T-Cell Responses to *Treponema pallidum* subsp. *pallidum* Antigens during the Course of Experimental Syphilis Infection

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In this study we describe the development of the T-cell response to a panel of *Treponema pallidum* antigens over the course of syphilis infection in the rabbit and determine whether these antigens induce the expression of Th1 cytokines. It was determined that the membrane proteins TpN17 and TpN47, as well as the endoflagellar sheath protein TpN37, induce strong proliferation responses through most of syphilis infection; Tromp1 induced only weak proliferative responses. An unexpected drop in proliferative response to these antigens at day 90 of infection, followed by a dramatic increase in response at day 180, suggests that there may be a secondary dissemination of *T. pallidum* which induces a recall response. Crude epitope mapping of TpN17 and TpN37 showed that multiple epitopes may be present on both antigens, which is likely a contributing factor in the immunodominance of these antigens. The T-cell response to the TpN37 molecule shows acquisition of newly recognized epitopes during the course of infection. Sonicated *T. pallidum* was found to induce the expression of interleukin-2 (IL-2) and gamma interferon and not IL-10 mRNA, showing that the general T-cell response to *T. pallidum* antigens in syphilis infection is biased towards the Th1 phenotype. Of the antigens tested, TpN37 appears to contribute the most to the Th1 cytokine response and therefore may play a key role in the clearance of *T. pallidum* from lesions.

Infection with the spirochete *Treponema pallidum* subsp. *pallidum* results in the development of syphilis which, in untreated humans, can progress through multiple symptomatic and latent stages. The natural history of syphilis begins with the development of a primary lesion or chancre at the site of infection (primary syphilis). These lesions, which contain large numbers of treponemes, heal without antibiotic treatment. During or following the resolution of primary syphilis, the rash of secondary syphilis develops containing large numbers of viable *T. pallidum*. As in primary syphilis, these lesions will also heal spontaneously. After the clearance of secondary lesions, a long period of latency ensues and, in two-thirds of untreated individuals, the infection remains latent lifelong. These clinical observations suggest that a vigorous, but imperfect, immune response develops during syphilis infection that is successful in clearing most but not all of the treponemes infecting the host.

Our knowledge of the nature of the immune responses involved in lesion clearance is far from complete. Histologic studies of human and rabbit syphilis lesions have shown that the inflammation resembles a delayed-type hypersensitivity response. The predominant cell types infiltrating syphilis lesions are T lymphocytes (CD4+ and CD8+) and macrophages (8, 24). Activated macrophages play a major role in the clearance of *T. pallidum* from early syphilis lesions (19, 21).

Evidence of macrophage activation during syphilis infection was provided by Lukehart et al. (11), who demonstrated that *T. pallidum* antigen-sensitized lymphocytes from syphilitic rabbits produce macrophage-activating factors (MAFs) that stimulate increased macrophage killing of *L. monocytogenes*. Furthermore, it was shown that the peak production of MAF by lymphocytes correlates with bacterial clearance in primary lesions of experimentally infected rabbits (12). Although the identity of the MAFs produced by the *T. pallidum*-specific lymphocytes was not determined in that study, the cytokine gamma interferon (IFN-γ) is likely to be involved. A recent study showed that the infiltrating cells in both primary and secondary human syphilis lesions predominantly express the Th1 cytokines interleukin-2 (IL-2), IFN-γ, and IL-12 (23). Similar observations have been made in the rabbit showing an increase in expression of IL-2 and IFN-γ mRNA by infiltrating cells in resolving primary syphilis lesions (7). However, in the guinea pig model, only IL-10 synthesis was found to be significantly stimulated after intradermal inoculation of *T. pallidum*, and there was no increase in the synthesis of IL-1α, tumor necrosis factor alpha, or IL-12 p40 (25).

These studies provide compelling evidence that one of the actions of the T-cell response during early syphilis infection is the development of a Th1 cytokine environment which promotes macrophage activation and bacterial clearance. T-cell responses later in syphilis are believed to play a role in the development of immunity to reinfection. Although there are only limited experimental data in human syphilis, there is evidence that partial resistance to reinfection develops after long-term infection (17). Studies performed in rabbits are more conclusive, showing a gradual development of resistance to reinfection which becomes complete between 3 and 6 months postinfection (22). Resistance is found to result from cell-mediated immunity (CMI), based on the failure of passive transfer of immune serum to protect naive rabbits against syphilis infection (5). The late development of immunity in the rabbit appears to be a process separate from the bacterial clearing immune response, which develops at 13 to 17 days after infection (15). Two possible hypotheses might explain the dichotomy of these responses: (i) the antigenic targets of the T-cell response do not change throughout infection, but the magnitude of the response intensifies, resulting in the development of resistance, or (ii) the targets of the T-cell response change over the course of infection, resulting in the develop-
ment of resistance via these newly expressed or recognized antigens.

One strategy to attempt to answer these questions about the nature and role of T-cell responses in syphilis infection is to identify the antigenic targets recognized by T cells throughout the course of infection. One such study was carried out by Baker-Zander et al. (4), who screened sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-fractionated T. pallidum proteins for induction of proliferation of cultured splenic lymphocytes from infected rabbits. This study revealed that a number of previously identified proteins elicited robust T-cell proliferative responses, namely, TpN15, TpN17, TpN37, and TpN47, as well as the endoflagellar sheath protein TpN17(FL) and TpN17(2/3) (6). The outer membrane location of this molecule has been demonstrated to be a target of opsonization (1). Furthermore, we demonstrate the expression of Th1-type cytokines in response to stimulation with these specific antigens.

**MATERIALS AND METHODS**

Rabbits. Adult male New Zealand White rabbits were obtained from R & R Rabbity (Stanwood, Wash.). Rabbits were tested for evidence of Treponema parahuscincaii infection by the Venereal Disease Research Laboratory (Atlanta, Ga.), fluorescent treponemal antibody-absorbed, and Western blot tests. Only rabbits that were seronegative by these tests were included in this study. Rabbits paraluiscuniculi in infection by the Venereal Disease Research Laboratory (Atlanta, Ga.), fluorescent treponemal antibody-absorbed, and Western blot tests. Only rabbits that were seronegative by these tests were included in this study. Rabbits were housed individually at 18 to 20°C and given antibiotic-free food and water. Rabbits were infected by intratesticular injection with 10^8 motile T. pallidum organisms.

Cloning, expression, and purification of recombinant proteins. The T. pallidum Nichols strain proteins TpN15, TpN17, and TpN47 were expressed as glutathione S-transferase (GST) fusion proteins by using a PCR cloning strategy. PCR primers were designed based on sequences published in GenBank (Table 1). To facilitate cloning, restriction endonuclease cleavage sites were included in all of the PCR primers. The PCR primer pairs TpN17(full length [FL]), Tromp1(FL), TpN37(FL), and TpN47(FL) (Table 1) were used to amplify the open reading frames (ORFs) from purified T. pallidum genomic DNA. The PCR reactions were performed in a 100-μl volume and contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 200 μM deoxynucleoside triphosphates (dNTPs), 1.5 mM MgCl₂, 2.5 U of Taq polymerase (Promega, Madison, Wis.), 1 μM concentrations each of the sense and antisense oligonucleotide primers, and 350 ng of T. pallidum genomic DNA. The cycling conditions for all PCR reactions were 94°C for 1 min, 55°C for 1 min and 30 s, and 72°C for 1 min and 30 s. The amplicons were cut with the appropriate restriction endonucleases and cloned into expression vectors. The Tromp1(FL) amplicon was cloned into the vector pGex5X-1 (Amersham Pharmacia Biotech) by standard molecular techniques (20). The TpN37(FL) and TpN47(FL) amplicons were cloned into the vector pGex5X-1 (Amersham Pharmacia Biotech) by standard molecular techniques (20). The TpN37(FL) and TpN47(FL) inserts, including the ORF of GST, were PCR amplified from the resulting pGex5X-1 clones with a GST-specific sense primer: 5'-GGGGATCCGATCGTCGAC-3' and TpN37(FL) and TpN47(FL) antisense primers by using the previously described reaction conditions. The GST-TpN37(FL) and GST-TpN47(FL) amplicons were also cloned into...
into the six-histidine fusion protein vector pRSSET C (Invitrogen, San Diego, Calif.).

Truncated fragments of TpN17 and TpN37 were cloned by PCR amplification of portions of the respective genes representing one-third of the coding sequence with 60-bp overlaps with the following primer pairs: TpN17(3/1), TpN17(2/3), TpN17(3/3), TpN37(3/1), TpN37(2/3), and TpN37(3/3) (Table 1). The amplicons were cut with BamHI and XhoI and cloned into BamHI/XhoI-cut pRSSET C vector modified by the insertion of the GST ORF of pGex-X1-1 into the BamHI site of the polylinker.

The plasmids GST-Tromp1(FL) and GST-T(FL) were transformed into Escherichia coli XL-1 Blue (Stratagene, La Jolla, Calif.) and GST-TpN37(FL), and GST-TpN47(FL) plasmids, as well as the truncated GST-TpN17 and GST-TpN37 plasmids, were transformed into E. coli BL21 pLysS (Novagen, Madison, Wis.). Recombinant proteins were expressed at 30°C in Luria-Bertani (Gibco/BRL, Grand Island, N.Y.) broth containing 2% glucose, ampicillin (50 μg/ml), and chloramphenicol (30 μg/ml) [for BL21 pLysS hosts only]. Cultures were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density at 540 nm of 0.6 to 0.8 and incubated for 3 h. The cells were harvested by centrifugation at 7,000 × g for 10 min and resuspended in lysis buffer containing 100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.0), aprotinin (2 μg/ml), leupeptin (2 μg/ml), and pepstatin (1 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). The bacterial suspensions were frozen at −20°C and thawed, and the cells were disrupted by intermittent sonication on ice for 2 min by using a probe sonicator (Sonomedics, Danbury, Conn.) set to 25 W. Soluble fusion proteins expressed by the GST-Tromp1 and GST-TpN17 clones, as well as pGEX-X1-1, were purified twice from bacterial lysates with glutathione-agarose (Sigma) affinity chromatography according to manufacturer’s instructions. Fusion protein inclusion bodies produced by the truncated GST-TpN17 and GST-TpN37 clones were isolated as described by Harlow and Lane (10). The inclusion bodies were dissolved in buffer containing 8 M urea, 0.1 M NaH₂PO₄, and 10 mM Tris-HCl (pH 8.0) and centrifuged at 100,000 × g for 20 min to remove the insoluble material. The inclusion protein fractions were purified twice with Ni-NTA Superflow Resin (Qiagen, Santa Clarita, Calif.) according to manufacturer’s instructions, dialyzed exhaustively into 10 mM Tris-HCl (pH 8.0), quantified, and stored at −70°C. The purity of antigens was monitored by SDSPAGE, and endotoxin was detected by using the Limulus amebocyte lysate test (BioWhittaker, Walkersville, Md.), which detects endotoxin in excess of 0.1 endotoxin unit/ml.

Lymphocyte proliferation. Splens were harvested from groups of uninfected rabbits or rabbits infected with T. pallidum for 10, 30, 90, or 180 days. Splicenic lymphocytes were cultured and tested with antigens by using previously published protocols (4, 15). The cells were resuspended in RPMI (Gibco/BRL) containing penicillin, streptomycin, and glutamine, and 1% heat-inactivated pooled rabbit serum and then cultured in flat-bottomed 96-well tissue culture plates (Costar, Cambridge, Mass.) at 37°C and 5% CO₂. Optimal culture conditions and antigen concentrations for maximum proliferative response were determined by titration (data not shown). Quadruplicate cultures at 2.5 × 10⁶ cells/well in 200 μl received 10 μg of concanavalin A (ConA; Sigma) per ml or sonicated T. pallidum (10⁷ T. pallidum/well, 6 μg of protein per ml) (15). For recombinant antigens, quadruplicate cultures at 5 × 10⁶ cells/well in 200 μl received 50 μg of GST, 10 μg of GST-Tromp1, 50 μg of GST-TpN17, 5 μg of GST-TpN37, or 5 μg of GST-TpN47 per ml. For epitope-mapping experiments the antigens were used at 2 μg/ml for the full-length and truncated TpN37 fusion proteins and 5 μg/ml for the full-length and truncated TpN37 fusion proteins.

Cultures containing sonicated T. pallidum and ConA were pulsed with 0.5 μCi of [³H]thymidine/well on day 3 of culture, whereas cultures containing recombinant antigens were pulsed on day 4 of culture. Exactly 24 h after [³H]thymidine addition, the cells were harvested by using a 96-well cell harvester (Tomtech, Orange, Conn.), and [³H]thymidine incorporation was measured by using an automated liquid scintillation counter (Beckelatube 1205; Wallac, Turku, Finland). The results are displayed as the geometric mean of counts per minute minus the geometric mean of background counts per minute measured in control wells containing no antigen.

Cytokine detection. Splenic lymphocytes were cultured from four uninfected rabbits and four rabbits each infected for 10, 30, 90, and 180 days in 1-cm-diameter-well culture plates (Costar) at a concentration of 6.25 × 10⁶ cells in 500 μl. Optimal antigen concentrations and culture conditions were determined in preliminary studies (data not shown). Each well received control or test antigens at the concentrations listed above. The cultures were incubated for 8 h at 37°C and 5% CO₂. Cells were pelleted by centrifugation, and RNA was isolated by using Ultraspec RNA isolation reagent (Biotex, Houston, Tex.) according to manufacturer’s instructions. The resulting RNA was repurified by using an RNaseasy RNA purification kit (Qiagen) according to manufacturer’s instructions. To synthesize cDNA, RNA from 3 × 10⁶ splenocytes in 16 μl of water was incubated in the presence of 2 U of amplification-grade DNase I (Gibco/BRL), 10 mM Tris-HCl (pH 8.4), 2 mM MgCl₂, and 50 mM KCl for 15 min at 25°C to remove contaminating genomic DNA. The reactions were stopped by the addition of 2.5 mM EDTA followed by heating at 65°C for 10 min. Random hexanucleotides (125 ng; Gibco/BRL) were added to the reactions, heated at 70°C for 15 min, and then placed on ice. Reverse transcription (RT) was initiated by addition of 500 μM dNTPs, 10 μM dithiothreitol, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM MgCl₂, 400 U of Superscript II (Gibco/BRL), and 40 U of RNasin (Promega) and then incubated at 43°C for 50 min. The reaction was terminated by heating it at 70°C for 15 min. The cDNA was then quantified by hyponixanthine phosphoribosyl transferase (HPRT) competitive PCR and normalized to 2.5 fg of competitor HPRT product with an MboI 89-bp internal deletion.

IFN-γ, IL-2, and IL-10 signal was detected by PCR techniques by using the oligonucleotides listed in Table 2. PCR was performed under the conditions described above except for an initial denaturation step of 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min. The resulting cytokine PCR products were quantified by scanning ethidium bromide-stained DNA bands on agarose gels with a UV scanner (Bio-Rad). Pixel data measured from PCR products were used to calculate the DNA band concentration based on standard curves generated by using PCR product DNA of known concentrations.

### RESULTS

#### Fusion protein expression

The T. pallidum proteins of interest were expressed as fusion proteins in E. coli in order to obtain sufficient amounts of purified protein for use in immunologic assays. The amino terminus fusion protein partner GST was included in all of the constructs to facilitate the expression T. pallidum proteins in E. coli and to serve as a target for affinity purification by glutathione-agarose chromatography. The TpN17-GST and Tromp1-GST fusion proteins were expressed as soluble cytoplasmic proteins, which allowed purification with glutathione-agarose (Fig. 1). The Tromp1-GST fusion protein contained the peptide portion of the Tromp1 molecule without the signal peptide, whereas the TpN17-GST fusion protein included the entire ORF of TpN17, including the signal peptide.

The remaining proteins were expressed as fusions containing both GST and six-histidine tags. The GST was included because it was found in preliminary cloning and expression experiments that these proteins were poorly expressed without the addition of this fusion molecule. It was also determined that the full-length TpN37 and TpN47 molecules, as well as the truncated TpN17 and TpN37, when fused to GST were expressed as fusion proteins in E. coli in order to obtain sufficient amounts of purified protein for use in immunologic assays. The amino terminus fusion protein partner GST was included in all of the constructs to facilitate the expression T. pallidum proteins in E. coli and to serve as a target for affinity purification by glutathione-agarose chromatography. The TpN17-GST and Tromp1-GST fusion proteins were expressed as soluble cytoplasmic proteins, which allowed purification with glutathione-agarose (Fig. 1). The Tromp1-GST fusion protein contained the peptide portion of the Tromp1 molecule without the signal peptide, whereas the TpN17-GST fusion protein included the entire ORF of TpN17, including the signal peptide.

### TABLE 2. Oligonucleotides used for cytokine PCR reactions

<table>
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<tr>
<th>Target mRNA</th>
<th>Sequence (5’-3’) (sense/antisense)</th>
<th>GenBank accession no.</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>HPRT</td>
<td>CTCGCTGATGATACATCAAAGACCTG</td>
<td>M31642</td>
<td>288 (native); 199 (competitor)</td>
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<tr>
<td>IFN-γ</td>
<td>TTCCTGCAGCTCACCTTCC</td>
<td>D84216</td>
<td>224</td>
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<tr>
<td>IL-2</td>
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<td>Z36904</td>
<td>203</td>
</tr>
<tr>
<td>IL-10</td>
<td>GAGAACCAGCTGCACGCCAT</td>
<td>D84217</td>
<td>179</td>
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pressed as insoluble inclusions. Since GST does not bind to its ligand glutathione when denatured, the six-histidine tag was added to these constructs to facilitate affinity purification by nickel column chromatography under denaturing conditions. The TpN37-GST fusion protein contained the entire ORF of TpN37, and the TpN47-GST contained the mature peptide portion of TpN47 without the signal peptide. All of the resulting fusion proteins were determined to have molecular masses that matched predicted molecular masses (Fig. 1). After two sequential purifications by either glutathione agarose or nickel resin chromatography, the proteins were found to be free of measurable or mitogenic amounts of LPS.

**Splenocyte response to T. pallidum proteins.** The proliferative response to fusion proteins was measured over a period of 180 days of infection to monitor the T-cell response during the course of experimental syphilis in the rabbit. The results of these time course experiments are summarized in Fig. 2. Splenocytes from uninfected animals showed no appreciable response to either the sonicated T. pallidum or the recombinant proteins. The response to ConA was consistently greater than 70,000 cpm at all time points (data not shown). The proliferative response to sonicated T. pallidum began to develop at day 10 of infection and increased throughout the course of the experiment, a result which mimics the response to TpN37-GST reported in previous studies (4, 15).

The proliferative response to all recombinant antigens was detectable by day 10 of infection except for the negative control GST, which failed to induce a response at any point. The TpN17-GST and TpN37-GST elicited the highest proliferative responses at all time points tested, whereas TpN47-GST induced only a weak response. Surprisingly, the proliferative response to the TpN17 fragments showed a markedly different pattern compared to that of the TpN17 fragments. A response to TpN37(2/3)-GST was present by day 10 of infection with no appreciable response to the flanking fragments. By day 180 the maximum response was directed to TpN37(3/3)-GST, with TpN37(2/3)-GST and TpN37(1/3)-GST showing appreciable but lower responses.

**Cytokine expression in response to T. pallidum antigens.** mRNA coding for the cytokines IL-2, IL-10, and IFN-γ was measured by RT-PCR in splenocytes incubated with T. pallidum antigens. The goal of these experiments was to determine whether the antigens under investigation are involved in eliciting the Th1 response detected in resolving syphilis lesions (7). The cytokines IL-2 and IFN-γ were studied because they are indicators of Th1 response, whereas IL-10 served as an indicator.
cator of a Th2 response or the presence of macrophages. Splenocytes cultured with sonicated T. pallidum produced IFN-γ mRNA as early as 10 days after infection (Fig. 4). IFN-γ mRNA expression increased at day 30, was reduced to baseline at day 90, and peaked at day 180. The responses to the recombinant T. pallidum proteins showed a similar temporal pattern of IFN-γ mRNA expression. This bimodal pattern of IFN-γ mRNA expression mimics the proliferative response to the recombinant antigens (Fig. 2). Generally, the magnitude of the IFN-γ mRNA expression correlates with the magnitude of proliferation for a given recombinant protein. One exception to this is the IFN-γ mRNA expression in response to TpN17-GST, which is notably lower than the response to TpN37-GST (Fig. 4) despite the fact that the proliferative responses are nearly identical (Fig. 2).

The IL-2 mRNA expression induced by the sonicated T. pallidum closely resembles the proliferative response, showing a generally increasing trend beginning at day 10 and peaking at day 180 (Fig. 5). The responses to the recombinant proteins were considerably different, the peak response occurring at day 10, with a subsequent downregulation over time toward day 90. The IL-2 mRNA expression increased again at day 180 but to a lower level than at day 10. There were no appreciable changes in the expression of IL-10 mRNA between the control and T. pallidum-infected animals at days 10, 30, 90, or 180 after infection with all the antigens tested (data not shown).

**DISCUSSION**

The T-cell response associated with healing T. pallidum lesions in both humans and rabbits produces a Th1 predominant cytokine pattern (7, 23). T. pallidum-specific T cells can be detected as early as 6 days after infection in the rabbit and are found at peak levels during clearance of bacteria from lesions (15). A number of major T-cell antigens have been identified by screening fractionated T. pallidum proteins, including TpN17, TpN37, and TpN47 (4). These proteins have been determined by SDS-PAGE fractionation to be major components of T. pallidum (13). The current study demonstrates that recombinant TpN17, TpN37, and TpN47 antigens induce a robust T-cell response, thus confirming the observations of Baker-Zander et al. (4). By using affinity-purified recombinant proteins we were able to determine with a higher degree of certainty that the proliferative responses observed were induced by the target molecules and not by trace proteins contaminants, as can occur with proteins isolated directly from T. pallidum. The proliferative responses to the recombinant antigens, like that of sonicated T. pallidum, were detectable by day 10 of infection and increased appreciably by day 30. This pattern of T-cell response development corresponds with the clearance of the majority of T. pallidum from primary lesions.
This temporal correlation suggests that the robust T-cell responses to TpN17, TpN37, and TpN47 may be involved in the clearance of bacteria. The decrease in the proliferative responses to the recombinant antigens at day 90 of infection was not expected, based on earlier observations that the response to sonicated T. pallidum is maintained at high levels in infected rabbits for as long as 2 years. One possible explanation for this finding is that the reduction in response to individual antigens is a normal downregulation of the T-cell response which occurs as a result of the clearance of bacteria.

The reappearance of the proliferative responses to the recombinant antigens at day 180 raises further questions. The functional significance of this epitope spreading has yet to be determined, but it is possible that a late T-cell response to a separate subset of antigens masks the downregulation observed with the TpN17, TpN37, and TpN47 antigens.

The possibility that the multiphasic T-cell responses observed in this and previous studies are the result of periodic T. pallidum bacteremia is intriguing but difficult to test experimentally. The difficulty in detecting T. pallidum has hampered efforts to monitor asymptomatic infection. Although there have been many detailed studies monitoring T. pallidum in lesions and organ tissues over the course of early syphilis in the rabbit, there have only been limited studies investigating the numbers of T. pallidum present in latent infection.

The reappearance of the proliferative responses to T. pallidum-GST fusion proteins may be subclinical. The existence of bimodal T-cell responses to individual antigens in syphilis infections has been reported previously. The study of Baker-Zander et al. (4) showed that the T-cell response to TpN47 and a complex containing the two flagellar proteins TpN33 and TpN35 is reduced to baseline at day 30 of infection and then increase dramatically at day 210. Splenocytes from day 90-infected rabbits were not tested in these experiments, but nevertheless there appears to be a comparable downregulation followed by a recall response. In a separate study, Baker-Zander and Sell (3) showed a drop in the splenic and lymph node lymphocyte response to sonicated T. pallidum at day 90 of infection, followed by a secondary peak at day 180. Furthermore, it was shown that the splenocyte response declined again at 12 months of infection and resurged at 24 months.

The question of whether T. pallidum is periodically reactivated in rabbits could be answered by monitoring for the presence of T. pallidum DNA in blood or other tissues by PCR amplification. If there are no T. pallidum bacteraemia in the rabbit, this would provide valuable insight into the immune evasion strategies utilized by this pathogen. Antigenic variation of outer membrane proteins, as seen in African trypanosomes and many Borrelia species, has long been suspected in T. pallidum but no variable outer-membrane-expressed antigens have been identified to date.

Because TpN17 and TpN37 elicited the strongest T-cell responses observed in this study, we were interested in determining whether these antigens possess multiple T-cell epitopes and whether there might be a shift or spreading of epitope recognition during the course of infection. The epitope-mapping studies showed that multiple epitopes are recognized on TpN17 and TpN37. It is possible that some of the proliferative responses were directed to the 20-amino-acid overlap included in the truncated fragments, so there may be as few as one T-cell epitope on TpN17 and as few as two T-cell epitopes on TpN37; alternatively, there may be several epitopes on each stimulatory fragment. The recognition of multiple epitopes on these molecules is likely to be one factor that contributes to the immunodominance of these two molecules. Antigenic spreading was observed in the response to TpN37, showing an initial predominant response to the central portion of the molecule followed by the development of responses to the first and third fragments of the proteins later in infection. This shows that an initial focused proliferative response can be detected to an immunodominant portion of TpN37, even in outbred rabbits. The functional significance of this epitope spreading has yet to be determined, but it is possible that the late-developing response to epitopes may contribute to the immunity to reinfection that becomes complete by day 90.

After characterizing the proliferative responses to this panel of proteins, we were interested in assessing the functional significance of these T-cell responses by measuring IFN-γ, IL-2, and IL-10 mRNA synthesis in response to the individual antigens. The expression of IFN-γ and IL-2 mRNA generally followed the patterns of proliferation, with production during the first 30 days of infection, a reduction between 30 and 90 days, and a secondary increase at day 180. This pattern of response provides additional evidence for the existence of a
recall response, possibly induced by a secondary *T. pallidum* bacteremia. The induction of IL-2 and IFN-γ mRNA and the lack of induction of IL-10 by sonicated *T. pallidum* shows that the T-cell responses generated during syphilis infection are biased toward the Th1 phenotype. Lacking information on IL-4 expression, we cannot formally exclude the existence of a Th2-type response. Of the antigens tested, TpN37 appears to have the greatest influence on the development of the Th1-type T-cell response, showing the strongest induction of both IFN-γ and IL-2 mRNA. The intensity of the proliferation and Th1 cytokine response to the TpN37 correlates with both lesion-clearing response (days 10 to 30) and resistance to reinfection (day 180). Thus, TpN37 merits further investigation as a CMI-inducing component of a multivalent vaccine for syphilis.

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**REFERENCES**