INFECTION AND IMMUNITY, Apr. 1997, p. 1440–1444 Vol. 65, No. 4
0019-9567/97/$04.00+0

Conservation of the 15-Kilodalton Lipoprotein among *Treponema pallidum* Subspecies and Strains and Other Pathogenic Treponemes: Genetic and Antigenic Analyses

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Received 28 October 1996/Returned for modification 12 December 1996/Accepted 23 January 1997

The 15-kDa lipoprotein of *Treponema pallidum* is a major immunogen during natural syphilis infection in humans and experimental infection in other hosts. The humoral and cellular immune responses to this molecule appear late in infection as resistance to reinfection is developing. One therefore might hypothesize that this antigen is important for protective immunity. This possibility is explored by using both genetic and antigenic approaches. Limited or no cross-protection has been demonstrated between the *T. pallidum* subspecies and strains or between *Treponema* species. We therefore hypothesized that if the 15-kDa antigen was of major importance in protective immunity, it might be a likely site of antigenic diversity. To explore this possibility, the sequences of the open reading frames of the 15-kDa gene have been determined for *Treponema pallidum* subsp. *pallidum* (Nichols and Bal-3 strains), *T. pallidum* subsp. *pertenue* (Gauthier strain), *T. pallidum* subsp. *endemicum* (Bosnia strain), *Treponema paraluiscuniculi* (Cuniculi A, H, and K strains), and a little-characterized simian isolate of *Treponema* sp. (Fribourg-Blanc strain). No significant differences in DNA sequences of the genes for the coding region of the 15-kDa antigen were found among the different species and subspecies studied. In addition, all organisms showed expression of the 15-kDa antigen as determined by monoclonal antibody staining. The role of the 15-kDa antigen in protection against homologous infection with *T. pallidum* subsp. *pallidum* Nichols was examined in rabbits immunized with a purified recombinant 15-kDa fusion protein. No alteration in chancre development was observed in immunized, compared to unimmunized, rabbits, and the antisera induced by the immunization failed to enhance phagocytosis of *T. pallidum* subsp. *pallidum* by macrophages in vitro. These results do not support a major role for this antigen in protection against syphilis infection.

The different *Treponema* species and subspecies produce distinct clinical diseases in humans and in other animal hosts. *Treponema pallidum* subsp. *pallidum*, *T. pallidum* subsp. *pertenue*, and *T. pallidum* subsp. *endemicum* infect humans and are the etiological agents of venereal syphilis, yaws, and endemic syphilis, respectively. *Treponema paraluiscuniculi* causes naturally occurring venereal syphilis in rabbits but is not infectious to humans (10); the simian isolate was obtained from skin lesions of a monkey (9) in a region of Africa where yaws is endemic. None of these pathogenic treponemes can be cultured continuously in vitro, and there is no morphological or laboratory-based method to differentiate them. The only recognized differences, and the basis for subspecies or species differentiation, are their host ranges and the varied clinical manifestations that they produce.

The 15-kDa lipoprotein is a major immunogen in natural and experimental syphilis infection. (In previous publications from our laboratory, this molecule has been called the 12-kDa molecule. In this publication, we change the name to be consistent with the standardized nomenclature described by Norris et al. [15].) In the rabbit model, it induces strong antibody (12) and cellular responses (3) late in infection as resistance to reinfection develops (5). This suggests that the 15-kDa antigen may be a factor in protective immunity. Limited or no cross-protective immune response can be demonstrated between *T. pallidum* subspecies and other pathogenic treponemes (19, 20). Therefore, if the 15-kDa antigen was a major protective antigen, one would expect to find differences in antigenic epitopes or differences in expression of the antigen among subspecies and species. Baker-Zander et al. (1) have shown some differences in the reactivity of Western blots (immunoblots) of total lysates of *T. pallidum* subsp. *pallidum* (Nichols strain) and *T. pallidum* subsp. *pertenue* (Gauthier strain) when tested with rabbit antisera raised against homologous organisms. Weak reactivity against the 15-kDa antigen was observed in the immunoblots of crude lysates of both subspecies when rabbit antiserum against *T. pallidum* subsp. *pertenue* was used to determine immune response patterns (1), while strong reactivity was observed with antiserum from *T. pallidum* subsp. *pallidum*-infected rabbits. This observation suggests that there might be some determinants in the 15-kDa protein present in one species or subspecies that induce dissimilar humoral responses in the host. Similar findings were made when antisera raised against *T. pallidum* subsp. *pallidum* and *T. paraluiscuniculi* were compared (10a).

To examine the 15-kDa antigen for species and subspecies diversity, we have sequenced the 15-kDa gene from three different subspecies of *T. pallidum* that induce clearly distinct infections in humans, one species (*T. paraluiscuniculi*) that produces infection only in rabbits, and an uncharacterized simian isolate, and we have examined expression of the 15-kDa antigen in these organisms. To examine the role of the 15-kDa antigen in protective immunity, we have immunized rabbits

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with a purified recombinant 15-kDa fusion protein, examined the immune sera for opsonic activity, and tested the immunized animals for resistance to homologous *T. pallidum* subsp. *pallidum* Nichols infection.

MATERIALS AND METHODS

Bacterial species, subspecies, and DNA. All *T. pallidum* subspecies, *T. paraluiscuniculi*, and the simian isolate treponeme were propagated in New Zealand White rabbits by intratesticular inoculation as described before (11). *T. pallidum* subsp. *pallidum* (Nichols strain) was originally sent to the University of Washington by James N. Miller (University of California, Los Angeles) in 1979, and *T. pallidum* subsp. *pertenue* (Gauthier strain) was supplied by Peter Perine (Centers for Disease Control, Atlanta, Ga.) in 1981. The following strains were supplied by Paul Hardy (Johns Hopkins University, Baltimore, Md.): *T. pallidum* subsp. *pallidum* (strain Bal-3), *T. paraluiscuniculi* (strains Cuniculi A, H, and K), *T. pallidum* subsp. *endemicum* (strain Bosnia A), and the simian treponeme originally isolated by Fribourg-Blanc et al. (9). To ensure that the correct strain was propagated and extracted, only one strain of treponeme was handled at any time during the propagation and freezing process, and labels on tubes were double-checked. Rabbit eartag numbers were also double-checked. Bacteria were extracted from infected rabbit testes in sterile saline, collected in DNase- and RNase-free 1.7-ml microcentrifuge tubes, taking careful precautions to avoid contamination by other DNA, and spun immediately in a microcentrifuge at 12,000 × *g* for 30 min at 4°C. The pellet was resuspended in 200 μl of 1× lysis buffer (10 mM Tris [pH 8.0], 0.1 M EDTA, 0.5% sodium dodecyl sulfate), and DNA was extracted with the Qiagen (Chatsworth, Calif.) kit for genomic DNA extraction as described in the manufacturer's instructions, but adding 50 μl of proteinase K (100-mg/ml stock solution) and incubating the sample for 2 h at 65°C. After the final elution step in 200 μl of H_2O, DNA was used for further analysis by PCR.

PCR amplification of the 15-kDa lipoprotein gene. Primers for amplification of the 15-kDa gene were designed based on the published sequence (GenBank accession number M30941) (16): the sense primer sequence is 5′CGGAATTC GCCTTCTCGCGTTTCTCG (an *Eco*RI site was introduced at the 5′ end), and the antisense primer sequence is 5′CGGGATCCGCATGCGAGGGTAGTTG CTGCTTCG (with a *Bam*HI restriction site at the 5′ end). PCR amplification was performed in a 100-μl reaction mixture containing 200 μM deoxynucleoside triphosphates, 50 mM Tris-HCl (pH 9.0 at 20°C), 1.5 mM $MgCl_2$, 200 mM NH_4SO_4, 1 μM each primer, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). The cycling conditions were as follows: denaturation at 94°C for 3 min, 30 to 40 cycles of 94°C for 1 min, 65°C for 2 min, 72°C for 1 min, and a final elongation step at 72°C for 10 min; the PCR tubes were kept at 4°C for immediate cloning into TA cloning vectors and also for preparative analysis in agarose gels.

DNA sequencing of clones and PCR products. Sequencing in both directions was performed with PCR products and clones. Sequencing of both PCR products and multiple clones was performed for *T. pallidum* subsp. *pallidum* Nichols, *T. pallidum* subsp. *pertenue* Gauthier, and *T. paraluiscuniculi* Cuniculi A. Clones were obtained by direct cloning of PCR products from *T. pallidum* subsp. *pertenue* Gauthier and *T. paraluiscuniculi* Cuniculi A into the PCR-3 TA-cloning vector (Invitrogen, San Diego, Calif.) and from *T. pallidum* subsp. *pallidum* Nichols into the PT7 Blue vector (Novagen, Madison, Wis.). Single colonies were selected for plasmid DNA extraction. Plasmid DNA was digested with *Eco*RI to identify the clones having inserts. Double-stranded plasmid DNA was extracted with the Qiagen plasmid kit from multiple clones containing inserts: four clones from *T. pallidum* subsp. *pertenue*, six clones from *T. paraluiscuniculi*, and two clones from *T. pallidum* subsp. *pallidum*.

Double-stranded plasmid DNA (500 ng) was used for automated sequencing by the dye terminator method (Perkin-Elmer, Foster City, Calif.) as described in the manufacturer's instructions but adding 1 μl of molecular-grade dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) to all sequencing reactions, giving a final concentration of 5% (vol/vol). Four clones (two from *T. pallidum* subsp. *pallidum*, one from *T. pallidum* subsp. *pertenue*, and one from *T. paraluiscuniculi*) were sequenced in their full length in both directions by using the primer walking approach. The 5′ and 3′ ends of the 15-kDa gene were sequenced first with the T7 primer, 5′TAATACGACTCACTATAGGG, the antisense SP6 primer, 5′-A TTTAGGTGACACTATAG, and the PCR3 reverse primer, 5′TAGAAGGCA CAGTCGAG, and then the following internal primers in the inserts were designed based on the initial sequences: primer 284-S, 5′GGCGAGAAGGAAG CCATT; primer 598-S, 5′GGGCGACGTATCAGGATT; primer 397-As, 5′GA CCATTACACCACGACC; and primer 592-As, 5′GACTTAAACCGCCCTT CT. The remaining eight clones (three from *T. pallidum* subsp. *pertenue* and five from *T. paraluiscuniculi*) were partially sequenced with the T7 and PCR3 reverse primers. In addition, complete sequences for the three strains described above were verified by direct sequencing of PCR products, as described below.

Direct sequencing in both directions from PCR products from at least two separate PCRs for all strains was performed as follows: after PCR amplification of the 15-kDa gene, amplicons were run in a 1% low-melting-point agarose gel in 1× TAE (20 mM Tris, 2 mM sodium acetate, 1 mM EDTA) buffer. Bands of the expected molecular weight were excised and DNA purified with the GeneClean Kit (Bio 101, Vista, Calif.). Two hundred nanograms of purified DNA was then used for sequencing as mentioned above but replacing the T7, SP6, and PCR3 reverse primers with the sense and antisense primers used for initial PCR amplification. Internal primers were the same.

GenBank accession numbers for the sequences are as follows: *T. pallidum* subsp. *pallidum* Nichols, U73115; *T. pallidum* subsp. *pallidum* Bal-3, U73116; *T. pallidum* subsp. *pertenue* Gauthier, U73117; *T. pallidum* subsp. *endemicum* Bosnia A, U73118; *Treponema* sp. simian strain, U73122; *T. paraluiscuniculi* Cuniculi A, U73119; *T. paraluiscuniculi* Cuniculi H, U73120; *T. paraluiscuniculi* Cuniculi K, U73121.

Cloning and expression of 15-kDa lipoprotein gene. The open reading frame (ORF) encoding the full-length mature 15-kDa lipoprotein was PCR amplified from *T. pallidum* subsp. *pallidum* Nichols genomic DNA. Primers were designed based on a published sequence (GenBank accession number M30941) (16). The sense primer sequence is 5′CGGAATTCATGGTGAAAAGAGGTCGGTTC, and the antisense primer sequence is 5′GCTCTAGACTACCTGCTAATAAT GGCTTC; these sequences include *Eco*RI and *Xba*I sites, respectively. PCR amplification was as described above, except the cycling conditions were as follows: denaturation at 94°C for 3 min, 31 cycles of 94°C for 1 min, 58°C for 1.5 min, 72°C for 1.5 min, and a final elongation step at 72°C for 10 min. The resulting PCR product was cloned into the PT7 Blue vector digested with *Eco*RI and *Xba*I. The 15-kDa insert was excised, gel purified, and ligated into the maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England Biolabs, Beverly, Mass.) which was cut with *Eco*RI and *Xba*I.

A pMAL-c2 clone harboring the 15-kDa lipoprotein gene was inoculated into 500 ml of LB broth containing 2% glucose and 100 μg of ampicillin per ml. The culture was grown on a shaker at 34°C to an optical density at 540 nm of 0.5 to 0.7, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The bacteria were incubated for an additional 2 to 3 h at 37°C and pelleted by centrifugation at 4,000 × *g* for 20 min. The pellet was resuspended in 10 ml of lysis buffer containing 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 μg of pepstatin A per ml (pH 7.5), frozen at −20°C, and thawed at room temperature. The bacteria were lysed by sonication for eight 15-s bursts on ice with a 50-W probe sonicator set on 60% power setting. The resulting lysate was centrifuged at 8,000 × *g* for 20 min at 4°C. The pellet containing insoluble MBP–15-kDa antigen fusion protein inclusion bodies was resuspended in 10 ml of wash buffer containing 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 μg of pepstatin per ml. The mixture was incubated on an end-over-end mixer at 4°C for 2 h following the addition of 8 mM $MgCl_2$, 10 μg of DNase I per ml, and 0.1% sodium deoxycholate. The inclusions were washed once with wash buffer containing 1% Nonidet P-40 and twice with wash buffer alone. The washed fusion protein inclusions were made soluble by resuspension in 10 ml of 8 M urea followed by incubation on a rocker at 4°C for 18 h. The urea was removed by dialysis against four 2-liter changes of Dulbecco's phosphate-buffered saline (PBS; pH 7.4). Insoluble protein was removed by centrifugation at 8,000 × *g* for 20 min at 4°C. The soluble protein was affinity purified with amylose resin chromatography medium as described in the manufacturer's instructions (New England Biolabs). Protein purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein content was determined by the Bio-Rad (Hercules, Calif.) protein assay.

Immunization with the fusion protein. Two adult male New Zealand White rabbits (one each) were immunized with 500 μg of purified protein in either incomplete Freund's adjuvant or the Ribi (monophosphoryl lipid A, trehalose dicorynomycolate, and cell wall skeleton [MPL+TDM+CWS] adjuvant system (Sigma) as described in the manufacturer's recommendations, with identical booster injections at 3 and 6 weeks. One week following the final boost, blood was collected for immune serum for testing in opsonization assays. Two animals were identically immunized with purified MBP for use as controls; blood was also collected from these animals. All sera demonstrated the expected reactivity by immunoblot (data not shown).

Opsonic activity of antisera. All antisera were tested for their ability to enhance phagocytosis of *T. pallidum* subsp. *pallidum* Nichols by the rabbit peritoneal macrophage assay previously described by our laboratory (13). The degree of opsonization with the 15-kDa antisera was compared with that of normal (uninfected) and immune (pool of sera from rabbits infected with *T. pallidum*) sera.

Infectious challenge. Following blood collection, the immunized rabbits were challenged intradermally on their shaved backs with 10^3 infectious *T. pallidum* subsp. *pallidum* Nichols bacteria at each of four sites. Two normal (unimmunized) rabbits were also infected (at eight sites each) for comparison. The animals were kept clean shaven, housed at 18 to 20°C, and examined daily for lesion development. Protection is indicated by failure to develop skin lesions or by significant delay in the time to lesion development.

Monoclonal antibody staining of *Treponema* species and subspecies. Immunofluorescent staining with monoclonal antibody F5 was performed on all strains as described previously (14). Briefly, slides were prepared from treponemes in saline, allowed to air dry, and fixed in acetone for 10 min; the F5 mouse monoclonal antibody was then added, and the slides were incubated for 30 min at 37°C in a humid chamber. The slides were washed three times in PBS, air dried, and then incubated with the second antibody, fluorescein isothiocyanate-

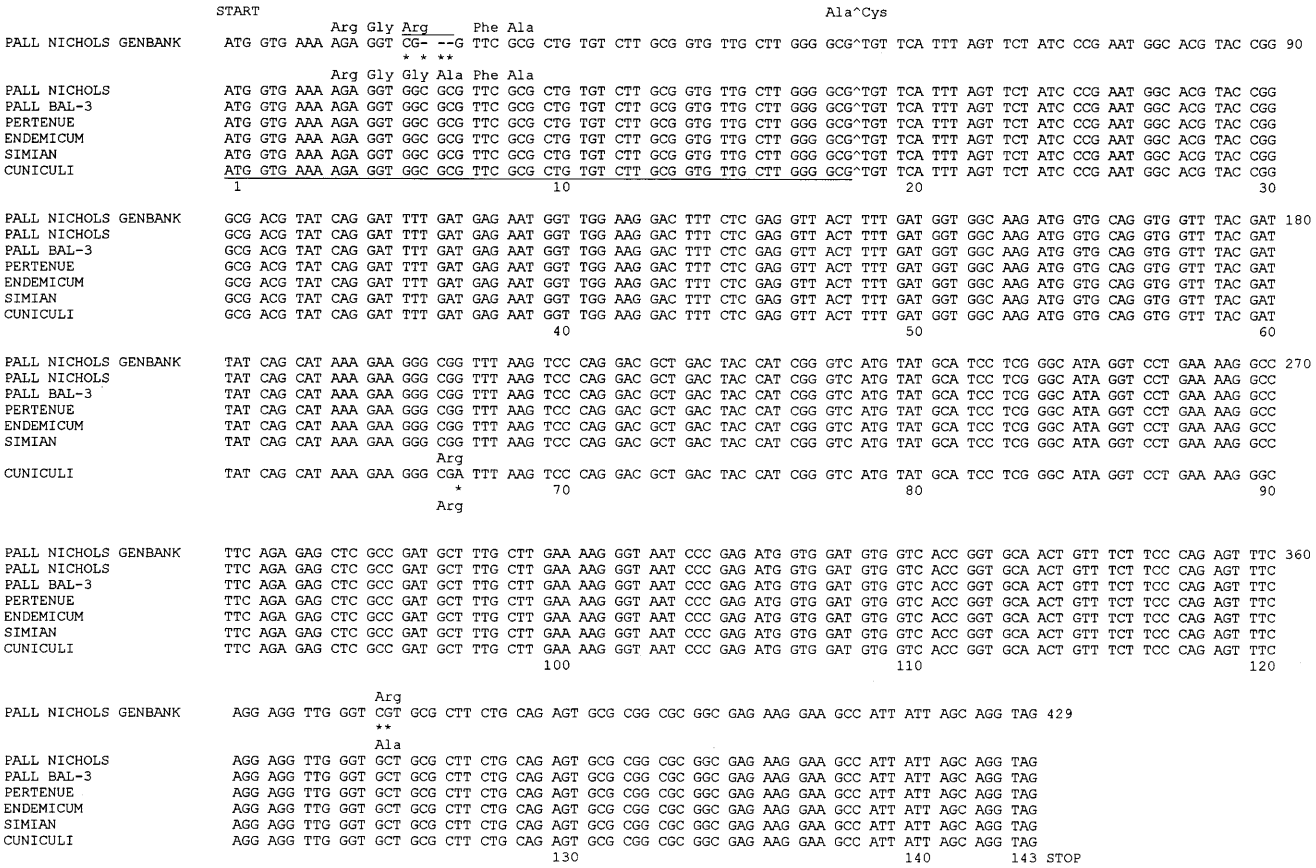

FIG. 1. Sequence alignment of the ORFs of the 15-kDa lipoprotein gene from three *T. pallidum* subspecies, *T. paraluiscuniculi*, and an uncharacterized simian isolate. The numbers on the right indicate the nucleotide positions; the numbers below the sequences indicate the amino acid positions. Sequence alignments were done with the Clustal W program (20). Abbreviations: PALL NICHOLS GENBANK, *T. pallidum* subsp. *pallidum* Nichols, sequence obtained from GenBank (accession number M30941); PALL NICHOLS, *T. pallidum* subsp. *pallidum* Nichols, sequenced in our laboratory; PALL Bal-3, *T. pallidum* subsp. *pallidum* Bal-3; PERTENUE, *T. pallidum* subsp. *pertenue* Gauthier; ENDEMICUM, *T. pallidum* subsp. *endemicum* Bosnia A; SIMIAN, uncharacterized simian isolate; and CUNICULI, *T. paraluiscuniculi* consensus sequence from Cuniculi A, Cuniculi H, and Cuniculi K strains. Nucleotide positions: 1 to 3, start codon; 427 to 429, stop codon; 1 to 54, signal peptide; 55 to 429, mature peptide-encoding region. *, mismatch in base composition; ^, peptidase II cleavage site.

labeled goat anti-mouse immunoglobulin G (Tago, Camarillo, Calif.), at a 1:100 dilution in PBS–2% Tween 80. Following three washes in PBS, the slides were air dried and coverslips were applied with GVA mounting fluid (Zymed, South San Francisco, Calif.). Slides were observed with a Zeiss fluorescence microscope at ×400.

RESULTS

Comparison of sequences of the 15-kDa ORFs. The sequences of the 15-kDa lipoprotein genes from each of the strains were determined. Figure 1 shows an alignment of the ORFs of the six sequences in comparison with the published sequence (GenBank accession number M30941). All six sequences determined in our laboratory are identical, with the exception of an A-for-G substitution in *T. paraluiscuniculi* at nucleotide position 201. This change is silent, however, in that this one nucleotide does not change the amino acid in the mature protein (in both cases, arginine). All three strains of *T. paraluiscuniculi* are identical.

When the sequences obtained by us were compared with the published sequence (16), we consistently found six mismatches at our nucleotide positions 16, 18, 19, 20, 373, and 374. A triplet (CGC) is missing from the sequence available from GenBank (at our positions 18 to 20). Because the Nichols strain used in our laboratory and that used in the laboratory that first published the 15-kDa gene sequence (16) were originally obtained from the same source, it is likely that the differences seen between our sequences and the published sequence are due to earlier sequencing errors rather than actual differences between the organisms.

These nucleotide differences produce a change in the corresponding amino acid sequences in the signal peptide and mature peptide-encoding regions. In all six sequences from our laboratory, there is a glycine and an alanine instead of an arginine at amino acid positions 6 and 7 in the signal peptide region and an alanine instead of an arginine at amino acid position 125 in the mature peptide region.

Expression of the 15-kDa antigen by different strains, species, and subspecies. Monoclonal antibody F5 (14), which identifies an epitope on the 15-kDa lipoprotein antigen, was used to stain the following treponemes by immunofluorescence: *T. pallidum* subsp. *pallidum* Nichols, *T. pallidum* subsp. *pallidum* Bal-3, *T. pallidum* subsp. *pertenue* Gauthier, *T. pallidum* subsp. *endemicum* Bosnia A, the simian strain, and *T. paraluiscuniculi* Cuniculi A. The same degree of reactivity is demonstrated in all strains (data not shown), indicating that the 15-kDa lipoprotein is expressed in all treponemes tested and contains, at minimum, the F5 epitope.

Opsonization by anti-15-kDa lipoprotein antisera. One of the functions of the antibodies induced during syphilis infection is the opsonization of the bacteria for phagocytosis and

TABLE 1. Opsonic activity of anti-15-kDa lipoprotein antiserum

Antiserum	*n*	% Phagocytosis[a]	*P* value[b]
Normal	5	10.4 ± 1.5	
Immune	6	53.3 ± 4.1	<0.001
Anti-MBP	3	11.0 ± 3.5	NS[c]
Anti-15-kDa	6	13.6 ± 1.3	NS

[a] Percentage of macrophages containing phagocytized *T. pallidum* (mean ± standard error).
[b] Compared to normal serum by Student's *t* test.
[c] NS, not significant.

killing by macrophages. This can be clearly demonstrated in vitro (4, 13), and histologic studies strongly suggest that it also occurs in vivo during healing of the primary lesions. We tested the anti-15-kDa lipoprotein antisera obtained by immunization (Table 1) and demonstrated no opsonic activity, compared to that of normal and immune serum. As expected, the control anti-MBP serum also failed to opsonize *T. pallidum* in this assay.

Protection against infectious challenge. To determine whether immunization with the 15-kDa antigen could protect rabbits against homologous infection with *T. pallidum* subsp. *pallidum* Nichols, immunized and control animals were infected intradermally with 10^3 *T. pallidum* bacteria at four or eight sites on their shaved backs. As shown in Table 2, lesions appeared at all sites in both immunized and control rabbits, and no delay in lesion development nor change in lesion character (size or severity) was noted. The slight acceleration in lesion development observed in the 15-kDa-antigen-immunized rabbits has also been found following challenge of rabbits immunized with a number of other antigens. This may be indicative of an induced, though nonprotective, specific immune response to the 15-kDa antigen. Although not included in this experiment, it is well documented that infectious challenge of rabbits immunized by infection of 3 months or longer with the homologous strain results in complete protection (no lesions appearing at challenge sites). Our findings indicate that immunization with the 15-kDa recombinant fusion protein provides no protection in rabbits.

DISCUSSION

The 15-kDa lipoprotein of *T. pallidum* is a strong immunogen in natural syphilis infection in humans and in experimental syphilis in the rabbit model. Previous observations that antisera to *T. pallidum* subsp. *pertenue* and *T. paraluiscuniculi* react poorly with the 15-kDa antigen of nonhomologous pathogenic treponemes suggested possible antigenic diversity in this molecule (2). The 15-kDa antigen has also been speculated to play an important role in protective immunity, since both humoral (12) and cellular (3) immune responses to this antigen appear late in infection, coincident with development of resistance to reinfection (5). The positive reactivity of the immunofluorescence staining with the F5 anti-15-kDa lipoprotein monoclonal antibody in all the treponemes studied demonstrates the universal expression and universal accessibility to the F5 antibody of the 15-kDa lipoprotein, although levels of expression cannot be quantitated by this means. Because this molecule is a relatively minor element in *T. pallidum*, the precise role of the strong cellular and humoral immune response against the 15-kDa lipoprotein is not known.

TABLE 2. Intradermal infection of immunized rabbits

Rabbit immunization	Time to lesion development (days)[a]	No. lesions/no. sites injected
None (normal rabbits)	15.4 ± 0.2	16/16
MBP (control)	14.1 ± 0.4	8/8
15-kDa lipoprotein–MBP fusion protein	12.8 ± 0.2	8/8

[a] Mean ± standard error.

Immunization of rabbits with recombinant pMAL–15-kDa lipoprotein fusion peptides failed to protect the rabbits against homologous challenge with the Nichols strain. In addition, antiserum raised against these molecules, while strongly reactive by immunoblot to recombinant and *T. pallidum*-derived molecules, failed to opsonize *T. pallidum* for phagocytosis by rabbit macrophages. It is possible that failure to induce protection and opsonic antibody might be due to a lack of appropriate conformation in the recombinant molecule, although the induced antiserum did react with native *T. pallidum* by immunofluorescence (data not shown). The immunization protocol, though successful in inducing antibody, may not have induced the appropriate cellular immunity necessary for protection, although similar immunization protocols have induced T-lymphocyte-proliferative responses in rabbits in our laboratory in the past. In addition, the MPL+TDM+CWS form of Ribi adjuvant used in some of our immunized rabbits is designed to induce T-cell responses.

Because cross-protection between subspecies or strains of *T. pallidum* is limited or nonexistent, while homologous protection is complete (19, 21), the protective antigen is likely to demonstrate diversity between species, subspecies, or strains. This study was intended to examine diversity in the 15-kDa lipoprotein but shows almost complete identity of the coding region sequence for the 15-kDa antigen in all examined *T. pallidum* subspecies and strains, *T. paraluiscuniculi*, and a poorly characterized simian isolate; complete amino acid identity is found in the mature peptide. The remarkable conservation of this gene sequence in the different pathogenic *Treponema* species and subspecies and the failure of immunized rabbits to demonstrate either protection or opsonic antibody strongly suggest that the 15-kDa antigen is unlikely to be important as a protective antigen against infection.

Little is known about the 15-kDa lipoprotein molecule. It and several other lipoprotein antigens have been shown to be localized to the inner membrane of *T. pallidum* (6, 7, 17) and are hypothesized to be anchored via their lipid tails, while the peptide portion is thought to extend into the periplasmic space (8). The fact that this molecule is so highly conserved among the pathogenic treponemes suggests that it may have a critical role in pathogenesis. In this regard, there is evidence that the lipoproteins, including the 15-kDa molecule, may initiate the early inflammatory changes that are seen during infection (18). Our finding that the deduced amino acid sequences are identical for the human (*T. pallidum* subspecies) and strict rabbit (*T. paraluiscuniculi*) pathogens suggests, however, that the 15-kDa molecule is not an important factor in distinguishing between the ability to infect humans and the ability to infect other animals.

ACKNOWLEDGMENTS

This work was supported by grants AI 18988 and AI 34615 to S.A.L. and an STD Cooperative Research Center (AI 31448) New Investigator Award to W.V.V.

We thank Sally Post for manuscript preparation and James N. Miller, Peter Perine, and Paul Hardy for donation of strains.

REFERENCES

1. **Baker-Zander, S. A., and S. A. Lukehart.** 1983. Molecular basis of immunological cross-reactivity between *Treponema pallidum* and *Treponema pertenue*. Infect. Immun. **42:**634–638.
2. **Baker-Zander, S. A., and S. A. Lukehart.** 1984. Antigenic cross-reactivity between *Treponema pallidum* and other pathogenic members of the family *Spirochaetaceae*. Infect. Immun. **46:**116–121.
3. **Baker-Zander, S. A., M. J. Fohn, and S. A. Lukehart.** 1988. Development of cellular immunity to individual soluble antigens of *Treponema pallidum* during experimental syphilis. J. Immunol. **141:**4363–4369.
4. **Baker-Zander, S. A., and S. A. Lukehart.** 1992. Macrophage-mediated killing of opsonized *Treponema pallidum*. J. Infect. Dis. **165:**69–74.
5. **Bishop, N. H., and J. N. Miller.** 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. Immunol. **117:**197–207.
6. **Blanco, D. R., K. Reimann, J. Skare, C. I. Champion, D. Foley, M. M. Exner, R. E. Hancock, J. N. Miller, and M. A. Lovett.** 1994. Isolation of the outer membrane from *Treponema pallidum* and *Treponema vincentii*. J. Bacteriol. **176:**6088–6099.
7. **Cox, D. L., D. R. Akins, S. F. Porcella, M. V. Norgard, and J. D. Radolf.** 1995. *Treponema pallidum* in gel microdroplets: a novel strategy for investigation of treponemal molecular architecture. Mol. Microbiol. **15:**1151–1164.
8. **Cox, D. L., P. Chang, A. W. McDowall, and J. D. Radolf.** 1992. The outer membrane, not a coat of host proteins, limits antigenicity of virulent *Treponema pallidum*. Infect. Immun. **60:**1076–1083.
9. **Fribourg-Blanc, A., G. Niel, and H. H. Mollaret.** 1963. Note sur quelques aspects immunologiques du cynocephale africain. Bull. Soc. Pathol. Exot. **56:**474–485.
10. **Graves, S., and J. Downes.** 1981. Experimental infection of man with rabbit-virulent *Treponema paraluis-cuniculi*. Br. J. Vener. Dis. **57:**7–10.

10a. **Lukehart, S. A., et al.** Unpublished data.

11. **Lukehart, S. A., S. A. Baker-Zander, and S. Sell.** 1980. Characterization of lymphocyte responsiveness in early experimental syphilis. I. In vitro response to mitogens and *Treponema pallidum* antigens. J. Immunol. **124:**454–460.
12. **Lukehart, S. A., S. A. Baker-Zander, and S. Sell.** 1986. Characterization of the humoral immune response of the rabbit to antigens of *Treponema pallidum* after experimental infection and therapy. Sex. Transm. Dis. **13:**9–15.
13. **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.
14. **Lukehart, S. A., M. R. Tam, J. Hom, S. A. Baker-Zander, K. K. Holmes, and R. C. Nowinski.** 1985. Characterization of monoclonal antibodies to *Treponema pallidum*. J. Immunol. **134:**585–592.
15. **Norris, S. J., and the *Treponema pallidum* Polypeptide Research Group.** 1993. Polypeptides of *Treponema pallidum*: progress toward understanding their structural, functional, and immunologic roles. Microbiol. Rev. **57:**750–779.
16. **Purcell, B. K., M. A. Swancutt, and J. D. Radolf.** 1990. Lipid modification of the 15 kilodalton major membrane immunogen of *Treponema pallidum*. Mol. Microbiol. **4:**1371–1379.
17. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56:**490–498.
18. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. J. Immunol. **147:**1968–1974.
19. **Schell, R. F., A. A. Azadegan, S. G. Nitskansky, and J. L. LeFrock.** 1982. Acquired resistance of hamsters to challenge with homologous and heterologous virulent treponemes. Infect. Immun. **37:**617–621.
20. **Thompson, J. D., D. G. Higgins, and J. T. Gibson, and W. Clustal.** 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties, and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
21. **Turner, T. B., and D. H. Hollander.** 1957. Antigenic relationship between strains of treponemes, p. 214–234. *In* Biology of the Treponematoses, World Health Organization monograph series no. 35. World Health Organization, Geneva, Switzerland.

Editor: J. G. Cannon