



Antigenic Variation in *Treponema pallidum*: TprK Sequence Diversity Accumulates in Response to Immune Pressure during Experimental Syphilis

This information is current as of June 26, 2020.

Lorenzo Giacani, Barbara J. Molini, Eric Y. Kim, B. Charmie Godornes, B. Troy Leader, Lauren C. Tantaló, Arturo Centurion-Lara and Sheila A. Lukehart

J Immunol 2010; 184:3822-3829; Prepublished online 26 February 2010;
doi: 10.4049/jimmunol.0902788
<http://www.jimmunol.org/content/184/7/3822>

Supplementary Material <http://www.jimmunol.org/content/suppl/2010/03/01/jimmunol.0902788.DC1>

References This article **cites 33 articles**, 19 of which you can access for free at:
<http://www.jimmunol.org/content/184/7/3822.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Antigenic Variation in *Treponema pallidum*: TprK Sequence Diversity Accumulates in Response to Immune Pressure during Experimental Syphilis

Lorenzo Giacani,* Barbara J. Molini,* Eric Y. Kim,* B. Charmie Godornes,*
B. Troy Leader,* Lauren C. Tantalo,[†] Arturo Centurion-Lara,* and Sheila A. Lukehart*[‡]

Pathogens that cause chronic infections often employ antigenic variation to evade the immune response and persist in the host. In *Treponema pallidum* (*T. pallidum*), the causative agent of syphilis, the TprK Ag undergoes variation of seven V regions (V1–V7) by nonreciprocal recombination of silent donor cassettes with the *tprK* expression site. These V regions are the targets of the host humoral immune response during experimental infection. The present study addresses the causal role of the acquired immune response in the selection of TprK variants in two ways: 1) by investigating TprK variants arising in immunocompetent versus immunosuppressed hosts; and 2) by investigating the effect of prior specific immunization on selection of TprK variants during infection. V region diversity, particularly in V6, accumulates more rapidly in immunocompetent rabbits than in pharmacologically immunosuppressed rabbits (treated with weekly injections of methylprednisolone acetate). In a complementary experiment, rabbits preimmunized with V6 region synthetic peptides had more rapid accumulation of V6 variant treponemes than control rabbits. These studies demonstrate that the host immune response selects against specific TprK epitopes expressed on *T. pallidum*, resulting in immune selection of new TprK variants during infection, confirming a role for antigenic variation in syphilis. *The Journal of Immunology*, 2010, 184: 3822–3829.

Pathogens frequently use antigenic variation mechanisms to evade the adaptive immune response, resulting in persistent infection. Syphilis is a lifelong infection in the absence of antibiotic treatment, and the mechanisms of persistence have been the subject of speculation for decades. Syphilis manifests in distinct clinical stages, of which the primary (ulcerative lesion) and secondary (skin rash, mucosal lesions) stages are infectious. The infectious lesions contain large numbers of treponemes but, after weeks to months, heal spontaneously after local immune clearance of the bacteria by opsonophagocytosis (1–3). The subsequent years to decades of persistent syphilis infection are usually asymptomatic, but ~30% of untreated individuals with latent infection develop tertiary syphilis (4). The manifestations of tertiary syphilis include gummas, cardiovascular syphilis, and late neurosyphilis, which can cause insanity, blindness, paralysis, or death.

Suggested mechanisms of *T. pallidum*'s persistence despite the host's efforts to eradicate the infection include residence within intracellular or immune privileged niches to hide from the immune effectors (5–9), *T. pallidum*'s ability to cloak its surface with a coat of host

serum proteins or mucopolysaccharides to avoid immune recognition (10–12), and immunosuppression of the host caused by syphilis infection (13, 14). All of these theories have lost favor in light of subsequent investigations. Freeze-fracture electron microscopy of *T. pallidum* revealed a paucity of integral membrane proteins in the *T. pallidum* outer membrane (OM), perhaps accounting for the relatively poor antigenicity of this spirochete's surface (15, 16). However, because *T. pallidum* can be phagocytized in the presence of opsonic Ab, Ab targets must be present on the surface of the bacterium. Furthermore, the finding that treponemes harvested from infected tissues later in infection, following the clearance of the majority of treponemes from the early lesions, are resistant to opsonophagocytosis raised the possibility that antigenic variation might occur in *T. pallidum*, but no specific variable Ag was identified (17). The subsequent identification and investigation of TprK provided the first candidate Ag of *T. pallidum* that might function in immune evasion. TprK is highly heterogeneous among and within *T. pallidum* strains, with sequence diversity localized in seven discrete variable regions (V1–V7) flanked by conserved domains (18, 19). Centurion-Lara et al. (20) recently proposed a model whereby sequence diversity is generated by nonreciprocal recombination (gene conversion) between multiple silent donor cassette segments and the single *tprK* expression site, similar to other bacterial variable Ags (21–23). This mechanism could theoretically generate millions of chimeric TprK variants that, if surface-exposed, could continuously alter *T. pallidum*'s surface antigenic profile.

During infection, TprK is targeted by the host immune response, with T cell responses directed at epitopes located primarily in the conserved regions of the Ag, whereas the humoral response targets the V regions (24). LaFond and colleagues (25) demonstrated that, although Abs against the V region sequences of the infecting inoculum develop during infection, little or no Ab reactivity against newly arising V region variants is detectable.

The present study addresses the direct causal role of acquired immunity in variant selection in two complementary ways: 1) by investigating the *T. pallidum* TprK variants arising in immunocompetent

*Department of Medicine, [†]Department of Neurology, and [‡]Department of Global Health, University of Washington, Seattle, WA 98104

Received for publication August 24, 2009. Accepted for publication January 11, 2010.

This work was supported by Grants R01 AI63940 and R01 AI42143 (to S.A.L. and A.C.-L.) from the National Institutes of Health.

Address correspondence and reprint requests to Dr. Sheila Lukehart, Department of Medicine, Box 359779, Harborview Medical Center, 325 Ninth Avenue, Seattle, WA 98104-2499. E-mail address: lukehart@u.washington.edu

The online version of this article contains supplemental material.

Abbreviations used in this paper: DS, diversity score; FLA, fragment length analysis; HPRT, hypoxanthine phosphoribosyltransferase; ID, intradermal; KLH, keyhole limpet hemocyanin; OM, outer membrane; ORF, open reading frame; qRT-PCR, quantitative real-time PCR; RSDI, reciprocal of Simpson's diversity index; RT, reverse transcription; VDRL, Venereal Disease Research Laboratory.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/\$16.00

versus immunosuppressed hosts; and 2) by investigating the effect of prior specific immunization on selection of *T. pallidum* variants during infection. These studies provide evidence for the role of the acquired immune response in the selection of TprK variants during the course of infection and confirm that TprK variation is central to immune evasion during syphilis.

Materials and Methods

T. pallidum Chicago strain propagation and derivation of the clonal Chicago C isolates *T. pallidum* subsp. *pallidum*, Chicago strain, originally obtained from Paul Hardy and Ellen Nell (Johns Hopkins University, Baltimore, MD), was propagated intratesticularly in New Zealand white rabbits as previously reported (17). Because the Chicago strain treponemes have diverse sequences in the *tprK* locus, two clonal isolates (for our purposes, a clonal isolate is defined as an isolate with very limited or no detectable *tprK* sequence diversity) were derived in our laboratory as previously described (25). The resulting isolates, obtained after two intratesticular expansions of the clonal population in the initial skin biopsy (25), were called Chicago C1 and Chicago C2 and used as inocula for the experimental infections (described below) without any further passage. Aliquots of Chicago C1 and C2 treponemes were stored as viable frozen stocks in liquid nitrogen and in lysis buffer for DNA sequence analysis. The level of sequence homogeneity of the *tprK* locus in both isolates was assessed by fragment length analysis (FLA; described below) and DNA sequencing. Approval of the protocols involving animal use was obtained in advance from the University of Washington Institutional Animal Care and Use Committee.

Experimental infection of pharmacologically immunosuppressed and control rabbits with the Chicago C1 strain

The Chicago C1 strain was used to intradermally (ID) infect five pharmacologically immunosuppressed rabbits and five control rabbits at 10 sites each on their clipped backs; 10^6 treponemes were injected per site. Pharmacological immunosuppression was achieved by weekly i.m. injections of 20 mg methylprednisolone acetate (Sicor, Irvine, CA). Optimal dosage was determined in a pilot experiment. Treatment was started 3 d before experimental infection, and doses were administered weekly for a total of six injections. A lesion biopsy (4-mm punch biopsies taken under local lidocaine anesthesia) was harvested from each rabbit weekly for a period of 5 wk and used for both DNA and RNA extraction. Blood samples were also collected weekly to measure Abs in the two groups. Venereal Disease Research Laboratory (VDRL) titers were compared between treated and control rabbits as a measure of the effectiveness of the immunosuppressive treatment. Immunosuppression was further evaluated by quantification of rabbit IFN- γ mRNA levels (normally the predominant cytokine in syphilis lesions) and *T. pallidum* burden in skin lesions by quantitative real-time PCR (qRT-PCR) on reverse-transcribed RNA samples. *tprK* mRNA levels were also evaluated by qRT-PCR to determine whether there were any effects of immunosuppression on *tprK* expression by *T. pallidum*. Generation of sequence diversity in the *tprK* locus during the course of infection was determined using FLA (described below) of individual *tprK* V regions and by sequence analysis.

Table I. Primers and synthetic peptides used in this study

| Purpose | Name | 5' to 3' Sequence | Size (bp) |
|--|----------------------------------|--|-----------|
| Primers | | | |
| Full-length <i>tprK</i> amplification and sequencing | <i>tprK</i> -S ^a | ACCGGGCATGAATTTTCTTT | 1593 |
| | <i>tprK</i> -As ^a | CCATACATCCCTACCAAATCA | |
| | <i>tprK</i> -int-As ^b | CCTACCCGCTGATACACCAC | |
| | M13rev ^b | CAGGAAACAGCTATGAC | |
| <i>tprK</i> fluorescent FLA ^c | M13for ^b | GTAAACGACGGCCAG | |
| | FAM-V1-S | GTGGGCTCAGGTTTTCGTTT | 187 |
| | V1-As | CGCATAGACATTTCCCTCAC | |
| | FAM-V2-S | GGGGCTCAGTTTGATATTG | 183 |
| | V2-As | CCGGTGAGCTCCACTTTAAT | |
| | FAM-V3-S | GAGCGTACGCGTGAAGATG | 166 |
| | V3-As | TAGCAGCCAGAGCACACAGA | |
| | FAM-V4-S | CTTTGGGGTCTGTGTGCTCT | 112 |
| | V4-As | AACGATACCCCAACGTCAAC | |
| | FAM-V5-S | TTGGGGTATCGTTGGTTCTC | 173 |
| | V5-As | CCCAAATCAAGACCTCAAG | |
| | FAM-V6-S | AAACCAAGGGTCTGATCCT | 188 |
| | V6-As | TAGACGATACGAACCCAG | |
| | FAM-V7-S | TGGGTGAGTATGGTTGGGTTA | 159 |
| | V7-As | GCCGAATCTCCACCTTCTCT | |
| qRT-PCR | TP0574-S | CGTGTGGTATCAACTATGG | 313 |
| | TP0574-As | TCAACCGTGTACTCAGTGC | |
| | RT- <i>tprK</i> -S | AGTTTGCGTCTAACACCGACTG | 410 |
| | RT- <i>tprK</i> -S | TCGCATGGCCATGTTGAGAAAT | |
| | rHPRT-S | TGATAGATCCATTCTATGACTGTAGA | 265 |
| | rHPRT-As | GGGTCTTTTCCACGACAG | |
| | rIFN- γ -S | TTCTTCAGCCTCACTCTCTCC | 224 |
| | rIFN- γ -As | TGTTGTCACTCTCTCTTTCC | |
| Purpose | Name | NH ₂ - to C-terminus Sequence | Size (aa) |
| Synthetic peptides | | | |
| ELISA | ChicC1-V6 | VHYKVLKARAQAPAAVPAADDIYF | 25 |
| Immunization | ChicC2-V5 | ASQASNVFGVFLTPMQKDDC ^d | 22 |
| | ChicC2-V6 | MPVHYKVLKARARAGAAVPAADDIYFPVC | 29 |

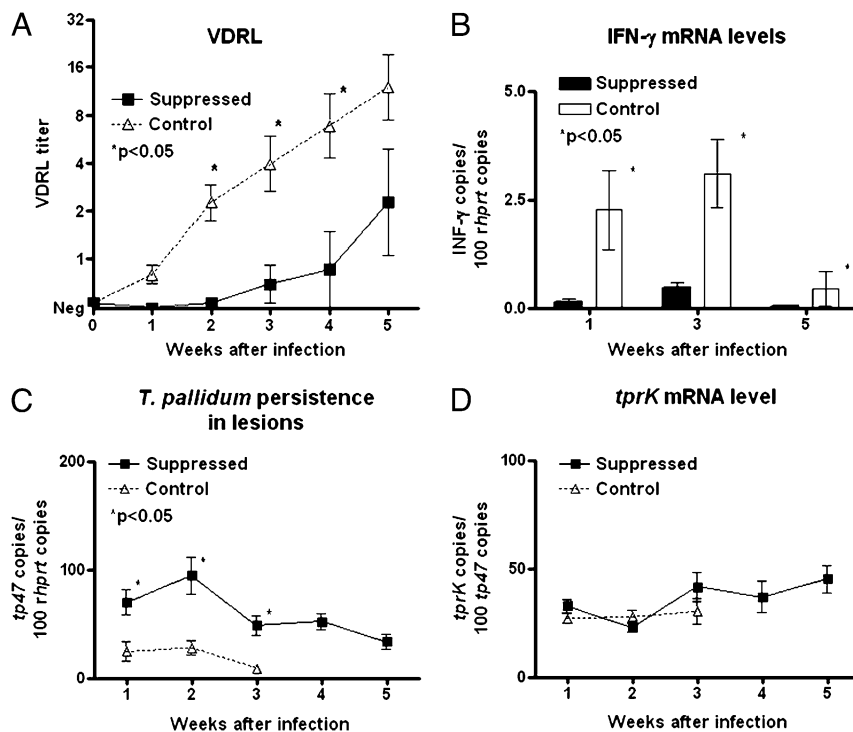
^a*tprK*-S/As primers were used for both full-length ORF amplification and sequencing after cloning.

^bSequencing primer. M13for and M13rev primers are PCR-II TOPO vector primers flanking the cloning site.

^cAll sense primers are FAM labeled.

^dThe final C residue was added during synthesis to allow conjugation with the KLH carrier.

FIGURE 1. Effectiveness of pharmacological treatment and *tprK* expression. Significant delay in development of VDRL Ab titers (A), reduced IFN- γ expression (B), and longer persistence of *T. pallidum* cells in lesions from suppressed rabbits (C) confirmed the effectiveness of pharmacological immunosuppression. Pharmacological treatment did not alter *tprK* mRNA levels in treponemes from control and treated rabbits (D).



Experimental infection of V region-immunized and control rabbits with the Chicago C2 strain

Synthetic peptides based on the Chicago C2 V5 and V6 sequences were purchased from GenScript Corporation (Piscataway, NJ) and conjugated to the keyhole limpet hemocyanin (KLH) carrier protein using the Imject Maleimide Activated mcKLH Kit (Pierce, Rockford IL) according to the manufacturer's protocol. Because maleimides reacts with sulfhydryl groups, a cysteine was added at the C-terminal of the Chicago C2 V6 peptide during the synthesis process, whereas the V5 peptides naturally end with a cysteine and no further modification was required. Peptide-carrier conjugates were separated from free carrier molecules using desalting columns (provided with the kit), and conjugate concentration was determined using the BCA Protein Assay Kit (Pierce). Peptide-carrier complexes were stored at -20°C until use. Two groups of three adult male rabbits each were immunized with the Chicago C2 V5-KLH and V6-KLH conjugates, respectively, whereas a third group was immunized with the KLH carrier alone. Prior to injection, Ags (200 $\mu\text{g}/\text{dose}$) were emulsified in Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO). A total of six doses were administered i.m. at 20-d intervals; efficacy of immunization was measured by ELISA detection of specific Abs. At the end of the immunization cycle, the three groups of immunized rabbits, plus three unimmunized control rabbits, were infected ID at 10 sites per rabbit with 10^5 Chicago C2 treponemes per site. Following ID infection, tissue biopsies from the leading edge of one lesion in each rabbit were taken at days 12 and 20 using a 4-mm biopsy punch (Miltex, York, PA) under local lidocaine anesthesia.

Specific procedures

Ab testing. VDRL titers were determined weekly on sera obtained from immunosuppressed and control rabbits; reagents (VDRL Ag and buffered saline) were purchased from Becton Dickinson (Sparks, MD) and used according to the manufacturer's instructions. The development of Abs to Chicago C1 V6 was determined by ELISA using a synthetic peptide representing the predominant Chicago C1 V6 sequence, as previously described (25). Sera obtained from rabbits immunized with Chicago C2 V5-KLH, V6-KLH, and KLH alone were similarly tested by ELISA for their ability to recognize the respective immunogens (synthetic V5 and V6 peptides and unconjugated KLH). All ELISA results represent the mean OD \pm SE of results for all animals in the group; individual sera were tested in triplicate wells per serum sample.

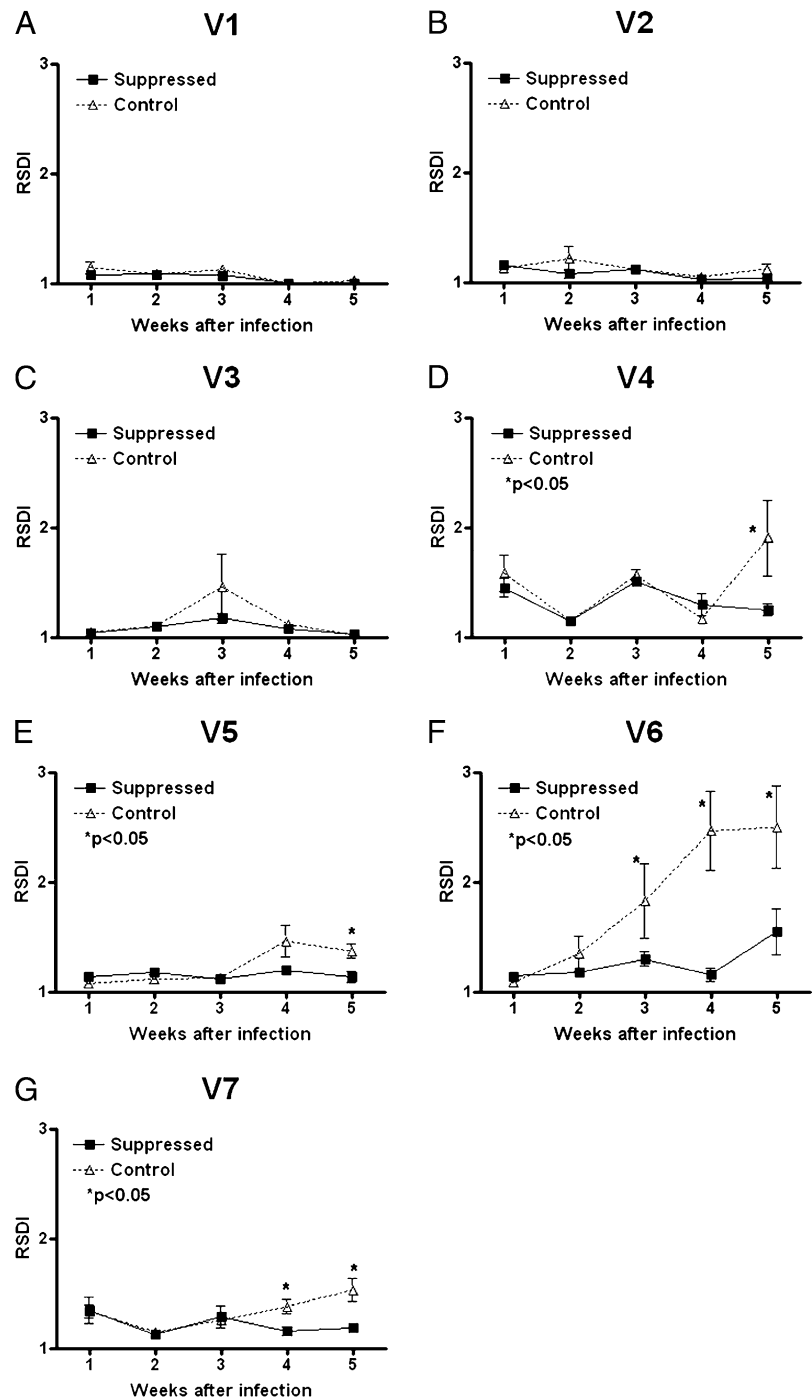
Nucleic acid extraction and manipulation. Immediately upon harvest, each biopsy was minced with a sterile blade. Half of the lesion tissue was resuspended in 400 μl $1\times$ lysis buffer [10 mM Tris (pH 8), 0.1 M EDTA, 0.5% SDS] for DNA extraction, and the other half in 400 μl Ultraspec buffer (Biotex Laboratories, Houston, TX) for total RNA isolation. DNA

extraction was performed as previously described (26) using the QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA), according to the manufacturer's protocol. Extracted samples were stored at -80°C until use. RNA isolation was performed following the Ultraspec manufacturer's instructions as already described (27); postextraction, DNaseI treatment was performed as reported (28). Reverse transcription (RT) of DNA-free RNA was performed using the Superscript II First Strand Synthesis Kit (Invitrogen, Carlsbad, CA) with random hexamers according to the provided protocol. cDNA sample preparation and storage for real-time amplification were also previously reported (28).

Real-time quantification of *T. pallidum* and rabbit mRNA. A relative quantification protocol using external standards was chosen to analyze mRNA levels at the time of biopsy harvest. This approach normalizes the amount of message from one or more target genes to the mRNA of a reference gene. TP0574 (the 47 kDa lipoprotein) was used as reference when the target to be measured was *tprK* mRNA; rabbit hypoxanthine phosphoribosyltransferase (HPRT) was chosen to normalize levels of rabbit IFN- γ mRNA. The quantity of *T. pallidum* in skin lesions was determined as the ratio between TP0574 and rabbit HPRT mRNA levels. The rationale behind the use of the TP0574 (over several other candidates) as a reference gene for *T. pallidum* has been discussed in detail (28), and real-time amplification protocols for TP0574, *tprK* (28) (primers in Table I), rabbit HPRT, and IFN- γ (29) have previously been described. Amplification reactions and data collection were carried out using the LightCycler 1.0 (Roche, Basel, Switzerland) instrument. All reactions were performed following the manufacturer's instructions with the Roche FastStart DNA Master Plus SYBR Green Kit (Roche). Triplicate amplifications were performed for each gene per sample using 3 μl of the cDNA preparations; a known concentration of a linear plasmid DNA containing all of the targets in its polylinker was amplified concurrently in each run as an internal standard and amplification control. Results were analyzed using the LightCycler 3.5 software (Roche). Differences in levels of gene expression between groups were compared using the Student *t* test with significance set at $p < 0.05$.

***tprK* amplification, FLA, cloning, and sequencing.** DNA extracted from lesion biopsies was used to amplify the *tprK* gene to evaluate presence of sequence diversity by: 1) FLA of amplicons of individual V regions; and 2) sequencing of the full-length *tprK* open reading frame (ORF). To perform FLA analysis, each of the seven *tprK* V regions was amplified using a fluorescent (FAM-labeled) sense primer and an unlabeled antisense primer (primer sequences and amplicon sizes are shown in Table I) complementary to the unique conserved sequences flanking each *tprK* V region. Amplifications were performed using the AccuPrime Pfx DNA Polymerase (Invitrogen) with ~ 100 ng DNA template in each reaction and primers at a final concentration of 400 nM. Pfx Polymerase is supplied with a $10\times$ reaction mixture containing 10 mM MgSO_4 and 3 mM of each 2'-deoxynucleoside 5'-triphosphate. A

FIGURE 2. Accumulation of diversity in *tpk* V regions determined by FLA analysis. RSDI values show that, in immunocompetent rabbits infected with the Chicago C1 strain, sequence diversity tends to accumulate more rapidly in V6, V4, V5, and V7 than in treated rabbits (D–G). No difference in diversity was seen for V1, V2, and V3 (A–C) between the two groups of rabbits.



touchdown amplification protocol was adopted to minimize nonspecific amplification products: during the first 10 cycles, the annealing temperature was decreased by 1°C per cycle until the optimal temperature of 55°C was reached. Initial denaturation and final extension steps were 10 min each; denaturation, annealing, and extension times were 1 min, 30 s, and 30 s, respectively, for a total of 45 cycles. Amplification and amplicon sizes were checked on 2% agarose gels and products purified using the QIAquick PCR purification kit (Qiagen). Concentrations were measured with an ND-1000 instrument (NanoDrop Technologies, Wilmington, DE), and all samples diluted to 0.2 ng/μl final concentration. One microliter of each sample was mixed with 15.4 μl Hi-Di Formamide (PerkinElmer/Applied Biosystems, Foster City, CA) and 0.1 μl MapMarker400 Rox-labeled DNA ladder (Bioventures, Murfreesboro, TN); samples were transferred to a 96-well plate and denatured by incubation at 95°C for 2 min, briefly chilled on ice, and loaded on an ABI3730xl DNA analyzer (PerkinElmer/Applied Biosystems). Graphically, the resulting electropherograms contain red peaks generated by the ROX-labeled DNA ladder and blue peaks representing amplification

products for a V region. Because changes in length of the V regions do not modify the *tpk* reading frame, the blue peaks will necessarily be three nucleotides apart from each other when length diversity is present within the V region. Electropherograms were analyzed using the GeneMapper 4.0 software package (PerkinElmer/Applied Biosystems); data relative to V region fragment length (determined by comparison with the ROX-labeled marker) and intensity (measured by area under the peak) were collected. For the inoculum and for each rabbit lesion at each time point, V region diversity observed by FLA was calculated as the reciprocal of Simpson's diversity index (RSDI) (30). The RSDI value takes into account not only the number of different V region sizes represented, but also their relative proportions; therefore, it is the most appropriate to interpret the FLA results. In this context, $RSDI = 1/\sum p_x^2$, where p_x = (area underneath the peak with size x /sum of the areas underneath all peaks). Using this interpolation, an RSDI value of 1 indicates the presence of a single V region size, whereas values >1 indicate increasing diversity in V region sizes, with higher values seen when higher proportions of different sizes are present.

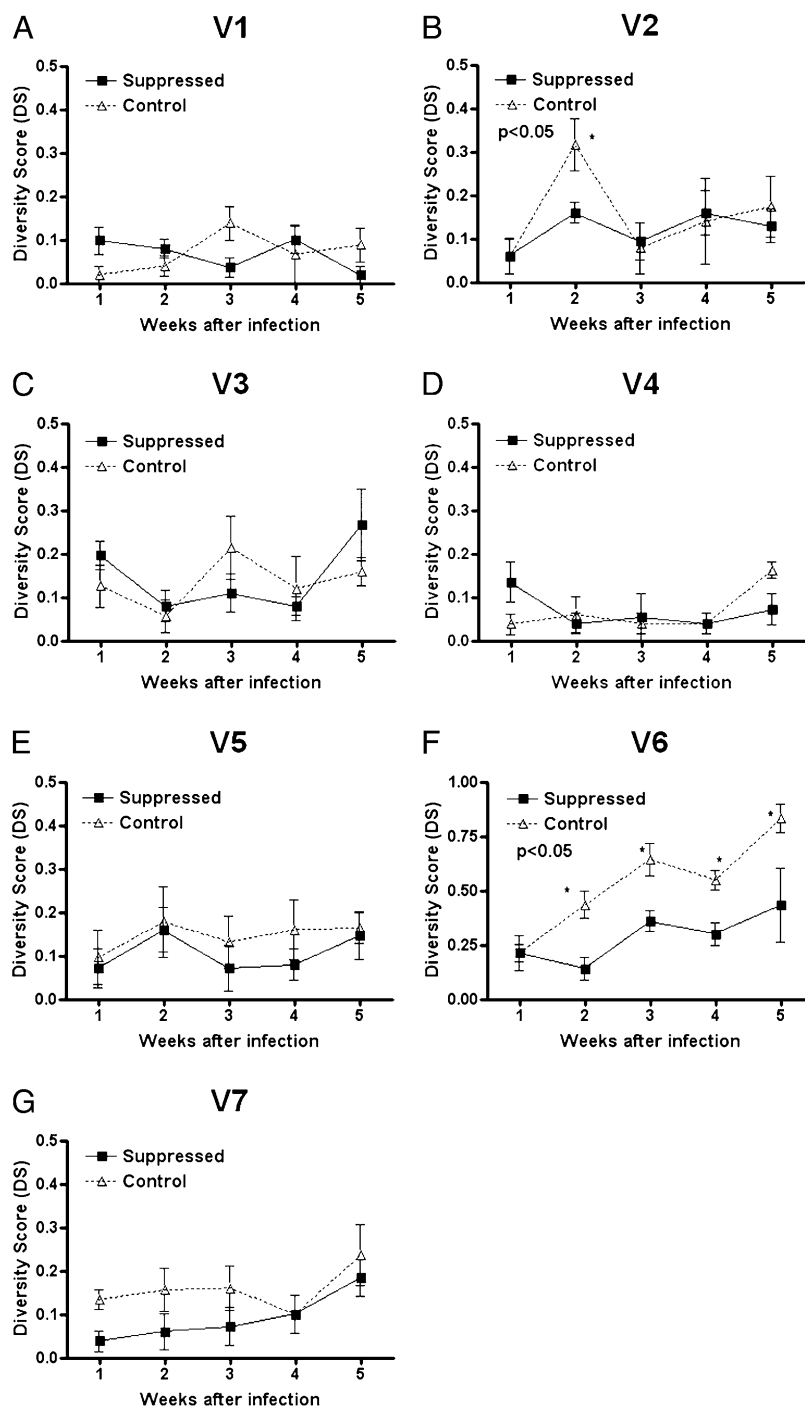


FIGURE 3. Accumulation of diversity in *tprK* V regions determined by sequence analysis. DS values for TprK V1-V7 (A-G) at weeks 1–5 postinfection with the Chicago C2 strain. The sequences of at least 10 TprK variants were determined for each lesion per time point. For each group of sequences, DS is calculated as the number of new V regions detected divided by the total number of sequences determined. When DS = 0, no new variants are detected compared with the inoculum, whereas a value of 1 indicates that none of the sequences is identical to the known inoculum sequences. DS values were found to be significantly higher ($p < 0.05$) in controls with respect to suppressed rabbits for V6 (F) at week 2 of infection.

Sequencing of the *tprK* ORF from the expression site was conducted as previously described (31), with the PCR amplicons being cloned into *Escherichia coli* to permit sequencing of genes from individual *T. pallidum* cells. Plasmid extraction was carried on using either the Plasmid Mini Kit (Qiagen) or the Montage Plasmid Miniprep96 Kit (Millipore, Billerica, MA). Approximately 10 *tprK* clones from each amplification were sequenced. Sequencing primers are listed in Table I. Nucleotide sequences were translated and analyzed using the BioEdit Sequence Alignment Editor program, available at www.mbio.ncsu.edu/BioEdit/bioedit.html. A V region was considered diverse if it differed from any sequence seen in the original founder Chicago C1 inoculum. Sequence diversity was calculated using the predicted amino acid sequence for each V region compared with the inoculum (founder) sequences as follows: diversity score (DS) = number of clones with a V region sequence not seen in the inoculum/number of clones sequenced. Thus, a diversity of 0 would result from a sample in which no new sequences were identified compared with the

inoculum; a DS of 1 would indicate that all of the sequences obtained were different from those seen in the inoculum.

Results

Derivation of the Chicago C1 and C2 clonal isolates

Both FLA and sequence analysis (all primers shown in Table I) showed that the *tprK* locus in the Chicago C1 and C2 clonal isolates used as the inocula for the studies reported in this study are characterized by very low diversity. V region sequences and RSDI values for these isolates are provided in Supplemental Table I. For each V region, the inoculum sequences were designated as the founder sequences and used to calculate the DS for each V region from samples obtained during experimental infection.

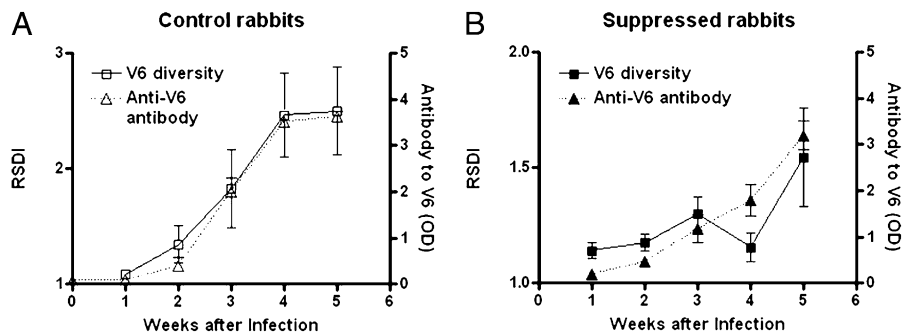


FIGURE 4. Correlation of development of anti-V6 Ab with presence of V6 variants. In immunocompetent rabbits, development of Abs against the predominant Chicago C1 V6 variant steadily increases during experimental infection (A, \triangle symbol). Anti-V6 Ab, however, fails to develop until later in infection and is significantly lower in titer in treated rabbits than in controls ($p < 0.05$) at week 3 and 4 postinfection (B, \blacktriangle symbol). In both groups, developing anti-V6 Ab parallels the accumulation of new V6 variants seen by FLA analysis (reported in Fig. 2F and here again for comparison purposes) and sequencing (Fig. 3F), suggesting a role for Ab in selection of new TprK variants in vivo.

Immunosuppression reduces selection for TprK sequence variants during syphilis infection

Effectiveness of immunosuppressive treatment. The effectiveness of methylprednisolone treatment was measured by comparison of serum VDRL Ab titers and lesion IFN- γ mRNA levels in treated rabbits compared with controls. As shown in Fig. 1A, treated animals had a significant delay in development of VDRL Ab, with lower overall Ab titers postinfection with the Chicago C1 strain, confirming immunosuppression in these rabbits. It should be noted, however, that the VDRL titer in the treated rabbits was modestly increased at week 5, suggesting that a mild immune response might have developed at that point despite the methylprednisolone treatment. Rabbit IFN- γ expression, which is robust during syphilis infection in rabbits and humans, was found to be significantly reduced in immunosuppressed rabbits compared with controls at each time point (Fig. 1B), further confirming the effectiveness of immunosuppression in the treated group. Also, because immunocompetent subjects are able to resolve early syphilis lesions by clearance of *T. pallidum*, we quantified viable *T. pallidum* (measured by mRNA), showing that *T. pallidum* could be detected in lesion biopsies from suppressed rabbits for at least 2 wk longer than in controls (Fig. 1C). *T. pallidum* 47-kDa lipoprotein (TP0574) mRNA (normalized to the rabbit HPRT message) could be detected up to week 3 after experimental infection in controls compared with at least week 5 in treated rabbits. These data confirm the efficacy of methylprednisolone for inducing an immunosuppressed state in these rabbits.

To be certain that any observed reduction in proportion of TprK variants in immunosuppressed rabbits was not due to downregulation of *tprK* expression in those animals, we measured *tprK* mRNA by qRT-PCR. As shown in Fig. 1D, immunosuppressive treatment did not alter the level of *tprK* expression per treponeme, with no significant difference between treated and control groups of rabbits during the time that *T. pallidum* mRNA could be detected by amplification.

Pharmacological immunosuppression reduces selection for TprK variants during syphilis infection. The accumulation of diversity in each TprK V region during the course of infection was investigated by both FLA and full-length *tprK* DNA sequencing. FLA results, reported as RSDI values, show that V region diversity increases with time in immunocompetent rabbits in V4, V5, V6, and V7 compared with immunosuppressed animals (Fig. 2D–G). In contrast, no difference in sequence diversity was seen for V1, V2, and V3 (Fig. 2A–C) between the two groups of rabbits. V6 RSDI values start to differ significantly ($p < 0.05$) at week 3 postinfection, whereas a significant

difference was seen for V7 at week 4 and at week 5 postinfection for V4 and V5. Very modest increases in V6 sequence diversity were seen at week 5 of infection in immunosuppressed animals. This may correspond to developing breakthrough specific immunity in the methylprednisolone-treated rabbits at week 5, as suggested by a slight increase in VDRL (Fig. 1A).

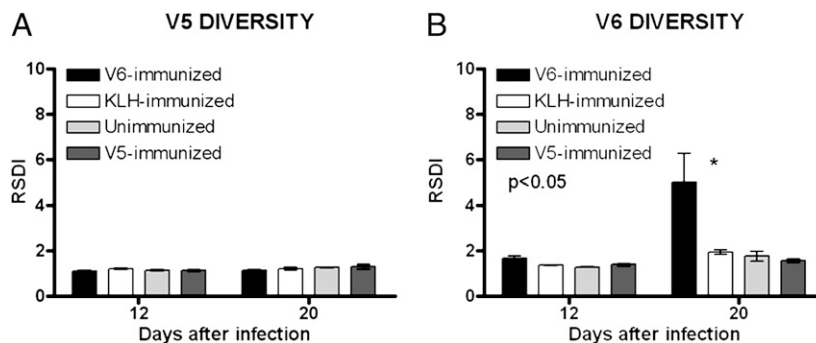
These same lesion samples were also analyzed by gene sequencing to determine whether any V region had diversified from the founder amino acid sequences for this isolate. DS was calculated as described above for each V region at weeks 1–5 postinfection (Fig. 3). Similar to the FLA results, sequencing data showed absence of significant differences in DS values for V1, V2, and V3 during the course of infection (Fig. 3A–C), whereas V6 was found to be significantly more diverse by week 2 postinfection in control compared with treated rabbits (Fig. 3F). In contrast to FLA analysis results, DS values for V4, V5, and V7 did not show significant differences in sequence diversity between treated and control rabbits (Fig. 3D–G), likely reflecting the limited sampling that is practicable with sequencing.

Specific Ab titer correlates with immune selection for TprK V6 variants. In control rabbits infected with the Chicago C1 strain, measurable Abs against the predominant Chicago C V6 peptide steadily increased postinfection (Fig. 4A); however, anti-V6 Ab titer was essentially unchanged through week 4 of infection in the immunosuppressed rabbits (Fig. 4B), with the titer increasing only at week 5. In both groups, Ab titer paralleled RSDI (reported in Fig. 2F and again, separately, in Fig. 4 for comparison purposes) and full-length *tprK* sequencing, suggesting a role for specific anti-V6 Ab in selection of TprK variants in vivo.

Prior immunization with TprK V6 selects for V6 variants in rabbits infected with Chicago C2. To complement the results described above, we asked whether prior immunization with V5 and V6 peptides (conjugated to KLH) would increase selection for TprK variants following infection with the Chicago C2 isolate. Efficacy of immunization was demonstrated by ELISA, showing that specific Abs to V5 and V6 were induced by the immunization protocol (data not shown).

FLA analysis of *tprK* V5 and V6 in lesions from immunized rabbits infected with Chicago C2 showed that accumulation of diversity in V6, but not in V5, is influenced by the presence of Ab against the original V region sequence (Fig. 5). V6 diversity significantly increased only in V6-immunized rabbits, but not in unimmunized, KLH-immunized, or V5-immunized controls (Fig. 5B), confirming the specificity of the selective effect on V6. In contrast, V5 diversity was not shown to be significantly affected by the presence of pre-existing anti-V5 Ab (Fig. 5A).

FIGURE 5. Accumulation of sequence diversity in *tprK* V5 and V6 regions in preimmunized rabbits. In Chicago C2-infected rabbits, V5 diversity is not significantly different in rabbits previously immunized with V5-KLH, V6-KLH, or KLH alone or in unimmunized controls (A). In contrast, V6 variants more readily are seen in rabbits previously immunized with V6-KLH compared with V5-KLH- or KLH-immunized rabbits or in unimmunized controls (B).



Discussion

Antigenic variation of the TprK Ag of *T. pallidum* is hypothesized to explain the persistence of *T. pallidum* in the host despite a robust immune response (18, 19, 25, 31, 32). An essential role for TprK in syphilis pathogenesis is strongly supported by the fact that its seven V regions are targeted by the humoral immune response during experimental infection (24) and also by the fact that, despite the high recombination rate between over 50 donor sequences and the *tprK* coding sequence, such rearrangements always result in an intact *tprK* ORF.

The involvement of TprK in immune evasion has not been directly addressed until now. A role in immune evasion implies that the target Ag is accessible to immune components and that an immunological function affects the survival of the individual bacterial cell expressing the Ag. Although the function of the TprK protein is unknown, computer prediction (pSORTb; www.psort.org/psortb/) suggests that the protein is located in the OM. Three-dimensional structural predictions of TprK (not shown) yield a tertiary structure typical of Gram-negative porins that reside in the bacterial OM. However, given the very fragile nature of the *T. pallidum* cell, surface exposure of TprK has yet to be experimentally demonstrated by biochemical analysis. We previously reported (18) that Abs raised against the *T. pallidum* Nichols strain TprK are opsonic, which strongly supports surface exposure. Recent experiments (unpublished) conducted in our laboratory have confirmed these results using a number of different antisera raised against recombinant TprK or TprK fragments; analogous opsonization studies with the Chicago strain ongoing in our laboratory confirm this finding for this strain. These studies support the surface exposure of TprK and a potential role for variation of this Ag as a means of immune evasion in syphilis. In complementary studies reported in this study, we demonstrated the direct effect of acquired immunity in selection of TprK variants during the course of infection. The naturally developing immune response during infection and pre-existing specific anti-V6 immunity both resulted in the accumulation of a higher proportion of TprK variants than seen in comparison rabbits.

We used two methods for quantitating V region sequence change in these studies: FLA reported as reciprocal of the RSDI and direct sequencing of ~10 clones per *tprK* amplicon. Each of these methods has advantages and disadvantages. Sequence analysis is useful in that it provides the actual sequence data, which are essential for analyses beyond the scope of diversity evaluation (i.e., epitope identification and donor site usage). However, the number of sequences that can practically be obtained per sample is limited by cost and time, and thus, the results do not fully reflect the extent of the sequence diversity of the original sample. FLA analysis potentially compensates for these limitations by providing analysis of all DNA species in the V region amplicons, thus giving a more comprehensive snapshot of the entire V region population in the sample

at a given time. It is likely that FLA analysis would identify variants that are infrequently represented in the sample and are thus not likely to be identified by limited sequencing. This may account for the observed higher sensitivity of FLA, compared with sequencing, for detecting variation in V4, V5, and V7 in the Chicago C1-infected rabbits (Figs. 2, 3). However, two sequence variants with the same V region length would not be distinguished by FLA, and thus, even this method likely underestimates the true magnitude of V region diversity in a given sample.

Using both methods for measuring diversity, our analyses showed a remarkable accumulation of diversity in V6 during the course of experimental infection with the Chicago C1 strain in immunocompetent rabbits, with a significantly higher number of variants generated compared with immunologically suppressed rabbits. FLA analysis results for V4, V5, and V7 suggest that these V regions might also be involved in immune evasion, even though differences between the control and treated rabbits become significant much later than for V6. In contrast, both analytical methods showed no difference between groups for V1, V2, and V3. It could be hypothesized that V4–V7 may be more accessible to the host's Abs, which could therefore more easily facilitate the clearance of *T. pallidum* cells carrying the original V region sequences. We have noted in the past that V1, V2, and V3 are less likely to vary in sequence than the other V regions and also are less likely to induce specific Abs than are other V regions (25).

Although pre-existing immunity to V6 selected for V6 variants following infection with Chicago C2, immunity to V5 had no such effect. This was puzzling in light of our finding that V5 variants appeared during the course of Chicago C1 infection, as described above. It is possible that the anti-V5 Abs evoked by immunization with synthetic V5 peptide-KLH conjugate did not reflect Abs induced during infection by the natural conformation of the V5 region in the context of the mature TprK protein in *T. pallidum* cells and were thus not functional in selection of V5 variants in our studies.

In all *T. pallidum* strains examined to date, V6 is the most variable of the V regions. Although no experimental evidence is available on TprK protein structure, V6 could occupy a key location that would be highly susceptible to Ab binding, consistent with the seemingly requisite high level of diversity exhibited by this V region.

Because *T. pallidum* appears to go to great lengths to preserve the ability to express TprK, one could postulate other biological roles for TprK in addition to altering *T. pallidum* surface antigenicity. Because of the nature of syphilis infection, sequence diversity could favor adaptation to changing microenvironmental conditions that *T. pallidum* encounters in the dissemination from the site of primary infection to distant body locations. LaFond et al. (19), using clustering analysis, demonstrated that *tprK* sequences from treponemes in primary chancres are more likely to cluster within a patient than among patients, and therefore, that *tprK* sequence variability is more limited within a *T. pallidum* isolate than among isolates. This suggests that different strains might express disparate repertoires of

tpk genes and could help to explain the biological basis for the pathogenetic differences exhibited by *T. pallidum* strains during experimental infection as, for instance, propensity to invade the host CNS (33) or ability to cause more or less severe lesions in the rabbit model (34). If TprK functions as a porin, sequence diversity could influence the specificity with which metabolic intermediates are translocated across the OM, potentially affecting the survival of variants in particular anatomical niches.

TprK clearly undergoes antigenic variation, with immune selection being evident as variants are able to evade the host immune response. We postulate that immune selection occurs via opsonophagocytosis, mediated by anti-TprK Abs, but it is unclear which specific epitopes are functional in this setting. It is important to remember as well that there are undoubtedly other surface-exposed Ags on *T. pallidum*, and the role of these molecules in pathogenesis and persistence also requires further study.

Acknowledgments

We thank Dr. Rebecca E. LaFond for suggestions to improve the manuscript, Prof. Ram Samudrala of the Department of Microbiology at the University of Washington for help with the TprK structural predictions (not shown), Bruce Godfrey of the Comparative Genomics Center at the University of Washington for assistance with the FLA experiments, and Elizabeth Miko Robertson for manuscript preparation.

Disclosures

The authors have no financial conflicts of interest.

References

1. Lukehart, S. A., and J. N. Miller. 1978. Demonstration of the in vitro phagocytosis of *Treponema pallidum* by rabbit peritoneal macrophages. *J. Immunol.* 121: 2014–2024.
2. Shaffer, J. M., S. A. Baker-Zander, and S. A. Lukehart. 1993. Opsonization of *Treponema pallidum* is mediated by immunoglobulin G antibodies induced only by pathogenic treponemes. *Infect. Immun.* 61: 781–784.
3. Baker-Zander, S. A., and S. A. Lukehart. 1992. Macrophage-mediated killing of opsonized *Treponema pallidum*. *J. Infect. Dis.* 165: 69–74.
4. Gjestland, T. 1955. The Oslo study of untreated syphilis. *Acta Derm. Venereol.* 35: 11–368.
5. Azar, H. A., T. D. Pham, and A. K. Kurban. 1970. An electron microscopic study of a syphilitic chancre. Engulfment of *Treponema pallidum* by plasma cells. *Arch. Pathol.* 90: 143–150.
6. Sykes, J. A., J. N. Miller, and A. J. Kalan. 1974. *Treponema pallidum* within cells of a primary chancre from a human female. *Br. J. Vener. Dis.* 50: 40–44.
7. Sykes, J. A., and J. N. Miller. 1971. Intracellular location of *Treponema pallidum* (Nichols strain) in the rabbit testis. *Infect. Immun.* 4: 307–314.
8. Medici, M. A. 1972. The immunoprotective niche—a new pathogenic mechanism for syphilis, the systemic mycoses and other infectious diseases. *J. Theor. Biol.* 36: 617–625.
9. Sell, S., J. Salman, and S. J. Norris. 1985. Reinfection of chancre-immune rabbits with *Treponema pallidum*. I. Light and immunofluorescence studies. *Am. J. Pathol.* 118: 248–255.
10. Christiansen, S. 1963. Protective layer covering pathogenic treponemata. *Lancet* 1: 423–425.
11. Alderete, J. F., and J. B. Baseman. 1980. Surface characterization of virulent *Treponema pallidum*. *Infect. Immun.* 30: 814–823.
12. Fitzgerald, T. J., and R. C. Johnson. 1979. Surface mucopolysaccharides of *Treponema pallidum*. *Infect. Immun.* 24: 244–251.
13. Wicher, V., and K. Wicher. 1977. In vitro cell response of *Treponema pallidum*-infected rabbits. II. Inhibition of lymphocyte response to phytohemagglutinin by serum of *T. pallidum*-infected rabbits. *Clin. Exp. Immunol.* 29: 487–495.
14. Wicher, V., and K. Wicher. 1977. In vitro cell response of *Treponema pallidum*-infected rabbits. III. Impairment in production of lymphocyte mitogenic factor. *Clin. Exp. Immunol.* 29: 496–500.
15. Radolf, J. D., M. V. Norgard, and W. W. Schulz. 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. *Proc. Natl. Acad. Sci. USA* 86: 2051–2055.
16. Walker, E. M., L. A. Borenstein, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1991. Analysis of outer membrane ultrastructure of pathogenic *Treponema* and *Borrelia* species by freeze-fracture electron microscopy. *J. Bacteriol.* 173: 5585–5588.
17. Lukehart, S. A., J. M. Shaffer, and S. A. Baker-Zander. 1992. A subpopulation of *Treponema pallidum* is resistant to phagocytosis: possible mechanism of persistence. *J. Infect. Dis.* 166: 1449–1453.
18. Centurion-Lara, A., C. Godornes, C. Castro, W. C. Van Voorhis, and S. A. Lukehart. 2000. The *tpk* gene is heterogeneous among *Treponema pallidum* strains and has multiple alleles. *Infect. Immun.* 68: 824–831.
19. LaFond, R. E., A. Centurion-Lara, C. Godornes, A. M. Rompalo, W. C. Van Voorhis, and S. A. Lukehart. 2003. Sequence diversity of *Treponema pallidum* subsp. *pallidum* *tpk* in human syphilis lesions and rabbit-propagated isolates. *J. Bacteriol.* 185: 6262–6268.
20. Centurion-Lara, A., R. E. LaFond, K. Hevner, C. Godornes, B. J. Molini, W. C. Van Voorhis, and S. A. Lukehart. 2004. Gene conversion: a mechanism for generation of heterogeneity in the *tpk* gene of *Treponema pallidum* during infection. *Mol. Microbiol.* 52: 1579–1596.
21. Zhang, J. R., and S. J. Norris. 1998. Genetic variation of the *Borrelia burgdorferi* gene *vsxE* involves cassette-specific, segmental gene conversion. *Infect. Immun.* 66: 3698–3704.
22. Barbour, A. G. 1990. Antigenic variation of a relapsing fever *Borrelia* species. *Annu. Rev. Microbiol.* 44: 155–171.
23. Barbet, A. F., A. Lundgren, J. Yi, F. R. Rurangirwa, and G. H. Palmer. 2000. Antigenic variation of *Anaplasma marginale* by expression of MSP2 mosaics. *Infect. Immun.* 68: 6133–6138.
24. Morgan, C. A., B. J. Molini, S. A. Lukehart, and W. C. Van Voorhis. 2002. Segregation of B and T cell epitopes of *Treponema pallidum* repeat protein K to variable and conserved regions during experimental syphilis infection. *J. Immunol.* 169: 952–957.
25. LaFond, R. E., B. J. Molini, W. C. Van Voorhis, and S. A. Lukehart. 2006. Antigenic variation of TprK V regions abrogates specific antibody binding in syphilis. *Infect. Immun.* 74: 6244–6251.
26. Giacani, L., E. S. Sun, K. Hevner, B. J. Molini, W. C. Van Voorhis, S. A. Lukehart, and A. Centurion-Lara. 2004. Tpr homologs in *Treponema paraluiscluniculi* Cuniculi A strain. *Infect. Immun.* 72: 6561–6576.
27. Giacani, L., K. Hevner, and A. Centurion-Lara. 2005. Gene organization and transcriptional analysis of the *tpj*, *tpi*, *tpg* and *tpf* loci in *Treponema pallidum* strains Nichols and Sea 81-4. *J. Bacteriol.* 187: 6084–6093.
28. Giacani, L., B. Molini, C. Godornes, L. Barrett, W. C. Van Voorhis, A. Centurion-Lara, and S. A. Lukehart. 2007. Quantitative analysis of *tpj* gene expression in *Treponema pallidum* isolates: Differences among isolates and correlation with T-cell responsiveness in experimental syphilis. *Infect. Immun.* 75: 104–112.
29. Godornes, C., B. T. Leader, B. J. Molini, A. Centurion-Lara, and S. A. Lukehart. 2007. Quantitation of rabbit cytokine mRNA by real-time RT-PCR. *Cytokine* 38: 1–7.
30. Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26: 2465–2466.
31. LaFond, R. E., A. Centurion-Lara, C. Godornes, W. C. Van Voorhis, and S. A. Lukehart. 2006. TprK sequence diversity accumulates during infection of rabbits with *Treponema pallidum* subsp. *pallidum* Nichols strain. *Infect. Immun.* 74: 1896–1906.
32. Morgan, C. A., S. A. Lukehart, and W. C. Van Voorhis. 2003. Protection against syphilis correlates with specificity of antibodies to the variable regions of *Treponema pallidum* repeat protein K. *Infect. Immun.* 71: 5605–5612.
33. Tantalos, L. C., S. A. Lukehart, and C. M. Marra. 2005. *Treponema pallidum* strain-specific differences in neuroinvasion and clinical phenotype in a rabbit model. *J. Infect. Dis.* 191: 75–80.
34. Turner, T. B., and D. H. Hollander. 1957. *Biology of the Treponematoses*. World Health Organization, Geneva.