INFECTION AND IMMUNITY, July 1996, p. 2457–2466 Vol. 64, No. 7
0019-9567/96/$04.00+0

Epitope Mapping of B-Cell Determinants on the 15-Kilodalton Lipoprotein of *Treponema pallidum* (Tpp15) with Synthetic Peptides

ROBERT E. BAUGHN,[1,2,3]* MATTHEW DEMECS,[3] LARRY H. TABER,[4] AND DANIEL M. MUSHER[1,3,5]

Departments of Microbiology and Immunology,[1] Dermatology,[2] Pediatrics,[4] and Medicine,[5] Baylor College of Medicine, and the Syphilis Research and Infectious Diseases Laboratories, Veterans Affairs Medical Center,[4] Houston, Texas 77030

Received 12 February 1996/Returned for modification 28 March 1996/Accepted 22 April 1996

The antigenicity of the 15-kDa lipoprotein of *Treponema pallidum* (Tpp15 or TpN15) was comprehensively evaluated in epitope-scanning studies with overlapping deca- and octapeptides and polyclonal rabbit and human infant immunoglobulins (Igs) and antisera. This approach enabled us to identify potentially important regions and to determine the optimal dilutions of Igs or antisera for use in further studies. IgM and IgG from both species were capable of recognizing multiple, continuous epitopes. A total of 13 peptides, principally clustered in the central regions of the protein, were recognized by all syphilitic sera and Ig fractions. On the basis of window analyses, frequency profiles, and alanine substitution studies, five heptapeptides were selected for mimetic studies. Two of these five immunodominant, continuous epitopes initially appeared to be species specific; however, antisera elicited against mimetics of all five epitopes were polyspecific, recognizing similar motifs on several other treponemal proteins, including those of avirulent organisms. The only mimetic which yielded positive reactions with infant IgM and syphilitic sera in the absence of cross-reactions with rabbit antisera to avirulent treponemes was the variant of the VMYASSG motif. These findings are relevant to the development of simple, inexpensive assays for the serodiagnosis of active syphilis.

Treponema pallidum subsp. *pallidum* is one of a few select pathogenic bacteria that have not been cultured continuously in vitro. This obstacle has impaired our knowledge of structure-physiologic function relationships, the contribution of individual polypeptides to the immunopathogenesis of syphilis, and the design and development of both suitable vaccine candidates and specific diagnostic reagents. With respect to the latter, congenital disease in the newborn often presents as a diagnostic dilemma in that ≥50% of infants are asymptomatic and conventional assay methods fail to distinguish between the infant's antibody response and transplacentally acquired maternal immunoglobulin G (IgG). Thus, a need exists for a simple and sensitive, yet inexpensive immunoassay for the detection of early congenital syphilis.

In recent years, investigators have recommended the use of IgM-immunoblot techniques (33, 46) as an adjunct for the diagnosis of problem cases. Although IgM reactions with the 47-, 17-, and 15-kDa lipoproteins of *T. pallidum* on immunoblots appear to correlate well with active infection, with a low incidence of false-positive reactions, the test is rather costly and not widely available. These considerations, together with stated objectives of the Sexually Transmitted Disease Diagnostics Initiative Group (49), led us to undertake epitope-mapping studies of *T. pallidum's* membrane-associated lipoproteins in the hope that synthetic peptides corresponding to immunodominant epitopes of those antigens might be useful for diagnostic purposes. This approach seemed warranted in that synthetic peptides have been used successfully as diagnostic tools for assessment of responses to various viral and bacterial agents (16, 22, 34, 53, 57).

* Corresponding author. Mailing address: Syphilis Research Laboratory, Bldg. 109, Room 234A, VA Medical Center, 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 791-1414, ext. 5876. Fax: (713) 790-9141.

With the intent of eventually mapping B-cell epitopes on all three of these diagnostically relevant proteins, we began with the smallest, Tpp15. We report here the results of the study. Identification and mapping of the immunodominant B-cell epitopes of Tpp15 was carried out with an overlapping peptide approach. Other aims of this study included (i) determining whether differences between isotypes or between species in terms of peptide recognition existed, (ii) determining if any cross-reactive epitopes were shared by Tpp15 and endogenous host proteins, and (iii) analyzing the IgM immune responses of infants with congenital syphilitic infection by using synthetic peptides corresponding to potentially relevant sequences.

MATERIALS AND METHODS

Organisms. The Nichols strain of *T. pallidum* subsp. *pallidum* was propagated by intratesticular passage in New Zealand White male rabbits. Organisms for intravenous (i.v.) and intradermal infection and for immunologic studies were extracted from infected testes and separated from host tissue contaminants by gradient density centrifugation with 40% Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) containing 0.2 mM dithiothreitol as previously described (4). Suspensions of *T. pallidum* proteins in phosphate-buffered saline (PBS [pH 7.2]) for preparation of immunosorbent columns and for use in microassays were allowed to stand overnight at 4°C in the presence of an enzyme inhibitor mixture containing 200 kallikrein inhibitor units of aprotinin (Trasylol; FBA Pharmaceuticals, New York, N.Y.) per ml and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) prior to sonication (5). Solubilized treponemal antigens for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as previously described (4–7).

Suspensions of avirulent spirochetes of *Treponema refringens*, *Treponema vincentii*, and *Treponema phagedenis* biotype Reiter were prepared by cultivation in Spirolate brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.), supplemented with 10% normal rabbit serum or with 10% fetal calf serum, at 35°C for 5 to 7 days (5). Organisms were harvested with PBS (pH 7.2) and finally resuspended in either Tris hydrochloride reducing sample buffer or 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer.

Peptide synthesis. The amino acid sequence of Tpp15 as deduced from the DNA sequence by Purcell et al. (44 [NBRF-PIR accession no. S12777]) was used to synthesize overlapping 10-mer or decapeptides (overlap, 9; offset, 1) covering the entire sequence, which included the leader peptide. This lipoprotein, which consists of a 17-amino-acid signal peptide, a consensus signal peptidase II cleav-

TABLE 1. Amino acid sequences of mimetics on the basis of selected TpN15 motifs

Parent motif	Peptide for[a]:		
	Immunization and biotinylation	ELISA testing	Affinity purification
^{53}MVQVVYD^{59}	acMVQVVYDC	acMVQVVYDGSGSK	acMVQVVYDK
^{73}DYHRVMY^{79}	acDYARVMYC	acDYARVMYGSGSK	acDYARVMYK
^{77}VMYASSG^{83}	acVMYASSGC	acVMYASSGSGSK	acVMYASSGK
^{87}EKAFREL^{93}	acEAAFRELC	acEAAFRELGSGSK	acEAAFRELK
^{109}VTGATVS^{115}	acVTGATVSC	acVTGATVSGSK	acVTGATVSK

[a] Mimetic changes, in addition to acetylation of all amino-terminal residues, are underlined. All of these mimetics are protected by U.S. patent application no. 08/452,409.

age site, and a mature protein containing 124 amino acids, also is frequently designated TpN15 (41). This "general net" synthesis was carried out with commercially available epitope-scanning kits (Chiron Mimotopes Peptide Systems, Raleigh, N.C.) (23–25). The chemicals used were of high-performance liquid chromatography grade or the best available grade. The synthesis design and the detailed procedure were carried out according to the software and manuals supplied. A total of 132 overlapping decapeptides were synthesized in duplicate with Fmoc (9-fluorenylmethoxycarbonyl) chemistry. The success of the syntheses was monitored by the simultaneous synthesis of positive (PLAQ) and negative (GLAQ) control peptides and subsequent testing of their reactivity with test monoclonal antibodies supplied by the manufacturer. Another peptide set consisting of 134 overlapping octapeptides homologous with the sequence and again offset by 1 amino acid was synthesized for confirmational and verifactory purposes.

After scanning studies by indirect enzyme-linked immunosorbent assay (ELISA) (described below), which were aimed at identifying reactive epitopes, the second phase of our strategy involved the synthesis of other peptide sets by a "window net" approach. Overlapping 5-, 6-, 7-, and 8-mer peptides spanning potentially important epitope regions were synthesized to identify the precise boundaries of the antigenic sites upon subsequent ELISA testing. The third strategic phase involved the synthesis of peptide sets comprising alanine-substituted analogs of selected, parent peptides. For each reactive peptide, alanine was substituted individually for each residue one at a time along the reactive peptide sequence, and alanine in the original sequence was replaced by glycine. The replacement peptide sets also were subjected to repeated ELISA testing with animal and human sera or Igs.

On the basis of the results of the ELISA with each of the peptide sets described above, individual peptide motifs that appeared to be important because of their reaction frequencies with rabbit and infant Ig (Table 1) were selected for further study by a mimetic approach. For animal immunization studies and for chemical cross-linking to HiTrap *N*-hydroxysuccinimidyl (NHS)-activated Sepharose (Pharmacia Biotech, Inc., Piscataway, N.J.), which required small-scale production, these mimetic motifs were synthesized with cleavable peptide kits (Chiron). In these syntheses, cysteine (C) or lysine (K) residues were placed at the carboxyl termini to facilitate coupling to tetanus toxoid (TT) or NHS-activated Sepharose and bovine serum albumin (BSA), respectively. Large-scale synthesis (100 to 300 mg) of selected mimetic motifs with spacer arms (such as Gly-Ser-Gly) and either a carboxyl-terminal C or K residue was performed by the Protein Chemistry Core Facility at Baylor College of Medicine. Mimetic peptides prior to chemical cross-linking were purified by fast-performance liquid chromatography (FPLC) with a PepRPC HR 5/5 column (Pharmacia).

Chemical cross-linking or coupling of mimetics to carrier proteins. The heterobifunctional reagent MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester [22310; Pierce Chemical Co., Rockford, Ill.]) was used to couple selected mimetics (Table 1) containing carboxyl-terminal Cys residues (no spacer arm) to TT (Wyeth Laboratories, Inc., Marietta, Pa.) for immunization according to the instructions of the manufacturer. Briefly, conjugation was a two-step procedure in which the carrier protein (TT) was first linked to MBS (30, 35). The MBS-TT conjugate was purified on prepacked PD-10 columns (Pharmacia) and then individually cross-linked to each of the Cys-containing mimetics.

For ELISA testing, mimetic variants (Table 1) were coupled to another carrier by means of a different coupling agent, to avoid the possible interference of linker-specific antibodies (15, 45). For conjugation, mimetic variants with spacer arms and a carboxyl-terminal Lys were first modified with the heterobifunctional cross-linker SPDP [*N*-succinimidyl 3-(2-pyridyldithio) propionate; 21557; Pierce] according to the manufacturer's instructions. Traut's reagent (2-iminothiolane; 26101; Pierce) was used at a 5× molar excess to introduce sulfhydryls on BSA. Conjugates of the two derivatized materials were then prepared by applying the 2-iminothiolane-treated carrier to a PD-10 column and allowing the eluant to drip into a tube containing the SPDP-treated mimetic. After incubation overnight at room temperature, uncoupled peptide was removed by repeated dialysis. Quantitative protein assays were performed with each of the conjugates (14) prior to ELISA testing.

Mimetics with spacer arms and carboxyl-terminal Lys residues (10 mg/ml) were also coupled to Hi-Trap NHS-activated 1-ml columns (Pharmacia) at a neutral pH according to the manufacturer's recommendations. These columns were then used to purify anti-peptide antibodies. FPLC runs employed an isocratic gradient with 75 mM Tris–0.15 M NaCl (pH 8.0) as buffer A and 100 mM glycine-HCl–0.5 M NaCl (pH 2.7) as the elution buffer, buffer B. Eluted fractions were immediately neutralized and stored at −20°C.

Immunization, infection, and bleeding of rabbits. Outbred New Zealand White male rabbits were acclimated to a room temperature of 18°C for 2 weeks prior to any test procedures. Baseline sera were obtained during this time period for Venereal Disease Research Laboratory testing and for the preparation of baseline IgM and IgG fractions. In each experimental series, a group of three rabbits received two intradermal injections (0.1 ml at each site) of 100 μg of an individual mimetic peptide coupled to TT and emulsified in an equal volume of Freund's complete adjuvant. These initial injections were followed by three sets of booster injections, each consisting of two injections of 100 μg of the same mimetic peptide coupled to TT and emulsified in an equal volume of Freund's incomplete adjuvant. These booster injections were administered at 14-day intervals. All animals were bled 7 days after each booster. Ten days after the last set of booster injections, rabbits were infected i.v. with 4×10^7 *T. pallidum* cells (5). Control animals for these infection studies underwent a series of four injections with Gly-Gly-Cys coupled to TT, the first emulsified in Freund's complete adjuvant and the last three emulsified in Freund's incomplete adjuvant. After infection, animals were maintained at 18°C, and their backs were shaved at least three times per week. Blood samples were obtained at 14-day intervals, and each rabbit was observed daily for lesion development, progression, and resolution (5). Serum and/or plasma (7 ml of blood per kg of body weight) samples were obtained at regular intervals after infection.

Antisera, Ig fragments, and affinity chromatography. In addition to the anti-peptide sera, described above, a variety of animal and commercial sera and Ig fragments were used in the present study. Rabbit sera stored in our laboratory at −80°C since 1982 include the following: serum, IgM, and IgG fractions from syphilitic rabbits bled weekly throughout the first 6 months of infection; post-reinfection samples; antisera to each of the avirulent treponemes; and a variety of antisera to human, rabbit, and bovine endogenous host proteins (4, 5, 52). IgG was purified from these samples by FPLC with HiTrap protein A affinity columns (Pharmacia) according to the manufacturer's recommendations. For the purification of IgM from these rabbit sera, a goat anti-rabbit μ-chain-specific IgG (Cappel Organon Teknika Corp., Durham, N.C.) was first chemically coupled to a protein A-agarose affinity column with dimethyl suberimidate (Schleicher & Schuell, Keene, N.H.). After application of serum samples, elution and reequilibration conditions were initiated according to the manufacturer's recommendations. Serum samples from infants with symptomatic congenital syphilis have also been stored at −80°C. These samples, which have been described previously (20, 32), were collected from September 1987 through December 1992. The QuikSep IgM rProtein G affinity method (Isolab, Akron, Ohio) was used to obtain IgM fractions from infant sera. Commercial monoclonal and polyclonal antibodies to bovine and human Fn and different collagens were obtained from Calbiochem (San Diego, Calif.) or Sigma Chemical Co. (St. Louis, Mo.).

Indirect peptide ELISAs. The four sets of immobilized peptides (overlapping deca-, octa-, window-, and alanine-substituted peptides) were assayed by ELISAs according to the instructions of the manufacturer, with polyclonal antisera or Ig fractions as first antibodies. Briefly, blocks of pins were first subjected to a blocking step by incubation for 1 h at room temperature with supercocktail PBST (2% BSA–0.1% Tween 20 in PBS [pH 7.2]). The blocks were then incubated overnight at 4°C with appropriate dilutions of antisera or Ig fractions in the supercocktail. After four washes with PBST, pins were exposed to appropriate dilutions of horseradish peroxidase-conjugated goat anti-rabbit subclass-specific secondary antibodies in the supercocktail. After four washes with 0.01 M PBST, the pins were placed in wells containing the substrate 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (Sigma Chemical Co.), and color development was allowed to proceed for approximately 1 h in the dark. Reactions were stopped by removing the blocks of pins, and the plates were then read at 405 nm in a microplate reader. Results were expressed in terms of optical density (OD) values. After the testing, antibodies bound to the peptides were removed by

ultrasonication (Branson model 3200) in hot (60°C) 0.1 M PBS containing 1% (wt/vol) SDS and 0.05% (vol/vol) 2-mercaptoethanol (pH 7.2).

Because of the complex nature of the polyclonal responses observed in divergent epitope-scanning studies, we applied the following algorithm to determine which peptides exhibited significant reactivities with syphilitic IgM and IgG. With each set of results, values were sorted into two groups: the 50% most reactive and the 50% least reactive. The mean for the least reactive (or lower half of all values) was then calculated. That value plus 3 standard deviations was considered the cutoff value for that set of OD readings, and all values above the cutoff, irrespective of dilution or time of sampling, were considered significant.

Mimetic peptide ELISAs. An extensive series of preliminary studies were conducted to determine the most ideal methodology for measuring antibody responses to synthetic mimetics. Those studies included the following initial or coating steps: (i) chemically coupling each of the peptides to Covalink (Nunc, InterMed, Kamstrup, Denmark) plates and to Costar plates with an *N*-oxysuccinimide–amine binding surface (Fisher Scientific, Pittsburgh, Pa.), (ii) allowing the peptides to simply adsorb to microtiter plates, (iii) biotinylation of peptides for use with streptavidin-coated plates, and (iv) chemically coupling peptides to protein carriers. Because the latter appeared to yield the most sensitive (low levels of nonspecific binding) and reproducible results, mimetics of selected Tpp15 motifs (Table 1) after synthesis were coupled to BSA as described above. These mimetope-BSA conjugates, in concentrations ranging from 2 to 100 μg/ml in 0.05 M carbonate buffer (pH 9.6), were adsorbed to microtiter plates (100 μl per well overnight at 4°C) in order to establish the optimal concentrations for testing. Unsaturated protein binding sites were blocked with 3% BSA in PBS for 1 h at room temperature. All of the following incubations were performed for 1 h at room temperature, and the plates were washed four times with PBS between each step. Duplicates of rabbit syphilitic sera in twofold serial dilutions (1:100 through 1:1,600) in PBS with 0.5% BSA were added to the plates for "checkerboard" analysis. Alkaline phosphatase-conjugated goat anti-rabbit γ- or μ-chain-specific Ig and *p*-nitrophenyl phosphate were used to detect specific antibody binding. Cutoff values for positive samples were calculated as the mean OD value plus 3 standard deviations of 10 baseline serum samples drawn from uninfected rabbits or five IgM fractions from normal infants. After the optimal concentration of each mimetic-BSA conjugate for ELISA testing had been established, the sera and Ig fractions were subjected to end point dilution ELISA analysis.

Other procedures. Dot immunoblotting of mimetic-BSA conjugates was carried out according to the procedure described by Berzins et al. (11). For detection of antibodies reacting with protein residues modified by the coupling reagents, the tripeptide GGK coupled to BSA was used as a control antigen. Immunoblotting of soluble treponemal proteins and endogenous host antigens separated by SDS-PAGE (reducing conditions) was performed as previously described (4, 6). Serologic tests for syphilis (rapid plasma reagin; Venereal Disease Research Laboratory) and fluorescent treponemal antibody-adsorbed tests) were carried out according to standard procedures (51). Levels of IgM and IgG in FPLC-purified fractions were quantified by μ- and γ-capture ELISAs, which have been described previously (5, 32).

Statistics and computer-assisted protein analysis. All data were stored in SigmaPlot for Windows, Origin, and Microsoft Excel databases, and statistical analyses were performed with those software programs. Hydrophobicity analysis, motif identity searches, protein subsequence searches, and secondary structure predictions were carried out with HIBIO DNASIS/PROSIS Software (Hitachi Software Engineering America, Ltd., Brisbane, Calif.) or, alternatively, by accessing tools such as BLASTP (1) via links available on the worldwide web (www) at the site http://www.bichem.purdue.edu/rt95.06.16/research_tools.html.

RESULTS

Comprehensive scanning of the immunogenicity of Tpp15. To analyze the complex polyclonal antibody response to Tpp15, antisera and Ig fractions from *T. pallidum*-infected or syphilitic rabbits, obtained at various time intervals after i.v. infection, were assayed with overlapping decapeptide homologs of the Tpp15 sequence in a solid-phase ELISA. One of the major problems associated with the initial or preliminary scans with the decapeptides was the fact that discordant ELISA results were obtained with respect to IgM reactivity between whole sera and IgM fractions, with the latter yielding not only fewer reactions to individual peptides but significantly reduced (>50%) OD readings. Representative results with the 132 overlapping decapeptides, such as those presented in Fig. 1A to C, illustrate this discordance. IgM and IgG fractions were prepared from the serum samples of six rabbits 21 days post-i.v. infection and were standardized by class-specific capture assays to contain 10 mg/ml. As shown in Fig. 1A and B, the individual isotype pools of those fractions were capable of recognizing multiple decapeptides; application of the algorithm to determine cutoff levels for significance yielded OD values of 0.048 and 0.07, respectively. However, when those peptides were retested for IgM reactivity with a mixture of the two subclass pools (Fig. 1C), many of the reactions were substantially lower, especially the reactions to peptides with their origin start points between positions 72 and 91. The reductions seen with peptides 77 to 79, for instance, were approximately 60% of those in Fig. 1A despite a rather strong correlation ($\rho_{A,C} = 0.839$). Overall, these results suggested that IgG antibodies to a particular peptide could interfere with and reduce IgM binding. In contrast, IgM did not seem to impact IgG reactivity (data not shown). IgG reactivity with the mixture at the same dilution or concentration yielded results similar to those in Fig. 1B ($\rho = 0.941$).

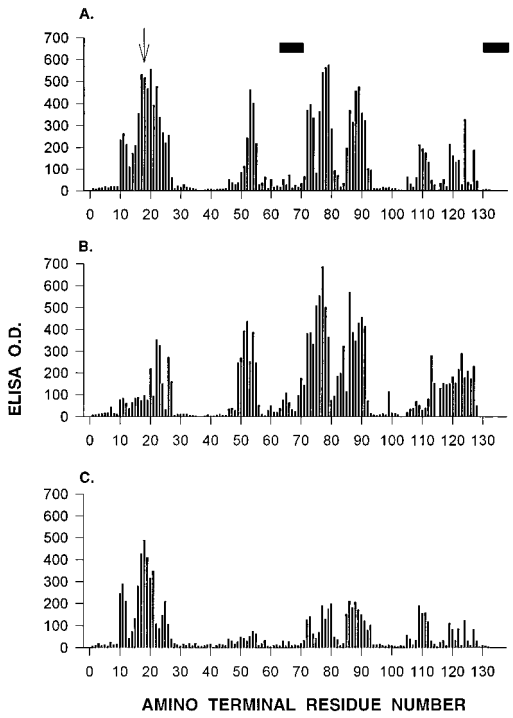

FIG. 1. Reactivities of syphilitic rabbit Igs with 132 overlapping decapeptides spanning the entire sequence of Tpp15. (A) Fourteen-day post-i.v. infection IgM (1:200 dilution); (B) 21-day post-i.v. infection IgG (1:200 dilution); (C) Mixture of 14-day IgM and 21-day IgG each at a final dilution of 1:200. The arrow in panel A indicates the consensus signal peptidase II cleavage site, which gives rise to a signal peptide and mature protein consisting of 17 and 124 residues, respectively, and the black bars represent the two predicted regions of B-cell determinants determined by the algorithm of Chou and Fasman (18) as described in reference 37. Values are expressed as 10^{-3} OD units.

Upon completion of the epitope mapping studies with the decapeptides, ELISAs with octapeptides were used to reexamine rabbit sera and Ig fractions in both individual and pooled samples. As would be expected, dramatic variations were seen with respect to the frequencies and titers of the two isotypes at different time points after infection. The IgG reactivity of pooled serum samples from the same six rabbits at 28 days post-i.v. infection is shown in Fig. 2A. Despite the time and dilution differences between the subclass fractions used in the construction of Fig. 1B and 2A, the results were quite similar. Statistically, the correlation between these two sets of data was $\rho = 0.864$.

The immunoreactive B-cell epitopes consistently fell into six discrete regions, irrespective of whether deca- or octapeptides were used in the scanning studies. Those regions spanning the

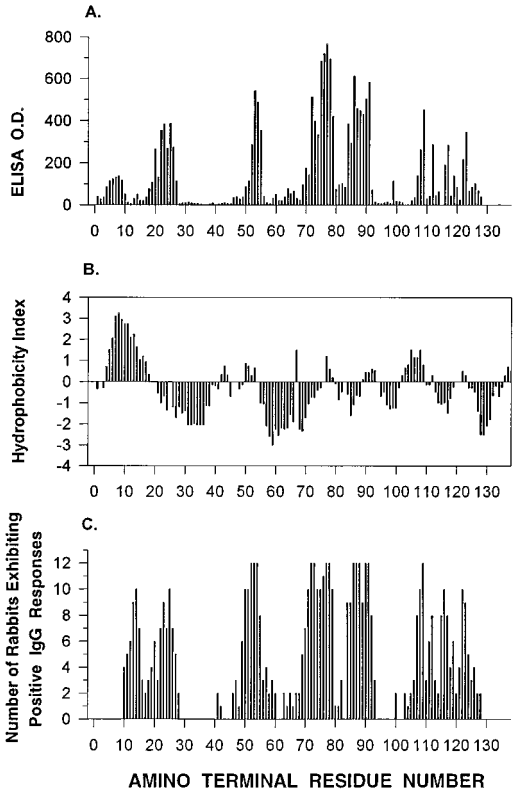

FIG. 2. Reactivities of syphilitic rabbit sera with 134 overlapping octapeptides spanning the entire sequence of Tpp15. (A) Twenty-eight-day post-i.v. infection IgG (1:400 dilution). Values are expressed as 10^{-3} OD units. (B) hydrophobicity plot with the algorithm of Kyte and Doolittle (31) and a window of 8. (C) Frequency profile of 12 individual 35-day post-i.v. infection serum samples (1:400 dilutions).

following amino acid start positions were as follows: I, 10 to 17; II, 20 to 27; III, 49 to 60; IV, 69 to 79; V, 84 to 92; VI, 107 to 113; and VII, 115 to 128. Negative stretches were at positions 1 to 9, 28 to 48, 61 to 68, 80 to 83, 94 to 104, and 129 to 132 or 134. Two regions of B-cell determinants, earlier predicted by Purcell et al. (reference 43 and shown by the black bars at the top of Fig. 1C), were actually within two of these negative stretches. Furthermore, there was no correlation between these results and hydrophobicity indices such as the one shown in Fig. 2B.

Serum samples from 12 individual rabbits, obtained 35 days post-i.v. infection, were then separately assayed at 1:400 dilutions with the overlapping octapeptide homologs. For each experiment, the algorithm ($\bar{x}$ the lower one-half of all values + 3 standard deviations) was applied to determine which ELISA values were significant. The combined results obtained in those experiments were then used to construct the frequency profile plot shown in Fig. 2C. Thirteen peptides were recognized by all 12 serum samples tested. The amino acid start positions for these octapeptides were 52, 53, 54, 72, 73, 77, 78, 86, 87, 88, 90, 91, and 109. Twelve other peptides were recognized by 10 or 11 of the 12 serum samples tested. Both the mean titer and frequency of those 25 most reactive peptides exceeded those of the 25 least reactive ($P < 0.001$ and $P < 0.001$, respectively). Despite the fact that titers and reaction frequencies appeared to be closely associated, exceptions did occur. Of the 36 octapeptides recognized by only one to three of the serum samples, 13 had high titers (OD value, >0.4). Collectively, 45

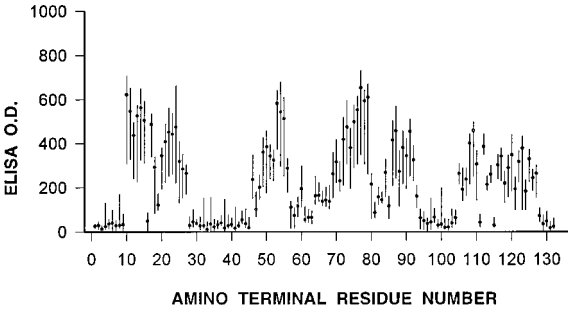

FIG. 3. Reactivities of IgM fractions from six infants with symptomatic congenital syphilis and the 134 overlapping octapeptides spanning the entire sequence of Tpp15. IgM fractions were each tested at a 1:200 dilution. Results are expressed as the geometric mean OD values (10^{-3}) of antibody responses and the ranges of reactivity.

peptides were recognized by more than one-half of the serum samples tested, while 41 peptides (approximately one-third) were not recognized by any of the 12 serum samples.

The peptide sets also were tested for IgM reactivity in six separate experiments with IgM fractions obtained from the sera of infants with symptomatic congenital syphilis. Figure 3 shows the geometric mean antibody responses and the ranges of reactivity for the octapeptides based on the results of those experiments. Although the cutoff values of those six experiments differed slightly, the highest OD value obtained was 0.064. More importantly, each of the infant IgM fractions reacted with the 13 octapeptides recognized by all 12 rabbits (Fig. 2C). The only major discrepancy with respect to species differences was that three additional octapeptides (start positions 10, 11, and 14) were recognized by all six infant IgMs. None of the octapeptides exhibited positive reactions in assays employing IgM fractions from normal infants (data not shown). A good correlation was noted between these geometric mean responses and rabbit IgM (Fig. 1A; $\rho = 0.71$) and IgG (Fig. 2A; $\rho = 0.74$) responses measured 14 or 28 days postinfection, respectively. Exclusion of the first 17 peptides, corresponding to the leader peptide of 17 amino acids (44), from all data calculations resulted in an improved correlation between IgM (Fig. 1A; $\rho = 0.83$) and IgG (Fig. 2A; $\rho = 0.88$).

Testing of deca- and octapeptides with IgGs from the sera of animals immunized against *T. phagedenis* biotype Reiter, *T. vincentii*, and *T. refringens* indicated that species-nonspecific motifs did exist (data not shown). Reactions occurred with peptides in regions II, III, and VII despite the fact that they did not react with the 15-kDa lipoprotein of *T. pallidum* in immunoblots. Correlation values (ρ) between the responses with these IgG and anti-*T. pallidum* responses, irrespective of source, were <0.34. Baseline Igs and sera, in addition to antisera to fibronectins, collagens, and cardiolipin, failed to yield significant IgM or IgG reactions with either deca- or octapeptides.

To confirm these results and narrow the windows of potentially important epitopes, 5-, 6-, 7- and 8-mer peptides within each of the immunoreactive regions were synthesized for further ELISA testing. Representative results with the peptides within regions IV and V and pooled rabbit sera 35 days postinfection are shown in Fig. 4. With few exceptions, significant binding differences ($P < 0.05$) were not seen between the 7- and 8-mers of the most reactive peptides. Heptapeptides uniformly exhibited stronger, though not significant ($P > 0.05$) reactions when reacted with pooled infant IgM. As shown in the figure, the general trend observed in ELISA studies with

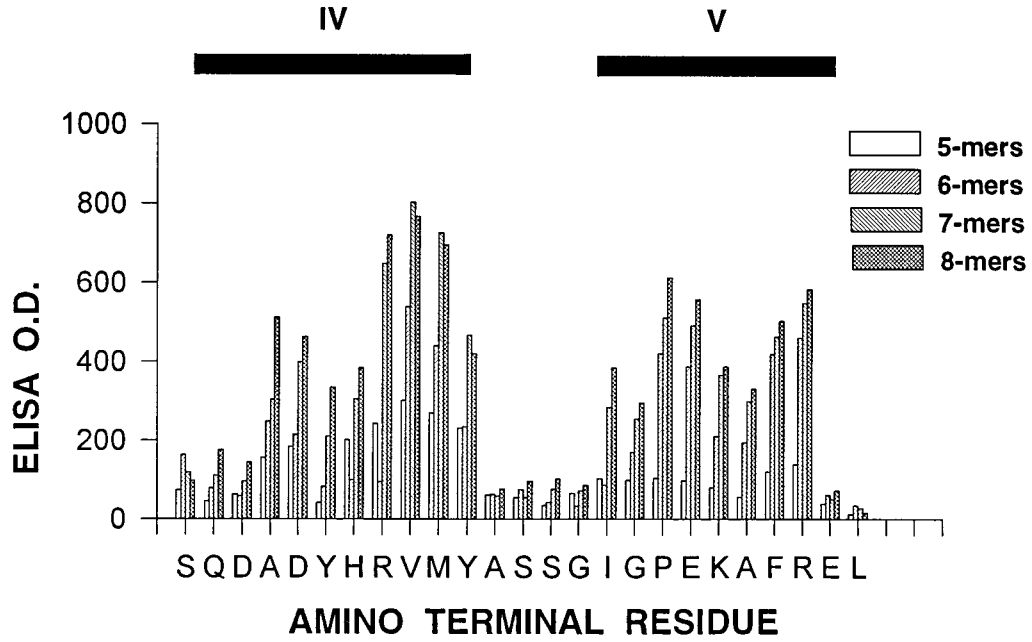

FIG. 4. IgG reactivities of pooled 35-day post-i.v. infection rabbit sera with penta-, hexa-, hepta-, and octapeptides within regions IV and V of Tpp15. Values are expressed as 10^{-3} OD units.

these window peptides was that 6-mers uniformly yielded lower OD readings, and 5-mers, when reactive, were significantly ($P < 0.05$) less so than the 7- and 8-mers.

On the basis of the window analyses and frequency profiles, five sequences were deemed core motifs and selected for alanine substitution studies: ^{53}MVQVVYD^{59}, ^{73}DYHRVMY^{79}, ^{77}VMYASSG^{83}, ^{87}EKAFREL^{93}, and ^{109}VTGATVS^{115}. Despite the fact that both the first and last sequences did not appear to be species specific, heptapeptides of these core motifs were resynthesized with Ala replacements in each of the positions and subjected to repeated ELISA testing. Representative results of four separate experiments (each with a different serum or IgM fraction pool) with three of these sequences are presented in Table 2. As disclosed in these and other ELISAs, substitution of Ala for His in the third position of the first motif unexpectedly resulted in increased antibody binding in all experiments. With this motif, only the substitution of Asp with Ala was associated with significant (>20%) decreases in reactivity. The substitution of Ala for Met, Tyr, or the second Ser in the motif ^{77}VMYASSG^{83}, in contrast, resulted in decreased antibody binding, suggesting that all three of those amino acids were critical for antibody recognition. Alanine replacements at the other positions had essentially no effect on antibody binding. Replacement of the Lys with Ala in the ^{87}EKAFREL^{93} motif in these and other ELISAs resulted in increased antibody binding, whereas substitutions of the Phe or the second Glu residues led to decreased antibody binding. ELISA testing also was carried out with the Ala substitution peptides of the other two motifs. With respect to ^{53}MVQV VYD^{59} and ^{109}VTGATVS^{115}, the sequences that appeared critical for antibody binding were M-Q--YD and -T--TVS, respectively (data not shown).

Mimetic studies with selected motifs. Upon completion of the studies described above, mimetics of each of the five core motifs were synthesized so that we might develop reagents for solid-phase peptide-ELISA testing, including rabbit antibodies for a capture assay and for standardization. Each mimetic along with a control trimer (GGC) was purified by FPLC on a PepRPC column and chemically coupled with MBS (via the carboxyl-terminal Cys which had been added) to TT. Each preparation was then used to immunize three rabbits. Rabbits were bled 7 days after the final booster and then infected with 4×10^7 *T. pallidum* cells. Compared with the controls, who developed rashes 19 to 22 days postchallenge and lesions by day 23, lesion development was accelerated in all immunized test animals. Rashes and lesions were evident by days 14 and 17, respectively. No differences were noted with respect to the numbers of disseminated lesions or their progression to induration, ulceration, and necrosis.

Serum samples from the test animals obtained 7 days after the last mimetic booster were examined by a variety of immunological procedures. In an attempt to quantify the anti-peptide responses, end point dilution ELISAs were constructed with each of the motif mimetics (Table 1, column 3) cross-linked to derivatized BSA. Earlier dose-response ELISAs with each of the peptide-BSA conjugates indicated that 100 μl of concentrations between 20 and 40 μg/ml was optimal for the coating of wells (data not shown). Control wells were coated with acGGK-derivatized BSA to determine background values, which were then subtracted from test values. The results of representative experiments with the core motifs ^{77}VM YASSG^{83} and ^{109}VTGATVS^{115} are shown in Fig. 5. Experiments with these and the other mimetics were similar in that 1:800 to 1:1,600 dilutions of each serum yielded results within the linear portion of the dilution curve with the respective homolog of the variant used for immunization. As shown in both of these representative examples, cross-reactions did not occur at dilutions greater than 1:200.

Sera and affinity-purified IgG fractions also were used to develop immunoblots of treponemal and endogenous host proteins. None of the sera or their respective IgG fractions were monospecific for a single *T. pallidum* antigen. As shown in Fig. 6, representative IgG fractions at concentrations of 12.5 μg per strip (roughly equivalent to a 1:800 serum dilution) yielded

TABLE 2. Effect of alanine replacements on antibody binding by different motifs

Ala replacement peptide[a]	Effect (10^{-3} OD units) on binding in fraction at dilution given[b]			
	IgG postinfection			Infant IgM (1:200)
	21 days (1:200)	28 days (1:400)	35 days (1:400)	
^{73}DYHRVMY^{79} (parent)	312	348	406	445
AYHRVMY	228 (−27)	264 (−24)	328 (−19)	320 (−28)
DAHRVMY	306 (NC)	314 (−10)	370 (−9)	531 (+19)
DYARVMY	397 (+27)	453 (+30)	359 (+38)	559 (+38)
DYHAVMY	296 (−5)	320 (−8)	406 (NC)	495 (+11)
DYHRAMY	281 (−10)	327 (−6)	359 (−12)	415 (−6)
DYHRVAY	291 (−7)	350 (NC)	413 (NC)	406 (−9)
DYHRVMA	268 (−14)	288 (−17)	347 (−15)	427 (−4)
^{77}VMYASSG^{83} (parent)	515	756	810	686
AMYASSG	489 (−5)	701 (−7)	745 (−8)	651 (−5)
VAYASSG	384 (−25)	335 (−56)	412 (−49)	412 (−40)
VMAASSG	249 (−52)	455 (−40)	466 (−42)	507 (−26)
VMYGASG	493 (NC)	780 (NC)	752 (−7)	661 (NC)
VMYAASG	489 (−5)	805 (+6)	769 (−5)	719 (+5)
VMYASAG	368 (−29)	514 (−32)	454 (−44)	548 (−20)
VMYASSA	493 (−4)	796 (+5)	748 (−8)	658 (−4)
^{87}EKAFREL^{93} (parent)	412	467	503	309
AKAFREL	408 (NC)	449 (NC)	538 (+7)	321 (NC)
EAAFREL	493 (+20)	663 (+42)	825 (+64)	408 (+32)
EKGFREL	450 (+9)	490 (+5)	528 (NC)	334 (+8)
EKAAREL	305 (−26)	281 (−40)	332 (−34)	230 (−26)
EKAFAEL	354 (−14)	397 (−15)	512 (NC)	265 (−14)
EKAFRAL	307 (−25)	318 (−32)	313 (−38)	197 (−36)
EKAFREA	397 (NC)	411 (−12)	468 (−7)	304 (NC)

[a] Positions in which Ala substitutions were made are underlined.
[b] Numbers in parentheses represent the percent positive or negative change in binding. NC, no change (<4% increase or decrease).

multiple reactions with several *T. pallidum* antigens. In each instance, the most intense reactions noted were to the 47- and 37-kDa proteins. The most disturbing aspect of the immunoblot studies was that reactions to the 15-kDa lipoprotein were faint or absent. Despite the fact that more intense reactions were noted with the 15-kDa antigen when the IgG fractions from all 15 test animals were tested at a concentration of 50 μg of IgG per strip, consistent reactions also were seen with 94-, 83-, 60-, 47-, 44.5-, 42-, and 37-kDa proteins of *T. pallidum*. Reactions with the 33- and 30-kDa materials were not consistently noted (5 of the 15 test fractions). At the higher concentration (50 μg per strip), IgG reactions did not differ from those seen with the respective sera from which they were obtained. All test IgGs cross-reacted with proteins present in

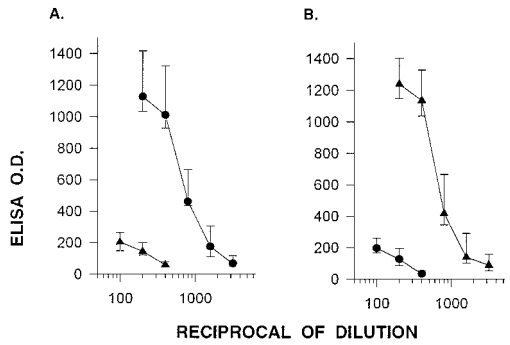

FIG. 5. End point dilution IgG ELISA with serum samples from six rabbits immunized with mimetics of either the VMYASSG or VTGATVS motif. Test wells of microtiter plates were coated with 100 μl of acVMYASSGSGSK-BSA (A [40 μg/ml]) or acVTGATVSGSGSK-BSA (B [40 μg/ml]). Control wells (A and B) to control for background (<0.07 OD units in all instances) were coated with acGGK-derivatized BSA (40 μg/ml). Responses at each dilution are expressed as the geometric mean and ranges of three animals immunized with acVMYASSGC-TT (●) and three animals immunized with acVTGATVSC-TT (▲). Values are expressed as 10^{-3} OD units.

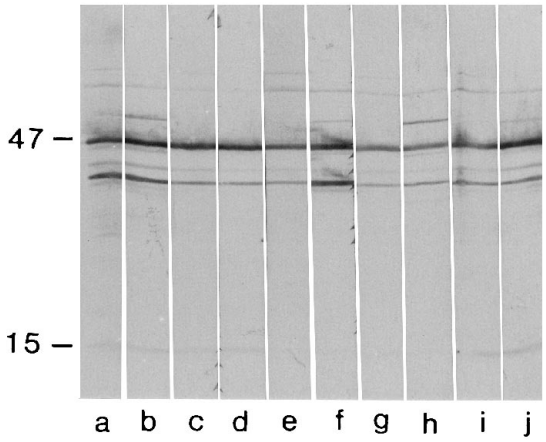

FIG. 6. Immunoblots of *T. pallidum* reacted with IgG fractions from animals immunized with mimetics of selected Tpp15 motifs cross-linked to TT. Each strip was reacted with 12.5 μg of affinity-purified IgG and subsequently developed with horseradish peroxidase-conjugated goat anti-rabbit IgG. Lanes: a and b, two rabbits immunized with acVMYASSGC-TT; c and d, two rabbits immunized with acDYARVMYC-TT; e and f, two rabbits immunized with MVQVVYDC-TT; g and h, two rabbits immunized with VTGATVSC-TT; i and j, two rabbits immunized with EAAFRELC-TT. Molecular mass markers (47 and 15 kDa) are shown on the left.

TABLE 3. ELISA results with mimetics of selected TpN15 motifs coupled to BSA

Mimetic used for coating of plates (20 μg of protein/ml)	Effect (10^{-3} OD units) with fraction[a]:				
	14-day postinfection IgM (n = 6)	28-day postinfection IgG (n = 12)	Anti-*T. phagedenis* IgG (n = 6)	Anti-*T. refringens* IgG (n = 6)	Congenital syphilis infant IgM (n = 6)
acMVQVVDGSGSK-BSA	123 (94–212)	340 (263–519)	327 (233–460)	269 (187–378)	178[b] (64–279)
acDYARVMYGSGSK-BSA	268 (152–369)	248 (187–403)	67 (28–104)	138 (19–186)	273 (139–408)
acVMYASSGSGSK-BSA	324 (197–411)	486 (280–564)	44 (17–83)	51 (21–99)	296 (178–512)
acEAAFRELGSGSK-BSA[c]	255 (134–386)	356 (175–497)	143 (68–186)	108 (25–164)	235 (151–365)
acVTGATVSGSK-BSA[d]	165 (123–259)	59 (24–165)	210 (146–318)	164 (72–239)	114 (60–243)

[a] Results are expressed as the geometric mean of all OD (10^{-3}) values. Ranges of the ODs for each test group are in parentheses.
[b] One of the six infant IgM values was negative with this mimetic (i.e., below the cutoff value).
[c] Position in which Ala substitution was made is underlined.
[d] Five of the 28-day postinfection IgG values and two of the six infant IgM values were negative with this mimetic (i.e., below the cutoff values).

immunoblots of each of the three avirulent treponemes (data not shown). None of the IgG fractions yielded positive reactions with immunoblots of endogenous host proteins, including human and bovine fibronectin, fibronectin fragments, and type IV and VI human collagen. Baseline or preimmunization sera and sera from the three rabbits immunized with the control trimer GGC linked to TT were negative in each type of immunoblot.

Attempts to purify peptide-specific IgGs from these sera by FPLC affinity chromatography with mimetics (Table 1) cross-linked to HiTrap NHS columns were only moderately successful on the basis of immunoblot results. At concentrations of 50 μg per strip, all peptide-specific IgGs reacted with the 15-kDa antigen; however, faint reactions were also noted with the 94-, 83-, 60-, and 37-kDa antigens (data not shown). At concentrations of 12.5 μg per strip, the only peptide-specific IgGs yielding reactions with the 15-kDa lipoprotein were from the six animals immunized with variants of the DYHRVMY and VM YASSG motifs.

Mimetics also were individually coupled to BSA with SPDP and Traut's reagent for use in solid-phase microtiter ELISAs. After establishing the optimal conditions for ELISA testing with a checkerboard (criss-cross) approach with 28- and 35-day postinfection sera, efforts were directed at assessing the antibody responses of other infected and immunized animals to each of the motifs. Representative results with 36 serum samples or Ig fractions are shown in Table 3. For these studies, a control trimer (GGK) coupled to BSA served as the background control. At 1:100 dilutions, 10 baseline rabbit serum samples yielded OD readings of <0.07 with the derivatized control (data not shown). Diluent control values, additionally were in the range of 0.00 to 0.043 OD unit. The cutoff values (mean of normal values + 3 standard deviations) after subtraction of the derivatized control OD values for rabbit IgM, rabbit IgG, and infant IgM were 0.094, 0.083, and 0.089, respectively. The only mimetic which consistently yielded positive reactions with rabbit IgM, rabbit IgG, and infant IgM in the absence of cross-reactions with rabbit antibodies to avirulent treponemes was the variant of the VMYASSG motif. Rabbit antibodies to both avirulent strains of treponemes exhibited positive reactions with the other four mimetics. Five of the 12 28-day postinfection IgG fractions and 2 of the 6 infant IgM fractions did not react with the mimetic of VTGATVS. Rabbit antiserum to bovine and human fibronectin, human collagen (types IV and VI), and cardiolipin did not show a cross-reaction with any of these mimetics, either in ELISA or dot immunoblots.

DISCUSSION

The goals of this study were (i) to define linear B-cell determinants on the 15-kDa lipoprotein of *T. pallidum* recognized by both rabbit and human Igs and to determine whether (ii) isotypes from rabbits and infants recognized different epitopes, (iii) cross-reactive epitopes were shared by Tpp15 and endogenous host proteins such as fibronectin and collagen, and (iv) mimetics of potentially relevant epitopes could be adapted for use in the serodiagnosis of congenital syphilis. The lipoprotein Tpp15 was chosen for two reasons. First, this lipoprotein has been recognized as a sensitive antigenic target for IgM responses in congenital infection (33, 46). Second, Tpp15 is the smallest of the sequenced lipoproteins, so that mapping studies required the synthesis of fewer overlapping peptides, making it feasible to test decapeptides and confirm those findings with octapeptides. With this overlapping peptide approach, our data show that a number of different antigenic regions, each containing multiple epitopes, were detected when sera from infected rabbits and infants with symptomatic congenital syphilis were tested against both sets of peptides. This was not unexpected, because proteins in general display a variety of linear or continuous epitopes in polyclonal responses (3, 24, 28, 32, 48, 56). Clearly, both in the rabbit model and in infants with congenital disease, infections appear to stimulate the formation of several populations of antibodies to a variety of epitopes of Tpp15.

Overall, epitope scans with both deca- and octapeptides yielded unambiguous results, irrespective of the serum or Ig fraction tested. In most instances (>95%), there was no need to apply the algorithm to determine which responses were significant. Altogether, 13 peptides were recognized by sera (including IgG and IgM fractions) from all syphilitic rabbits studied as well as by IgM fractions of sera from all infants with congenital syphilis. Except for peptide 109 in region VI, these immunoreactive octapeptides were clustered in pairs or triplets in the central portion of the protein: peptides 52, 53, and 54 in region III-peptides 72, 73, 77, and 78 in region IV; and peptides 86, 87, 88, 90, and 91 in region V. The only species difference consistently noted was that all six infant IgM fractions also reacted with peptides 10, 11, and 14. Because these were within the region of the signal peptide immediately prior to the consensus signal peptidase II cleavage site and were not a part of the mature protein, they were excluded from subsequent mimetic work. Antibodies to those epitopes, which would not be present in the mature lipidated molecule, probably arise as a result of either extensive motif identity or intra- and intermolecular spreading (described below). Efforts to

demonstrate correlations between our data and hydrophilicity, probability of β-turn occurrence, or estimates of surface exposure were unsuccessful (data not shown). As noted by other investigators (47, 54), this simply may reflect the fact that predictive algorithms designed for water-soluble proteins are less than ideal for predictions with integral membrane proteins and are not much better than chance alone.

High interstudy correlation values for both deca- and octapeptides, irrespective of test dilution or serum or Ig source, were considered evidence that little allelic diversity exists in B-cell epitope preference either within or between species. In this respect, Tpp15 appears to differ from Tpp17 and several immunodominant regions of the larger Tpp47. Unpublished preliminary data (8) suggest that both of those proteins contain IgM-, IgG-, and species-restricted epitopes.

Baseline sera and Igs of both species, in addition to antisera to fibronectins, collagens, and cardiolipin did not show any IgM or IgG reactivity with either deca- or octapeptides. Thus, even though other linear peptide-based studies have shown high degrees of nonspecific IgM activities with irrelevant control sera (22, 32, 53), this did not appear to constitute a problem in the present study. The fact that IgG antibodies could block and significantly interfer with IgM reactivity was not unexpected on the basis of earlier treponemal ELISA studies (40). It did, however, indicate that test samples for IgM reactivity in subsequent epitope scans and mimetic ELISA should be fractionated to avoid competing reactions with higher-affinity IgGs.

Although 12 of the 13 high-frequency (100%) immunoreactive peptides were clustered in pairs or triplets, we felt that additional ELISAs with the peptides created in a window net synthesis were necessary to accurately identify core motifs for animal immunization and subsequent peptide-based solid-phase assays. Those studies enabled us to evaluate net absorbances as a function of relevant reactive and flanking negative peptides and to determine which amino acids in each case were common to all reactive peptides. On the basis of these studies, five core motifs were selected for Ala substitution studies so that we could determine which amino acids were critical for antibody recognition. As expected, decreases in antibody binding were observed with some of the replacement peptides. However, we did not expect increases in antibody binding as were seen with two of the five motifs: replacement of His with Ala in position 75 (DYHRVMY motif) and substitution of Ala for Lys in position 89 (EKKFREL motif). Although this phenomenon might have reflected the presence of heteroclitic antibodies in syphilitic sera, it is more likely that the mimetic changes allow for enhanced detection of cross-reactive antibodies. Despite extensive motif identity searches, however, we have no explanation for why this would occur with respect to the DYHRVMY motif. On the other hand, the change in position 89 would permit cross-reactive antibodies directed at the bacterial EAA motif to result in increased binding. This motif is highly conserved in hydrophobic membrane proteins of bacterial ABC transporters (10, 19, 27, 47) and has actually been postulated to contain signals of functional importance, such as a recognition site for an ATP-binding protein (47). In *Escherichia coli* Mg1C, RbsC, and AraH, this tripeptide is located at positions 218 to 220, 206 to 208, and 209 to 211, respectively. Thus, if antibodies were directed against the MgIC of *T. pallidum* or even against related proteins of unrelated bacteria, they might be expected to cross-react with the mimetic variant. Although Becker et al. (9) have shown similarity between the 38-kDa lipoprotein of *T. pallidum* and MgIB of *E. coli*, additional molecular studies are necessary to document the presence of the EAA motif in other treponemal sequences. Work is currently in progress in our laboratory to discern the extent of cross-reactions between antibodies directed at the EAAFREL motif and variant homologs in ABC transporters.

Because none of the mimetics were considered potential vaccine candidates, immunization studies with each of the individual mimetics were conducted primarily for the production of peptide-specific IgGs. Nonetheless, we were interested in determining the effect, if any, of peptide immunization on subsequent i.v. challenge. As in previous immunization experiments with endoflagella and antigen 4D (TpN19) (13, 17), lesion development in all instances was accelerated. However, unlike those studies, the lesions in our rabbits could not be described as atypical, nor could we detect substantial changes in end point anti-peptide IgG titers after challenge. Accelerated lesion development has been ascribed to an enhanced cellular immune response (13, 41). However, accelerated lesion development may be much more complicated. We have had no problem in demonstrating classical Arthus reactions at 6 to 24 h to a variety of different peptides after immunization (8). Thus, one can certainly question the role of insoluble complexes, complement fixation, and other early events in accelerated inflammatory reactions. An accelerated response seen after challenge also would be consistent with intra- and intermolecular spreading to T-cell epitopes of avirulent treponemes (12, 39).

In the present study, the IgG responses, irrespective of the immunizing mimetic, were clearly polyspecific. Immunization of rabbits with single peptide motifs in each instance led to polyspecific responses to a variety of *T. pallidum* antigens and proteins of the avirulent treponemes. These results suggest that extensive sequence identity between the immunizing mimetic and other treponemal proteins may exist, despite the lack of direct molecular evidence. At the present time, data bank information, especially for the proteins of the avirulent treponemes, is incomplete or nonexistent. Ohno's early theoretical study (42) suggested that "two totally unrelated proteins, on the average, share 30 identical tripeptides, two tetrapeptides, and one pentapeptide per 500 residues." For related proteins, particularly within a genus, this is probably an underestimation, and one cannot simply assume that an oligopeptide exists only at a single site on a single protein species (21). In fact, oligopeptides composed of similar residues can occur at greater frequencies than expected. Sequence analyses involving comparisons between the core motifs of Tpp15 chosen for mimetic work and Tpp47, however, failed to reveal any identical matches, despite the fact that 10 tripeptides are shared by both proteins. Additionally, cross-reactions were minimal in end point dilution ELISA studies with each of the motifs.

The IgM reactions with peptides within the signal peptide, a portion of the protein not present in the mature lipidated molecule, may reflect natural cross-reactive antibodies, especially in infected infants. Variants of the ^{14}LLGACSF^{20} motif, for instance, are ubiquitous on the basis of motif searches. Extensive matches occur with several proteins, including protein-tyrosine kinases, fibroblast growth factor receptors, keratinocyte growth factor receptors, and *N*-methyl-D-aspartate receptors. If natural IgM antibodies to any of these were present in serum, they could conceivably cross-react with peptides of unrelated proteins in epitope-scanning ELISA. Clearly, much additional work is necessary to resolve this issue. In the absence of replacement analyses, we do not even know which residues are critical for antibody recognition.

The expanded responses after immunization with Tpp15 motifs, especially the intense immunoblot reactions seen with

the 47- and 37-kDa proteins (Fig. 6), are difficult to explain in the absence of extensive motif identity. Baseline or preimmunization sera and sera from animals immunized with the control trimer GGC linked to TT were nonreactive in immunoblotting studies. Furthermore, because different lots of enzyme-labeled secondary antibody and their respective substrates failed to alter the results, it is unlikely that our findings represent immunoblotting artifacts. It is plausible that these polyspecific responses are due to intra- and intermolecular epitope spreading involving group-specific or function-specific motifs, such as those of ABC transporters of normal flora. Intramolecular spreading and intermolecular spreading, which appear to involve both B and T cells, have been described in persistent autoimmune responses and chronic infections to explain why initiation of immunity to a single component leads to persistent responses involving additional linked components (12, 29, 38, 39, 50). The diversity of the antibody responses after peptide immunization and the fact that those antibodies cross-reacted with proteins of all three avirulent organisms would be consistent with epitope spreading. However, much additional work is needed to determine if related organisms in the oral cavity and gastrointestinal tract are capable of contributing to and perpetuating epitope spreading to other motifs, including those that are highly conserved or that exhibit extensive identity.

Results obtained in the mimetic ELISA constitute additional evidence that the immunodominant motifs of Tpp15 examined were not species specific, nor were the antibodies directed against them. The only possible exception is the VMYASSG mimetic, which yielded negative reactions with antisera to both avirulent organisms. This conflicts with early studies which suggested that Tpp15 might represent a pathogen-specific protein (2, 26, 36, 55, 56). Such a conclusion was later supported by Tpp15's reaction with monoclonal antibodies and the absence of hybridization with genomic DNA from *T. phagedenis* biotype Reiter (43). These differences, however, are easy to explain. In the present study, relatively short peptides consisting of eight or fewer amino acid residues were used to define determinants recognized by antibodies from rabbits infected with *T. pallidum* as well as those from infants with symptomatic congenital syphilis. In contrast to polyclonal responses, monoclonal antibodies directed against conformational (discontinuous) epitopes or a core epitope larger than eight amino acids would in all likelihood not bind to short sequences on other treponemal proteins, including those of the avirulent organisms.

Despite the fact that the immunodominant motifs did not appear to be pathogen restricted, with the possible exception of VMYASSG, the results obtained with infant IgM in the mimetic ELISAs are encouraging. For detection by ELISA, however, it was necessary to covalently link each mimetic to a carrier protein such as BSA. This approach was taken upon completion of a series of preliminary studies, all of which yielded essentially negative results (data not shown). Those preliminary studies included attempts to chemically couple each of the peptides to Covalink and *N*-oxysuccinimide–amine plates and also simply allowing the mimetics to adsorb to microtiter plates. When rabbit sera containing anti-Tpp15 antibodies were examined by both of these methods, IgM or IgG reactivities with the peptides were either very low or nonexistent. Additional attempts were made to biotinylate each of the peptides for use in ELISAs with streptavidin-coated plates. Although the latter initially appeared promising, the majority of normal and test sera (>80%) yielded high background values between 0.15 and 0.30 OD units, which adversely impacted assay sensitivity.

The use of peptide-based ELISAs for diagnosis of virus infections (16, 22, 34, 37, 53) attests to the fact that a similar approach for the detection of congenital syphilis and early, primary infection is achievable and worthy of further consideration. The large-scale production of high-purity, synthetic peptides is relatively inexpensive, so that the actual cost per test would be less than a few cents per mimetic. As opposed to expensive recombinant protein approaches, this would certainly be within the guidelines of the Sexually Transmitted Diseases Diagnostics Initiative Steering Committee (49). The other economic advantage of a peptide-based assay is that it would eliminate the need to propagate treponemes in rabbits for hemagglutination and fluorescent treponemal antibody-adsorbed confirmatory tests. On a per-test-cost basis, fractionation of infant IgM is unreasonable. For this reason, efforts currently under way in our laboratory are directed at a μ-capture IgM ELISA with a combination of mimetics covalently coupled to a carrier and an enzyme-labeled anti-peptide rabbit IgG for detection. On the basis of the present findings, additional studies, including the assessment of sensitivities and specificities with a larger population base, including infants with asymptomatic disease, with these and other mimetics from Tpp17 and Tpp47 alone and in combination would seem feasible and justified.

ACKNOWLEDGMENTS

We thank Berma Kinsey for helpful advice concerning the chemical cross-linking of mimetic peptides to carrier proteins.

This work was supported by research funds from the Department of Veterans Affairs.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403.
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. **Batteiger, B. E.** 1996. The major outer membrane protein of a single *Chlamydia trachomatis* serovar can possess more than one serovar-specific epitope. Infect. Immun. **64:**542–547.
4. **Baughn, R. E.** 1986. Antibody-independent interactions of fibronectin, C1q, and human neutrophils with *Treponema pallidum*. Infect. Immun. **54:**456–464.
5. **Baughn, R. E.** 1990. Demonstration and immunochemical characterization of natural, autologous anti-idiotypic antibodies throughout the course of experimental syphilis. Infect. Immun. **58:**766–773.
6. **Baughn, R. E., M. C. McNeely, J. L. Jorizzo, and D. M. Musher.** 1986. Characterization of the antigenic determinants and host components in immune complexes from patients with secondary syphilis. J. Immunol. **136:** 1406–1414.
7. **Baughn, R. E., and D. M. Musher.** 1983. Isolation and preliminary characterization of circulating immune complexes from rabbits with experimental syphilis. Infect. Immun. **42:**579–584.
8. **Baughn, R. E., and D. M. Musher.** Unpublished data.
9. **Becker, P. S., D. R. Akins, J. D. Radolf, and M. V. Norgard.** 1994. Similarity between the 38-kilodalton lipoprotein of *Treponema pallidum* and the glucose/galactose-binding (MgIB) protein of *Escherichia coli*. Infect. Immun. **62:**1381–1391.
10. **Bell, A. W., S. D. Buckel, J. M. Groarke, J. N. Hope, D. H. Kingsley, and M. A. Hermodson.** 1986. The nucleotide sequences of the *rbsD*, *rbsA*, and *rbsC* genes of *Escherichia coli* K12. J. Biol. Chem. **261:**7652–7658.
11. **Berzins, K., H. Perlman, B. Wahlin, J. Carlsson, M. Wahlgren, R. Udomsangpetcch, A. Bjorkman, M. E. Patarroyo, and P. Perlman.** 1986. Rabbit and human antibodies to a repeated amino acid sequence of a Plasmodium falciparum antigen, Pf 155, react with the native protein and inhibit merozoite invasion. Proc. Natl. Acad. Sci. USA **83:**1065.
12. **Bockenstedt, L. K., R. J. Gee, and M. J. Mamula.** 1995. Self-peptides in the initiation of lupus autoimmunity. J. Immunol. **155:**3516–3524.
13. **Borenstein, L. A., J. D. Radolf, T. E. Fehniger, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1988. Immunization of rabbits with recombinant *Treponema pallidum* surface antigen 4D alters the course of experimental syphilis. J. Immunol. **140:**2415–2421.
14. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.

15. **Briand, J. P., S. Muller, and M. H. V. Van Regenmortel.** 1985. Synthetic peptide as antigens: pitfalls of conjugation methods. J. Immunol. Methods **78:**59–69.
16. **Broliden, P. A., U. Ruden, A. S. Ouattara, G. de The, E. Solver, J. Trojnar, and B. Wahren.** 1991. Specific synthetic peptides for detection of and discrimination between HIV-1 and HIV-2 infection. J. Acquired Immune Defic. Syndr. **4:**952–958.
17. **Champion, C. I., J. N. Miller, L. A. Borenstein, M. A. Lovett, and D. R. Blanco.** 1990. Immunization with *Treponema pallidum* endoflagella alters the course of experimental rabbit syphilis. Infect. Immun. **58:**3158–3161.
18. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47:**45–148.
19. **Dassa, E., and M. Hofnung.** 1985. Sequence of the *malG* gene in *E. coli* K12: homologies between inegral membrane components from binding protein-dependent transport systems. EMBO J. **4:**2287–2293.
20. **Dobson, S. R. M., L. G. Taber, and R. E. Baughn.** 1988. Recognition of *Treponema pallidum* antigens by IgG and IgM antibodies in congenitally infected newborns and their mothers. J. Infect. Dis. **157:**903–910.
21. **Doi, H., M. Kitajima, I. Watanabe, Y. Kikuchi, F. Matsuzawa, S.-I. Aikawa, K. Takiguchi, and S. Ohno.** 1995. Diverse incidences of individual oligopeptides (dipeptidic to hexapeptidic) in proteins of human, bakers' yeast, and Escherichia coli origin registered in the Swiss-Prot data base. Proc. Natl. Acad. Sci. USA **92:**2879–2883.
22. **Fridell, E., B. J. Cohen, and B. Wahren.** 1991. Evaluation of a synthetic peptide enzyme-linked immunosorbent assay for immunoglobulin M to human parvovirus B19. J. Clin. Microbiol. **29:**1376–1381.
23. **Geysen, M. H.** 1990. Molecular technology: peptide epitope mapping and the pin technology. Southeast Asian J. Trop. Med. Public Health **21:**523–533.
24. **Geysen, M. H., R. H. Meloen, and S. J. Barteling.** 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81:**3998–4002.
25. **Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. G. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods **102:**259–274.
26. **Hensel, V., H.-J. Wellensiek, and S. Bhakdi.** 1985. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting as a serologic tool in the diagnosis of syphilis infection. J. Clin. Microbiol. **21:**82–87.
27. **Hogg, R. W., C. Voelker, and I. Von Cariovitz.** 1991. Nucleotide sequence and analysis of the *mgl* operon of *Escherichia coli* K12. Mol. Gen. Genet. **229:**453–459.
28. **Innis, L. B., V. Thirawuth, and C. Hemachudha.** 1989. Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus. Am. J. Trop. Med. Hyg. **40:**676–687.
29. **James, J. A., T. Gross, R. H. Scofield, and J. B. Harley.** 1995. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B′-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. J. Exp. Med. **181:**453.
30. **Kitagawa, T., and T. Aikawa.** 1976. Enzyme coupled immunoassay of insulin using a novel coupling reagent. J. Biochem. **79:**233.
31. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
32. **Lachumanan, R., S. Devi, Y.-M. Cheong, S. J. Rodda, and T. Pang.** 1993. Epitope mapping of the Sta58 major outer membrane protein of *Rickettsia tsutsugamushi*. Infect. Immun. **61:**4527–4531.
33. **Lewis, L. L., L. H. Taber, and R. E. Baughn.** 1990. Evaluation of immunoglobulin M Western blot analysis in the diagnosis of congenital syphilis. J. Clin. Microbiol. **28:**296–302.
34. **Linde, A., B. Kallin, J. Dillner, et al.** 1990. Evaluation of enzyme-linked immunosorbent assays with two synthetic peptides of Epstein-Barr virus for diagnosis of infectious mononucleosis. J. Infect. Dis. **161:**903–909.
35. **Liu, F. T., M. Zinnecker, T. Hamaska, and D. H. Katz.** 1979. New procedures for the preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18:**690.
36. **Lukehart, S. A., S. A. Baker-Zander, and E. R. Gubish, Jr.** 1982. Identification of *Treponema pallidum* antigens: comparison with a non-pathogenic treponeme. J. Immunol. **129:**833–838.
37. **Lundkvist, A., S. Björsten, B. Niklasson, and N. Ahlborg.** 1995. Mapping of B-cell determinants in the nucleocapsid protein of Puumala virus: definition of epitopes specific for acute immunoglobulin G recognition in humans. Clin. Diagn. Lab. Immunol. **2:**82–86.
38. **Mamula, M. J., and C. A. Janeway, Jr.** 1993. Do B cells drive the diversification of immune responses. Immunol. Today **14:**151–152.
39. **Mamula, M. J., R.-H. Lin, C. A. Janeway, Jr., and J. A. Hardin.** 1992. Breaking T cell tolerance with foreign and self co-immunogens. A study of autoimmune B and T cell epitopes of cytochrome c. J. Immunol. **149:**789–795.
40. **Mueller, F., M. Moskophidis, and H.-L. Borkhardt.** 1987. Detection of immunoglobulin M antibodies to *Treponema pallidum* in a modified enzyme-linked immunosorbent assay. Eur. J. Clin. Microbiol. **6:**35–39.
41. **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.
42. **Ohno, S.** 1991. Many peptide fragments of alien antigens are homologous with host proteins, thus canalizing T-cell responses. Proc. Natl. Acad. Sci. USA **88:**3065–3068.
43. **Purcell, B. K., N. R. Chamberlain, M. S. Goldberg, L. P. Andrews, E. J. Robinson, M. V. Norgard, and J. D. Radolf.** 1989. Molecular cloning and characterization of the 15-kilodalton major immunogen of *Treponema pallidum*. Infect. Immun. **57:**3708–3714.
44. **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.
45. **Richards, F. M., and J. R. Knowles.** 1968. Glutaraldehyde as a protein cross-linking reagent. J. Mol. Biol. **37:**231–235.
46. **Sanchez, P. J., G. H. McCraken, Jr., G. D. Wendel, K. Olsen, N. Threlkeld, and M. V. Norgard.** 1989. Molecular analysis of the fetal IgM response to *Treponema pallidum* antigens: implications for improved serodiagnosis of congenital syphilis. J. Infect. Dis. **159:**508–517.
47. **Saurin, W., W. Koster, and E. Dassa.** 1994. Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins. Mol. Microbiol. **12:**993–1004.
48. **Schulz, E. G.** 1988. A critical evaluation of methods for prediction of protein secondary structures. Annu. Rev. Biophys. Biochem. **17:**1–21.
49. **Sexually Transmitted Disease Diagnostics Initiative Steering Committee.** 1993. Sexually transmitted disease diagnostics initiative. Sex. Transm. Dis. **20:**120.
50. **Topfer, F., T. Gordon, and J. McCluskey.** 1995. Intra- and intermolecular spreading of autoimmunity involving the nuclear self-antigens La (SS-B) and Ro (SS-A). Proc. Natl. Acad. Sci. USA **92:**875–879.
51. **U.S. Department of Health, Education, and Welfare.** 1969. National Communicable Disease Center, Venereal Disease Program Manual of tests for syphilis. U.S. Public Health Service publication no. 988. Center for Disease Control, Atlanta.
52. **Versalovic, J., Z.-D. Nash, R. Carinhas, D. M. Musher, and R. E. Baughn.** 1990. Immunoglobulin class restriction of autoimmune responses in secondary syphilis. Clin. Exp. Immunol. **80:**381–386.
53. **Wahren, B.** 1991. Synthetic peptides for viral serology and analysis. Rev. Med. Microbiol. **2:**153–160.
54. **Westhof, E., D. Altschuuh, D. Moras, A. C. Bloomer, A. Mondragon, A. Klug, and M. H. V. V. Regenmoral.** 1984. Correlation between segmental mobility and the location of antigenic determinants in proteins. Nature (London) **311:**123.
55. **Wicher, V., J. Zabek, and K. Wicher.** 1989. Kinetics of pathogen-specific humoral response in *Treponema pallidum*-infected young and old inbred strain 2 guinea pigs. Clin. Exp. Immunol. **77:**144–150.
56. **Wicher, V., J. Zabek, and K. Wicher.** 1991. Pathogen-specific humoral response in *Treponema pallidum*-infected humans, rabbits, and guinea pigs. J. Infect. Dis. **163:**830–836.
57. **Zhong, G., R. E. Reid, and R. C. Brunham.** 1990. Mapping antigenic sites on the major outer membrane protein of *Chlamydia trachomatis* with synthetic peptides. Infect. Immun. **58:**1450–1455.

Editor: R. E. McCallum