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The Surface of Virulent *Treponema pallidum*: Resistance to Antibody Binding in the Absence of Complement and Surface Association of Recombinant Antigen 4D

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The binding of immunoglobulin G present in syphilitic immune rabbit serum, syphilitic human serum, and rabbit antiserum to purified recombinant *Treponema pallidum* antigen 4D by *T. pallidum*, Nichols strain, was studied by immunoelectron microscopy. Treponemes were incubated with antiserum under the conditions of the *T. pallidum* immobilization test, in which *T. pallidum*-specific antibody renders the organism nonmotile and avirulent only in the presence of complement after a 16-h incubation period in an anaerobic environment. Antibody was not demonstrable on the surface of *T. pallidum* incubated with nonimmune rabbit serum or normal human serum in the presence of complement. Similarly, in the absence of complement, little or no antibody was found on the treponemal surface after incubation with syphilitic immune rabbit serum, syphilitic human serum, or rabbit antiserum directed against the recombinant 4D antigen. The addition of complement to syphilitic immune rabbit serum, syphilitic human serum, and anti-4D antibody resulted in immobilization and the deposition of antibody on the entire surface of the immobilized organisms. These results corroborate earlier work by other investigators demonstrating the resistance of freshly isolated *T. pallidum* to antibody binding in a variety of serological tests. Detection of 4D antigen on the surface of immobilized *T. pallidum* strongly implies that the use of *T. pallidum* immobilization test conditions provides a means to demonstrate the association of individual surface antigens on virulent *T. pallidum*. The resistance of *T. pallidum* to antibody binding may be relevant to the pathogenesis of syphilis.

Isolation and characterization of the surface molecules of *Treponema pallidum*, which are important in the pathogenesis of syphilis, have been hindered by several interrelated problems. The inability to continuously passage the organism in vitro has limited the quantities of treponemal components available for analysis (9, 24). Because the organism must be passaged in a mammalian host, contaminating substances must be carefully removed to avoid confusion with authentic treponemal constituents. The in vitro fragility of the organism, and of its outer membrane in particular, may result in loss during routine experimental manipulation of exactly those components which are the object of investigation (14, 32).

An additional consideration in defining surface molecules of *T. pallidum* is that the surface of virulent *T. pallidum* appears resistant in a variety of serologic tests to the binding of specific antibodies present in syphilitic serum. For example, Hardy and Nell found that organisms required "aging" (prolonged incubation at 4°C) before they could be agglutinated by syphilitic serum (15). Indirect immunofluorescence of freshly extracted *T. pallidum* is unsuccessful without prior fixation (26) or aging (21) of organisms. Assays of complement-dependent antibody activity, such as immobilization (22) or the in vitro-in vivo neutralization test of Bishop and Miller (3, 4), require a minimum of 4 h of incubation at 35°C for reactivity to occur. These findings have given rise to the hypothesis that an outer coat (6), composed of substances such as serum proteins (1), fibronectins (11, 28, 33), and mucopolysaccharides (10, 34), which are known to adhere to the *T. pallidum* surface, lies external to the outer membrane and masks surface antigens from antibody recognition. Processes such as aging and fixation, which destroy the virulence of the organism (20) and lead to seroreactivity (15, 21, 26), may remove or alter the outer layer and enable antigen-antibody interaction to occur (27).

Expression of *T. pallidum* antigens in *Escherichia coli* (23, 31, 35, 36) has provided a means of circumventing the limited availability of treponemal antigens due to the cultivation problem. We previously described our initial characterization of a protease-resistant recombinant *T. pallidum* antigen designated "4D" (8). The 4D molecule is a 190-kilodalton (kDa) ordered-ring structure composed of 19-kDa monomers (6a). Antibody to the purified 4D molecule can immobilize *T. pallidum* in the complement-dependent *T. pallidum* immobilization (TPI) test (8), suggesting the surface location of the native 4D molecule on *T. pallidum*.

In this study, we established by immunoelectron microscopy the native surface association of the 4D antigen with antiserum raised to the purified recombinant antigen. Our micrographs provide further demonstration of the striking resistance of virulent *T. pallidum* to antibody binding in the absence of complement during incubation under TPI test conditions. They also corroborate an earlier electron microscopy study of Hovind-Hougen et al. (16, 17) which showed that complement is essential for demonstrable binding of human syphilitic antibody to the surface of virulent organisms in the TPI test.

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MATERIALS AND METHODS

Treponemes. *T. pallidum*, Nichols strain, was maintained by intratesticular passage in adult male New Zealand White rabbits without the use of corticosteroids. Rabbits were individually housed at 18 to 20°C and fed antibiotic-free food and water ad libitum. Ten days after intratesticular inoculation, rabbits were sacrificed by the intracardiac injection of 1 ml of sodium pentabarbitol (Euthanol; Western Medical Supply Co. Inc., Arcadia, Calif.), and the testes were aseptically removed as previously described (14).

TPI testing. TPI tests were performed, with minor modifications, as described in the *Manual of Tests for Syphilis* (5). Suspensions of virulent *T. pallidum* were combined with heat-inactivated (56°C for 30 min) test and control sera (11% final serum concentration) and fresh guinea pig serum as a source of complement. Final treponemal concentrations were adjusted to approximately 6×10^7 to 8×10^7 organisms/ml (final volume, 0.55 ml) rather than the usual concentration of approximately 1.5×10^7 organisms/ml. Incubation was carried out for 16 h at 34°C in an atmosphere of 95% N_2 and 5% CO_2. Twenty-five organisms from each specimen (with and without complement) were scored for motility under high dry dark-field microscopy.

If the difference in motility between the test specimens with and without complement was greater than or equal to 50%, the test serum was considered to be reactive. Differences between 21 and 49% indicated minimal but definite immobilizing activity (weakly reactive), and differences of $\leq$20% indicated the absence of immobilizing antibody (nonreactive). On each occasion, the TPI assay of test sera with complement was performed in duplicate.

Immunoelectron microscopy. TPI test incubation mixtures were transferred to Microfuge (Eppendorf) tubes with siliconized Pasteur pipettes and centrifuged at 12,800 × *g* for 4 min. The pellets were washed with 0.5 ml of phosphate-buffered saline, pH 7.2, and resuspended in the same volume of phosphate-buffered saline. A 2-μl amount of ferritin-conjugated goat anti-rabbit or anti-human immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) was added to each of the appropriate tubes (1:250 dilution), and the specimens were incubated for 30 min at room temperature with frequent, gentle agitation. The treponemal suspensions were centrifuged at 12,800 × *g* for 4 min, the pellets were washed in 0.5 ml of phosphate-buffered saline, and the final pellets were resuspended in 25 μl of phosphate-buffered saline. The final suspensions were examined under dark-field microscopy to ensure a minimum concentration of 100 organisms per high dry (40×) objective (about 10^8 organisms/ml).

Specimens were applied to 400-mesh Parlodion (Mallinckrodt, Inc., St. Louis, Mo.)-coated copper grids (Ted Pella Inc., Tustin, Calif.) by the single-droplet method. Grids were stained with 1% uranyl acetate and examined in a Zeiss EM 109 electron microscope at 80-kV accelerating voltage. Organisms were scored for immunoferritin labeling. Ultrastructural integrity was judged by the presence or absence of an easily discernable outer envelope external to the periplasmic flagella. More than 500 intact organisms in three separate experiments were scored.

4D antigen. The recombinant 4D antigen was purified to homogeneity from *E. coli* RR1 containing plasmid pAW329 as previously described (8).

Antiserum. Rabbit antiserum to the purified 4D antigen was raised as previously described (8). Syphilitic immune rabbit serum was prepared from male New Zealand White rabbits bled 12 to 18 months post-intratesticular infection with 2.5×10^7 *T. pallidum* cells. The donors were shown to be resistant to an intradermal challenge with 10^6 treponemes at each of four sites 3, 5, and 12 months postinfection. Nonimmune rabbit serum was prepared from normal adult animals with Venereal Disease Research Laboratory (VDRL)-nonreactive tests. Human syphilitic serum was obtained from a patient with a characteristic palmar and plantar rash and reactive nontreponemal and treponemal serological tests. Normal human serum was obtained from laboratory personnel with VDRL nonreactive tests.

RESULTS

Freshly extracted *T. pallidum* cells were incubated under TPI test conditions with nonimmune rabbit serum, normal human serum, syphilitic immune rabbit serum, syphilitic human serum, and rabbit anti-4D serum in the presence or absence of guinea pig complement and were prepared for electron microscopy as described in Materials and Methods.

T. pallidum cells incubated with either normal rabbit serum or normal human serum and complement retained both motility and structural integrity of the outer membrane (Fig. 1A and B). These organisms did not demonstrate significant amounts of staining with the ferritin conjugate. Identical results were obtained with these sera in the absence of complement and with complement alone in the absence of serum (data not shown). Similarly, *T. pallidum* cells incubated with either syphilitic immune rabbit serum or syphilitic human serum with heat-inactivated guinea pig serum retained motility and failed to demonstrate immunoferritin staining (Fig. 2A and B). In contrast, 100% of treponemes incubated with either syphilitic immune rabbit serum or syphilitic human serum and guinea pig complement (Fig. 2C and D) were immobilized and, after reaction with immunoferritin, demonstrated heavy uniform labeling with the ferritin conjugate. Thus, both immobilization and immunoferritin staining required the presence of complement in addition to specific *T. pallidum* antibodies.

T. pallidum cells incubated with 4D antiserum without complement were not immobilized and failed to demonstrate any surface ferritin binding (Fig. 3A). In contrast, an average of 95% of treponemes incubated with 4D antiserum and complement were immobilized, and approximately 50% of treponemes were uniformly coated with ferritin in a manner indistinguishable from those incubated with syphilitic immune rabbit serum or syphilitic human serum and complement (Fig. 3B). An additional 30% of the treponemes demonstrated patchy ferritin staining (Fig. 3C). The remaining 20% of treponemes did not exhibit significant ferritin staining. Many of the unstained treponemes appeared to have lost their outer membranes (Fig. 3D), suggesting that loss of 4D and bound antibody may have occurred during the washing procedures.

DISCUSSION

This study confirmed, by immunoelectron microscopy, that virulent *T. pallidum* is strikingly resistant to specific-antibody binding when incubated without complement under the conditions of the TPI test. Treponemes incubated with syphilitic human serum or immune rabbit serum without complement retained their motility (and presumably their virulence) and were free of demonstrable surface immunoglobulin (Fig. 2A and B). On the other hand, organisms immobilized by treponemal antibodies and complement were heavily and uniformly stained along their entire surface with the immunoferritin label (Fig. 2C and D). We conclude that

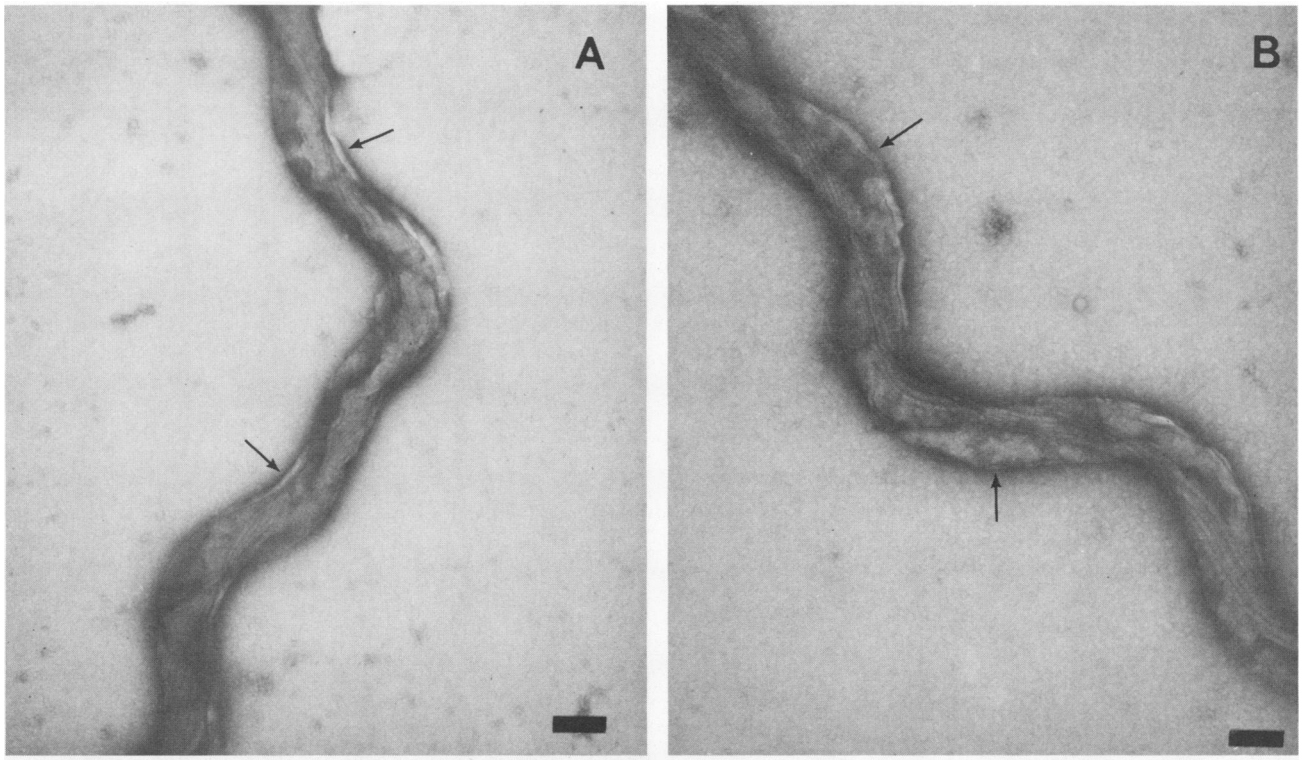

FIG. 1. Interaction of *T. pallidum* with normal human serum and normal rabbit serum. Electron micrograph of *T. pallidum* incubated under TPI test conditions with active complement and normal rabbit serum (A) and normal human serum (B). Ferritin-conjugated goat anti-rabbit and anti-human immunoglobulin G were used to visualize bound antibody. →, Outer membrane. Bar, 0.25 μm.

complement-mediated immobilization of *T. pallidum* under conditions of the TPI test exposed surface antigens for antibody interaction and that the ability to resist antibody binding is a characteristic of virulent *T. pallidum*, at least under these in vitro conditions.

Our micrographs indicate that the accessibility of *T. pallidum* surface antigens to specific antibodies at the outset of the TPI test incubation is limited and below the level of detection by electron microscopy (though still adequate for specific complement fixation). During the subsequent antibody-dependent, complement-mediated immobilization process, possibly as a direct result of the lytic action of complement on the *T. pallidum* outer membrane, "masked" determinants may become exposed. It is likely that the heavily ferritin-coated organisms in Fig. 2C and D are the final products of this process. Evidence that virulent *T. pallidum* can interact, at least to a limited extent, with anti-treponemal antibodies without active complement has been provided by Fitzgerald et al. (11) and Thomas et al. (33), who reported inhibition of *T. pallidum* attachment to epithelial monolayers or fibronectin-coated cover slips after incubation with immune rabbit serum.

These results confirm and extend the work of Hovind-Hougen et al. (16, 17), who found that an 8-h incubation period under TPI test conditions was necessary to observe complement-mediated treponemal antibody binding to the surface of *T. pallidum*. These investigators also demonstrated with thin sections that ferritin label was found exclusively on the surface and that outer membranes of labeled organisms remained structurally intact. Our inclusion of syphilitic immune rabbit serum and 4D antiserum with immobilizing activity has demonstrated the general applicability of these conditions for study of the *T. pallidum* surface. We have demonstrated the native surface location of two recombinant *T. pallidum* antigens, the 4D antigen and a 38-kDa antigen (7) with TPI test conditions in conjunction with immunoelectron microscopy.

Treponemes immobilized with rabbit anti-4D serum fell into three categories after immunoferritin labeling (Fig. 3). Of the labeled organisms, 50% were heavily and uniformly labeled in a fashion indistinguishable from that of organisms immobilized with human or rabbit immune syphilitic serum. Of the labeled organisms, 30% had ferritin label distributed unevenly along their surface. The remaining 20% of the organisms had no discernible ferritin labeling. Many of the unlabeled organisms apparently had been stripped of outer membrane and possibly of bound 4D antibody and conjugate by the washing procedures. However, the fact that an average of 5% of organisms in a TPI incubation mixture failed to become immobilized by 4D antiserum and complement suggests that some of the differences in surface labeling reflect true heterogeneities in a population of *T. pallidum* with respect to surface expression or antigenic availability of 4D.

There have been relatively few published studies describing immunoelectron microscopy of *T. pallidum* surface molecules. Marchitto et al. (19) have described a monoclonal antibody to a 47-kDa *T. pallidum*-specific antigen which also has TPI reactivity. They localized their 47-kDa antigen to the *T. pallidum* surface with a protein A-colloidal gold label, although their surface labeling was not noted to be complement dependent.

A variety of other techniques have been used to identify *T. pallidum* surface molecules. Baseman and Hayes (2) defined

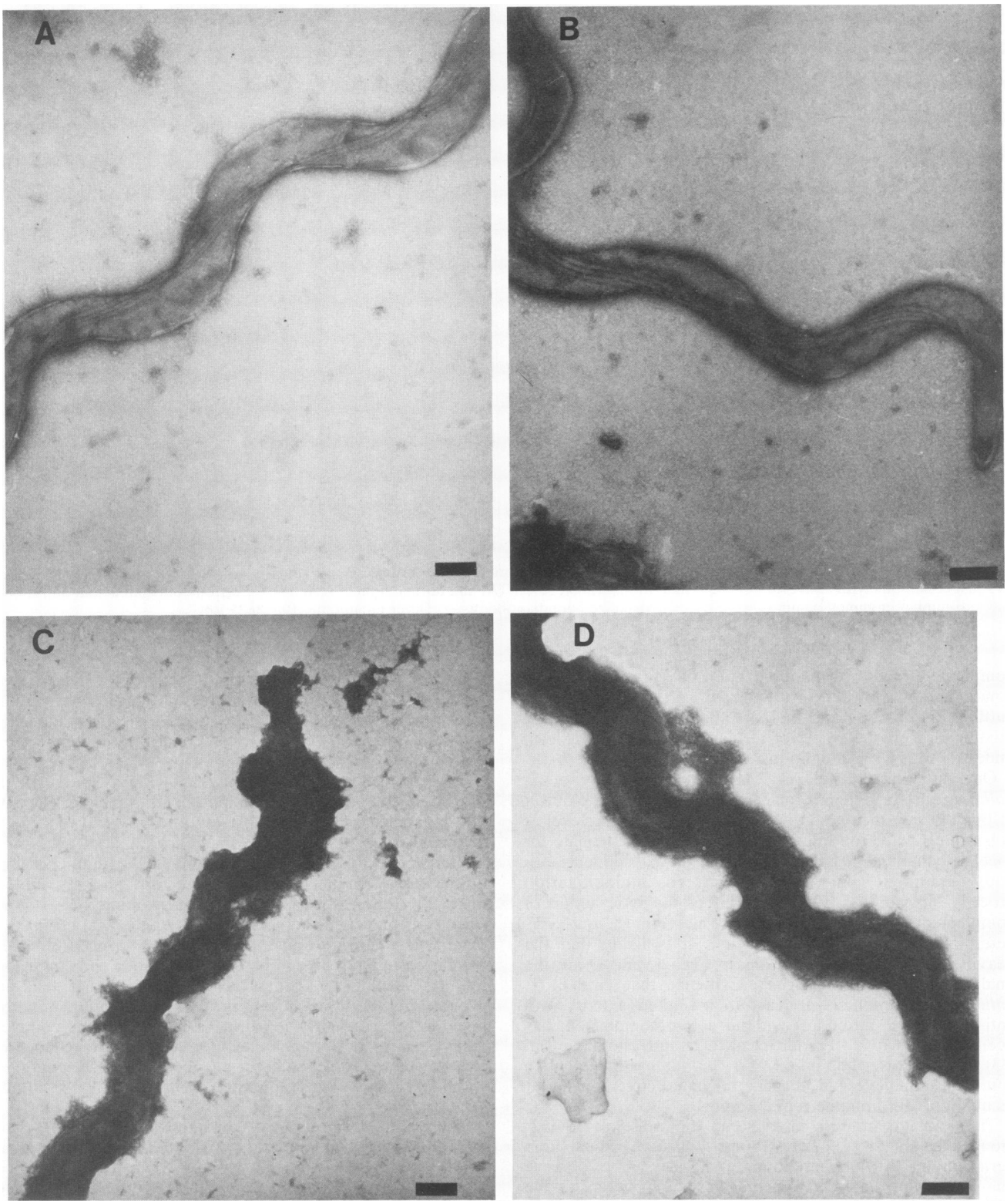

FIG. 2. Interaction of syphilis antibodies with *T. pallidum*. Immunoelectron micrographs of *T. pallidum* incubated under TPI test conditions with heat-inactivated complement and syphilitic immune rabbit serum (A) or syphilitic human serum (B), and with active complement and syphilitic immune rabbit serum (C) or syphilitic human serum (D). Bars, 0.25 μm.

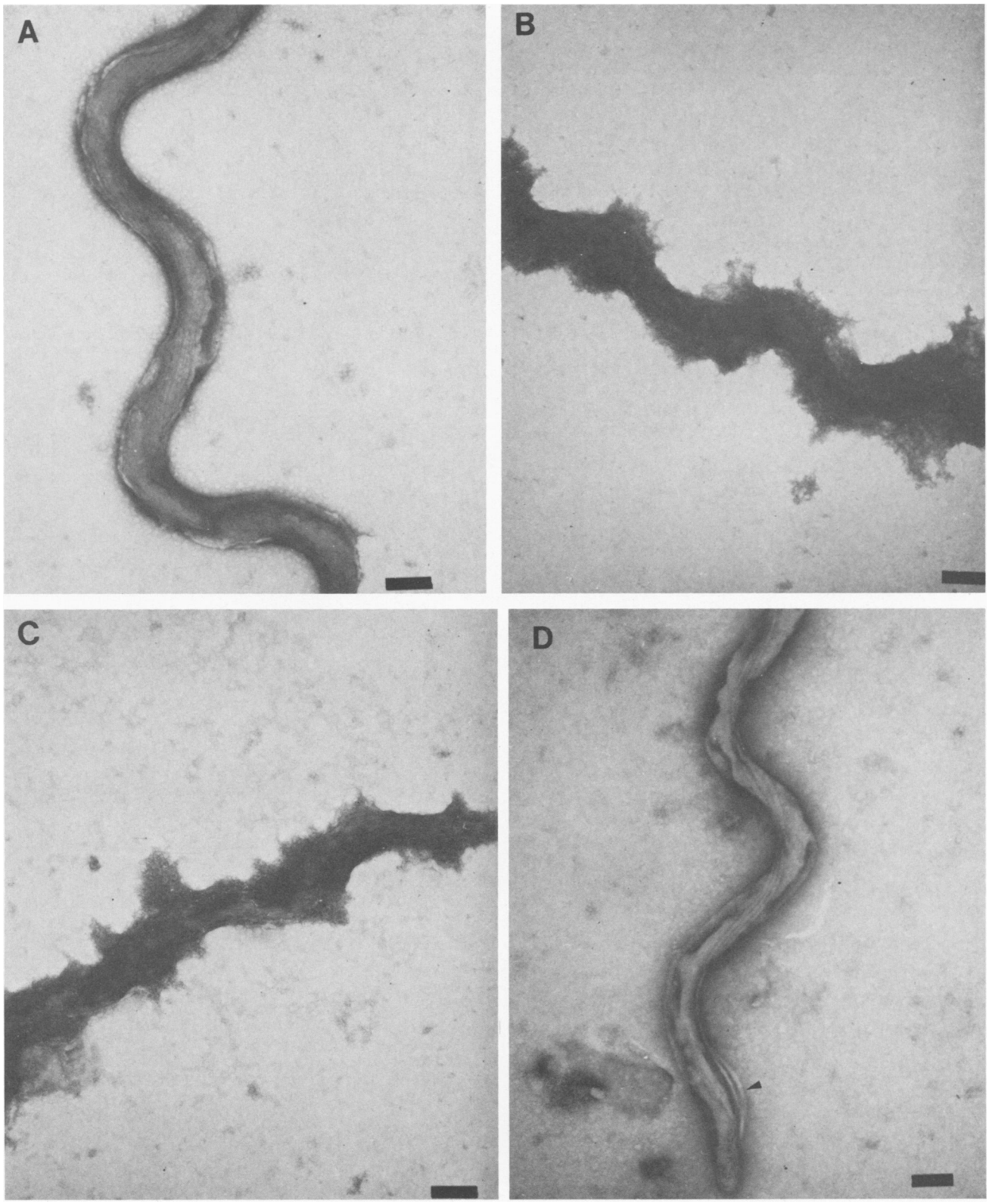

FIG. 3. Interaction of *T. pallidum* with monospecific 4D rabbit antiserum. Immunoelectron micrographs of *T. pallidum* incubated under TPI test conditions with heat inactivated 4D antiserum (A) and 4D antiserum with active complement (B to D). Organisms with bound 4D antibody were labeled in a uniform (B) or patchy (C) manner. Many of the unlabeled organisms appeared to have lost their outer membrane (D) as determined by the presence of exposed periplasmic flagella (◀). Bars, 0.25 μm.

T. pallidum proteins which interact specifically with fibronectin (33) and which have been implicated as attachment ligands. They, as well as Norris and Sell (25) and Jones et al. (18), have used surface-radiolabeling procedures to define surface proteins. Stamm and Bassford (30) have recently defined a sonication-resistant fraction of in vitro-labeled *T. pallidum*. The disparate conclusions reached by these investigators regarding the identity of *T. pallidum* surface proteins probably reflect the fragility and unusual properties of the treponemal surface as well as the different techniques used.

We believe that immunoelectron microscopy offers several advantages over less-direct techniques. It enables the investigator to judge the ultrastructural integrity of individual organisms, especially with regard to the fragile outer membrane, and to determine the specific structures interacting with antibody. Furthermore, the TPI test conditions we used are known to preserve motility (22) and virulence (unpublished observations) of *T. pallidum* in the presence of treponemal antibody alone. The preparation of *T. pallidum* antigen suspensions for use in treponemal antibody assays by procedures such as aging (15, 21), washing (29), and fixation (26, 32) has the potential to significantly alter outer membrane structure (27) and to render the organisms avirulent (20).

The mechanism by which *T. pallidum* masks its surface molecules from antibody interaction is unknown. We have not been able to detect by negative-staining transmission electron microscopy a surface coat external to the outer membrane of washed treponemes (unpublished observations). An alternative explanation is that the native organization of the *T. pallidum* outer membrane allows only limited exposure of surface-associated antigens such as 4D and the 38-kDa recombinant antigen (7). Transient gaps in the fluid outer membrane could then permit limited access of antibody to unmasked determinants. Penn and Rhodes (27) have also proposed that the *T. pallidum* outer membrane functions as an inert outer layer which conceals surface-associated antigens. Data obtained with another pathogen, *Coxiella burnetti*, have proven that integral membrane components such as lipopolysaccharide can mask other immunogenic components of the surface of the organism (12). *T. pallidum* does not contain detectable, smooth lipopolysaccharide (8), and definitive proof of the existence of rough lipopolysaccharide has not been provided.

Although the importance of the masking phenomenon to in vivo host-pathogen interactions is speculative, there are features of syphilitic infection which suggest that the resistance of *T. pallidum* to antibody binding may occur in vivo as well as in vitro. For example, during early latency, recurrent dissemination of virulent organisms can occur despite the presence of high titers of circulating antibodies to virtually every treponemal polypeptide (13). Furthermore, we have not been able to detect by immunoelectron microscopy significant amounts of bound antibody on the surface of treponemes freshly extracted from non-cortisone-treated rabbits (data not shown). It is possible that *T. pallidum* is even more resistant to the effects of specific antibody and complement in vivo than during prolonged incubation in vitro.

Several physical properties of the 4D antigen suggest a functionally important role for this molecule on the surface of *T. pallidum*. The 190-kDa 4D molecule forms an ordered-ring structure 10 nm in diameter, consistent with potential porin activity (6a). The ordered-ring structure is resistant to proteolysis with proteinase K, perhaps offering some degree of protection to *T. pallidum* against host proteases. Finally, the significant partial protection against dermal challenge exhibited by 4D-immunized rabbits (T. E. Fehniger, J. D. Radolf, T. M. Cunningham, H. Togashi, B. L. Johnson, L. A. Borenstein, D. R. Blanco, A. Urquart, J. A. Lake, J. N. Miller, and M. A. Lovett, Fed. Proc. **44**:596, 1985; L. A. Borenstein, T. E. Fehniger, J. D. Radolf, D. R. Blanco, J. N. Miller, and M. A. Lovett, manuscript in preparation) suggests that the 4D molecule of *T. pallidum* plays a role in the pathogenesis of syphilis.

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