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Immunological Evaluation and Cellular Location Analysis of the TprI Antigen of *Treponema pallidum* subsp. *pallidum*

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The TprI antigen of *Treponema pallidum* subsp. *pallidum* is a putative virulence factor predicted to be located in the outer membrane of the syphilis spirochete. In this study, we analyzed the immune response against TprI and its subunits in sera collected both from rabbits experimentally infected with the Nichols strain and from patients with syphilis, showing a different pattern of reactivity toward the antigen in these two groups of samples. The protective ability of recombinant TprI and its hypothetical outer membrane location were also investigated. Although no rabbit was protected after challenge, immunoelectron microscopy results, to be further investigated, were compatible with the outer membrane location of the antigen.

After the genome sequence of *Treponema pallidum* subsp. *pallidum* (Nichols strain) was released (9), research on syphilis focused on the *tpr* (*T. pallidum* repeat) genes (4–7, 10, 12, 17, 18, 25), a family of 12 paralogs homologous to the major surface protein genes of *Treponema denticola*. Except for TprK, which represents the hub of the antigenic variation model proposed for *T. pallidum* (5, 6), the function of the Tprs is still unknown. The Tpr antigens are divided into three subfamilies according to sequence homology (4). Subfamily I (TprC, -D, -F, and -I) members share conserved amino and carboxyl termini but are characterized by different central domains (Fig. 1) (4). Among them, TprI is predicted by PSORT analysis to harbor an amino-terminal cleavable signal peptide and to be surface exposed (5). Thus far, no surface antigen has been certainly identified in *T. pallidum*, due to the inability to culture the organism, the extreme fragility of its outer membrane (OM) (20), and the low density of integral proteins in this cellular compartment (8). However, because phagocytosis of opsonized treponemes is believed to be an important mechanism of clearance of *T. pallidum* from early lesions (1, 13, 14), potential outer membrane antigens became the object of intensive investigation, whether predicted to be virulence factors or not (3).

The recently published immunological data on TprI and subfamily I members (12, 25) highlighted their importance during the host immune response to syphilis infection. These studies suggest that the expression of these genes may be modulated and that different *T. pallidum* isolates may express different repertoires of *tpr* genes. Sun et al. (25) showed that rabbit immunization with a TprI paralog (TprF) strongly alters cutaneous-lesion development after intradermal homologous challenge.

The source, propagation, and extraction of *T. pallidum* subsp. *pallidum*, Nichols strain, were previously reported (16).

The QIAamp DNA Mini kit (QIAGEN) and TRIzol reagent (Invitrogen) were used to isolate DNA and RNA, respectively. cDNA was synthesized from DNase I-treated RNA with the SuperScript First-Strand Synthesis System (Invitrogen) using random primers according to the provided protocol. The *tprI* message was amplified using specific primers (Table 1). The amplification was performed in 50 μl, containing 0.5 μM primers, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 2 U of recombinant DNA polymerase (TaKaRa). PCR conditions were 2 min at 95°C, followed by 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C, for 35 cycles. Final extension was 72°C for 3 min.

The *tprI* open reading frame (without the first 17 codons representing the predicted signal peptide) was amplified using the *Pfu* DNA polymerase (Stratagene) in a 50-μl final volume containing 0.2 μM primers (Table 1), 200 μM deoxynucleoside triphosphates, and 1.25 U of enzyme. Cycling conditions were 3 min at 95°C, followed by 1 min at 95°C, 1 min at 53°C, and 1 min at 72°C for the first five cycles. The annealing temperature was raised to 64°C in the last 30 cycles. Final extension was 3 min at 72°C. After gel purification (using the QIAquick Gel Extraction kit [QIAGEN]), the amplicon was cloned into the TOPO-TA vector (Invitrogen) and sequenced (the primers are listed in Table 1) in both directions with the Applied Biosystems dye terminator sequencing kit (Perkin-Elmer) using the primer-walking approach. The sequences coding for the three TprI regions (amino terminus, amino acids [aa] 18 to 281; nonconserved central region, aa 282 to 393; carboxyl terminus, aa 394 to 609) were amplified from the plasmid containing the full-length *tprI* as a template. All four fragments were then subcloned into the pET22b(+) plasmid (Novagen), using suitable restriction sites to avoid the expression of the vector-encoded signal peptide.

Expression of the full-length recombinant TprI (rTprI) and its subunits was induced with IPTG (isopropyl-β-D-thiogalac-

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TABLE 1. Primers

Purpose	Sense primer	Antisense primer	Size (bp)
tprI transcriptional analysis	5′-GACCCTGCCGATGCAGGTAAT	5′-TAAGCACGATGTCCGACTGACT	336
tprI sequencing	5′-CCTTTCCAATGGTGCCTGGG	5′-CGAAGCCATACTTGCTG	
	5′-ACATCGTGCTTATCATGCT	5′-TTGAACCGGTCCAGCCGTG	
tprI cloning[a]			
Full-length TprI (aa 18–609)	5′-GGAATTCATATGGCTTCTGGTTATGCAGGCGT	5′-GCCCAAGCTTCCAAACCACCTTCACCCC	1,799
Amino terminus (aa 18–281)	5′-GGAATTCATATGGCTTCTGGTTATGCAGGCGT	5′-GCCCAAGCTTCTGGTGTTGGTTACCGGC	814
Central region (aa 282–393)	5′-GGAATTCATATGGACCCTGCCGATGCA	5′-GCCCAAGCTTTAAGCACGATGTCCGACTGACT	358
Carboxyl terminus (aa 394–609)	5′-GGAATTCATATGCTGCTAACCTGGCTTGAG	5′-GCCCAAGCTTCCAAACCACCTTCACCCC	664

[a] Underlined sequences represent NdeI (CATATG) and HindIII (AAGCTT) restriction sites in sense and antisense primers, respectively.

FIG. 1. Schematic representation of predicted TprI protein in comparison to the other subfamily I Tprs of *T. pallidum* subsp. *pallidum*, Nichols strain. Identical patterns indicate amino acid sequence identity among the members. TprI and -F amino-terminal regions are 98% identical to the corresponding sequences of TprC and -D. TprI and TprC and -D carboxyl termini are 94% identical. *, NH_2-terminal signal peptide (first 17 amino acids). [1], *tprF* presents deletions in the sequence coding for portions of the central region and the COOH-terminal region, with a resulting frameshift (§) and premature termination.

topyranoside) at 0.8 mM final concentration for 3 h at 37°C after the cultures entered log-phase growth and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell lysates using standard protocols (24). Lysates were obtained by resuspending cell pellets in 1× sample buffer (2% SDS, 10% beta-mercaptoethanol, 60 mM Tris [pH 6.8], 0.01% bromophenol blue) and boiling them for 5 min. Antigens were purified by nickel affinity chromatography under denaturing conditions with the His-Bind Purification kit (Novagen) according to the provided protocol. To solubilize the antigens from inclusion bodies, sonicated cultures were incubated in ice for 1 h in 1× binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) containing 6 M urea. Elution fractions were checked for purity by SDS-PAGE and dialyzed against saline containing decreasing concentrations of urea. Densitometric analysis with the Rainbow Marker (Amersham) as a standard was used for quantification. The antigens were stored in 75-μg/ml aliquots at −80°C.

Two New Zealand White rabbits that had previously tested seronegative for syphilis were infected intratesticularly with 5×10^7 treponemes. Sera were collected prior to infection, at day 10, and approximately every 30 days thereafter until day 120 postinfection. Sera collected at day 30 were tested for syphilis by Western blotting (WB), evaluating reactivity to the TpN47, TmpA, TpN17, and TpN15 antigens as already described (15).

To test reactivity against the recombinant antigens, nitrocellulose strips were prepared for each of them as previously described (22, 26). Sera from infected and rTprI-immunized (described below) animals, 150 sera from patients with syphilis at different stages (Table 2), and 50 control sera from blood donors were tested. Briefly, strips were incubated overnight either in rabbit or human sera diluted 1:100 in phosphate-buffered saline (PBS)-0.05% Tween 20. Antigen-antibody complexes were detected with a specific peroxidase-conjugated secondary antibody (either goat anti-rabbit immunoglobulin G [IgG] or rabbit anti-human IgG; Dako) diluted 1:500 in PBS-

TABLE 2. Immune response analysis of human sera to recombinant TprI antigens

Source of sera[a]	Full-length TprI no. positive/total (%)	TprI amino terminus no. positive/total (%)	TprI central region no. positive/total (%)	TprI carboxyl terminus no. positive/total (%)
Primary syphilis (n = 49)	47/49 (96)	47/49 (96)	0/49 (0)	8/49 (16.3)
Secondary syphilis (n = 40)	40/40 (100)	40/40 (100)	0/40 (0)	11/40 (27.5)
Late syphilis (n = 57)	57/57 (100)	57/57 (100)	0/57 (0)	5/57 (8.7)
Tertiary syphilis (n = 2)	2/2 (100)	2/2 (100)	0/2 (0)	2/2 (100)
Unknown stage (n = 2)	2/2 (100)	2/2 (100)	0/2 (0)	2/2 (100)
Total (n = 150)	148/150 (98.6)	148/150 (98.6)	0/150 (0)	28/150 (18.6)
Blood donors (n = 50)	0/50 (0)			

[a] The clinical and laboratory guidelines proposed by Norris and Larsen (19) were used to establish the stage of the disease.

0.05% Tween 20, and 4-chloro-1-naphthol (Bio-Rad) as already described (23). To avoid bias, investigators evaluating results were blinded to the group of sera being tested. The Human Subjects Committee of the University of Bologna approved the use of these sera for research purposes.

Of six rabbits, also seronegative for syphilis, three were immunized with 150 μg of rTprI in Ribi (monophosphoryl-lipid A plus trehalose dicorynomycolate plus cell wall skeleton) adjuvant (Sigma), while three control rabbits were immunized with an unrelated *Chlamydia trachomatis* recombinant protein (OMP2). Antigen administration was performed subcutaneously (15 μg), intradermally (45 μg), intramuscularly (60 μg), and intraperitoneally (30 μg). Three additional booster immunizations were given at 3-week intervals. Serum reactivity against either rTprI (and its regions) or OMP2 was assessed 1 week after the last immunization. All rabbits were then contemporaneously challenged intradermally at eight sites on the back with 10^5 *T. pallidum* organisms per site. Lesion development was observed daily for 5 weeks after the challenge. Rapid plasma reagin (RPR) (Radim), *Treponema pallidum* hemagglutination (TPHA) (AlfaWasserman), and WB (15) tests were performed 3 weeks after challenge to assess the state of the infection. Dark-field microscopy was performed only on control rabbit ulcerated lesions.

Sera collected from rTprI-immunized rabbits were pooled, and the IgG was purified using the ImmunoPure IgG Purification kit (Pierce). To minimize possible nonspecific binding of unrelated antibodies, solution collected after chromatography was further enriched in rTprI-specific antibodies by absorption and elution from an rTprI-coated nitrocellulose support following a standard protocol (21). The purified fraction was tested for retained ability to recognize the recombinant antigens as described above.

For electron microscopy (EM) investigation, treponemes were harvested in 0.15 M PBS supplemented with 2% (vol/vol) heat-inactivated noninfected rabbit serum and transported under anaerobic conditions to the electron microscopy facility without any further manipulation. After separation from host gross cellular debris by low-speed centrifugation (250 × *g* for 10 min at room temperature), the specimen was checked for motility and fixed in 0.1% (wt/vol) cacodylate buffer (pH 7.2) containing 2% paraformaldehyde and 0.5% glutaraldehyde for 1 h at 4°C. A sample was then rinsed in the same buffer, dehydrated in ethanol, and embedded in LR White (London Resin Co). Sections were prepared as previously reported (11), and immunostaining was performed following a reported protocol (2) using anti-rTprI as the primary antibody and goat anti-rabbit IgG conjugated with 12-nm colloidal gold particles (Jackson ImmunoResearch) as the secondary antibody.

Control immunostaining reactions were performed with a rabbit anti-47-kDa lipoprotein primary antibody purified from a Nichols-infected animal (native 47-kDa antigen used in the absorption-elution protocol was previously isolated by SDS-PAGE and blotting from cultured treponemes) and with secondary antibody alone.

tprI transcriptional analysis resulted in a specific amplicon when cDNA was analyzed (Fig. 2). Control amplifications confirmed the correct execution of the protocol. All of the sera collected from rabbits experimentally infected with *T. pallidum* failed to recognize the full-length rTprI and its regions. Sera collected prior to infection were equally nonreactive (data not shown). However, a serological test for syphilis (WB) confirmed the infection was established, because sera were reactive to all four *T. pallidum* antigens (TpN47, TmpA, TpN17, and TpN15).

rTprI was recognized by 98.6% of human sera (Table 2); only two samples from individuals with early disease were

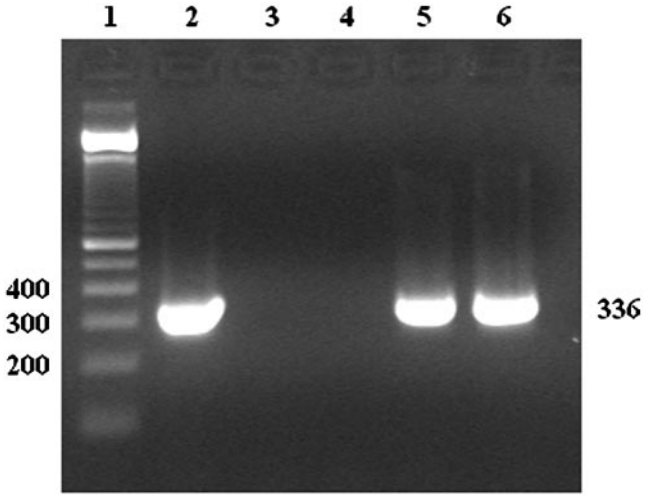


FIG. 2. Amplification of *T. pallidum* Nichols strain cDNA using primers specific for the central nonconserved region of *tprI*. Lanes: 1, molecular weight marker; 2, cDNA; 3, DNase-treated RNA; 4, no template; 5, non-DNase-treated RNA; 6, DNA. All sizes are in bp.

TABLE 3. Protection against experimental infection after immunization with recombinant full-length TprI

Immunization status	Total no. rabbits	No. of RPR-positive rabbits (%)	No. of TPHA positive rabbits (%)	No. of WB positive[a] rabbits (%)	No. of dark-field positive lesions/ total (%)	No. of ulcerative lesions/ total (%)	Status of rabbits after challenge (%)
C. trachomatis OMP2 immunized	3	3/3 (100)	3/3 (100)	3/3 (100)	16/20 (80)	20/24 (83.3)	3/3 infected (100)
Full-length rTprI immunized	3	3/3 (100)	3/3 (100)	3/3 (100)	Not determined	0/24 (0)	3/3 infected (100)

[a] All four antigens (TpN47, TmpA, TpN17, and TpN15) (15) were recognized by all samples when sera from TprI-immunized and OMP2-immunized rabbits were tested.

negative. The TprI amino terminus was recognized by every serum that was reactive against the full-length peptide. No reactivity was seen against the central nonconserved region, while 18.6% of sera recognized the TprI carboxyl terminus. None of the control sera was reactive.

Comparison of immunological parameters and lesion development between rTprI-immunized and control rabbits was used to investigate rTprI protective ability. Prior to challenge, sera from rTprI-immunized rabbits were shown to be reactive to the full-length antigen and its three subunits (data not shown). Reactivity against recombinant OMP2 was seen in sera from the control rabbits. Cutaneous lesions of rTprI-immunized rabbits appeared flatter and paler and healed faster because they did not ulcerate (Table 3). Erythema at the challenge sites was visible within an average of 3 and 4 days in rTprI- and OMP2-immunized rabbits, respectively, and average dimensions of lesions 2 weeks after challenge were 9.2 mm in rTprI-immunized rabbits and 8.8 mm in control animals. Starting at day 16, lesions of rTprI-immunized rabbits gradually healed, while lesions in control rabbits progressed to ulceration. At the end of the observation period, average dimensions were 4.2 mm in rTprI-immunized rabbits and 19.3 mm in control rabbits. Overall, immunization with rTprI was not protective, because all of the rabbits challenged seroconverted (Table 3).

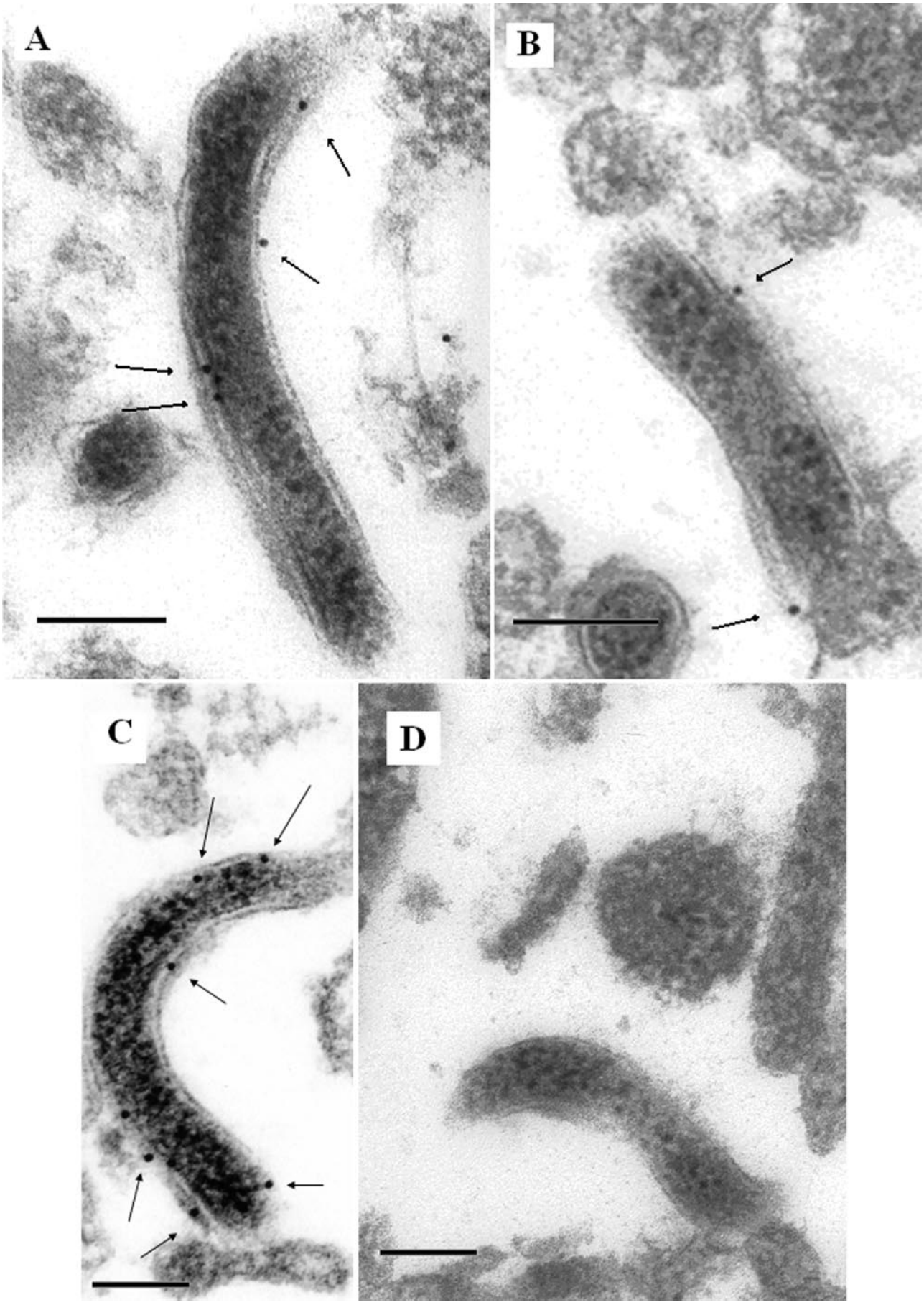
At the moment of fixation, >97% of spirochetes were motile. Nine pictures were taken to investigate antigen locations. The results (Fig. 3A and B) were compatible with both the paucity of this antigen on *T. pallidum* cells and its outer membrane location. Gold particles marked, however, both the external layer of the OM and the periplasm. Quantitative assessment of the particle number showed that 39.6% of particles were directly outside the OM, while 60.4% were periplasmic; 47-kDa lipoprotein immunostaining resulted in a clear periplasmic location (Fig. 3C). No staining was observed in the absence of primary antibody (Fig. 3D).

Absence of immune reactivity to the rTprI and its subunits in sera collected from Nichols-infected rabbits is in general agreement with data already reported (25, 12), which show either weak or absent responsiveness to TprI and subfamily I antigens in the Nichols-infected rabbit model with respect to other strains of *T. pallidum*. Even in the presence of specific message, at posttranscriptional level, attenuation mechanisms could prevent the mRNA from being efficiently translated, and by inference, could prevent the synthesis of enough protein to elicit a detectable immune response. Nichols is also believed to be a strain highly adapted to the rabbit host, where it has been propagated since its isolation in 1912, and this strain might use a peculiar repertoire of Tprs during the establishment of the infection. In this context, TprI synthesis (along with that of other subfamily I members) might be down-regulated, and the immune response could remain unseen or become detectable over a considerable time after infection.

Immune response characterization using a panel of human sera from syphilis patients showed an early reactivity to the full-length rTprI (basically directed against its amino-terminal region and partially against the carboxyl terminus); only two cases of primary syphilis whose onset was less than 1 week before the blood sample was taken were negative. This result is partially in agreement with the reported data on the immunogenicity of subfamily I Tprs during experimental infection with non-Nichols strains (12). In that case, however, reactivity against the TprI central region could also be detected, as well as an early immune response against the conserved amino terminus when some strains were used (12, 25). Our data suggest either that, during the course of natural infection, the TprI central region is not immunogenic or that the antigen synthesis in vivo is not sufficient to induce a measurable immune response. On the other hand, the signal detected against the amino and carboxyl termini could also be generated by the immune response against other subfamily I members, which share those regions.

Although no protective capability can be attributed to this antigen, we showed that immunization with full-length rTprI significantly altered lesion development after challenge. This supports the actual translation of subfamily I antigens (and possibly TprI) in the Nichols strain during the course of infection in rabbits, although antibody response against these proteins may be difficult to detect with the system we adopted. A possible explanation for this discrepancy may involve the purification protocol of rTprI, which implies denaturation of the antigen from *Escherichia coli* inclusion bodies. No renaturation

FIG. 3. Immunoelectron microscopy analysis of TprI antigen locations on *T. pallidum* cells. (A, B) Pictures from two different immunostaining reactions performed with anti-rTprI primary antibody. (C) Analysis of the 47-kDa lipoprotein location on *T. pallidum* cells. (D) Negative control (no primary antibody). Arrows indicate colloidal gold positions on bacterial cells. Bar length is 0.3 μm.



protocol (dialysis against decreasing concentrations of denaturing agent and ion-exchange or hydrophobic interaction chromatography) attempted in our laboratory allowed the purification of an antigen potentially more similar in structure to the native protein. During immunization, we likely induced a response mainly directed toward linear epitopes of rTprI, due to the fact that the conformational epitopes were unavailable to the immune system. It is possible that lack of immune response to conformational epitopes might have partially influenced the outcome of the protection assay and the results of serum reactivity analysis.

EM results confirmed that translation of subfamily I Tprs occurs during experimental infection with the Nichols strain, also showing a situation consistent with the paucity of these antigens on the *T. pallidum* cell. Colloidal gold particles, however, specifically marked both the external layer of the outer membrane and the cellular periplasmic space, calling into question any conclusion regarding antigen location. Because the primary antibody used was directed toward the full-length antigen, the concurrent identification of the other subfamily I members (TprF shares the hypothetical location of TprI) besides TprI cannot be ruled out, and a signal within the periplasm could also be attributed to an inner membrane protein protruding toward this cellular compartment (TprC and -D are predicted to be inner membrane proteins). Although the TprI location will be further investigated using a specific antiserum against the central region of the protein (possibly obtained with a recombinant peptide in its native conformation), the EM approach seems to promise to achieve this goal.

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