Antigenic and Structural Characterization of *Treponema pallidum* (Nichols Strain) Endoflagella

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Purified endoflagella from *Treponema pallidum*, Nichols strain, were characterized both structurally and antigenically. Structural analysis showed *T. pallidum* endoflagella are composed of 35- and 33-kilodalton (kDa) subunits which lack cysteine and do not share N-terminal amino acid sequence homology (20 residues). Intact endoflagella were dissociated into the composite subunits by incubation, which disrupts noncovalent bonds. Antiserum raised against purified *T. pallidum* endoflagella identified shared epitopes on the endoflagellar polypeptides of the nonpathogen, *Treponema phagedenis* biotype Reiter. Pathogen-specific epitopes were also found on the 35- and 33-kDa polypeptides by using affinity-purified endoflagellar antibodies. The pathogen-specific epitopes were localized by immunoblotting analysis of chymotryptic digests of the endoflagellar subunits; 18- and 26-kDa fragments derived from the 35-kDa subunit were found to possess a majority of the pathogen-specific epitopes. Both the 35- and 33-kDa subunits had surface exposure, as determined by immunoelectron microscopy, although additional immunochemical data indicated that the surface exposure of the 35-kDa subunit was greater.

Translational and axial motility are striking and common properties among the spirochetes which are mediated by their periplasmic endoflagella (5). The ability of virulent *Treponema pallidum* to rapidly disseminate to a variety of tissues (25) suggests that endoflagella contribute to the pathogenesis of syphilis. Studies using syphilitic sera on immunoblots of *T. pallidum* have shown that during syphilitic infection antibodies are generated against many *T. pallidum* proteins, including endoflagellum-associated polypeptides (1, 9, 10, 18). These observations support the hypothesis that endoflagella are targets for immune mechanisms responsible for acquired resistance.

Due to the difficulties in obtaining sufficient quantities of *T. pallidum*, information regarding *T. pallidum* endoflagella has largely been obtained from studies using endoflagella prepared from the cultivatable, nonpathogenic *Treponema phagedenis* biotype Reiter (TPR). The endoflagella of *T. pallidum* and TPR share considerable structural and antigenic homology (2, 4, 12, 19, 20, 21). We have recently found that antibodies to epitopes shared by the endoflagella of these treponemes can immobilize *T. pallidum* in vitro and contribute to the in vitro treponemal activity present in normal human serum (4). This treponemal activity indicates the biological importance of these common endoflagellar epitopes and suggests that unique properties of *T. pallidum* endoflagella could be particularly relevant to syphilis pathogenesis. Our recent ability to isolate *T. pallidum* endoflagella in milligram quantities permitted a detailed structural and antigenic characterization of this organelle, which is presented in this report.

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MATERIALS AND METHODS

Source of treponemes. *T. pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described elsewhere (16). Animals used to prepare *T. pallidum* endoflagella and antigen for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were injected intramuscularly with 10 mg of cortisone acetate per kg of body weight (Merck Sharp & Dohme, Rahway, N.J.) from days 3 through 10 after infection. TPR was grown and maintained as previously described (4).

Preparation of *T. pallidum* endoflagella. *T. pallidum* endoflagella were isolated and purified by a modification of the method for TPR endoflagella previously described (4, 12). Because it was found that *T. pallidum* endoflagella are more sensitive to dissociation in SDS than TPR endoflagella are (data not shown), Triton X-114 (Sigma Chemical Co., St. Louis, Mo.) was substituted in this procedure. *T. pallidum* suspensions containing approximately 2 x 10^13 organisms were prepared by multiple extraction in phosphate-buffered saline (PBS; pH 7.2) of 11-day-infected testicles. Sodium citrate (final concentration, 0.06 M) was added to the PBS to inhibit fibrin formation. *T. pallidum* outer membranes were removed by incubation with 0.1% Triton X-114 in double-distilled water for 2 h at 37°C (T. M. Cunningham, J. N. Miller, and M. A. Lovett, submitted for publication). Subsequent isolation of endoflagella resulted in significantly higher yields (1 mg/10^13 treponemes) than previously reported (4). The purity of the preparations was confirmed by SDS-PAGE and electron microscopy. *T. pallidum* endoflagella were adjusted to a protein concentration of 1 mg/ml of PBS by optical density (280 nm), with an extinction coefficient of 11 (1% [wt/vol], 1 cm) that was determined previously with bovine serum albumin standards and purified TPR endoflagella (4) by using the Lowry protein assay.

Amino acid analysis and sequence determination of *T. pallidum* endoflagellar polypeptides. Samples containing approximately 5 μg of endoflagella in 2.5 μl of double-distilled water were either first incubated with performic acid for 30
min or directly hydrolyzed under vacuum for 18 h in 6 N HCl at 110°C. Derivation with phenylisothiocyanate was performed before reverse-phase high-pressure liquid chromatography separation (Nova-Pak column) and amino acid analysis with a 120A analyzer (Applied Biosystems, Foster City, Calif.). N-terminal amino acid sequence determination was performed on samples containing 100 μg of endoflagella by using a 470A protein sequenator (Applied Biosystems).

SDS-PAGE immunoblotting and epitope bridging. SDS-polyacrylamide slab gels were run by using the discontinuous buffer system of Laemmli (14). Samples of T. pallidum and TPR were prepared as previously described (4). After electrophoresis, gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose sheets (Sartorius, Westbury, N.Y.) for immunoblotting (24). Nitrocellulose strips were incubated with serum diluted 1:50 or 1:100 in Tris sodium azide (TSA) with 3% nonfat dry milk (TSA-M; Carnation Co., Los Angeles, Calif.), probed with 125I-labeled staphylococcal protein A (Amersham Corp., Arlington Heights, Ill.), and autoradiographed with Kodak X-AR5 film. All gels included a lane of low-molecular-weight marker proteins (Pharmacia Fine Chemicals, Piscataway, N.J.) which were boiled for 10 min in final sample buffer (FSB) containing 2% SDS, 5% mercaptoethanol, and 0.001% bromphenol blue in 62.5 mM Tris buffer (pH 6.8).

The technique of epitope bridging previously described (8, 21) was used to identify externally located epitopes on intact T. pallidum endoflagella. T. pallidum endoflagella (60 μg) in 200 mM phosphate buffer (pH 7.2) were iodinated with lactoperoxidase (17). Nitrocellulose strips containing either 10 μg of T. pallidum endoflagella or 3 × 10⁶ to 5 × 10⁷ Percoll (Pharmacia)-purified T. pallidum (11) were incubated with antiserum diluted 1:100 in TSA-M, probed with the radiolabeled T. pallidum endoflagella adjusted to 200,000 cpm/ml in TSA-M, and autoradiographed as described above.

Production of antiserum to T. pallidum endoflagella. Each of five rabbits initially received approximately 100 μg of T. pallidum endoflagella. In four of the five rabbits, T. pallidum endoflagella in 1 ml of PBS were mixed with 1 ml of Freund complete adjuvant and injected between two intramuscular and two subcutaneous sites. In the fifth rabbit, endoflagella in 1 ml of PBS were mixed with 1 ml of Ribi MPL/TDM adjuvant (Ribi Immunochem Research, Inc., Hamilton, Mont.) and injected by the same routes. Animals which received endoflagella in Freund complete adjuvant were boosted twice by the same routes at 4-week intervals with approximately 100 μg of antigen in 1 ml of PBS mixed with 1 ml of Freund incomplete adjuvant. The animal which received endoflagella in the Ribi adjuvant was boosted once after 4 weeks with a similar preparation by using the same routes. Rabbit anti-TPR endoflagellar serum was prepared as previously described (4).

Affinity purification of antibody to T. pallidum endoflagellar polypeptides. Antibodies were affinity purified to the T. pallidum 33- and 35-kilo dalton (kDa) polypeptides by the procedure of Thomas et al. (23) as follows. Vertical strips (1 cm; approximately 10 μg of electrophoresed T. pallidum endoflagella) were cut from amido black-stained nitrocellulose transfers. The 33- and 35-kDa T. pallidum endoflagellar polypeptides excised and preincubated for 2 h with TSA-M before overnight incubation with anti-T. pallidum endoflagellar serum (obtained from the animal immunized with Ribi adjuvant) diluted 1:10 with TSA-M. After incubation, the strips were washed 10 times with TSA over a 2-h period. To elute antibody, the strips were transferred to polyethylene tubes containing 1 ml of 0.1 M glycine hydrochloride buffer (pH 2.0) with 0.5% bovine serum albumin. The eluted antibody was neutralized with 200 μl of 1 M Tris (pH 9.0) and dialyzed against PBS for 24 h with three changes of buffer. Eluted antibody from the different strips was then diluted 1:11 in TSA-M and used to identify T. pallidum endoflagellar polypeptides as described above for immunoblotting.

T. pallidum endoflagellar dissociation experiments. The ability of different incubation conditions to dissociate T. pallidum endoflagella was investigated as follows. T. pallidum endoflagella (10 μg) in 10 μl of PBS were added to 70 μl of 100 mM sodium citrate (pH 2.0), 100 mM sodium citrate (pH 4.0), 100 mM sodium phosphate (pH 6.0), or 100 mM sodium phosphate (pH 8.0); to 10 μl of 0.2 or 0.1% 2-mercaptoethanol in PBS (final concentrations, 0.1 and 0.05% [vol/vol], respectively); to 10 μl of 0.2, 0.1, or 0.5% SDS in PBS (final concentrations, 0.1, 0.05, and 0.025% [wt/vol], respectively); to 10 μl of 0.2 or 0.1% Nonidet P-40 (Shell Oil, Inc., Chemical Division, Lafayette, Calif.), N-lauryl sarcosine (sarcosyl; Sigma), or Triton X-114 (Sigma; final detergent concentrations, 0.1 and 0.05% [vol/vol], wt/vol, and vol/vol, respectively); to 70 μl of 9 M urea or 9 M guanidine hydrochloride in 200 mM phosphate buffer (final urea and guanidine hydrochloride concentrations, 8 M); and to 30 μl of FSB. Samples were incubated for 1 h at 37°C and mixed with 30 μl of 30% glycerol-0.001% bromphenol blue in PBS (pH 7.2) before SDS-PAGE. Because T. pallidum endoflagella were found to dissociate at relatively low concentrations of SDS, these experiments were performed with gels and running buffer containing 1/10 the usual SDS concentrations, i.e., 0.01% SDS. T. pallidum endoflagella (10 μg) were also added to 30 μl of FSB and 30% glycerol-0.001% bromphenol blue-PBS, boiled for 10 min, and electrophoresed as described above.

Chromotypic peptide mapping. One-dimensional antigenic peptide maps of T. pallidum endoflagellar polypeptides were constructed by a modification of the method described by Cleveland et al. (6). Two preparations of T. pallidum endoflagella (40 and 10 μg) were each boiled for 10 min in 40 μl of 2× and 1× FSB, respectively, and run in adjacent lanes on SDS-PAGE (12.5% polyacrylamide). After electrophoresis, the lane containing 10 μg of T. pallidum endoflagella was excised with a scalpel and stained for 15 min with Coomassie brilliant blue while the lane containing 40 μg was fixed for 2 h in 20% ethanol. The destained gel was carefully aligned with the ethanol-fixed gel, and the individual endoflagellar polypeptide bands of 33 and 35 kDa were excised with a scalpel. The excised gel fragments were then placed in separate wells of a second 15% polyacrylamide gel and overlaid with 1 μg of chymotrypsin in 10 μl of 30% glycerol-0.001% bromphenol blue-PBS. T. pallidum endoflagella (10 μg) boiled for 10 min in FSB were also included as a control. After the dye front had progressed two-thirds of the way through the stacking gel, the current was turned off for 45 min, after which electrophoresis was completed. The gel was then Western blotted, and individual strips of 33- and 35-kDa proteins partially digested with chymotrypsin were cut. The two strips were first reacted with anti-TPR endoflagellar serum diluted 1:100 in TSA-M, probed with 125I-labeled staphylococcal protein A, and autoradiographed. The same strips were then reincubated with the appropriate T. pallidum endoflagellar affinity antibodies diluted 1:11 in TSA-M. The strips were probed and autoradiographed as described above.

IEM. Specimens were prepared for immunoelectron microscopy (IEM) by the single droplet method (26). IEM of T.
**pallidum** endoflagella was performed on purified endoflagella and suspensions of freshly extracted organisms (approximately 10⁹ organisms per ml) from which gross testicular tissue debris was removed by two low-speed centrifugations for 10 min each at 500 x g. Treponemal fractions (1 ml) were centrifuged at 13,000 x g for 10 min in microcentrifuge tubes. The pellets were suspended in 1 ml of double-distilled water and incubated for 1.5 h at 37°C to remove T. pallidum outer membranes. The organisms were centrifuged again for 10 min at 13,800 x g, and the final pellets were suspended in 250 μl of PBS. For IEM, Parlodion (Mallinkrodt, Inc., St. Louis, Mo.) and carbon-coated 400-mesh copper grids were glow discharged immediately before use and floated onto 30-μl drops containing either the T. pallidum suspension or purified T. pallidum endoflagella (100 μg/ml of PBS). Grids washed once in double-distilled water were then floated for 30 min in a humidified chamber on 30-μl drops containing 1:100 dilutions of normal rabbit serum or anti-T. pallidum endoflagella serum and 1:2 dilutions of affinity antibodies to T. pallidum endoflagellar proteins in PBS-0.5% bovine serum albumin.

After three washes, the grids were floated for 20 min on drops containing protein A-colloidal gold (Janssen Pharmaceutical, Beerse, Belgium) diluted 1:40 with PBS-0.01% bovine serum albumin. After seven washes, the grids were negatively stained with 1% uranyl acetate and examined in an electron microscope (Jeol 100 CX) at 80 kV of accelerating voltage.

**RESULTS**

**T. pallidum** endoflagellar protein composition. Amino acid composition and the N-terminal sequence were determined for purified preparations of T. pallidum endoflagella. Compositional analysis revealed the complete absence of cysteine residues from T. pallidum endoflagella, a finding consistent with other bacterial flagella (3, 7, 13). Sequence determination identified 20 residues and the N-terminal position of both a major and a minor protein component; sequence comparison showed only 10% homology between the two proteins (Fig. 1). The protein composition of T. pallidum endoflagella was also shown by SDS-PAGE (Fig. 2). Heavily stained protein bands were identified at 35 and 33 (doublet) kDa. Further, several bands of less-intense staining were occasionally observed on some gels at 31, 29, and 27 kDa. It was also found that endoflagella, when electrophoresed in gels and running buffer containing 0.01% SDS, generated, in addition to 35- and 31-kDa bands, a single 33-kDa band and consistently lacked the bands at 29 and 27 kDa (Fig. 3).

**Physical stability of T. pallidum endoflagella.** Samples of purified T. pallidum endoflagella were incubated under a variety of conditions and then applied directly to the sample wells of SDS-polyacrylamide gels (Fig. 3). Undissociated endoflagella were unable to enter the gel, as shown by Coomassie brilliant blue staining at the origin of the stacking gel. Specimens in which dissociation had occurred produced endoflagellar protein bands at 35, 33, and 31 kDa.

Complete dissociation occurred after boiling in FSB (Fig. 3, lane 1) and incubation at 37°C in SDS concentrations as low as 0.05% (lane 4). In the absence of SDS, the reducing agent 2-mercaptoethanol did not produce dissociation (lanes 10 and 11). Endoflagella were also completely and partially dissociated by incubation at 37°C at pH 2 (lane 6) and pH 4 (lane 7), respectively, while incubation at higher pHs had little effect (lanes 8 and 9). Boiling in phosphate buffer (pH 8.0) partially dissociated endoflagella; stained material entered the stacking gel but not the separating gel (data not shown). The abilities of the ionic detergent sarcosyl and the nonionic detergents Nonidet P-40 and Triton X-114 to dissociate endoflagella at 37°C were also compared with the dissociation ability of SDS. At the concentrations tested (0.05 and 0.1%), only SDS was capable of dissociating intact endoflagella (data not shown). Complete dissociation was also produced by 37°C incubation in either 8 M urea or 8 M guanidine (data not shown).

**Epitope distribution on endoflagellar proteins.** Antisera to

![FIG. 2. Polypeptides of T. pallidum endoflagella (TP-EF) and T. pallidum (TP). Preparations were separated on an SDS-12.5% polyacrylamide gel and were stained with Coomassie brilliant blue. The numbers refer to the molecular weights (x10³) of the protein markers (Mk).](http://iai.asm.org/)
purified *T. pallidum* endoflagella were raised in rabbits with Freund and Ribi adjuvant preparations. Serum from an animal immunized with endoflagella and Freund adjuvant reacted with endoflagellar proteins of both *T. pallidum* (35, 33 [doublet], and 31 kDa) and TPR (37, 33 [doublet], 30, and 27 kDa) (Fig. 4), corroborating the presence of common epitopes between the endoflagellar proteins of these two treponemes, which had been previously identified with anti-TPR endoflagellar serum (4, 21). Several bands were weakly detected at lower molecular weights, which may represent endoflagellar degradation products as previously suggested (4). Serum from the animal immunized with endoflagella and Ribi adjuvant also reacted with endoflagellar proteins of *T. pallidum* (35, 33 [doublet], and 31 kDa) and TPR (33-kDa doublet) but failed to detect the TPR 27- and 30-kDa endoflagellar proteins and only weakly detected the TPR 37-kDa endoflagellar protein. Because the Ribi antiserum was either not reactive or weakly reactive with these TPR proteins, we used this antiserum to prepare *T. pallidum* endoflagellar affinity antibodies in an effort to create probes that would identify the presence and distribution of pathogen-specific epitopes. Affinity antibodies to the 35-kDa *T. pallidum* protein strongly recognized only the 35-kDa *T. pallidum* polypeptide (Fig. 5). Antibodies to the 33-kDa *T. pallidum* protein, while only weakly detecting the TPR 33-kDa polypeptides, strongly recognized both the 33- and 31-kDa polypeptides of *T. pallidum*, suggesting that these proteins are related. The results with the affinity antibodies demonstrate the presence of pathogen-specific epitopes on both the *T. pallidum* 33- and 35-kDa endoflagellar proteins.

The antibodies affinity purified to the *T. pallidum* endoflagellar subunits were used in combination with anti-TPR endoflagellar serum to probe immunoblots of chymotryptic partial digests of the 35- and 33-kDa *T. pallidum* endoflagellar polypeptides. Digests of each polypeptide were first probed with anti-TPR endoflagellar serum to detect peptides containing common epitopes and then with the corresponding affinity antibodies to detect peptides containing pathogen-specific epitopes. Undigested endoflagella were also included and probed with anti-*T. pallidum* endoflagellar serum to identify residual unproteolyzed material in each peptide digest. Antibodies affinity purified to the 33- and 35-kDa subunits did not detect any new peptide fragments that were not already identified by anti-TPR endoflagellar serum (Fig. 6), indicating that all of the antigenic peptides generated contained common epitopes. However, affin antibodies purified to the 35-kDa subunit were shown to detect peptides of 26 and 18 kDa (Fig. 6) with greater intensity than those detected with anti-TPR endoflagellar serum, suggesting that these peptides from the 35-kDa protein have a greater proportion of pathogen-specific determinants.

Identification of exposed determinants on intact *T. pallidum* endoflagella

**FIG. 3.** SDS-PAGE of intact *T. pallidum* endoflagella under different incubation conditions. Undissociated endoflagella did not enter the 4.5% stacking gel and can be seen as Coomassie brilliant blue-stained material caught in the sample wells of the stacking gel (*lane 1*). Lanes: 1, *T. pallidum* endoflagella boiled in FSB; 2, *T. pallidum* endoflagella in PBS (pH 7.2); 3, 4, and 5, 0.01, 0.05, and 0.1% SDS, respectively; 6 and 7, citrate buffer at pH 2 and 4, respectively; 8 and 9, phosphate buffer at pH 6 and 8, respectively; 10 and 11, 0.05 and 0.1% 2-mercaptoethanol, respectively. Specimens in lanes 2 through 11 were incubated for 1 h at 37°C.

**FIG. 4.** The detection of *T. pallidum* (Tp) and TPR endoflagellar proteins by antisera raised against *T. pallidum* endoflagella with Freund and Ribi adjuvants. Western blots of *T. pallidum* and TPR were incubated overnight with αTp-EF(Freund) diluted 1:250 or αTp-EF(Ribi) diluted 1:100 in TSA-M. Antigenic proteins were detected with 125I-labeled protein A and were autoradiographed with Kodak X-AR5 film. The numbers refer to the molecular weights (×10^3) of protein standards included but not shown.
endoflagella. Determinants exposed on intact T. pallidum endoflagella were identified by a modification of immunoblotting termed epitope bridging (8). By this technique, nitrocellulose transfers are probed with intact 125I-labeled T. pallidum endoflagella rather than with 125I-labeled staphylococcal protein A after reaction with the appropriate antiserum. Epitopes on the endoflagellar surface that cross-react with those of proteins on blots are bound by specific multivalent antibody. Immunoblots of T. pallidum and T. pallidum endoflagella probed with anti-T. pallidum endoflagellar serum and 125I-labeled staphylococcal protein A (Fig. 7, lanes WB) revealed all of the major endoflagellar polypeptides. However, the same antiserum recognized only the 35-kDa endoflagellar protein on blots reacted with 125I-labeled T. pallidum endoflagella (Fig. 7, lanes EB). These data indicate that the 35-kDa protein contains the principal surface-exposed epitopes on intact T. pallidum endoflagella.

IEM of T. pallidum endoflagella. Purified T. pallidum endoflagella and endoflagella exposed on T. pallidum by removal of outer membranes were incubated on copper grids with either normal rabbit serum, anti-T. pallidum endoflagellar serum, or the affinity antibodies to the 35- and 33-kDa T. pallidum endoflagellar proteins and probed with protein A-collodial gold. Anti-T. pallidum endoflagellar serum as well as affinity antibodies to the 35- and 33-kDa proteins produced specific colloidal gold labeling of both intact endoflagella on T. pallidum (Fig. 8B, C, and D) and purified endoflagella (data not shown). In contrast, normal rabbit serum did not produce labeling of either endoflagella on T. pallidum (Fig. 8A) or purified endoflagella (data not shown).

DISCUSSION

Evidence supporting the involvement of endoflagella in the pathogenesis of syphilis has been provided by the recent finding that anti-endoflagellar antibodies in both normal human serum and antiserum to purified TPR endoflagella possess complement-dependent in vitro treponemical activity against T. pallidum (4). These results suggest a transient surface exposure of this periplasmic organelle, a concept supported by the recent studies of Stamm et al. (22) which showed the immunoprecipitation of endoflagellar proteins from viable, structurally intact T. pallidum. Because endoflagella may contribute directly to the virulence and invasiveness of T. pallidum, molecular properties unique to T. pallidum endoflagella are likely to be important in syphilis pathogenesis. In this report, we have presented a structural and antigenic analysis of T. pallidum endoflagella.

The SDS-PAGE profile of purified T. pallidum endoflagella consisted of heavily stained bands at 35 and 33 (doublet) kDa, similar to those previously reported (4, 19), and occasional minor bands at 31, 29, and 27 kDa. These minor bands may represent degradation products since antibodies affinity purified to the 33-kDa subunit were shown to react with the 31-kDa band (Fig. 5). A similar result was observed

FIG. 5. Detection and relationship of T. pallidum-specific endoflagellar determinants. Rabbit anti-T. pallidum endoflagellar serum (αTp-EF) at a 1:100 dilution and antibodies against individual T. pallidum endoflagellar proteins (α35 and α33 kDa) diluted 1:11 were reacted with Western blots of T. pallidum (Tp) and TPR. The numbers refer to the molecular weights (×10^3) of protein standards included but not shown.

FIG. 6. Chymotrypsin antigenic peptide maps of T. pallidum endoflagellar polypeptides. The 35- and 33 (doublet)-kDa bands of T. pallidum endoflagella were excised as described in the text, digested with 1 μg of chymotrypsin before electrophoresis on a 15% polyacrylamide gel, and then Western blotted. Strips of digested 35- and 33-kDa polypeptides were sequentially probed and autoradiographed by using anti-TPR endoflagellar serum (αTPR-EF) followed by affinity antibodies to the T. pallidum endoflagellar proteins (α35 and α33). Undigested T. pallidum endoflagella (Tp-EF) reacted with anti-T. pallidum endoflagellar serum (αTp-EF) are included for comparison. Asterisks denote peptide fragments containing a majority of specific T. pallidum determinants. Numbers refer to the molecular weights (×10^3) of protein standards included but not shown.
guanidine hydrochloride, and particularly in SDS, but they were not dissociated in the ionic detergent sarcosyl or the nonionic detergents Nonidet P-40 and Triton X-114. Comparison with TPR endoflagella showed that T. pallidum endoflagella are more sensitive to dissociation by SDS and acidic pH (data not shown). The inability of 2-mercaptoethanol to dissociate T. pallidum endoflagella is consistent with the absence of cysteine residues capable of disulfide bonding. We conclude that the T. pallidum endoflagellar structure is not mediated by covalent bonds between the two constituent proteins, a finding similar to that reported for TPR endoflagella (21), endoflagella from other spirochetes (3, 13), and other purified bacterial flagella (7).

Antiserum against purified T. pallidum endoflagella identified all of the endoflagellar polypeptides of T. pallidum and TPR (Fig. 4), a result consistent with the cross-reactivity between each of the analogous proteins (21). However, the finding that antiserum from the animal immunized with T. pallidum endoflagella in Ribi adjuvant failed to identify the TPR 27- and 30-kDa proteins and only weakly identified the TPR 37-kDa protein was unexpected. It is unlikely that this result was due to differences in antibody titers since the Ribi antiserum was used at a dilution 2.5 times greater than that used for Freund antiserum and since the intensity of bands detected with either antiserum was comparable. In view of the result, we used the Ribi antiserum to prepare affinity antibodies against T. pallidum endoflagellar proteins with the objective of identifying the distribution of pathogen-specific determinants. Affinity antibodies to the 35- and 33-kDa proteins were found to recognize strongly only the homologous T. pallidum endoflagellar proteins (Fig. 5). Similar results were shown previously on T. pallidum immunoblots by using affinity antibodies purified to the analogous TPR endoflagellar proteins (21). These observations suggest that both the common and specific epitopes are not redistributed between the two endoflagellar subunits, a finding supported by the lack of significant N-terminal sequence homology between the 35- and 33-kDa proteins (Fig. 1).

Chymotryptic peptide digests in combination with affinity antibodies and anti-TPR endoflagellar serum revealed that peptides generated from the T. pallidum 35-kDa endoflagellar protein (18 and 26 kDa) contained a majority of the pathogen-specific epitopes (Fig. 6). This finding is consistent with the observation that T. pallidum surface accessibility is unaltered against the 35-kDa protein but markedly diminished against the 33-kDa proteins after TPR absorption of either anti-T. pallidum endoflagellar serum (data not shown) or syphilitic immune rabbit serum (15). These results indicate that the T. pallidum 35-kDa protein contains the principal pathogen-specific endoflagellar determinants.

We used a modification of immunoblotting called epitope bridging (8) to determine which of the endoflagellar proteins are located on the surface of intact endoflagella. By this technique, T. pallidum endoflagellar antiserum recognized only the 35-kDa polypeptide, a finding consistent with the preferential radiolabeling of the 35-kDa protein (Fig. 7, lane 125I-EF). These results indicated that the most externally located epitopes of T. pallidum endoflagella are located on the 35-kDa subunit. It is also possible that determinants on the 33-kDa proteins are accessible to antibody but not to the epitope-bridging reagent as a result of the surface geometry of intact endoflagella. Indeed, IEM with purified endoflagella and endoflagella exposed on T. pallidum (Fig. 8) showed significant binding of the affinity antibodies to both the 35- and 33-kDa proteins, thereby demonstrating the accessibility of these epitopes on intact endoflagella. These results also

FIG. 7. Identification of externally located epitopes on T. pallidum endoflagella by epitope bridging. Nitrocellulose transfers of purified T. pallidum endoflagella (Tp-EF) and T. pallidum (Tp) were incubated with a 1:100 dilution of anti-T. pallidum endoflagellar serum (αTp-EF) and probed with either 125I-labeled staphylococcal protein A by Western blot (WB) or 125I-labeled T. pallidum endoflagella by epitope bridging (EB) before autoradiography. An autoradiogram of 125I-labeled T. pallidum endoflagellar proteins (125I-EF) separated by SDS-PAGE is included. The numbers refer to the molecular weights (×103) of protein standards included but not shown.

previously with endoflagellar proteins of TPR (21). However, the possibility that these minor polypeptides are unique endoflagellar subunits and products of either separate genes or genes with internal promoter sites has not been eliminated. Reduction of the SDS concentration in gels did not affect the 35-kDa component but did result in the generation of a single 33-kDa band and the diminution or loss of the lower-molecular-weight bands. This observation suggested that T. pallidum endoflagella are composed of two proteins, a finding supported by N-terminal sequence analysis which revealed only a major and a minor component; high-pressure liquid chromatography elution patterns indicate that these components represent the 33- and 35-kDa proteins, respectively. Although N-terminal-sequence comparison to residue 20 of these two proteins showed little homology, it was found that the T. pallidum major component shared 85% sequence homology with the major endoflagellar component of TPR (data not shown), further demonstrating the similarities between the 33-kDa endoflagellar proteins of these organisms.

Purified T. pallidum endoflagella were dissociated by incubation at 37°C at acidic pH, in the chaotropes urea and
show that the procedure used for endoflagellum preparation did not alter the antigenic and structural properties of the molecule since IEM with whole antiserum and affinity antibodies did not detect any differences in binding between purified endoflagella and endoflagella exposed on *T. pallidum*.

Since common endoflagellar epitopes are capable of eliciting treponemical antibody against *T. pallidum* (4), antibodies specific to *T. pallidum* endoflagella may have greater treponemical activity by virtue of the pathogen-unique properties of this molecule. The demonstration that the *T. pallidum* 35-kDa protein possesses pathogen-specific epitopes and appears to be an external subunit of endoflagella suggests that this protein represents a principal target for immune mechanisms. Previous studies using immunoblotting have shown that *T. pallidum* endoflagellar components, particularly the 35-kDa protein, are strongly recognized by serum antibody early on and throughout the course of human and experimental rabbit syphilis (1, 9, 10, 18). These findings have been recently extended by S. A. Lukehart (personal communication) to include cellular mechanisms operative during experimental syphilis. *T. pallidum* proteins ranging from 30 to 37 kDa excised from SDS-polyacrylamide gels were shown to be potent blastogenic activators of lymphocytes from infected rabbits. These observations indicate a strong humoral and cellular response to *T. pallidum* endoflagella that may contribute to the development and persistence of immunity. Studies to determine the ability of purified *T. pallidum* endoflagella to elicit treponemical antibody and protective immunity in experimental syphilis are in progress.

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