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Changes in the Cell Surface Properties of *Treponema pallidum* That Occur during In Vitro Incubation of Freshly Extracted Organisms

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We previously reported that a number of *Treponema pallidum* membrane proteins appear to reside on the cell surface, since intact treponemes radiolabeled by overnight incubation in medium containing [^{35}S]methionine bind immunoglobulin G (IgG) antibodies directed against these proteins. In the present study, it was found that freshly extracted organisms radiolabeled in vitro for only 2 h inefficiently bound IgG antibodies directed against just two proteins of molecular weights 40,000 and 34,000. An in vitro incubation period of greater than 8 h was required before IgG antibodies present in rabbit syphilitic serum could recognize additional protein antigens on the cell surface. Treatment of aged treponemes, but not freshly extracted organisms, with 0.04% sodium dodecyl sulfate selectively removed a membranous layer from the treponemal surface. Only three treponemal proteins were found associated with this structure, including the same 40,000- and 34,000-molecular-weight proteins mentioned above. These two proteins most likely represent endoflagellar subunits, since they were precipitated with rabbit antisera prepared against purified endoflagellar subunits of the cultivable treponemal strain *Treponema phagedenis*. Further evidence also was obtained that cells of *T. pallidum* actively secrete into their extracellular environment a unique class of low-molecular-weight proteins.

Treponema pallidum is the etiologic agent of syphilis. A major goal of investigators has been to determine the particular antigens of this bacterium that are responsible for eliciting a protective immune response in the infected host. Since a protective response is presumably targeted against antigenic determinants residing on the cell surface, several different techniques have been used in an attempt to identify the surface-exposed protein antigens of *T. pallidum* (1, 2, 7, 14, 23, 26, 29, 32, 35, 38). The results obtained in different laboratories have shown considerable variation, and as yet, no consensus has emerged.

Although the organism cannot be continuously cultivated in vitro, treponemes harvested from infected rabbit testes can remain viable for considerable periods. We have previously found that such freshly extracted treponemes continue to synthesize a seemingly full complement of proteins that can be intrinsically radiolabeled with [^{35}S]methionine to very high specific activities (35). Using the whole-cell radioimmunoprecipitation procedure described by Hansen et al. (11), we reported that a number of *T. pallidum* membrane proteins appeared to be surface exposed, since intact treponemes radiolabeled by overnight incubation in medium containing [^{35}S]methionine bind immunoglobulin G (IgG) antibodies directed against these proteins (35). We also described a unique class of low-molecular-weight treponemal proteins that appear in the extracellular medium following overnight incubation (35).

Numerous studies have demonstrated that freshly extracted treponemes exhibit serological reactivity only after in vitro storage for some time period (4, 12, 19–21, 24, 27, 33). For this reason, we considered the possibility that our previous findings resulted from changes in the surface properties of *T. pallidum* that occur during overnight incubation in radiolabeling medium. In this study, we demonstrate that such surface changes did occur and that freshly extracted organisms radiolabeled in vitro for only 2 h inefficiently bound IgG antibodies directed against just two proteins, both of which are most likely endoflagellar subunits. We also found that 0.04% sodium dodecyl sulfate (SDS) removed from aged treponemes, but not from freshly extracted organisms, a membranous surface layer. Finally, further evidence is presented indicating that treponemes actively secrete several proteins into their extracellular environment.

MATERIALS AND METHODS

Cultivation and in vitro radiolabeling of treponemes. The source of *T. pallidum* (Nichols) and its cultivation in infected rabbit testes have been previously described (36). Treponemes were extracted into medium and intrinsically radiolabeled with [^{35}S]methionine as previously described (35). For experiments in which aliquots of treponemes were radiolabeled for different times and then directly compared (see Results), the concentration of [^{35}S]methionine in the radiolabeling medium was adjusted so that the level of incorporation of label into total treponemal protein in each aliquot was approximately equal. For example, in experiments in which treponemes were radiolabeled for 2, 4, or 18 h, the amount of [^{35}S]methionine stock solution (10.4 μCi/μl) added to each sample was 64, 45, or 32 μl, respectively. [^{35}S]methionine (1,154 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

Detection of cell surface-exposed antigenic proteins. A modification of the whole-cell radioimmunoprecipitation procedure of Hansen et al. (11) was used as previously described (35). In the present study, this procedure was further modified to obtain more efficient solubilization of antigen-antibody complexes. After being washed three times with phosphate-buffered saline to remove unbound antibodies and other serum components, treponemes were suspended in 0.7 ml of solubilization buffer (11) minus detergent and disrupted by brief sonication on ice (three 5-s bursts with the Microtip at 30% power) (model 300; Fisher Scientific Co., Pittsburgh, Pa.). Three detergents (0.1 ml of each) at a 10× concentra-

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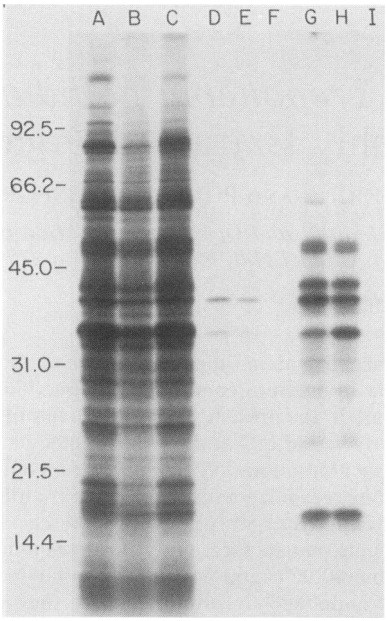


FIG. 1. Radioimmunoprecipitation of *T. pallidum* cell surface-exposed protein antigens. Freshly extracted treponemes that had been incubated in radiolabeling medium for either 2 or 18 h were incubated with ERSS. Cells were washed free of unbound antibody, and antigen-antibody complexes were solubilized and precipitated with protein A-Sepharose. Precipitates were analyzed by SDS-PAGE and fluorography. Lanes: A, treponemes labeled with [^{35}S]methionine for 18 h and solubilized in buffer containing 1% SDS; B and C, treponemes radiolabeled for 2 (B) or 18 (C) h and solubilized in buffer as described by Hansen et al. (11); D to I, treponemes radiolabeled for 2 (D to F) or 18 (G to I) h and precipitates obtained with serum from a rabbit 99 (D and G) and 133 (E and H) days postinfection and with preinfection control serum (F and I). The protein sizes are indicated in kilodaltons. See text for additional details.

tion were added to the sonicate, which was then processed as previously described (35).

Treatment of treponemes with 0.04% SDS. After incubation in radiolabeling medium for the indicated times (see Results), treponemes were pelleted by centrifugation at 20,000 × *g* for 15 min at 4°C, washed once in FTA-Abs buffer (41), and suspended in 0.5 ml of distilled H_2O at a concentration of 2×10^9 cells per ml. An equal volume of 0.08% SDS in distilled H_2O was added, and each sample was gently agitated at room temperature for 15 min. Treponemes were pelleted by centrifugation in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 15 min at 4°C. The cell-free supernatants were centrifuged at 100,000 × *g* for 90 min at 4°C. The small, clear, membranous pellet visible following centrifugation was solubilized in 132 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS.

Transmission electron microscopy. Pellets of whole untreated treponemes, treponemes treated with 0.04% SDS for 15 min, and the putative outer membrane fraction were fixed with 2% glutaraldehyde (EM grade; Polysciences, Inc., Rydal, Pa.) in 0.1 M Sorensen buffer (pH 7.2) for 1 h at room temperature (22°C). The specimens were then washed several times in Sorensen buffer and postfixed for 45 min at room temperature with 1% osmium tetroxide in the same buffer. Following dehydration in a graded series of ethanol and two changes of propylene oxide, the samples were infiltrated and embedded in a fresh mixture of Epon-Araldite (Polysciences) and cured at 60°C for 48 h. Silver-to-gray thin sections were prepared with a glass knife on a Reichardt ultramicrotome, counterstained with uranyl acetate followed by lead citrate, and examined in a Siemens 101A electron microscope operating at 60 kV.

Extracellular treponemal proteins. Treponemes that had been radiolabeled with [^{35}S]methionine for the indicated times were pelleted in a Microfuge, and the supernatant fraction containing the extracellular proteins was precipitated with ice-cold trichloroacetic acid (TCA) and processed as previously described (35).

Rabbit sera. The experimental rabbit syphilitic sera (ERSS) used in this study were previously described (36). Rabbit antisera directed against purified *Treponema phagedenis* biotype Kazan 5 endoflagellar proteins were obtained from Nyles Charon of West Virginia University (16, 17).

Radioimmunoprecipitation, SDS-PAGE, and fluorography. Radiolabeled treponemal proteins were solubilized in buffer containing 1% SDS and immunoprecipitated as previously described (35). The SDS-polyacrylamide gel electrophoresis (PAGE) system used also was previously described (36). Molecular weights were calculated on the basis of the positions of unlabeled known protein standards (Bio-Rad Laboratories, Richmond, Calif.) and are the same as those used in previous studies from this laboratory. For correlations of designations and molecular weights of treponemal proteins assigned by various investigators, see Norris et al. (25).

RESULTS

Cell surface proteins of *T. pallidum*. To initiate this study, we once again determined which treponemal protein antigens were precipitated when the whole-cell radioimmunoprecipitation procedure was used. Treponemes were harvested from infected rabbit testes and radiolabeled in vitro with [^{35}S]methionine for either 2 or 18 h. Immediately following the end of the radiolabeling period, cell surface proteins accessible to IgG antibodies present in high-titer ERSS were identified (see Materials and Methods). Intact treponemes radiolabeled for 18 h bound IgG antibodies directed against a number of distinct proteins (Fig. 1). Particularly prominent among the protein antigens that were precipitated by this procedure were those with approximate molecular weights of 48,000 (a doublet), 42,000, 40,000, 34,000, and 16,000. These results were very similar to those found previously (35). In marked contrast, only the 40,000-molecular-weight protein (40K protein) and the 34K protein were recognized by using intact treponemes radiolabeled in vitro for just 2 h. Furthermore, the efficiency of precipitation of these two proteins was considerably reduced when compared with treponemes labeled for the longer time period.

The finding that there was a very real difference in the results obtained, depending on the length of time treponemes were incubated in vitro in radiolabeling medium, was reproduced several times. Additional experiments (data not shown) demonstrated the following. (i) The results were not significantly altered if treponemes radiolabeled in vitro for 2 or 18 h were not washed or were washed one or two times prior to incubation with ERSS. (ii) If treponemes were radiolabeled for 2 h with [^{35}S]methionine and then incubated in the presence of an excess of unlabeled methionine for an additional 16 h, the results were the same as if treponemes had been radiolabeled throughout the 18-h incubation period. Likewise, if treponemes were incubated in radiolabeling medium for 16 h in the absence of [^{35}S]methionine and

then incubated for an additional 2 h with [^{35}S]methionine present, the pattern of radiolabeled protein antigens precipitated was essentially identical to that obtained by radiolabeling treponemes for 18 h. Thus, the differences observed above cannot be explained simply on the basis of the length of the radiolabeling period. (iii) An in vitro incubation period of longer than 8 h was required before IgG antibodies in ERSS gained ready access to the 48K, 42K, and 16K protein antigens.

Treatment of treponemes with 0.04% SDS. Johnson et al. (13) previously reported that treatment of cells of certain cultivable treponemal species with a low concentration of SDS (0.04%) selectively removed the outer envelope. We investigated the effect of 0.04% SDS on *T. pallidum*. Freshly extracted treponemes were radiolabeled for 18 h with [^{35}S]methionine, washed, and treated with 0.04% SDS for 15, 30, or 45 min (see Materials and Methods). At this point, the organisms were found to maintain their characteristic treponemal morphology when examined by dark-field microscopy. Next, treponemes were pelleted, and the cell-free supernatants were centrifuged at 100,000 × *g* for 90 min. This resulted in a small, clear, membranous pellet that was subsequently solubilized and analyzed by SDS-PAGE and fluorography (Fig. 2, lanes B to D). Three radiolabeled treponemal proteins of molecular weights 42,000, 40,000, and 34,000 were present in this pellet. These proteins appeared to correspond to proteins with identical molecular weights that were identified above, and this protein pattern did not appear to vary with the time of treatment. When treponemes were treated with different concentrations of SDS for 15 min, it was found that both 0.04 and 0.08% SDS resulted in similar high-speed pellets and similar protein patterns (Fig. 2, lanes E to H). In subsequent experiments, treponemes were treated with 0.04% SDS for 15 min.

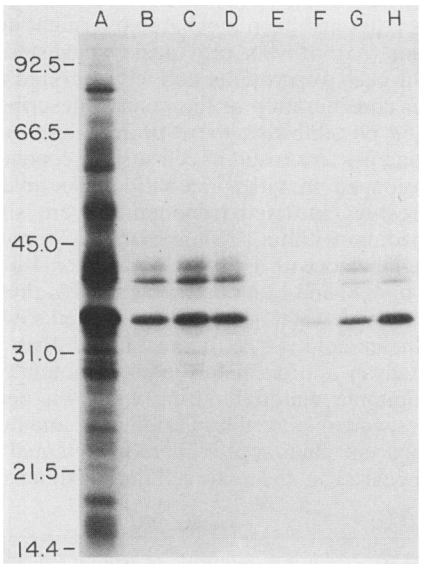


FIG. 2. Treatment of treponemes with 0.04% SDS. Freshly extracted treponemes were incubated for 18 h in radiolabeling medium with [^{35}S]methionine. Fractions were prepared as described in Materials and Methods, solubilized in buffer containing 1% SDS, and analyzed by SDS-PAGE and fluorography. Lanes: A, untreated treponemes; B to D, high-speed membranous pellets obtained after treating treponemes with 0.04% SDS for 15 (B), 30 (C), or 45 (D) min; E to H, high-speed pellets obtained after treating treponemes for 15 min without SDS (E) or with 0.02 (F), 0.04 (G), or 0.08% (H) SDS. Protein sizes are indicated in kilodaltons. See text for additional details.

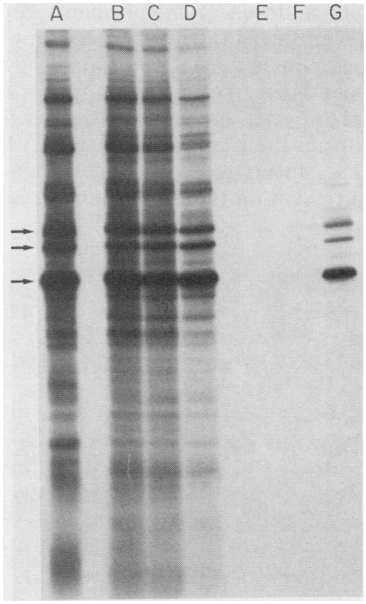
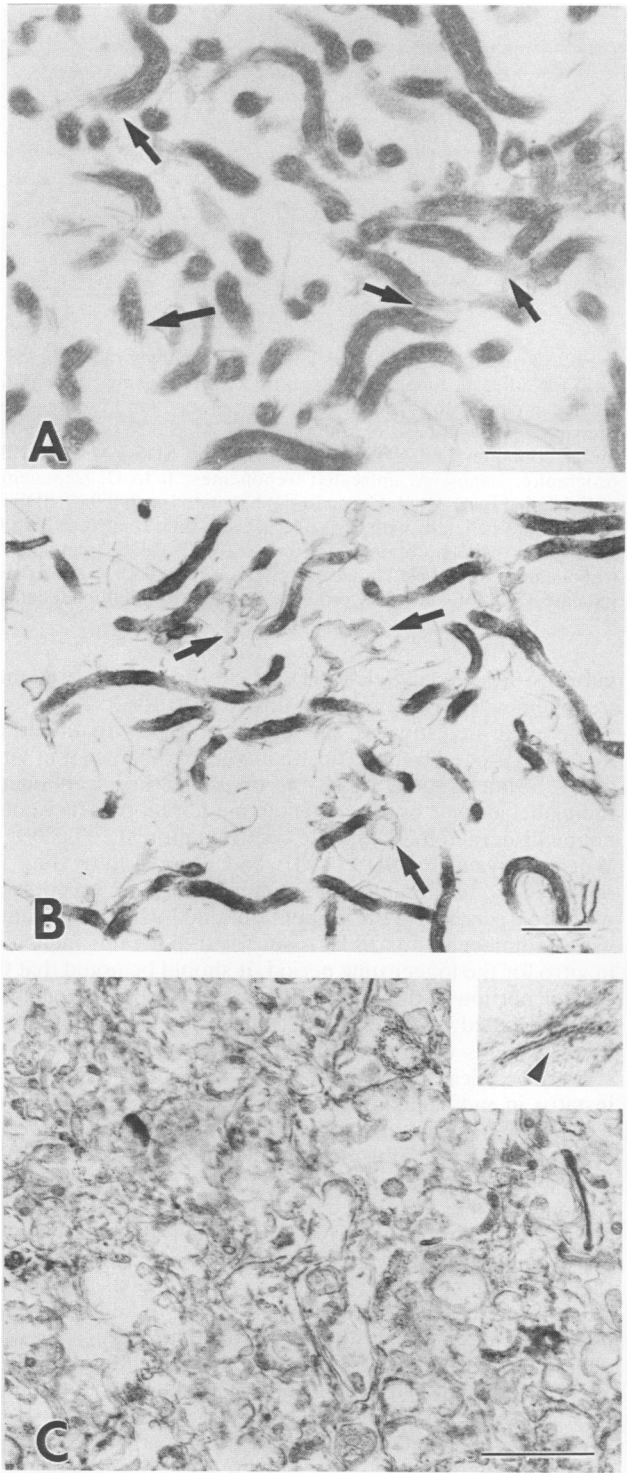


FIG. 3. Effect of SDS treatment on treponemes radiolabeled in vitro for different times. Freshly extracted treponemes were radiolabeled in vitro with [^{35}S]methionine for 2, 4, or 18 h. Fractions were prepared as described in Materials and Methods, solubilized in buffer containing 1% SDS, and analyzed by SDS-PAGE and fluorography. Lanes: A, untreated treponemes; B to D, treponemes radiolabeled for 2 (B), 4 (C), or 18 (D) h, treated with 0.04% SDS for 15 min, and pelleted, with the supernatants then removed; E to G, high-speed pellets obtained following 0.04% SDS treatment of treponemes radiolabeled in vitro for 2 (E), 4 (F), or 18 (G) h. Arrows indicate 42K, 40K, and 34K proteins. See text for additional details.

We next investigated the effect of SDS treatment on freshly extracted treponemes that were radiolabeled in vitro with [^{35}S]methionine for 2, 4, or 18 h. For treponemes radiolabeled for 2 or 4 h, a visible membranous pellet could not be discerned following the centrifugation at 100,000 × *g*. When analyzed by SDS-PAGE and fluorography (Fig. 3), only a very small amount of radiolabeled protein was present when compared with the amount obtained when treponemes were radiolabeled for 18 h. Also, for treponemes incubated in vitro for the longer time period, it should be noted that the greater portion of the 42K, 40K, and 34K proteins remained cell associated, despite the fact that a significant amount of these proteins was removed by the SDS treatment.

Electron microscopy. Treponemes that had been incubated in vitro in radiolabeling medium for 18 h and treated with 0.04% SDS for 15 min as described above were examined in cross section by transmission electron microscopy. The high-speed membranous pellet was also examined, as were treponemes that were not exposed to SDS. As shown in Fig. 4A, untreated treponemes remained intact; their endoflagella were easily discerned (arrows) and apparently bounded by the outer membrane. The outer membrane itself was not detectable. In past electron microscopy studies of *T. pallidum*, the outer membrane has proven to be a somewhat elusive structure that is not easily visualized (10, 15, 34, 39). Treponemes that had been treated with 0.04% SDS retained their characteristic spirochetal morphology (Fig. 4B). However, the endoflagella were clearly no longer constrained by an outer membrane and were seen to extend freely from the

cell surface. In addition, a membranous ribbon was seen attached to several cells (arrows), suggesting that the effect of the SDS treatment is to peel away some surface layer. The high-speed pellet (Fig. 4C) appears to be a concentrated preparation of the membranous structures that were seen peeling away from the treated organisms. A higher magnification (panel C, insert) clearly revealed that these represented structures with a typical membrane bilayer.



34K and 40K proteins are most likely endoflagellar subunits. The apparent molecular weights of the 34K and 40K proteins were very similar to proteins that have been suggested to be subunits of the *T. pallidum* endoflagella (28, 31). We previously observed some reactivity of the 40K and 34K treponemal proteins with rabbit antiserum prepared against *T. phagedenis* biotype Reiter (35). Despite the lack of significant genetic relatedness (22), it has been demonstrated that some *T. pallidum* protein antigens exhibit immunological cross-reactivity with *T. phagedenis* protein antigens, including proteins constituting the endoflagella (5, 6, 17, 31, 37). To test the possibility that the 40K and 34K proteins are endoflagellar subunits, we obtained rabbit antisera prepared against purified *T. phagedenis* biotype Kazan 5 flagellar proteins of molecular weights 39,800 and 33,000. It was previously demonstrated that antiserum to the 33,000-molecular-weight protein reacts with a *T. pallidum* protein of similar molecular weight (17). In the present study, it was found that these antisera specifically precipitated the 40K and 34K *T. pallidum* proteins from both the solubilized extracts of whole radiolabeled treponemes and the high-speed membranous pellet obtained from 0.04% SDS-treated organisms (Fig. 5). It should be noted that antiserum to the 39,800-molecular-weight protein was considerably less efficient at precipitating *T. pallidum* proteins than was antiserum to the 33,000-molecular-weight protein. Since both antisera react strongly with their corresponding *T. phagedenis* protein antigens (Nyles Charon, personal communication), this result suggests that there is considerably less cross-reactivity between the higher-molecular-weight endoflagellar protein of *T. phagedenis* and its putative *T. pallidum* counterpart.

Extracellular protein antigens of *T. pallidum*. We previously demonstrated the presence of several low-molecular-weight protein antigens in the cell-free supernatant of treponemes that had been incubated overnight in radiolabeling medium (35). It was previously suggested that these represent a class of proteins actively secreted by *T. pallidum*. After consideration of the results described above, it also seemed possible that these proteins are only released from treponemes as a result of cell surface changes occurring during prolonged in vitro incubation. To investigate this further, freshly extracted treponemes were suspended in labeling medium without [^{35}S]methionine. Treponemes were then pulse-labeled with [^{35}S]methionine for 4-h periods beginning at 0, 4, 8, and 12 h postextraction. At the end of each labeling interval, total supernatant proteins were precipitated with ice-cold TCA. It was found that treponemes incorporated an approximately equal amount of label into TCA-precipitable material during each 4-h period. When precipitates were analyzed by SDS-PAGE and fluorography, it was apparent that approximately an equal amount of protein appeared in the extracellular medium during each

FIG. 4. Ultrastructural features of treponemes treated with 0.04% SDS. See text for details. (A) Untreated treponemes. The organisms appear intact and exhibit characteristic treponemal morphology. Endoflagella are clearly visible (arrows) and are presumably constrained by the outer membrane. (B) Treponemes treated with 0.04% SDS for 15 min. Note the removal of a membranous structure (arrows), with the subsequent disarray and release of the underlying endoflagella. (C) Membrane preparation obtained by centrifugation of cell-free supernatant at 100,000 × *g*. Numerous morphologically distinct portions of intact multilayered membrane structures can be observed (arrowhead in insert). Magnification of insert, ×140,000. Bar, 0.5 μm.

time interval (Fig. 6, lanes A to D). In a separate experiment, freshly extracted treponemes were suspended in radiolabeling medium with [^{35}S]methionine for 1, 5, 15, 30, or 60 min and immediately analyzed for the presence of extracellular proteins (Fig. 6, lanes E to I). The results clearly indicate that radiolabeled proteins were detected in the supernatant fraction after only a 1-min labeling period and continued to accumulate throughout the 60-min labeling period.

DISCUSSION

In this study, we noted two major changes in the cell surface properties of *T. pallidum* that occurred during incubation of treponemes in vitro following their extraction from infected rabbit testes. First, we found that the number of protein antigens that could be recognized in intact cells by IgG antibodies present in ERSS markedly increased with time. Weak reactivity with only two protein antigens of molecular weights 40,000 and 34,000 was detected for freshly harvested organisms that were incubated in radiolabeling medium for 2 h. In contrast, intact treponemes incubated in radiolabeling medium for 18 h bound IgG antibodies directed against a number of protein antigens. Second, it was found that 0.04% SDS selectively removed a membranous surface layer from treponemes that had been incubated in radiolabeling medium for 18 h but not from treponemes incubated for only 4 h.

Our finding that very few, if any, protein antigens are IgG antibody accessible on the surface of freshly extracted treponemes is consistent with numerous observations that such treponemes are serologically nonreactive (4, 12, 19–21, 24, 27, 33). For example, Hardy and Nell (12) found that

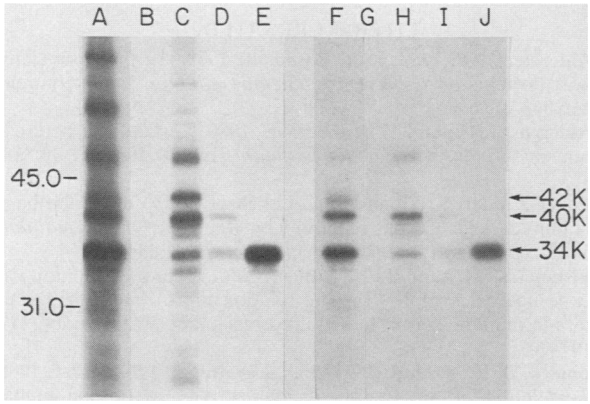


FIG. 5. Radioimmunoprecipitation of *T. pallidum* proteins with antisera prepared against purified *T. phagedenis* endoflagellar subunits. Immunoprecipitations with solubilized, [^{35}S]methionine-labeled extracts, rabbit serum, and protein A-Sepharose were performed as previously described (35). Precipitates were analyzed by SDS-PAGE and fluorography. Lanes: A, whole-cell antigen extract; F, high-speed membranous pellet extract; B to E and G to J, precipitates obtained by using the two respective extracts with normal rabbit serum (B and G), high-titer ERSS (C and H), antiserum prepared against purified 39,800-molecular-weight *T. phagedenis* endoflagellar subunit (16) (D and I), and serum prepared against purified 33,000-molecular-weight *T. phagedenis* endoflagellar subunit (17) (E and J). The 42K, 40K, and 34K treponemal proteins are indicated by arrows. Protein sizes are indicated in kilodaltons on the left. Note that rabbit serum prepared against the purified 39,800-molecular-weight *T. phagedenis* endoflagellar subunit also precipitates the 33,000-molecular-weight subunit (Nyles Charon, personal communication). Only the relevant portion of the gel is shown. See text for additional details.

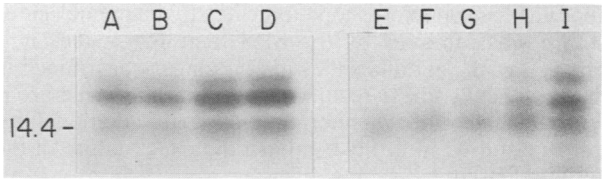


FIG. 6. SDS-PAGE of radiolabeled extracellular proteins of *T. pallidum*. Extracellular proteins in cell-free supernatants of radiolabeled treponemes were concentrated by TCA precipitation and analyzed by SDS-PAGE and fluorography, as previously described (35). Lanes: A to D, TCA precipitates obtained when treponemes were radiolabeled for 4 h with [^{35}S]methionine (final concentration, 65 μCi/ml) after preincubation without label for 0 (A), 4 (B), 8 (C), or 12 (D) h; E to I, TCA precipitates obtained when freshly extracted treponemes were incubated in medium containing [^{35}S]methionine (final concentration, 65 μCi/ml) for 1 (E), 5 (F), 15 (G), 30 (H), or 60 (I) min. Only the relevant portions of the gels are shown. See text for additional experimental details.

treponemes harvested from rabbit testes required considerable aging in vitro before they could be agglutinated by syphilitic serum. Indirect immunofluorescence with freshly extracted treponemes was unsuccessful without aging (21) or prior fixation with acetone (27). Complement-dependent antibody activity assays such as the *T. pallidum* immobilization (TPI) test and the in vitro-in vivo neutralization test were found to require 16 h of in vitro incubation of treponemes and antibody for maximal reactivity (4, 24).

In recent years, different techniques have been used to identify the cell surface proteins of *T. pallidum*. Several investigators have used surface iodination of treponemes with ^{125}I with widely varying results. Whereas three groups found from 6 to 13 different proteins radiolabeled by this procedure (2, 14, 23), Norris and Sell (26) reported the surface iodination of only one protein, and Penn et al. (29) failed to iodinate any proteins on freshly extracted organisms. Norgard and co-workers (14, 18, 33, 38) have produced monoclonal antibodies reactive with a number of *T. pallidum* proteins, many of which have been designated as cell surface proteins on the basis of several criteria. However, as correctly pointed out by these investigators (18), each of these criteria has inherent limitations which prevent one from making definitive conclusions. Lovett and coworkers (7, 32) have cloned *T. pallidum* protein antigens of 190,000 and 38,000 molecular weight. Rabbit IgG antibody prepared against the recombinant proteins can immobilize *T. pallidum* in the TPI test, and a surface association of the native treponemal antigen was demonstrated by immunoelectron microscopy under TPI test conditions. In the TPI test, organisms are incubated in vitro with antibody and complement for 16 h (41). Thus, reactivity of treponemes with these antibodies may result from cell surface changes similar to those reported here.

We believe that there is a direct correlation between the increase in reactivity of IgG antibodies with intact treponemes and the ability of 0.04% SDS to selectively remove a membranous layer from the treponemal cell surface. The evidence suggests that as treponemes are incubated in vitro, they undergo an aging process in which the outer membrane, considered to be a somewhat labile structure (6, 10, 34), eventually begins to lose its integrity. At this point, detergent molecules can intercalate between the outer membrane and the cytoplasmic membrane, causing this structure to peel away. Coincidentally, antigenic determinants normally concealed by the outer membrane become accessible to

antibody. Electron microscopy indicates that a membranous structure was removed by the SDS treatment. Since it is apparent that the endoflagella were no longer constrained in such treated cells, these results suggest that this structure is the treponemal outer membrane, although additional research is required to firmly establish the exact nature of the material released.

Alderete and Baseman (1) have reported that treponemes possess a surface coating of avidly bound host serum proteins that could hinder accessibility of antibodies to surface antigens. An antigenically inert mucopolysaccharide layer also has been reported to coat the treponemal surface (8). Thus, the possibility exists that it could be the removal of either or both of these layers that is responsible for the changes we have observed. However, the breakdown or removal of these layers alone could not account for our electron microscopy data. Penn and Rhodes (30) have previously suggested that the outer membrane is an antigenically inert layer that conceals the endoflagella and other treponeme-associated antigens. In addition, Blanco et al. (6) recently suggested that in vitro incubation of *T. pallidum* organisms may result in a slow deterioration in outer membrane integrity.

Penn et al. (29) described the selective solubilization by using Triton X-100 of the outer membrane from azide-killed treponemes that had been stored at 4°C for several days. Analysis of the solubilized material by SDS-PAGE indicated that a specific subset of treponemal proteins is significantly extracted into this fraction, including proteins of molecular weights 60,000, 47,000, 40,000, 33,000, and 16,000. The 47K protein is a highly immunogenic protein that is believed by several investigators to represent a major surface antigen of *T. pallidum* (3, 9, 14, 18, 40). It was found that none of these proteins can be surface iodinated in freshly extracted organisms unless detergent is present. Although it was suggested that these proteins are outer membrane components, the possibility that these are periplasmic or cytoplasmic membrane components released by detergent extraction cannot be discounted. We found only three treponemal proteins associated with the membrane fraction released from aged treponemes by treatment with 0.04% SDS. Since the major portion of these proteins remained cell associated and the 40K and 34K proteins were precipitated by antisera directed against purified *T. phagedenis* endoflagella, we suspect that these proteins are not integral components of the putative outer membrane fraction we prepared.

Although the 40K and 34K proteins are most likely endoflagellar subunits, this does not totally eliminate the possibility that these two proteins are partially surface exposed in intact organisms. First, we reproducibly encountered weak reactivity of ERSS with these two proteins in treponemes radiolabeled in vitro for just 2 h. This reactivity did not appear to result from a minor population of treponemes with permeable outer membranes, since reactivity with other proteins which are strongly recognized in aged treponemes was not detected at the earlier time. Second, Norris and Sell (26) reported that under their experimental conditions, just one 39,000-molecular-weight treponemal protein was surface iodinated in intact treponemes. This protein was subsequently identified as an endoflagellar protein that corresponds to the 40K protein we have identified (25). Third, it has been suggested that the treponemicidal activity of normal human serum is due to IgG antibody directed against cross-reacting endoflagellar antigens that is produced in response to the presence of cultivable treponemes in the normal human flora (5). Fourth, we previously reported that this same 40K protein represents the first protein against which an IgG antibody response can be detected in infected rabbits (35). The appearance of specific antibody coincides with the development of peak orchitis, after which point treponemes are rapidly cleared from infected tissues. The primary human syphilitic sera tested also strongly recognized this protein (35). Thus, it could be that antibodies directed against this protein are an important component of the host immune response.

Finally, we investigated further our previous finding that treponemes incubated in vitro secrete a unique class of low-molecular-weight proteins (35). We found that these proteins could be detected in the extracellular medium by using freshly extracted organisms and a labeling time as short as 1 min. Clearly, the appearance of these proteins in the medium did not correlate with the observed changes in treponemal cell surface properties. The finding that these proteins could be detected after such a short labeling interval strongly indicates that they are being actively secreted from the cell. These proteins can be precipitated by both rabbit and human syphilitic sera (35), indicating that they are synthesized during infection and therefore must be considered potentially important virulence determinants. The role of these secreted proteins in the pathogenesis and immunobiology of syphilis is currently under investigation.

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