Segregation of B and T Cell Epitopes of *Treponema pallidum* Repeat Protein K to Variable and Conserved Regions During Experimental Syphilis Infection[1]

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Robust immune responses clear millions of treponemes to resolve lesions of primary and secondary syphilis, but cannot clear the treponemes that lead to debilitating and sometimes fatal tertiary syphilis. It is also known that the rabbit model and humans can be reinfected with heterologous isolates. How some treponemes are able to escape the immune system is unknown. In our laboratories rabbits immunized with the Seattle Nichols strain *Treponema pallidum* repeat protein K (TprK) were previously shown to have attenuated lesion development following challenge. In other isolates, TprK was shown to have seven discrete variable regions, with sequence variation among and within isolates. Using overlapping synthetic 20-aa peptides, we demonstrate that during experimental infection with the Nichols strain, the T cell responses are directed to conserved regions, while the Ab responses are directed primarily to variable regions. Abs from rabbits immunized with recombinant TprK recognized conserved and variable regions, suggesting that the conserved regions are inherently as immunogenic as the variable regions. TprK variability may allow some treponemes to escape recognition from Abs. The variable region heterogeneity may help explain the lack of protection against heterologous isolates. ***The Journal of Immunology,*** **2002, 169: 952–957.**

Syphilis is a spirochetal infection with multiple manifestations of disease. Robust specific immune responses are able to resolve primary and secondary lesions, but are not able to clear all treponemes. Syphilitic lesions are infiltrated by T cells that activate macrophages to phagocytose opsonized treponemes, the major mechanism of treponemal clearance (1–4). Many manifestations of tertiary syphilis can be attributed to chronic inflammation thought to be triggered by persistent organisms in a variety of tissues. How these treponemes are able to escape the robust immune responses, invade a wide range of tissues, and persist is largely unknown. It is also unknown why infection-induced homologous protection in the rabbit model does not protect against heterologous isolates (5) and why humans can be infected multiple times (6).

In the search for *T. pallidum* subspecies *pallidum* virulence factors, members of a family of 12 genes called *Treponema pallidum* repeat (*tpr*)[3] genes A–L were identified (7–9). In an organism that lacks major biosynthetic capacity, devoting 2% of its small genome to the *tpr* gene family is quite remarkable and suggests the importance of the *tpr* gene family to the organism. We have reported that when one member of this family, *Treponema pallidum* repeat protein K (TprK), is used as an immunogen, lesion development is attenuated following homologous challenge in the rabbit model (7). Although Hazlett et al. (10) failed to corroborate these results, we have confirmed and expanded the original results to show that immunizing with the N-terminal portion (aa 37–273) of TprK retards lesion development (manuscript in preparation) as previously seen with a larger fragment (aa 37–348) (7). Therefore, there is evidence that TprK does play an important role in the immune response. Comparison of TprK sequences from various *T. pallidum* isolates has shown that TprK varies in seven discrete variable regions among and within all isolates examined except the Nichols strain (11). We hypothesize that TprK elicits a protective immune response, but, due to its variability, aids subsets of treponemes to escape the immune response. In support of our hypothesis we show here that during infection with *T. pallidum,* Abs are specifically directed toward the discrete variable regions of TprK. In contrast to the Ab response, the T cell response is directed to the conserved regions of TprK, which may be useful in a multicomponent vaccine.

Materials and Methods

Experimental infection with T. pallidum

Twenty outbred adult male New Zealand White rabbits (R & R Rabbitry, Stanwood, WA) were infected intratesticularly with 1×10^8 *T. pallidum*, Nichols strain; four uninfected rabbits were used as controls. The *T. pallidum* Nichols strain (obtained from J. N. Miller (University of California, Los Angeles, CA) and brought to Seattle in 1979) was propagated intratesticularly in rabbits as previously described (12). At 10, 30, 90, 175, and 280 days after infection, blood and splenocytes were harvested from groups of four animals for lymphocyte proliferation and Ab assays.

Immunization

The open reading frame of *tprK* was divided into three sections: fragment 1, encoding aa 37–273; fragment 2, encoding aa 274–348; and fragment 3, encoding aa 349–478. The amplicons from *T. pallidum* Seattle Nichols strain DNA were cloned into the pRSET expression vector (Invitrogen, Carlsbad, CA), the sequence was verified, and the peptide was expressed in *Escherichia coli* and purified as previously described (7, 13). The proteins were dialyzed into PBS, pH 7.2, size confirmation and purity were evaluated by SDS-PAGE, and concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). New Zealand White

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[3] Abbreviations used in this paper: *tprK*, *Treponema pallidum* repeat K gene; TprK, *Treponema pallidum* repeat K protein; MOMP, *Chlamydia trachomatis* major outer membrane protein; NFM, nonfat dry milk; Opa, neisserial opacity protein; Por, neisserial protein I; VlsE, *Borrelia burgdorferi* VMP-like sequence protein.

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male rabbits (R & R Rabbitry) were immunized with 125 μg of the three recombinant fragments in Ribi Adjuvant (Sigma-Aldrich, St. Louis, MO) administered s.c., intradermally, i.m., and i.p. every 3 wk for six consecutive immunizations. Four rabbits were immunized with each fragment except for fragment 2 ($n = 3$) due to an unrelated early death of one rabbit. Serum was collected from each rabbit 10–14 days after the last boost, pooled, and used in Ab assays.

Peptides for epitope mapping

Overlapping 20-aa synthetic peptides were designed based on the Seattle Nichols strain sequence (GenBank accession no. AF194369). Starting after the signal sequence cleavage site (10, 11) a total of 39 synthetic peptides (spanning aa 30–429; Table I) generally overlapping by 10 aa were made using a Rainin-PTI Symphony instrument and Sephadex desalting step to a minimum purity of 70% analyzed by HPLC trace and mass spectrometry (Fred Hutchinson Cancer Research Center, Seattle, WA) and rehydrated in PBS, pH 7.2. The conserved 3′ *tprK* region (encoding aa 425–478) was cloned, expressed in *E. coli*, and purified as described in the previous section.

Lymphocyte proliferation assays

Spleen cells obtained from uninfected and infected rabbits were tested for proliferative activity as previously described (12, 14). Quadruplicate cultures of 200 μl cells from each animal tested separately were inoculated with 5 μg synthetic peptides, 2.5 μg recombinant peptide, 10 μl sonicated *T. pallidum* (treponeme-specific positive control), 4 μg Con A (T cell mitogen used as a positive control; Sigma-Aldrich), or 10 μl PBS (control for background). Proliferation was measured as the amount of tritiated thymidine taken up by new cells. The mean ± SE of the quadruplicate test wells minus the mean of quadruplicate wells with no Ag from each animal was calculated. The data presented in Fig. 1 are the mean ± SE for each condition from four different animals per time point.

ELISAs

Ninety-six-well Maxisorb Immunoplates (Nunc, Naperville, IL) were coated with 50 μl of 10 μg/ml peptides in PBS and incubated at 4°C overnight. Plates were washed with PBS and 0.05% Tween 20 and blocked with 3% nonfat dry milk (NFM) in PBS. Sera were preadsorbed with crude lysate of *E. coli* expressing an unrelated recombinant protein to remove Abs directed against *E. coli* and vector-encoded peptides within recombinant peptide 40. One hundred microliters of serum diluted to a final concentration of 1/20 in PBS with 1% NFM were added to each well and incubated at 37°C for 1 h. The plates were washed as described above, and 100 μl goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/2000 in PBS and 1% NFM were added to each well and incubated for 1 h at room temperature. Plates were washed, developed with 50 μl/well of 1 mg/ml para-nitrophenylphosphate substrate (Sigma-Aldrich), for 15 min for recombinant peptide and 1 h for synthetic peptides, and absorbance was measured at OD_{405}. A bicinchoninic acid protein assay (Pierce) was performed in plates coated with Ag and washed to demonstrate that all peptides bound to the plates (data not shown). The mean ± SE of triplicate experimental wells minus the mean of the wells with no peptide was calculated for all conditions tested with serum from each animal. Each graph in Fig. 2 represents the mean ± SE for each condition from four different rabbits infected for the same amount of time. The graphs in Fig. 3 represent the mean ± SE for triplicate wells minus the mean of wells with no peptide for all conditions tested with sera that were pooled from immunized rabbits before testing in ELISAs.

Results

T cell epitopes are localized to the conserved regions

To determine the peptide targets of the T cell response, splenocytes were isolated from outbred rabbits that were not infected or were infected for varying lengths of time: peak orchitis was observed at 10 days, orchitis was resolving at 30 days, rabbits become immune to homologous challenge between 90–175 days, and 280 days represents long term infection (1, 5). Lymphocytes from uninfected rabbits did not proliferate significantly when exposed to any peptide (Fig. 1). At each time point postinfection, the lymphocytes from each of the four rabbits tested responded to peptides representing conserved regions of TprK. Some reactivity was seen to adjacent peptides 2 and 16, which contain portions of the conserved regions of reactive peptides 1, 3, and 15 (Table I). For peptides 15 and 40, proliferative responses were seen as early as 10 days postinfection and persisted until the last time point tested, 280 days (Fig. 1). Individual rabbits recognized the same subset of peptides, e.g., peptides 1, 3, 9, 10, 15, and 40, although the magnitude of the response varied among these outbred animals (data not shown). All infected rabbits developed responses equivalent to at least 20,000 cpm in response to sonicated *T. pallidum* and 30,000 cpm in response to Con A; the uninfected control rabbits developed responses of <1,000 cpm to sonicated *T. pallidum* and at least 30,000 cpm to Con A (data not shown).

Ab responses during infection are primarily directed to the variable regions

To identify B cell epitopes of TprK throughout infection, ELISAs were performed with sera from the same 20 experimentally infected and four uninfected outbred rabbits used in the T cell epitope-mapping experiments. No response was seen with sera from uninfected rabbits or rabbits infected for only 10 days (data not shown). At 30 days postinfection Ab responses were directed

Table I. *Peptide sequences*[a]

No.	Sequence
1	QVSFTPDIEGYAELAWGIAS
2	ELAWGIASDGGALKHGFKTT
3	LKHGFKTTTDFKIVFPIVAK
4	FKIVFPIVAKKDFKYRGEGN
5	KDFKYRGEGNVYAEINVKAL
6	VYAEINVKALKLSLESNGGA
7	KLSLESNGGAKFDTKGSAKT
8	KFDTKGSAKTIEATLHCYGA
9	IEATLHCYGAYLTIGKNPDF
10	YLTIGKNPDFKSTFAVLWEP
11	KSTFAVLWEPWTANGDYKSK
12	WTANGDYKSKGDKPVYEPGF
13	GDKPVYEPGFEGAGGKLGYK
14	EGAGGKLGYKQTDIAGTGLT
15	QTDIAGTGLTFDIAFKFASN
16	FDIAFKFASNTDWEGKDSKG
17	DSKGNVPAGVTPSKYGLGGD
18	TPSKYGLGGDILFGWERTRE
19	LGGDILFGWERTREDGVQEY
20	ILFGWERTREDGVQEYIKVE
21	IKVELTGNSTLSSDYAQARA
22	LTGNSTLSSDYAQARAPAAG
23	YAQARAPAAGAKVSMKLWGL
24	AKVSMKLWGLCALAATDVGH
25	CALAATDVGHKKNGAQGTVG
26	KKNGAQGTVGADALLTLGYR
27	ADALLTLGYRWFSAGGYFAS
28	WFSAGGYFASQASNVFGGVF
29	QASNVFGGVFLNMAMREHDC
30	LNMAMREHDCAAYIKLETKG
31	AAYIKLETKGSDPDTSFLEG
32	SDPDTSFLEGLDLGVDVRTY
33	LDLGVDVRTYMPVHYKVLKA
34	MPVHYKVLKALPRADIHFPV
35	LPRADIHFPVYGKVWGSYRH
36	YGKVWGSYRHDMGEYGWVKV
37	DMGEYGWVKVYANLYGGTNK
38	YANLYGGTNKKATPPAAPAT
39	KATPPAAPATKWSKEYCGYY
40	YCGYYECGVVVSPLEKVEIR
	LSWEQGKLQENSNVVIEKNV
	TERWQFVGACRLIW

[a] Sequences are numbered and listed from N to C terminus. The underlined amino acids represent variable amino acids.

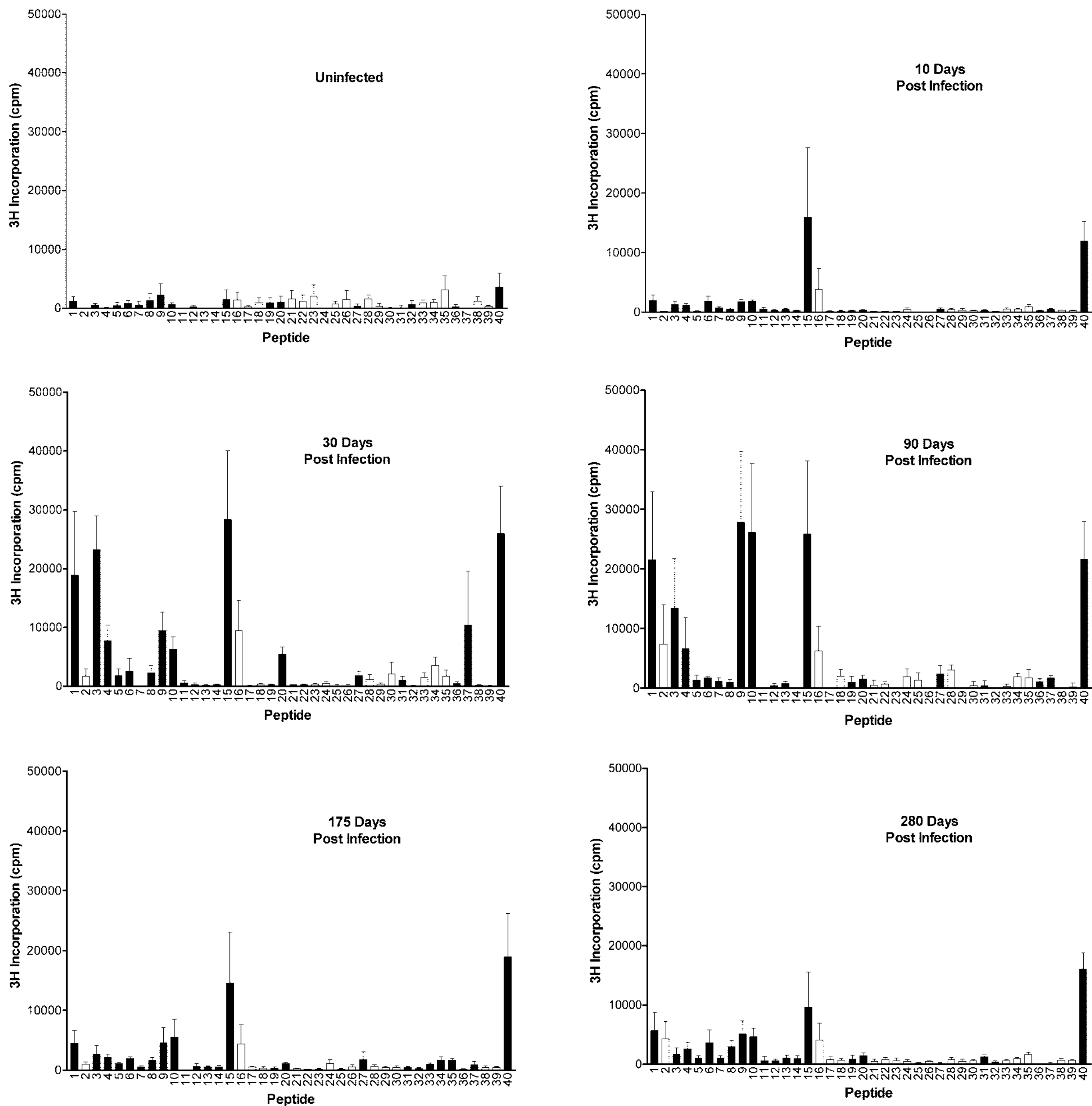

FIGURE 1. T cell epitopes. TprK peptides were evaluated for their ability to elicit lymphocyte proliferation from groups of four rabbits uninfected or infected with *T. pallidum* for varying lengths of time. Filled bars represent peptides containing only amino acids in the conserved regions. Open bars represent peptides containing amino acids in the variable regions. T cell responses throughout experimental infection in rabbits are directed toward the conserved regions of TprK.

to a conserved and several variable portions of TprK (Fig. 2). By day 90, however, all peptides recognized by Abs contained amino acids representative of variable regions, in contrast to conserved T cell epitopes. In confirmatory studies with two additional groups of rabbits, Abs to conserved peptides were also recognized only at 30 days postinfection and only to variable regions later in infection (data not shown). Ab responses to some peptides were detectable for at least 280 days postinfection (Fig. 2). Unlike the consistent T cell responses, Abs from individual rabbits recognized different subsets of variable region peptides (data not shown).

TprK immunized rabbits develop Abs to conserved and variable regions

To demonstrate which peptides were capable of eliciting Ab responses, sera were collected from rabbits immunized with recombinant TprK fragments, pooled, and tested in ELISAs. In contrast to Abs resulting from infection, both conserved and variable peptides throughout TprK were recognized by antisera (Fig. 3).

Discussion

Variable proteins from a number of bacteria, such as *Chlamydia trachomatis* major outer membrane protein (MOMP), nontypeable *Hemophilus influenzae* major outer membrane proteins P2 and P5, and *Neisseria gonorrhoeae* opacity proteins (Opa) and protein I (Por), have been shown to have critical functions in pathogenesis (such as pore forming or adhesion) and to be targets of variant-specific bactericidal, blocking, or opsonic Abs (15–25). In this study the T cell epitopes of TprK recognized during infection were shown to be limited to regions that are highly conserved among *T. pallidum* subspecies *pallidum* isolates, while the B cell epitopes

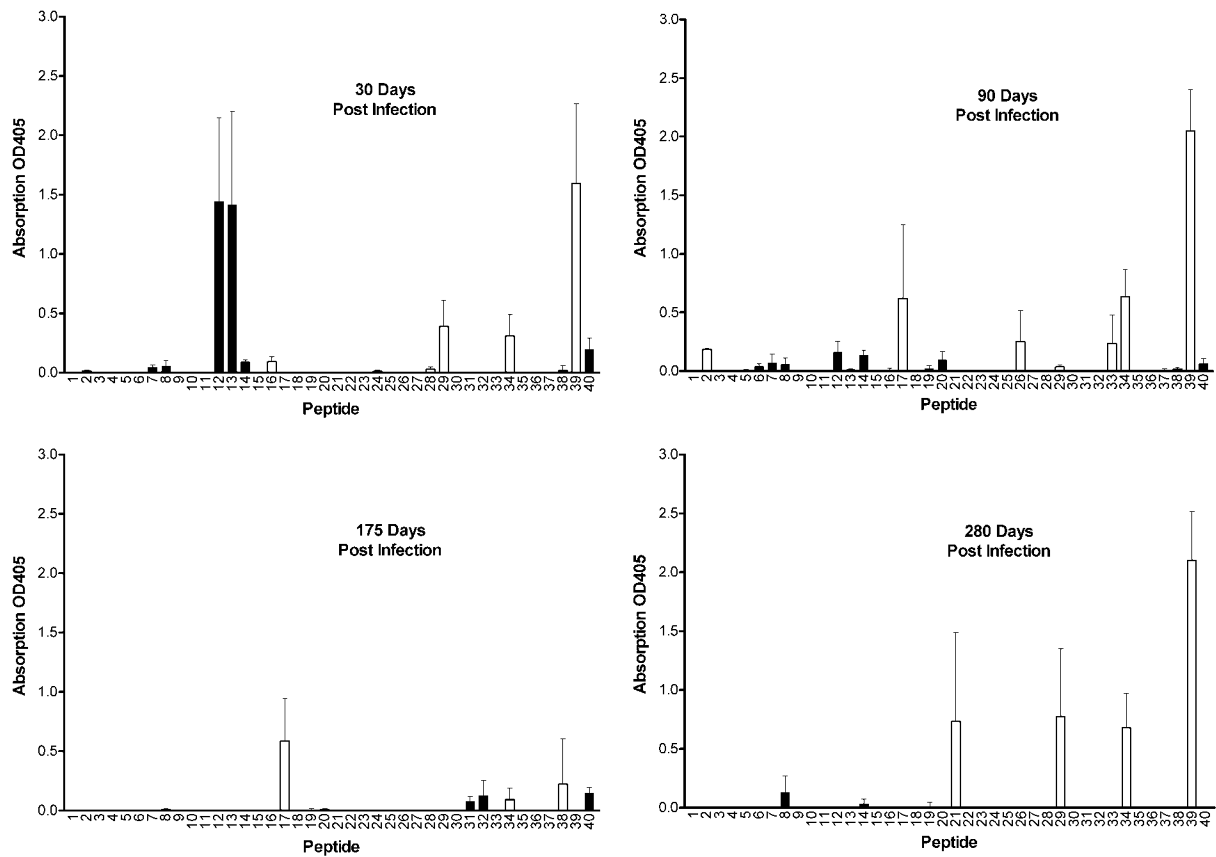

FIGURE 2. B cell epitopes throughout infection. Sera collected from groups of four rabbits infected with *T. pallidum* for varying lengths of time were tested in ELISAs to determine B cell epitopes. Filled bars represent peptides containing only amino acids in the conserved regions. Open bars represent peptides containing amino acids in the variable regions. Ab responses were primarily directed to the variable regions of TprK.

were focused primarily in the variable regions. Our results are consistent with a role for the variable regions in pathogenesis and a functional role for Abs to the variable regions, as seen in other bacterial infections (15–25).

After 30 days postinfection the Ab response is directed only against peptides that contain amino acids representing variable regions. It is likely that the Abs are reacting to the variable amino acids within these peptides because the adjacent peptides that overlap with those conserved sequences are not recognized (Table I and Fig. 2). In a few instances, however, such as Ab reactivity to peptides 21 and 26, both the conserved and variable amino acids within a peptide seem necessary to constitute the proper epitope, because neither the peptides overlapping only the variable or only the conserved amino acids are reactive. It is also possible that this striking lack of response to conserved sequences may be due in part to the fact that conformational epitopes are not detected by this peptide assay. Nevertheless, in distinct contrast to the T cell response, several peptides representing variable regions are targets of the humoral response.

Also in contrast to the T cell response, which consistently recognizes the same peptides, Abs recognize different epitopes at different times postinfection (Figs. 1 and 2). The diversity in the humoral response is probably not due to TprK variation in these studies, because the peptides are homologous to the Seattle Nichols laboratory strain that was used to infect the rabbits. Unlike most *T. pallidum* isolates that have heterogeneous TprK sequences within an isolate, the laboratory Nichols strain seems to have only one TprK sequence (8, 10, 11). In separate experiments groups of rabbits were infected, and serial sera were collected from each animal. In these studies after 30 days of infection there was no shift in peptides recognized during the course of infection by sera from each individual. There were, however, differences in the peptides recognized by sera from the different outbred rabbits. Similar variation might exist among genetically diverse humans.

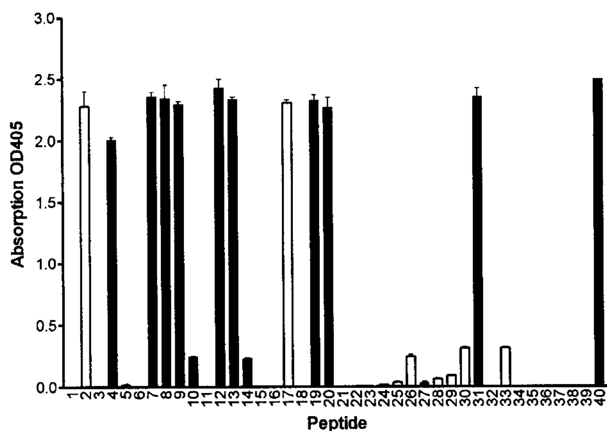

FIGURE 3. Immunogenic B cell epitopes. Pooled sera collected from rabbits immunized with the three TprK fragments were used in ELISAs to determine which regions of TprK are capable of eliciting Ab responses. Filled bars represent peptides containing only amino acids in the conserved regions. Open bars represent peptides containing amino acids in the variable regions. In contrast to infection, Abs that developed following immunization are directed against both conserved and variable regions throughout TprK.

In rabbits immunized with recombinant TprK, Abs are raised against peptides from both the conserved and variable regions (Fig. 3). The recombinant TprK fragments are most likely folded and exposed to the immune system in a different manner than native TprK, thus allowing the conserved regions of recombinant TprK to be more exposed. These data suggest that the conserved regions are inherently as immunogenic as the variable regions, but are not readily exposed later in infection. In infected rabbits, Abs to conserved regions are demonstrable only at 30 days postinfection, immediately following massive treponemal killing and clearance (Fig. 2). During bacterial clearance conserved regions of TprK may be available in larger quantity for B cell recognition, while the variable regions may be more accessible for B cell stimulation in intact organisms later in infection. After the antigenic mass is cleared during resolution of the primary stage, the B cell response may be refocused to those regions exposed on the surface of persistent, intact treponemes and thus more readily available to B cells. The fact that both T and B cell responses were detectable for at least 280 days suggests that TprK is expressed throughout the course of infection (Figs. 1 and 2).

That TprK diversity is limited to discrete regions of the protein is consistent with the hypothesis that the conserved regions may be essential for structural integrity. Analogies to other bacteria with molecules that have similar discrete variable regions suggest a possible surface exposure for the variable regions while the conserved regions are intramembrane domains. The discrete variable regions in neisserial Por and Opa, *H. influenzae* P2 and P5, *C. trachomatis* MOMP, and *B. burgdorferi* VlsE are all predicted to be surface-exposed loops (15, 21, 22, 25–27). Under immunological pressure it is advantageous for antigenic targets not essential for structural integrity to be hypermutable. The mutations may also give the pathogen functional advantages as seen with the surface-exposed variants of the neisserial Opa proteins that specifically bind to different tissues (23).

Because Abs are directed toward the variable regions of TprK, treponemes that express a new variant of TprK may escape recognition. The Ab response directed to variable regions of TprK may help explain the lack of heterologous protection in rabbits and humans (5, 6), as heterologous isolates do not have identical TprK variants (11). Another protein with similar discrete variable regions, *C. trachomatis* MOMP, has been implicated in conferring strain-specific immunity in humans (28).

It is known, however, that the Ab response alone does not confer complete protection. Passive Ab transfers from rabbits that are immune to homologous infection (infected for >3 mo) have indicated that Abs can delay and alter lesion development, but do not prevent infection (29). Although the lack of complete protection may in part be due to treponemes with new TprK variants escaping the Ab responses, it is likely that a T cell response is also important to protect against syphilis. Although adoptive transfer of T cells is impossible because the animal model is outbred, there is clear evidence of the involvement of a strong T cell response during infection. Activated T cells and macrophages infiltrate primary and secondary syphilitic lesions and clear opsonized treponemes, resulting in lesion resolution (1–4). The T lymphocyte responses to TprK were directed against conserved regions that were recognized by all infected rabbits (Fig. 1). These conserved TprK T cell epitopes may prove useful in a subunit vaccine. However, it is most likely that complete heterologous protection would require not only a T cell response, but also a cross-protective B cell response that would be unlikely with a single TprK.

Despite its small genome, *T. pallidum* evades the immune system and invades a variety of tissues. Perhaps because of its minimal genome and its metabolic dependence on the host, it is under great pressure to develop mechanisms for antigenic diversity, thus enhancing survival within the host. We demonstrated that the Ab responses during infection are directed to variable regions, which may affect tissue tropism, persistence, and the lack of heterologous protection. Our identification of vigorous T cell responses to conserved epitopes may be exploited in a multicomponent vaccine.

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