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Assessment of cell-surface exposure and vaccinogenic potentials of *Treponema pallidum* candidate outer membrane proteins

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Abstract

Syphilis, a sexually transmitted infection caused by the spirochetal bacterium *Treponema pallidum*, remains a global public health problem. *T. pallidum* is believed to be an extracellular pathogen and, as such, the identification of *T. pallidum* outer membrane proteins that could serve as targets for opsonic or bactericidal antibodies has remained a high research priority for vaccine development. However, the identification of *T. pallidum* outer membrane proteins has remained highly elusive. Recent studies and bioinformatics have implicated four treponemal proteins as potential outer membrane proteins (TP0155, TP0326, TP0483 and TP0956). Indirect immunofluorescence assays performed on treponemes encapsulated within agarose gel microdroplets failed to provide evidence that any of these four molecules were surface-exposed in *T. pallidum*. Second, recombinant fusion proteins corresponding to all four candidate outer membrane proteins were used separately, or in combination, to vaccinate New Zealand White rabbits. Despite achieving high titers (>1:50,000) of serum antibodies, none of the rabbits displayed chancre immunity after intradermal challenge with viable *T. pallidum*.

Keywords

TREPONEMA PALLIDUM; VACCINE; OUTER MEMBRANE PROTEIN

1. Introduction

Syphilis is a chronic, complex sexually transmitted infection of humans caused by the spirochetal bacterium *Treponema pallidum* subspecies *pallidum* (*Tp*). Although effective drug-treatment regimes exist, syphilis remains a pandemic disease [1] that contributes to the spread of HIV [2]. The development of an efficacious vaccine has remained an important research and public health goal [3-6].

Protective immunity against *Tp* infection is supported by clinical observations in both humans and animals. Although derived from a study in which ethics were compromised, humans with late latent syphilis appear to develop some immunity to subsequent infection [7]. Regarding animal models, rabbits are the preferred mammalian model for immunoprotection studies with *Tp* because they develop chancre-like lesions after intradermal injection [3,8]. Chancre

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immunity is achievable in rabbits systemically infected with *Tp* for 3-6 months and is characterized by the failure to produce lesions after intradermal challenge with virulent *Tp* [3,9]. Furthermore, a seminal study by J.N. Miller [4] showed that rabbits immunized over 37 weeks with multiple injections of gamma-irradiated (killed) *Tp* developed full immunity to subsequent challenge with viable *Tp*, thereby substantiating the feasibility of a syphilis vaccine.

Considering that *Tp* is believed to be an extracellular pathogen, it is plausible that antibodies play a pivotal role in the clearance of the spirochetes. The primary targets of these antibodies are thought to be surface-exposed, outer membrane proteins (OMPs). However, the identification of bona fide OMPs in *Tp* has remained elusive [5,10-12]. Towards that goal, four *Tp* proteins, TP0155, TP0326, TP0483, and TP0956, have recently emerged as potential OMP candidates. TP0155 and TP0483 were reported as fibronectin-binding proteins [13]. Antibodies against TP0326 were opsonic and enhanced the phagocytocis of *Tp* by macrophages [5]. Finally, rising antibody titers against TP0956 in rabbits infected with *Tp* correlated with the development of chancre immunity, implying a possible contribution of these antibodies to immune protection [14]. Unfortunately, despite these promising aforementioned results, the membrane topologies of these four proteins remain unknown, as well as their potential to serve as highly efficacious vaccinogens for the prevention of syphilitic infection. To further examine the potential cell-surface exposure of TP0155, TP0326, TP0483, and TP0956, antibodies directed against each respective protein were employed in the agarose gel microdroplet immunofluorescence assay [15]. We also evaluated each protein individually, or combined as a quadravalent vaccine, for immunoprotective capability in the rabbit model of experimental syphilis.

2. Materials and Methods

2.1. Cultivation and isolation of treponemes

All animal studies described herein were approved by the U.T. Southwestern Institutional Animal Care and Use Committee.

Tp subspecies *pallidum* (Nichols strain) was maintained and passaged by intratesticular inoculation of adult male New Zealand White rabbits as previously described [15]. Rabbit testicular debris was removed from treponemal suspensions by two successive rounds of slow-speed centrifugation (200 × *g* for 8 minutes).

2.2. Generation of histidine-tagged fusion proteins for vaccination studies

Individual open-reading frames lacking N-terminal signal sequences were amplified by PCR from *Tp* genomic DNA using the following primer pairs: 5'-TP0155 5'-ACGCGGATCCCCATTGACACCTGCCCTCAC-3' and 3'-TP0155 5'-ATCCAAGCTTTTACTACGGAAGGGTACGCATACG-3'; 5'-TP0483 5'-ACGCGGATCCAAGGAACTCGTCCACGTATCTCAG-3' and 3'-TP0483 5'-ATCCAAGCTTTTATCAGTTATGAAAGCGATAGCCG-3'; 5'-TP0956 5'-ACGCGGATCCCTCTCCCACACGCTCGCTC-3' and 3'-TP0956 5'-ATCCAAGCTTTTATCAATCCAAGAAAAAATCCTGC-3'; 5'-TP0326 5'-ACGCGGATCCGAGGGAAAGCCTATCTCTG-3' and 3'-TP0326 5'-ATCCAAGCTTTTACTACAAATTATTTACCGTGAAC-3'. Restriction sites (*Bam*H I or *Hin*d III) are underlined. N-terminal, histidine-tagged fusion proteins were generated using pProEX-HTb (Invitrogen, Calsbad, CA). Constructs were transformed into *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) and insert sequences verified via DNA sequencing. Recombinant fusion proteins were purified on nickel-NTA agarose (Qiagen, Valencia, CA) following manufacturer's directions. Further purification was achieved by electroeluction of SDS-PAGE-separated protein into SDS-PAGE running buffer (192 mM glycine, 25 mM Tris

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base, 0.1% SDS) using an Elutrap electroelution system (Schleicher and Schuell Inc., Keene, NH).

2.3. SDS-PAGE and immunoblotting

Proteins were separated on 12.5% polyacrylamide resolving gels and were transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell) for immunoblotting. Blots were blocked for 1 h in StartingBlock (Pierce, Rockford, IL), incubated for 1 h with a 1:1000-1:50,000 dilution of rabbit polyclonal antiserum or a 1:1000 dilution of rat polyclonal antiserum or mouse monoclonal, 11E3 [16]. After 3 washes in phosphate-buffered saline (PBS)/0.5%Tween, blots were incubated for 1 h with 1:1000-1:20,000 dilution of horseradish peroxidase conjugated goat anti-rabbit (rat or mouse as appropriate) immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA). Immunoblots were either developed with 4-chloro-1-naphthol as the substrate, or with the ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) for chemiluminescence detection.

2.4. Rat antisera generation

For the generation of polyclonal antisera, 20 μg of each recombinant protein (in 200 μl of PBS) was emulsified with an equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO) and injected intraperitoneally into 6-week-old, female Sprague-Dawley rats (Harlan, Indianapolis, IN). After 3 weeks, the rats were boosted with a similar amount of protein emulsified in incomplete Freund's adjuvant (Sigma). Two weeks after this boost, antiserum was collected and immunoblotted against recombinant protein and, if necessary, a second boost was performed to increase antiserum affinity and titer. Serum antibody titers were estimated by preparing 10-fold serial dilutions of each antiserum, and using them in colorimetric immunoblots against 500 ng (per gel lane) of each protein. Antibody titers were designated as greater than the lowest serum dilution that yielded a visible (positive) band on immunoblots.

2.5. Rabbit immunization and intradermal challenge

Each protein antigen was emulsified in a 1:1 mixture of the Ribi adjuvant system (Sigma). New Zealand White male rabbits were injected with 125 μg of test antigen at each immunization, divided equally among 6 intradermal, 1 subcutaneous, and 2 intramuscular injection sites. Each rabbit was immunized a total of 4 times at three-week intervals. Two rabbits were immunized with one of the following antigens Tp47, TP0155, TP0326, TP0483, or TP0956, and two rabbits were immunized with a mixture of TP0155, TP0326, TP0483, and TP0956. Finally, two rabbits were left unimmunized (negative controls). Antiserum was collected two weeks after the final immunization and titers were evaluated by immunoblot analysis using the relevant immunizing recombinant protein or protein mixture. Rabbits were challenged with 10^5 freshly-isolated *Tp* administered intradermally at 8 sites along shaved backs. Rabbits were examined daily and photographed to document lesion development. Darkfield microscopic examinations of lesion aspirates were preformed to confirm the presence of motile spirochetes.

2.6. *Encapsulation of* Tp *within agarose gel microdroplets and indirect immunofluorescence of treponemal antigens*

Spirochetes were harvested in *Tp* medium and flushed with 5% CO_2 / 95% N_2 [15]. The *Tp* medium consisted of a combination of Earle's balanced salt solution, MEM amino acids, MEM non-essential amino acids, and MEM vitamins with 2 mM L-glutamine, 550 μM D-mannitol, 320 μM L-histidine, 24 mM sodium bicarbonate, 25 mM MOPS (3-[N-Morpholino] propanesulfonic acid), 900 μM sodium pyruvate , 14 mM D-(+)glucose, 650 μM dithiothreitol, and 20% fetal bovine serum (Mediatech, Inc., Herndon, VA) with a final pH of 7.5. Spirochetes in suspension were enumerated by darkfield microscopy and diluted to a concentration of

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10^8/ml in *Tp* medium before being incorporated into 2% low-melting temperature agarose as previously described [15].

The agarose gel microdroplet preparation was separated into one ml aliquots for labeling with *Tp*-specific antibodies. The primary antibodies utilized were: 11E3, a mouse monoclonal antibody directed against Tp47 [16] (1:20 dilution), polyclonal rabbit anti-TP0326 (1:3 dilution), or polyclonal rat anti-TP0155, anti-TP0483 and anti-TP0956 (1:100 dilution). Polyclonal rat serum against *Borrelia burgdorferi* (*B. burgdorferi*) OspC (1:100) was used as a non-treponemal negative control. In addition, YOYO-1 (Molecular Probes) (1:1000 dilution), a nuclear stain with a similar staining pattern as Tp47, was also used as an additional negative control (data not shown)[15]. The microdroplets were incubated with primary antibody overnight at 4°C, followed by gentle centrifugation (500 x *g*, 5 min) and five washes with fresh *Tp* medium. Conjugated secondary antibodies, either 2 μg of goat anti-mouse IgG, or 2 μg of goat anti-rat or 1 μg of goat anti-rabbit Alexa Fluor 488 or 568 (Molecular Probes), were added to the agarose gel microdroplets and incubated for 2 h at 4°C, followed by washing in *Tp* medium. All secondary antibody probes (without primary antibody) were utilized alone in gel microdroplet preparations to assess non-specific labeling.

Spirochetes encapsulated within gel microdroplets were visualized on glass slides with an Olympus BH2 microscope (darkfield lens, BP545 [red], and BP490 [green] filter [Olympus America Inc, Melville, NY]). Slides were prepared from at least 3 independent microdroplet preparations, and more than 100 bacteria were counted per slide. Images were obtained using a SPOT Pursuit camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The ratio of fluorescent spirochetes to the total number of spirochetes (determined by darkfield microscopy) was calculated.

3. Results

3.1. Assessment of TP0155, TP0326, TP0483 and TP0956 cell-surface exposure in Tp

The agrose gel microdroplet method [15] was utilized to assess whether TP0155, TP0326, TP0483 or TP0956 are exposed on the surface of *Tp*. Monospecific rat- or rabbit-derived polyclonal antibodies were employed to detect TP0155, TP0326, TP0483, or TP0956. A monoclonal antibody directed against Tp47, a periplasmic lipoprotein of *Tp*, was used as a marker for subsurface localization [15]. We first characterized the antibodies used in the gel microdroplet assays by probing *Tp* whole cell lysates to detect native treponemal proteins. Non-rabbit antibodies are particularly advantageous as probes in gel microdroplet assays because they circumvent the need to use anti-rabbit-conjugated secondary antibody (which can react with rabbit antibody that may contaminate *Tp* preparations). However, in the case of TP0326, only rabbit antibodies were available. Sensitive immunoblotting (chemiluminescence) was used to assess antigen-specific antibodies in the sera of immunized animals after sera were diluted 1:1000. None of the antisera from unimmunized (pre-immune) animals showed evidence in immunoblots of antibodies directed against either the respective recombinant (immunizing) protein or the corresponding native molecule of predicted size in *Tp* (Fig. 1A-D, left column) (Table 1). When animals were immunized with each recombinant protein, all elicited antibodies that reacted with their corresponding immunizing recombinant antigen (Fig. 1A-D, right column, right-facing arrows). When these same sera were used in immunoblots with *Tp*, sera from animals immunized with rTP0483, rTP0956, or rTP0326 showed antibody reactivities with native treponemal polypeptides (Fig. 1B,C, left-facing arrows) that corresponded with the predicted molecular masses of each native protein in *Tp* (Table 1). Sera from rats immunized with TP0155 revealed a polypeptide in *Tp* (Fig. 1A, left-facing arrow) slightly higher in molecular mass than predicted to be the case for the cognate native molecule in *Tp* (Table 1); the reason for this difference remains unclear at this time. Of note, all sera (rat or rabbit, pre-immune or immune) blotted against *Tp* displayed reactivity

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with a polypeptide of ca. 50-kDa (rabbit) or ca. 54-kDa (rat). The prominent polypeptide in Fig. 1D is most likely rabbit IgG (50-kDa heavy chain) contaminating the *Tp* suspensions. However, sera from immunized rats (Fig. 1A-C) contained antibodies that reacted with a slightly larger (54-kDa) ubiquitous polypeptide; this reactivity was faint in pre-immune sera and appeared to increase in intensity after immunization. Finally, as expected [16], murine monoclonal antibody 11E3 detected Tp47 prominently within *Tp* whole cell lysates (not shown).

When the antibodies against Tp47, TP0155, TP0326 or TP0483 were used to probe intact *Tp* encapsulated within agarose gel microdroplets, spirochetes were not labeled (Fig. 2A). In contrast, spirochetes treated with Triton X-100 fluoresced when treated with the same antibody preparations (Fig. 2A). Quantitatively, less than 9% of spirochetes fluoresced when exposed to the antibodies, whereas in the presence of Triton X-100, immunolabeling was observed in no less than 86% of the spirochetes (Fig. 2B). Control labeling experiments using antibodies against an unrelated surface protein from *B. burgdorferi* (OspC) or only secondary antibodies (anti-mouse or rat) had minimal fluorescence (<1%) in the presence of Triton X-100 (data not shown). However, slightly higher labeling (~10%) was observed using the anti-rabbit secondary antibody alone (Fig. 2B), most likely due to the presence of rabbit host antibodies in the treponemal preparation (Fig. 1D). Interestingly, whereas treponemes did not substantially fluoresce in the absence of Triton X-100 when probed for TP0956 (4% labeling), the addition of detergent did not increase the amount of fluorescence to any appreciable degree (16% labeling).

3.2. Rabbit immunizations with histidine-tagged fusion proteins

Each of two rabbits was immunized with a total of 500 μg of each antigen, or a combination of TP0155, TP0326, TP0483 and TP0956, over a 9-week period. Assessment of antibody titers within the various immunized rabbit sera was used as a surrogate marker for the immunogenicity of each recombinant antigen preparation. All rabbits immunized with individual proteins or with the tetravalent combination demonstrated serum titers of greater than 1:50,000 for each antigen, as determined by immunoblotting 10-fold serial dilutions of each serum sample. A representative immunoblot from each rabbit pair is shown in Fig. 3. Smaller polypeptides on immunoblots most likely represented fusion protein degradation products.

The rabbit immune responses after receiving the various vaccine preparations were assessed by evaluating antibody reactivities of the pre- and post- immunization sera from each rabbit with recombinant immunogens or *Tp* whole cell lysates. Sensitive immunoblotting (chemiluminescence) again was used to aid in the detection of potentially low-abundance proteins in *Tp*. Representative immunoblots for each antigen are shown in Fig. 4. Sera from animals immunized with each respective immunogen showed their expected immunoreactivity with the corresponding recombinant antigen (Fig. 4A-D). Immunoblots using sera from the control immunization of animals with Tp47 yielded the well established pattern of immunoreactivity with Tp47 in *Tp* (not shown) [16]. Sera from all rabbit immunizations reacted with a protein in *Tp* preparations migrating at about 50 kDa, which also appeared in immunoblots of sera from non-immune animals, again representing rabbit IgG (heavy chain) within treponemal preparations. Sera from rabbits immunized with TP0326 (Fig. 4B), TP0483 (Fig. 4C), or TP0956 (Fig. 4D) detected native treponemal polypeptides of the expected molecular masses (Table 1), consistent with results of Fig. 1. In contrast, a native polypeptide of expected mass was not detected in *Tp* when the immunoblots were probed with TP0155-immunized rabbit serum (Fig. 4A). The combined data revealed that vaccination with the respective recombinant proteins gave rise to antibodies reactive with native TP0326, TP0483, and TP0956. Whereas the intrinsic immunogenicity of TP0155 was demonstrated (as per high

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titers of antibody in the sera of immunized animals) (**Figs.** 3 and 4A), the failure to identify the cognate polypeptide in *Tp* likely was due to low abundance [17].

The protective capacity of each fusion protein was tested by challenging immunized rabbits with live *Tp*. All rabbits developed red, raised, indurated lesions at all eight inoculation sites which eventually ulcerated (Fig. 5). Two antigens (TP0326 and TP0956) appeared to cause intensified lesions. Darkfield microscopy performed on aspirates of lesions from all animals revealed the presence of motile spirochetes. Ulcerations on the rabbits inoculated with the control immunogen, Tp47, appeared earlier than in the other immunized rabbits.

4. Discussion

Using the gel microdroplet assay, we were unable to garner evidence that either TP0155 or TP0483 is surface-exposed in *Tp* [15]. Our conclusion that TP0155 and TP0483 likely are not outer membrane proteins seemingly contradicts the previous report by Cameron et al. [13] that TP0155 and TP0483 are fibronectin-binding proteins, based on observations that antibodies directed against TP0155 or TP0483 partially block the attachment of *Tp* to fibronectin-coated microscope slides. If TP0155 and TP0483 bind to fibronectin as part of *Tp*'s dermal invasion strategy [18], then they should be outer membrane proteins with surface-exposed domains. However, TP0155, TP0483, or polypeptides with similar molecular masses have not been prominently noted in isolated *Tp* outer membranes [19], although their low abundance could explain a failure to detect them. Indeed, freeze-fracture freeze-etching electron microscopy of *Tp* has shown a paucity of proteins within treponemal outer membranes [20]. Furthermore, despite using high-titer rabbit antiserum directed against recombinant TP0155, we were unable to conclusively identify a protein of the correct molecular mass within whole-cell lysates of *Tp*, even though the rat-derived antibodies reacted with *Tp* in gel microdroplet assays. This discrepancy may reflect differences between Freund's adjuvant (used to generate rat antisera) and the Ribi adjuvant system (used for rabbit immunizations) [21]. Recent microarray data support the low abundance of TP0155 and TP0483, inasmuch as the ratios of mRNA-derived cDNA to DNA for both proteins are very low (0.333 for TP0155; 0.446 for TP0483) [17]. Finally, we were not able to demonstrate any protective effect of vaccinating rabbits with either TP0155 or TP0483, despite achieving high levels of serum antibodies against each protein after immunization.

The gel microdroplet assays also failed to indicate that TP0326 is surface-exposed in *Tp*. This finding contradicts protein homology searches [5] that assign TP0326 as a member of a family of porins [22] important in the assembly of Gram-negative bacterial outer membranes [5,22]. The issue of whether *Tp* produces porins has been controversial; although there have been reports of outer membrane porins in *Tp* [11], such reports have been refuted [23,24]. Although unlikely, it remains possible that TP0326 is a porin that lacks surface-exposed epitopes. On the other hand, Cameron et al. [5] reported that antibodies directed against TP0326 could serve as opsonins for the enhanced phagocytocis of *Tp* by macrophages, suggesting that TP0326 is surface-exposed. Pursuant to this line of reasoning, we immunized rabbits with recombinant TP0326 and failed to observe any degree of immunoprotection when these vaccinated animals were challenged intradermally with viable *Tp*.

Whereas the gel microdroplet assay currently is the most useful approach for investigating the cell-surface exposure of spirochetal antigens [15], it should be acknowledged that there are certain limitations to the technique. For example, not a single outer surface protein of *Tp* yet has been definitively identified, and thus there is not an antibody against a known surface protein of *Tp* that can be used as a positive control in the gel microdroplet assay. Thus, the overall sensitivity of the assay is not well established, especially for *Tp* outer membrane proteins that may be in low abundance or have few surface-exposed epitopes. Of note, a

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monoclonal antibody (M131) directed against treponemal phosphorylcholine has been shown to be reactive in gel microdroplet assays with *Tp* [25]. Indeed, we have used the M131 successfully in the gel microdroplet assay (not shown), although, in our hands, only about 50% of agarose gel-encapsulated spirochetes label, and the labeling is punctuate. Thus, whereas M131 may serve as a useful qualitative positive control in the gel microdroplet assay, its quantitative value in this assay remains unknown.

Although TP0956 has not been well studied, a Conserved Domain Database (CDD) search revealed that TP0956 is included within a family of 6 predicted integral membrane proteins (α-helical, not β-barrels) with unknown function. TP0956 has the highest identity (42%) with a probable lipoprotein in *Treponema denticola* (TDE1021) [26]. In our studies, antibodies directed against TP0956 and used in chemiluminescent immunoblots detected a polypeptide of the expected mass within whole-cell lysates of *Tp*, suggesting that TP0956 is readily expressed by *Tp*. The same antibody preparation, however, failed to react with *Tp* encapsulated in agarose gel microdroplets, even when mild detergent was used to disrupt the *Tp* outer membrane. The negative gel microdroplet results for TP0956 were surprising given that, as noted earlier, an antibody directed against TP0155 readily detected spirochetes in gel microdroplet assays but failed to detect its cognate antigen in chemiluminescent immunoblots. Although the reasons for positive reactivity of polyclonal antiserum against TP0956 in immunoblots but not in fluorescence gel microdroplet assays with *Tp* remain obscure, it is possible that recombinant protein immunization of rats elicited antibodies only to linear epitopes of the protein (plausible considering the protein purification techniques used), rather than to conformational or buried epitopes that might be more relevant to the actual cellular environment of TP0956. The notion of conformational-specific epitopes in *Tp* is not limited to TP0956 [27], and reports from other pathogenic bacteria support this possibility [28].

TP0956 also was previously hypothesized by McKevitt et al. to be a syphilis vaccine candidate [14]. This prediction was based on the correlation that chancre immunity in *Tp*–infected rabbits occurs between 60-120 days, and that the antibody response to TP0956 increases 30-fold at days 56-84 [14]. Thus, we proceeded with rabbit vaccination experiments. As with the other target antigens of this study, recombinant TP0956 failed to induce immunoprotection in the rabbit model of experimental syphilis.

Two additional aspects of our rabbit vaccination experiments are noteworthy. First, we remain cognizant that no single protein or lipoprotein may serve as a fully protective immunogen against *Tp* infection. For that reason, and recognizing that a degree of “partial protection” induced by any one immunogen may not be readily discernable in the rabbit model of experimental syphilis, we also immunized animals with a multivalent vaccine consisting of all four immunogens (TP0155, TP0326, TP0483, and TP0956). Although recently a multivalent vaccine has shown promise for immunoprotection against *Staphylococcus aureus* using four surface proteins [29], our multivalent vaccine failed to induce protective immunity in rabbits. Second, Cameron et al. [5] previously observed a decrease in the severity of rabbit dermal lesions after rabbits immunized with recombinant TP0326 were intradermally challenged with viable treponemes [5]. In our study, after comparable vaccination with recombinant TP0326, all rabbits developed ulcerated lesions. This result occurred despite our efforts to follow precisely the vaccination and challenge protocol of Cameron et al. [5]. On the other hand, any number of subtle parameters could have influenced our differing results, including the fact that New Zealand rabbits are outbred. Performing vaccine trials with results that are consistent from laboratory to laboratory (using the rabbit model of experimental syphilis) remains one of the formidable technical challenges in syphilis vaccine research. Perhaps more contemporary vaccinology strategies, such as genetic immunization, may help reduce potential variables such as protein misfolding, conformational epitopes, or ineffective protein processing/presentation.

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The quest to define the molecular component(s) of *Tp* that might induce protective immunity in rabbits should not be daunted. While the search for a bona fide surface-exposed OMP remains paramount, it is also arguable that a better understanding, in general, of syphilis immunology, particularly as it pertains to the development of chancre immunity in rabbits, can offer new insights into elucidating previously unknown immunological aspects of chronic bacterial infections. It is plausible that both humoral and cell-mediated immune mechanisms contribute to the clearance (resolution of lesions) and control (latency) of treponemal infection [30,31], and clarifying the roles of both of these arms of the immune system remains warranted.

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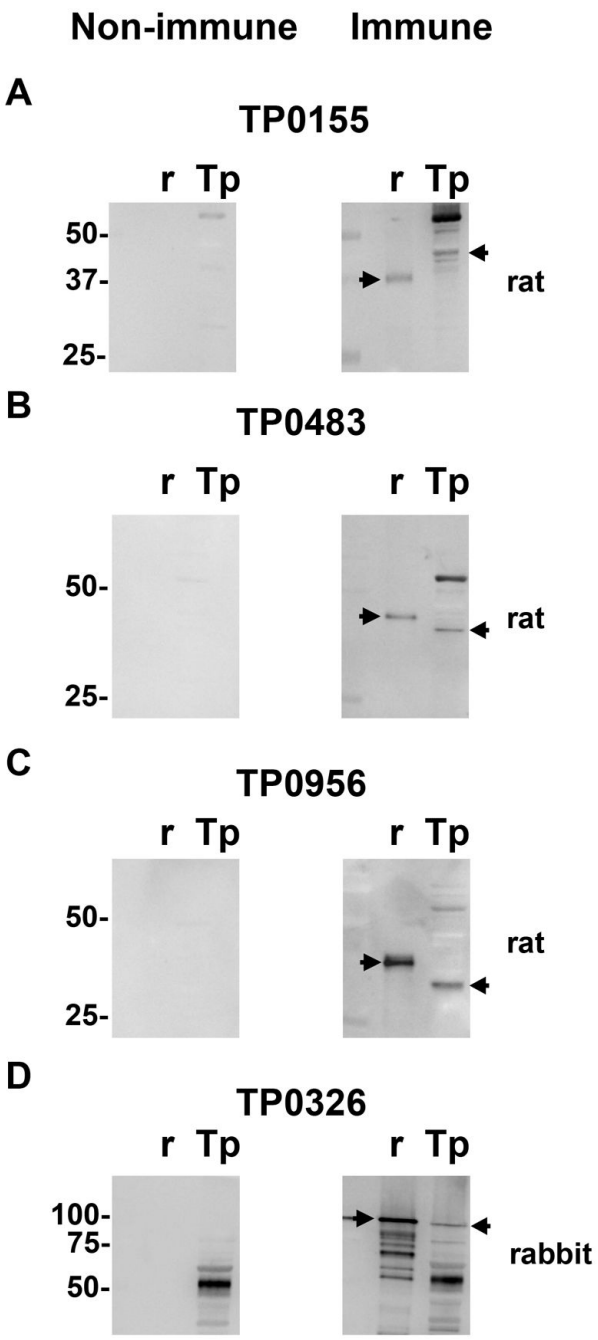

Fig. 1.

Chemiluminescent immunoblot analysis of sera from animals immunized with recombinant fusion proteins of TP0155, TP0483, TP0956, or TP0326. In the cases of TP0155 (A), TP0483 (B), and TP0956 (C), individual recombinant (r) proteins or whole cell lysates of $1 \times 10_8$ *Tp* (Tp) were probed using pooled non-immune (normal) rat sera or immune rat sera. In the case of TP0326 (D), recombinant protein or the lysate from 1×10^8 *Tp* were probed using non-immune rabbit sera or immune rabbit sera. The molecular mass standards (in kDa) are shown at the left of each matched set of images. Right-facing arrows delineate recombinant protein, whereas left-facing arrows highlight proteins in *Tp* whole cell lysates uniquely reacting with each antibody.

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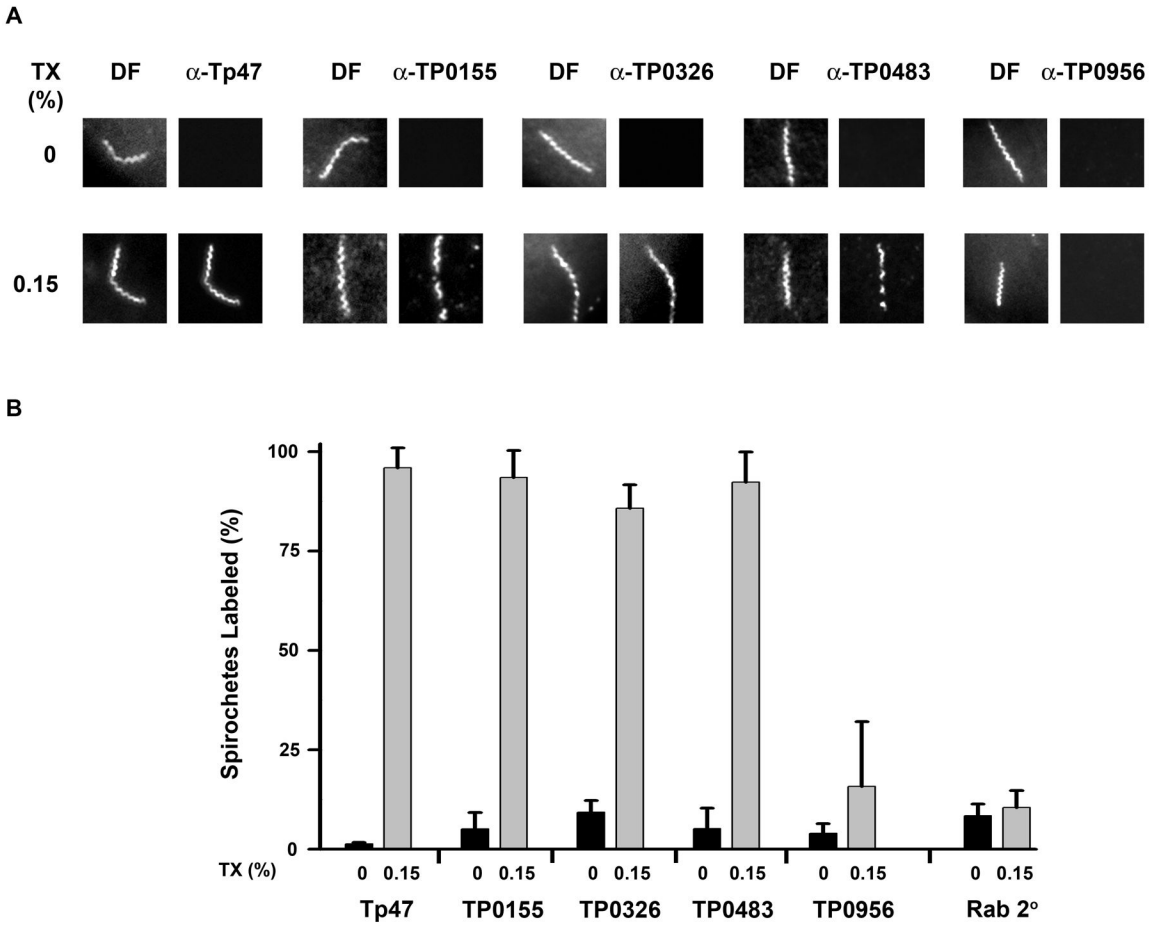

Fig. 2.

Membrane topology assessment of TP0155, TP0326, TP0483 and TP0956 in *Tp*. (A) *Tp* bacteria encapsulated in agarose gel microdroplets were exposed to antibodies (Fig. 1) directed against Tp47 (control), TP0155, TP0326, TP0483 or TP0956 in the presence or absence of 0.15% (v/v) Triton X-100 (TX). After secondary antibody treatment, spirochetes were examined by either darkfield (DF) or fluorescence microscopy. (B) Fluorescent treponemes were quantified from at least 3 independent microdroplet preparations and data were plotted. A control experiment utilizing anti-rabbit secondary antibody only was also plotted for comparison (Rab 2^{o}). Error bars represent standard deviations.

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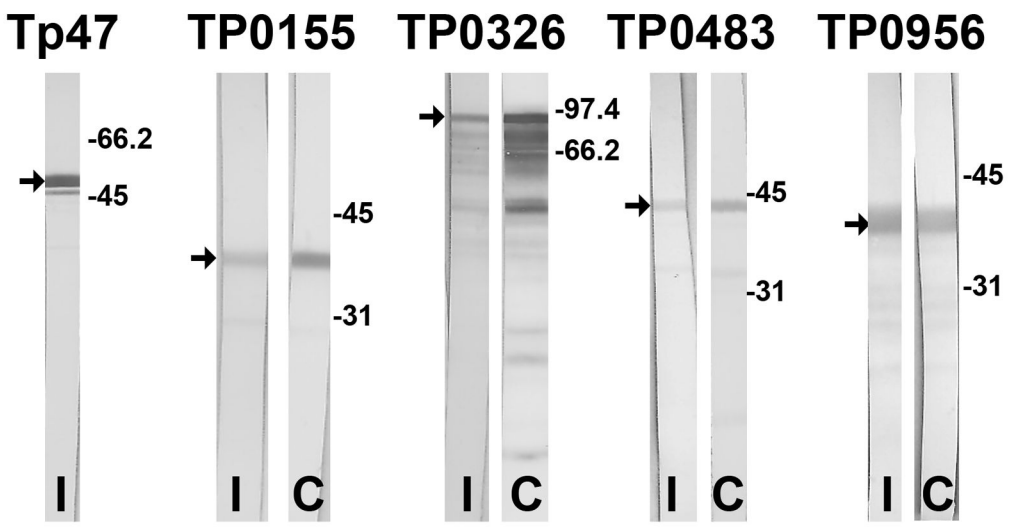

Fig. 3.

Representative immunoblots (colorimetric) of recombinant *Tp* proteins using sera from vaccinated rabbits. A 1:50,000 dilution of serum from vaccinated rabbits was used to probe for reactivity with recombinant *Tp* proteins. An immunoblot using serum from one rabbit immunized with Tp47, TP0155, TP0326, TP0483 or TP0956 (I) is compared to an immunoblot using sera from one rabbit immunized with the combination (C) vaccine consisting of TP0155, TP0326, TP0483 and TP0956. Arrows denote full-length recombinant protein (smaller polypeptides likely represent degradation products). Molecular mass standards (in kDa) are shown at the right of each set of immunoblots.

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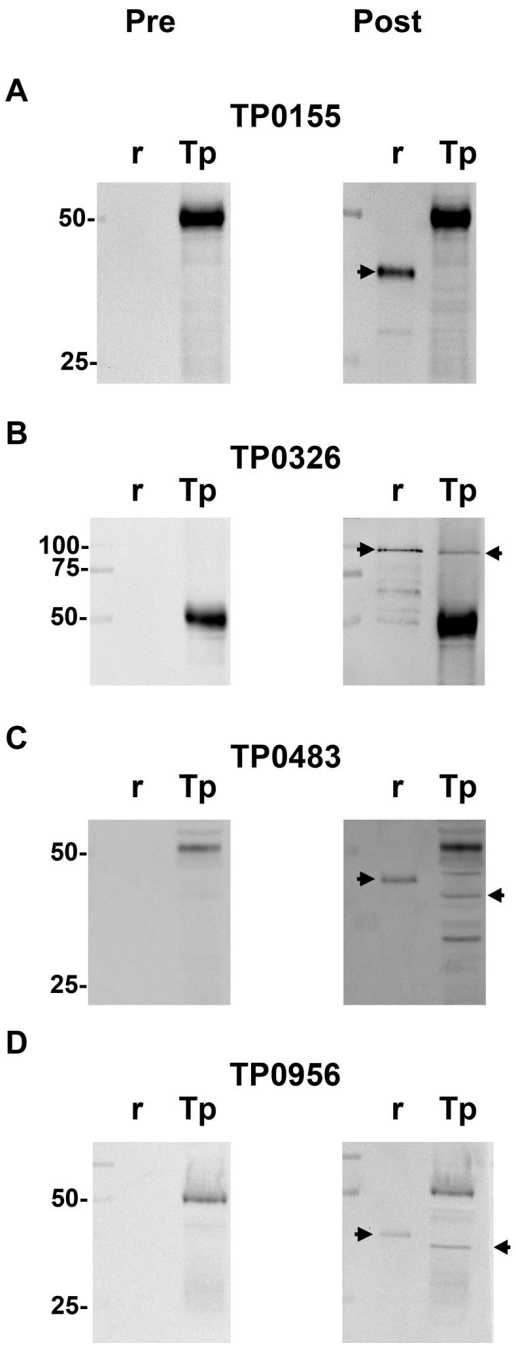

Fig. 4.

Chemiluminescent immunoblot analysis of *Tp* whole cell lysates using sera from rabbits vaccinated with recombinant proteins. Individual (r) recombinant proteins or lysates from 1×10^8 *Tp* (Tp) were probed using rabbit sera harvested before (Pre) or after (Post) vaccination with the recombinant proteins. The molecular mass standards (in kDa) are shown at the left of each matched set of images. Right-facing arrows denote recombinant protein. Left-facing arrows highlight proteins in treponemal whole cell lysates that react uniquely with antibody in the sera of vaccinated rabbits.

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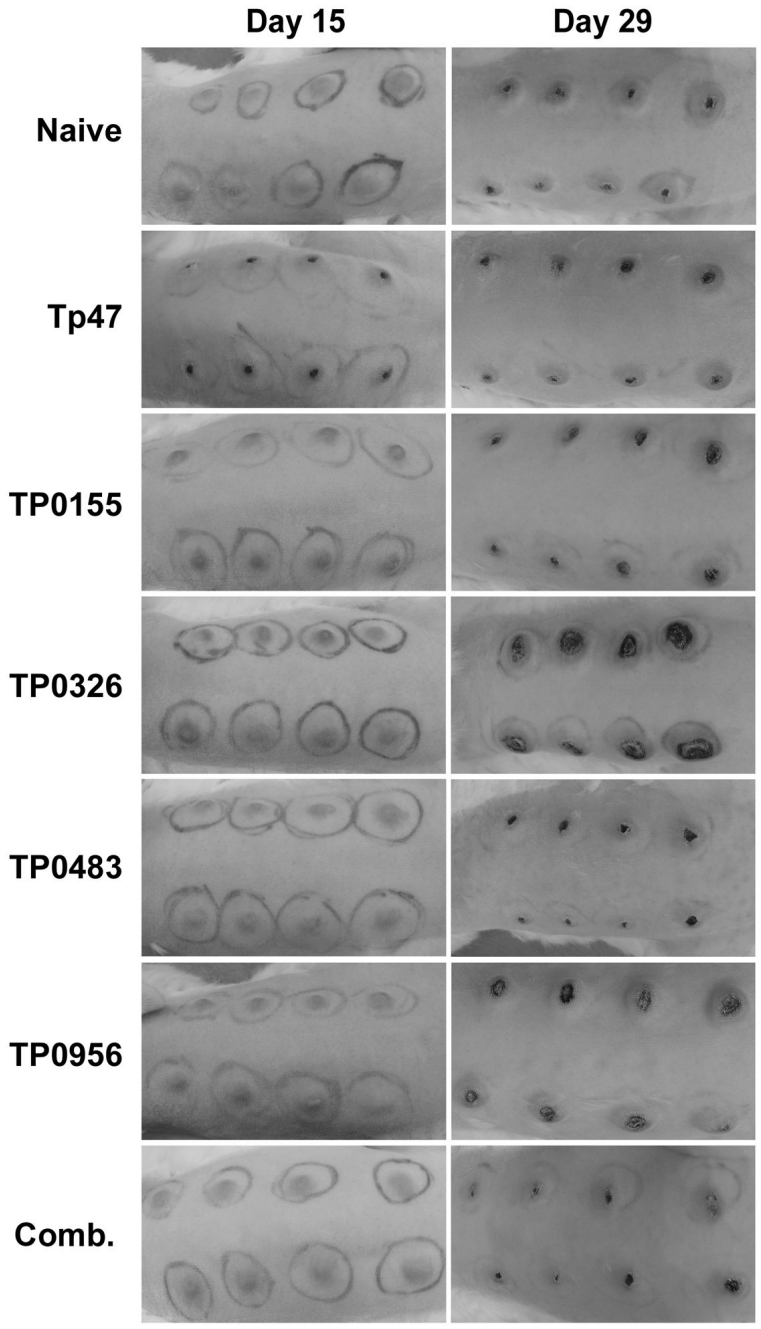

Fig. 5.

Intradermal challenge of vaccinated rabbits with *Tp*. Rabbits previously vaccinated with the recombinant *Tp* protein antigens Tp47, TP0155, TP0326, TP0483, or TP0956, or a combination (Comb.) of TP0155, TP0326, TP0483 and TP0956, were challenged intradermally with live *Tp*. A control rabbit was not vaccinated prior to challenge. 10^5 *Tp* were injected at each of 8 sites along shaved rabbit backs. Black pen marks encircle each inoculation site.

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Table 1

Sizes of *Tp* proteins.

Recombinant protein name	Amino acids in recombinant protein	Calculated molecular mass of recombinant (includes tag) (kDa) [1]	Estimated molecular mass of native mature protein (kDa) [2]
rTP0155	46-371	39.0	38.1
rTP0326	29-837	94.3	93.0
rTP0483	22-374	42.8	39.6
rTP0956	32-323	35.9	33.8

[1] An N-terminal histidine tag and amino acid linker adds an additional 3.2 kDa to each recombinant protein.

[2] This table does not take into account possible acylation of lipoproteins (i.e., TP0326 and TP0956).