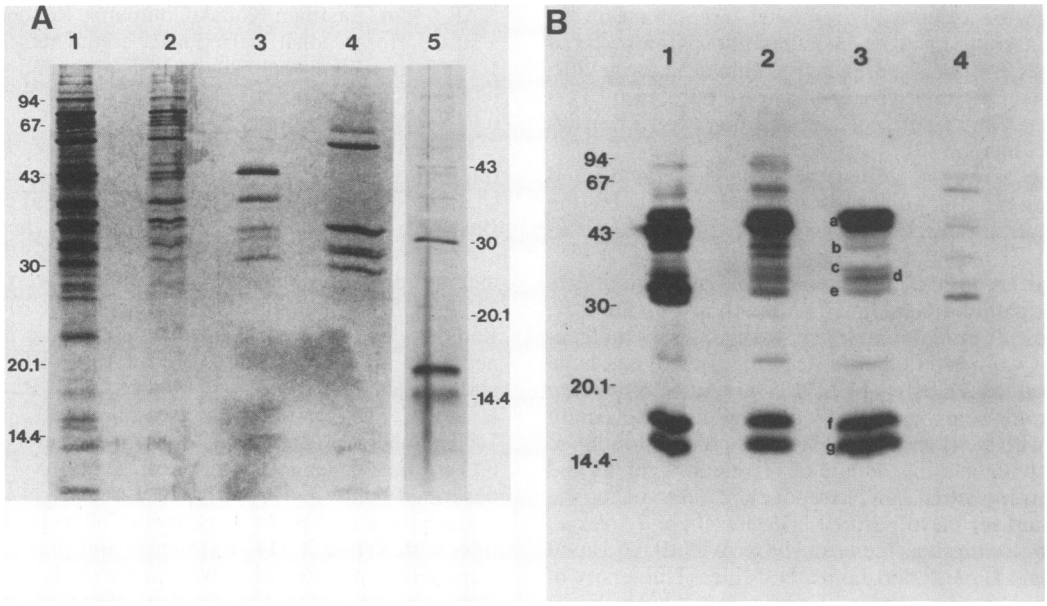

FIG. 1. SDS-PAGE and immunoblot analyses of *T. pallidum* polypeptides after Triton X-114 extraction of Percoll-purified organisms. Molecular size standards (in kilodaltons) are listed at the sides. (A) 12.5% SDS-polyacrylamide gel stained with Coomassie brilliant blue (lanes 1 to 4) or silver (lane 5). Lanes: 1, 3×10^8 whole, solubilized *T. pallidum* cells; 2, Triton X-114-insoluble material; 3, detergent-phase proteins; 4, aqueous-phase proteins; 5, detergent-phase proteins stained with silver. (B) Immunoblot with human syphilitic serum. Lanes 1 to 4 are the same as those in panel A. Major polypeptides detected in the detergent phase (lane 3) are labeled a through g.

tion of radiolabeled treponemal proteins by sequential addition of polyclonal antisera and protein A-Sepharose CL-4B (Sigma). Instead, 50-μl portions of protein A-Sepharose 4B were preincubated with 250 μl of human syphilitic serum (diluted 1:10 in PBS) before addition to the washed detergent and aqueous phases; the detergent phases were diluted with PBS to a final concentration of 1% before immunoprecipitation was done. After an overnight incubation at 4°C with gentle agitation, the slurries were collected by centrifugation at 13,000 × *g*, washed repeatedly in PBS, and boiled in the final sample buffer for electrophoresis on 12.5% SDS-polyacrylamide gels. Gel lanes for SDS-PAGE were loaded either with total immunoprecipitates from the detergent and aqueous phases or with equal proportions of the Triton X-114-insoluble material. Coomassie blue-stained gels were equilibrated in distilled water, soaked in fluorographic enhancer (Autofluor, National Diagnostics, Somerville, N.J.), dried under high vacuum, and exposed on XAR-5 film at –70°C.

Triton X-114 extraction of *T. pallidum* cells before and after removal of outer membranes. Approximately 5×10^9 virulent *T. pallidum* cells in PBS were divided into equal portions and collected by centrifugation at 20,000 × *g* for 20 min at 4°C. One pellet was gently suspended in 2 ml of ice-cold PBS to which 0.5 ml of 0.5% Triton X-114 (final Triton X-114 concentration of 0.1% [vol/vol]) was added. After a 20-min incubation on ice with gentle agitation, a 5-μl specimen was removed for electron microscopy to confirm that the outer membranes had been removed. The residual cytoplasmic bodies were then centrifuged again at 20,000 × *g* for 20 min at 4°C, and the supernatant containing the extracted outer membranes was removed. The pellets containing the cytoplasmic bodies and the organisms not extracted with 0.1% Triton X-114 were then each suspended in 250 μl of ice-cold PBS to which an equal volume of ice-cold 4% Triton X-114 in PBS (final Triton X-114 concentration of 2% [vol/vol]) was added. After incubation of the suspended pellets for 2 h at 4°C with gentle agitation, the Triton X-114-insoluble material was removed by a final centrifugation at 20,000 × *g* for 20 min at 4°C, and the supernatants were aspirated for phase partitioning. At a later time, the 0.1% Triton X-114 supernatant, containing extracted outer membrane material, was concentrated approximately fivefold on a Speed-Vac apparatus (Savant Instruments, Inc., Farmingdale, N.Y.); an equal volume of 4% Triton X-114 in PBS was added before phase partitioning. The detergent phases from the whole organisms and cytoplasmic bodies extracted with 2% Triton X-114 and from the 0.1% Triton X-114 supernatant were analyzed by SDS-PAGE and immunoblotting with human syphilitic serum.

Electron microscopy. Specimens were prepared for whole-mount electron microscopy by the single droplet method (36) on Parlodion (Ted Pella, Inc., Tustin, Calif.) and carbon-coated copper grids (400 mesh; Ted Pella) which were glow discharged immediately before use. Cells were floated on grids at 4°C, and the grids were washed with ice-cold PBS before negative staining was done at room temperature with 1% uranyl acetate (Sigma). Micrographs were taken at 80 kV of accelerating voltage on a JEOL 100C electron microscope.

RESULTS

SDS-PAGE and immunoblot analysis of *T. pallidum* polypeptides identified by phase partitioning with Triton X-114. Percoll-purified *T. pallidum* was extracted with 1% Triton X-114 at an approximate detergent-to-protein ratio of 10:1, and the solubilized material was phase partitioned. Figure 1A shows that the majority of treponemal proteins remained with the Triton X-114 insoluble material (lane 2). Proteins with apparent molecular masses of 47, 38, 35, 34, and 32 kDa

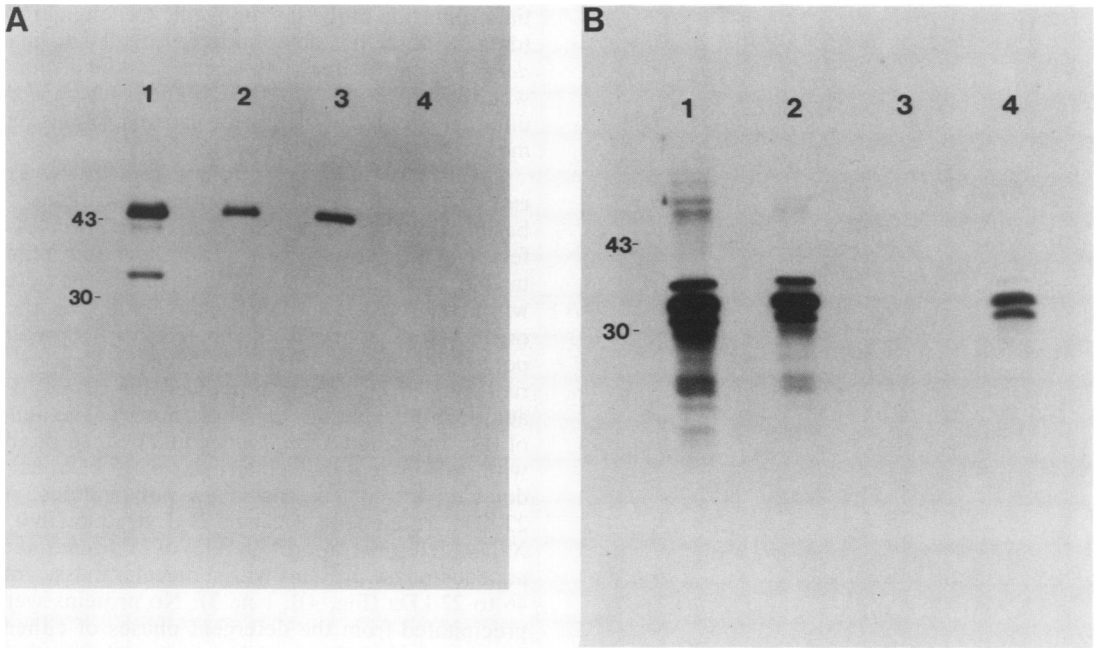

FIG. 2. Immunoblot analysis of *T. pallidum* fractions reacted with monoclonal antibody 11E3 directed against the 47-kDa protein (A) and monospecific antiserum directed against the endoflagellar proteins of the nonpathogenic *T. phagedenis* biotype Reiter (B). Lanes: 1, whole *T. pallidum* cells; 2, Triton X-114 insoluble material; 3, detergent-phase proteins; 4, aqueous-phase proteins. Size standards in kilodaltons are indicated on the left for both panels.

were identified by Coomassie brilliant blue staining of the detergent phase (lane 3); less abundant proteins with apparent molecular masses of 17 and 15 kDa also were detected. The polypeptides in the aqueous phase produced a profile distinctly different from that of the detergent-phase proteins (lane 4). With the exception of the 32-kDa polypeptide, all of the higher-molecular-mass detergent-phase proteins stained poorly with silver; in contrast, both the 17- and the 15-kDa proteins, which were barely detected with Coomassie brilliant blue, were readily identified by silver staining (lane 5).

Each of the detergent-phase proteins reacted to some extent by immunoblotting with human secondary syphilitic serum (Fig. 1B, lane 3). The 47-kDa protein was intensely antigenic, reacting as a 47-, 48-kDa doublet; the 15- and the 17-kDa polypeptides also were extremely immunoreactive. The four proteins which migrated between 38 and 32 kDa were significantly less antigenic. Weakly immunoreactive proteins with apparent molecular masses of 22 and 29 kDa were identified as well in the detergent phase. Immunoblots of the detergent-insoluble material suggested that significant amounts of each of the detergent-phase proteins were not solubilized by Triton X-114 (lane 2). The aqueous-phase proteins identified by Coomassie brilliant blue staining also reacted with human syphilitic serum (lane 4). Aqueous-phase antigens with apparent molecular masses of 46 and 43 kDa also were identified.

The intense immunoreactivity of the 47-kDa detergent-phase proteins suggested that they represented the well-characterized 47-kDa *T. pallidum* antigen (16, 18, 21, 35). This possibility was confirmed by immunoblotting the detergent-phase proteins with murine monoclonal antibody 11E3 which is directed against a pathogen-specific epitope of this immunogen (16, 19). Monoclonal antibody 11E3 reacted strongly with the detergent-phase 47-kDa protein (Fig. 2A, lane 3) but not with any of the proteins of similar apparent molecular masses in the aqueous phase (lane 4). Immunoblots done with the monoclonal antibody also confirmed that significant amounts of this antigen remained in the Triton X-114-insoluble material (lane 2). Monoclonal antibody 11E3 also recognized a 34-kDa breakdown product of the 47-kDa protein in whole cells and in Triton X-114-insoluble material (lanes 1 and 2), but it did not react with any other detergent-phase proteins (lane 3). To determine whether any of the detergent-phase proteins represented endoflagellar contaminants, immunoblots were reacted with monospecific antiserum directed against the antigenically conserved endoflagella of the nonpathogenic *T. phagedenis* biotype Reiter. This antiserum did not react with any of the proteins found in the detergent phase (Fig. 2B, lane 3). In contrast, polypeptides with the characteristic profile of *T. pallidum* endoflagellar proteins (28) were identified in whole cells, Triton X-114-insoluble material, and the aqueous-phase proteins (lanes 1, 2, and 4, respectively).

2DE and immunoblot analysis of detergent-phase proteins and polypeptides from solubilized, intact *T. pallidum*. The detergent-phase proteins were investigated further by 2DE and by immunoblotting with human syphilitic serum. In addition to the polypeptides previously identified by one-dimensional SDS-PAGE (Fig. 1B, lane 3), the detergent phase contained several minor, relatively acidic components (pI, approximately 5.5), with molecular masses ranging from 34 to 38 kDa and a strongly antigenic, more basic protein (pI, approximately 6.5) with an apparent molecular mass of 47 kDa (Fig. 3A). One antigen with a molecular mass centered at 34 kDa demonstrated a 2DE mobility identical to that shown by Norris et al. (23) for a 34-kDa protein recently cloned in *E. coli* by Swancutt et al. (34) and by van Embden and co-workers (12, 37). This particular protein did, in fact,

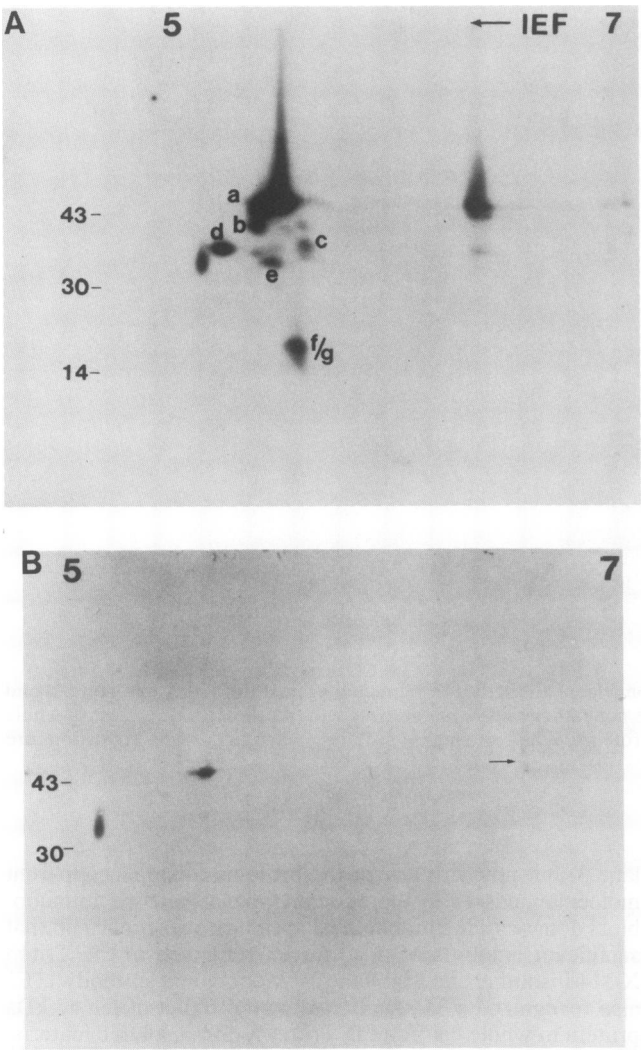

FIG. 3. Two-dimensional electrophoresis immunoblots of Triton X-114-extracted detergent-phase proteins. (A) Autoradiogram of detergent-phase proteins immunoblotted with human syphilitic serum and ^{125}I-staphylococcal protein A. Polypeptides detected by one-dimensional SDS-PAGE and immunoblotting are designated a through g, as in Fig. 1B, lane 3. Abbreviation: IEF, isoelectric focusing. (B) Detergent-phase proteins incubated sequentially with murine monoclonal antibodies 3B5 and 11E3, directed against the 34- and 47-kDa *T. pallidum* antigens, respectively (arrow indicates basic, 47-kDa material reacting with monoclonal antibody 11E3). Size standards in kilodaltons are indicated on the left for both panels.

react with murine monoclonal antibody 3B5 (34) directed against this antigen (Fig. 3B). The same 2DE immunoblot (Fig. 3B) was subsequently reacted with monoclonal antibody 11E3 and corroborated the 2DE mobility for the 47-kDa protein also published by Norris et al. (23). The basic 47-kDa material detected by human syphilitic serum (Fig. 3A) also reacted with monoclonal antibody 11E3 (Fig. 3B). Because it was not possible to distinguish clearly between the 15- and the 17-kDa proteins on 2DE immunoblots reacted with human syphilitic serum, the antigenic material in this molecular mass region was designated f/g (Fig. 3A). Comparison between the detergent phase (Fig. 3A) and *T. pallidum* 2DE transfers immunoblotted with human syphilitic serum revealed that the detergent phase contained a substantial proportion of the immunogenic treponemal polypeptides (data not shown). Rabbit antisera directed against *T. phagedenis* biotype Reiter endoflagella identified four proteins in whole *T. pallidum* with 2DE mobilities which were distinctly different from the detergent-phase proteins with similar molecular masses (data not shown).

Localization of detergent-phase proteins by Triton X-114 extraction of ^{35}S-radiolabeled organisms. Viable, ^{35}S-radiolabeled organisms were extracted with Triton X-114 at different detergent-to-protein ratios, and the immunoprecipitates from the detergent and aqueous phases were correlated with electron microscopic examination of the extracted organisms. Treponemes incubated in PBS produced a total polypeptide profile (Fig. 4A, lane 1) identical to that of radiolabeled organisms processed for SDS-PAGE without additional incubation (data not shown). The aqueous phase of PBS-incubated *T. pallidum* contained a prominent protein with an apparent molecular mass of 15 kDa, several faintly detected lower-molecular-mass polypeptides, and a faint 22-kDa protein (Fig. 4A, lane 3). Extraction in 0.02% Triton X-114 (detergent-to-protein ratio of 2:1) generated additional aqueous-phase proteins with molecular masses ranging from 46 to 22 kDa (Fig. 4B, lane 3). No proteins were immunoprecipitated from the detergent phases of either PBS-incubated or 0.02% Triton X-114 extracted organisms (Fig. 4A and B, lanes 2). The aqueous-phase proteins from organisms extracted in 0.1% Triton X-114 (detergent-to-protein ratio of 10:1) (panel C, lane 3), were essentially identical to, although more intense than, those identified in the 0.2% aqueous phase (panel B, lane 3). Proteins with molecular masses of 92, 75, 47, 38, and 32 kDa were detected in the 0.1% detergent phase. Extraction with 0.5% and 2.0% Triton X-114 (detergent-to-protein ratios of 50:1 and 200:1, respectively) resulted in progressive intensification of these detergent-phase proteins along with the appearance of the 17- and 15-kDa polypeptides identified on immunoblots (panels D and E, lanes 2). Aqueous-phase proteins showed little change with extraction in detergent concentrations above 0.1%.

Approximately 85% of radiolabeled organisms examined by electron microscopy immediately after overnight incubation were intact in that they possessed clearly discernible outer membranes and endoflagella tightly coiled about the cytoplasmic bodies (data not shown); this result corresponded well to the average motility (75%) after several radiolabeling experiments. Incubation in PBS resulted in a decreased proportion (approximately 70%) of intact treponemes, many of which demonstrated obvious disruptions in their outer membranes and exposure of endoflagella (Fig. 5A). In contrast, more than 90% of the organisms incubated in 0.02% Triton X-114 demonstrated more significant morphological derangements which consisted of apparent ballooning (Fig. 5B) or complete loss of outer membranes. Virtually all of the treponemes incubated with 0.1% Triton X-114 were without outer membranes (Fig. 5C), although occasionally, organisms demonstrated what appeared to be small blebs of adherent outer membrane material. Incubation in higher concentrations of detergent produced further ultrastructural derangements, including removal of large segments of the cytoplasmic membranes and extrusion of what appeared to be cytoplasmic contents (Fig. 5D).

Triton X-114 extraction of organisms before and after removal of the outer membrane. Organisms were extracted with 2% Triton X-114 before and after removal of their outer membranes. Electron microscopy confirmed that preincubation of virulent treponemes with 0.1% Triton X-114 selec-

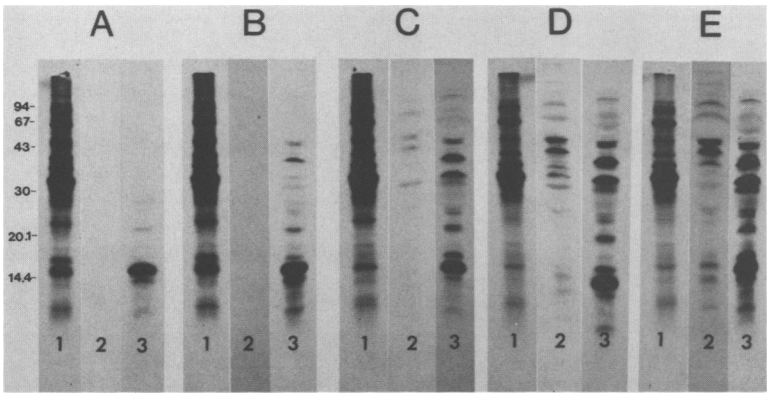

FIG. 4. Extraction of virulent, radiolabeled *T. pallidum* cells with PBS (A) or 0.02, 0.10, 0.50, or 2.0% Triton X-114 (panels B through E, respectively). Lanes: 1, Triton X-114-insoluble material; 2, detergent-phase proteins; 3, aqueous-phase proteins. Size standards in kilodaltons are indicated on the left.

tively removed their outer membranes (data not shown). The detergent-phase proteins obtained from both groups of treponemes were identical (Fig. 6, lanes 1 and 2). The 47-kDa protein was detected in the detergent phase from the 0.1% Triton X-114 supernatant which contained the solubilized outer membranes (lane 3).

DISCUSSION

In recent years, the application of modern molecular biological techniques has greatly facilitated the identification and functional characterization of important *T. pallidum* antigens. Despite these advances, uncertainty remains about

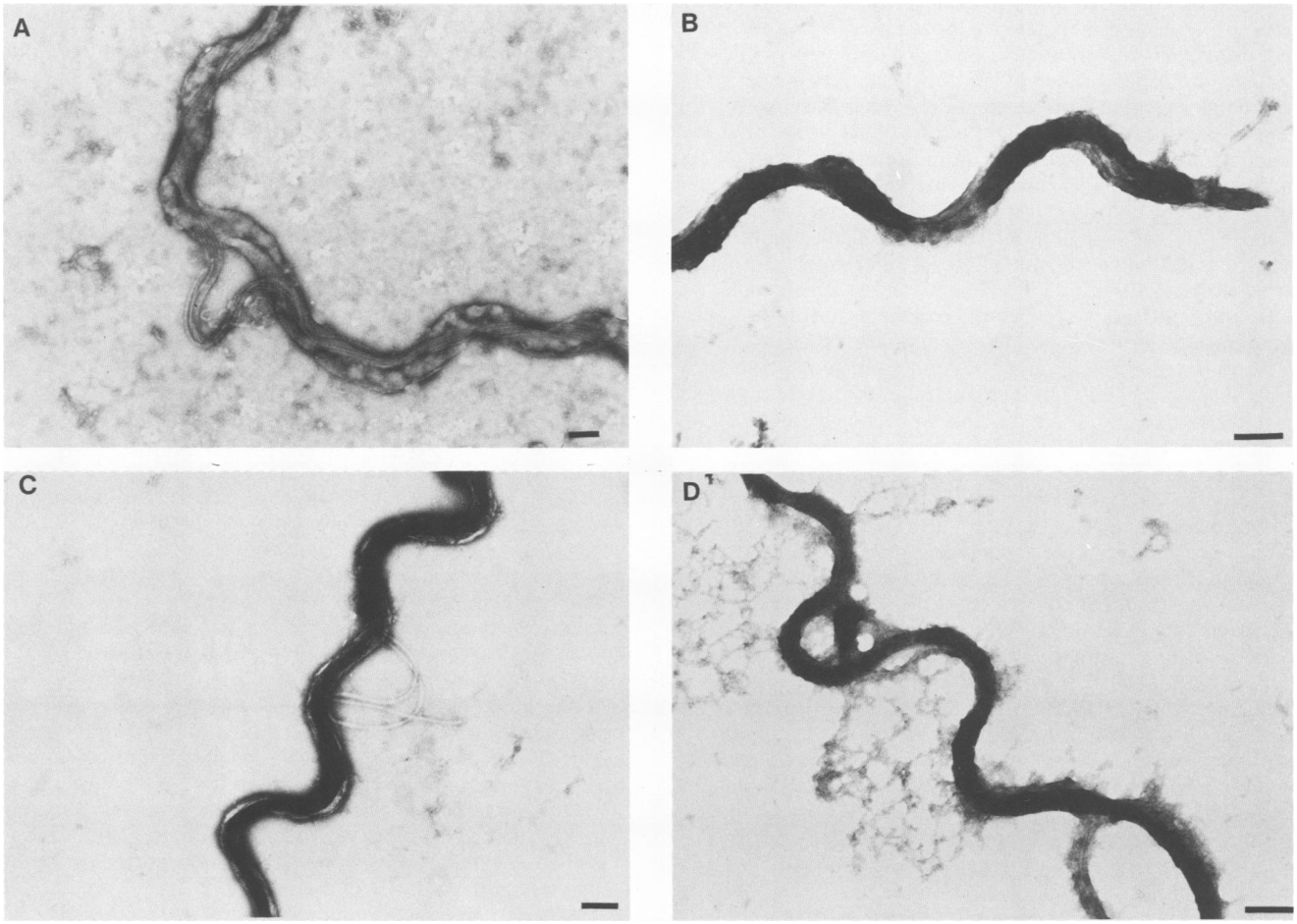

FIG. 5. Electron micrographs of negatively stained *T. pallidum* cells incubated with PBS (A) or 0.02 (B), 0.10 (C), or 0.5% (D) Triton X-114. Bars, 0.2 μm.

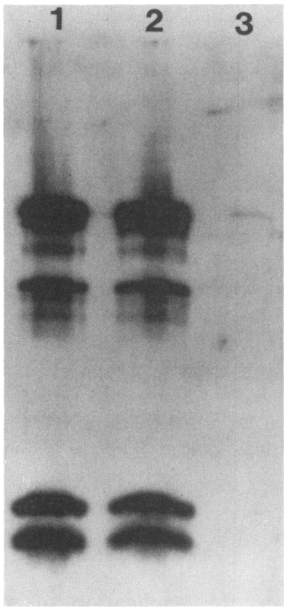

FIG. 6. Immunoblots of detergent-phase proteins from intact *T. pallidum* (lane 1), cytoplasmic bodies (lane 2), and solubilized outer membranes (lane 3) reacted with human syphilitic serum.

the identities of those polypeptides which are integral membrane proteins and their precise locations in either the outer or cytoplasmic membranes. Reports by other investigators that the outer membranes of spirochetes can be removed by detergents or chaotropic reagents provided the impetus for the current study (2, 15). Penn et al. (26) reported that low concentrations (0.2%) of the nonionic detergent Triton X-100 could solubilize *T. pallidum* outer membranes. We chose the closely related detergent, Triton X-114, for our experiments because its low cloud point (20°C) enabled separation of detergent-solubilized proteins into distinct aqueous and detergent phases (3).

A relatively small number of treponemal proteins segregated into the hydrophobic detergent phase. SDS-PAGE and immunoblot analysis revealed several pathogen-specific proteins that have been identified and characterized previously by other investigators. Among these proteins were the highly immunogenic 47-kDa protein described by Jones et al. (16) and others (18, 35), the 34-kDa protein described by Swancutt et al. (34) and designated TpD by van Embden and co-workers (12, 37), and two strongly antigenic 17- and 15-kDa proteins identified independently by Lukehart et al. (18) and Hensel et al. (11). 2DE and immunoblot analysis with murine monoclonal antibodies proved to be particularly useful for analyzing the detergent-phase proteins. This technique unequivocally identified the 34-kDa protein of Swancutt et al. (34) which was not expected on the basis of one-dimensional SDS-PAGE and immunoblotting with human syphilitic serum. Equally important was the demonstration by both one- and two-dimensional immunoblotting techniques that none of the proteins with apparent molecular masses between 32 and 38 kDa represented contaminating endoflagellar components. It also became apparent that *T. pallidum* contains both detergent- and aqueous-phase proteins with molecular masses of about 45 to 48 kDa; only the detergent-phase 47-kDa protein reacted with monoclonal antibody 11E3. Comigration of several immunogens probably explains the difficulty in establishing the pathogen specificity of this particular protein by one-dimensional immunoblotting techniques.

Phase partitioning of in vitro-radiolabeled organisms also identified several comigrating, highly antigenic proteins with molecular masses at and below 17 kDa; the complexity of this region of the SDS-PAGE profile of the organism has not been expressly noted previously. The radiolabeled aqueous-phase polypeptides with molecular masses at and below 15 kDa appeared to correspond to proteins that Stamm and co-workers (31, 32) proposed may represent extracellular secretion products of *T. pallidum* cells. However, in view of the fact that many of these organisms have suffered variable amounts of outer membrane damage, probably as a consequence of physical manipulations (e.g., centrifugation and resuspension) during the radiolabeling procedure, it is possible that these polypeptides represent soluble periplasmic components released from disrupted organisms.

To localize individual detergent-phase proteins to either the cytoplasmic or outer membranes, ^{35}S-radiolabeled organisms were extracted in different concentrations of Triton X-114 and examined by electron microscopy. Modest amounts of detergent-phase proteins were identified at concentrations which appeared to remove most of the outer membranes of the organisms. In contrast, greater amounts of detergent-phase proteins were extracted at detergent concentrations that resulted in more pronounced morphological alterations, including disruption of large portions of cytoplasmic membranes. The observation that all of the detergent-phase proteins, and not certain polypeptides, were progressively coextracted by increasing Triton X-114 concentrations suggested that all were solubilized from the same membranous compartment rather than from structures with different solubilization characteristics.

The experiments with ^{35}S-radiolabeled treponemes indicated that neither the majority of detergent-phase proteins, nor any individual polypeptide, could be localized entirely to the *T. pallidum* outer membrane. This conclusion was tested further by the extraction with 2% Triton X-114 of two portions of organisms, one of which was preincubated with 0.1% Triton X-114 (the detergent concentration which appeared sufficient for removal of most of the outer membranes). The detergent phases obtained from the two groups of organisms were qualitatively and quantitatively similar. In addition, the 47-kDa protein was identified in the detergent phase from the 0.1% Triton X-114 supernatant (which contained the solubilized outer membranes). Penn et al. (26) also identified the 47-kDa protein as the sole polypeptide component of outer membranes extracted with Triton X-100. The finding that the 47-kDa protein may be located in both the outer and cytoplasmic membranes of *T. pallidum* is consistent with results obtained recently for the recombinant 47-kDa antigen (4).

Our results support the hypothesis, proposed independently by Penn and Rhodes (27) and Radolf et al. (29), that the *T. pallidum* outer membrane is a protein-deficient lipid bilayer which is structurally and biochemically different from the outer membranes of conventional gram-negative bacteria. A growing body of evidence now exists to support this unorthodox proposal. First, it has long been appreciated that virulent organisms are antigenically less reactive until they are altered by fixation (27) or prolonged incubation at 4°C (10). Recently, this observation was supported by immunoelectron microscopy (7, 29). Although the presence of an outer coat of host or treponemal molecules traditionally has been offered as the explanation for this phenomenon (1, 8, 20), the ability of active complement to enhance antibody

binding is more consistent with this being an intrinsic property of the outer membrane (7, 29). Furthermore, removal of the outer membrane either by detergent solubilization or by physical manipulation results in a marked increase in antibody binding as determined by immunoelectron microscopy (27; J. D. Radolf, manuscript in preparation). In addition, Penn et al. (26) also have demonstrated that far fewer proteins are extrinsically radiolabeled on intact organisms in comparison with *T. pallidum* cells whose outer membranes are first removed by Triton X-100 extraction. Finally, Stamm et al. (32) were able to identify only endoflagellar proteins in the outer membrane material isolated from *T. pallidum* by using 0.04% SDS. A paucity of proteins in the outer membrane would readily explain all of these observations.

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