

MONOCLONAL ANTIBODY WITH HEMAGGLUTINATION,
IMMOBILIZATION, AND NEUTRALIZATION ACTIVITIES
DEFINES AN IMMUNODOMINANT, 47,000 MOL WT,
SURFACE-EXPOSED IMMUNOGEN OF *TREPONEMA PALLIDUM*
(NICHOLS)

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Syphilis is a chronic, complex, sexually transmitted disease of humans caused by *Treponema pallidum*. Despite intensive research, the parameters affecting the pathogenesis of syphilis remain substantially undefined, due primarily to the inability to culture large numbers of *T. pallidum* in vitro. Examination of the host response to *T. pallidum* has therefore been one of the few effective tools for studying the consequences of treponemal infection. Resistance to reinfection develops during the course of the disease and is elicited in human latent syphilitic patients (3–5) as well as in the rabbit (1–8) and hamster (9) experimental models. Evidence for the involvement of both humoral and cell-mediated immunity in the response of the host to infection has been presented (1, 2, 10, 11).

The fact that humoral immunity may play a significant role in the developing immune response is implicated by the ability of antibody to block the attachment of *T. pallidum* to tissue culture cells in vitro (12, 13), the immobilization of *T. pallidum* by antibody in vitro (14, 15), the in vitro–in vivo neutralization of *T. pallidum* (16), and immune passive transfer experiments using immune serum (17–23) or IgG purified from immune serum (24). The passive protection studies have shown either complete or partial but definitive protection against *T. pallidum* infection by reducing, delaying, and/or preventing lesions following the passive immunization of rabbits (17–24).

The persistence of the organism, despite a high “protective” antibody titer (1, 2), indicates that antibody alone cannot eradicate the organisms during *T. pallidum* infection (1, 2). Considerable controversy exists, however, concerning the role of cell-mediated immunity in syphilis. Several cell-mediated immune mechanisms have been suggested to have a role in the response of the host to *T.*

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pallidum infection. These mechanisms have been recently reviewed in detail (10, 11). Though the individual roles of cell-mediated and humoral immunity have not been precisely characterized, the host immune response to *T. pallidum* may very well include components of each.

Several investigators have exploited the humoral immune response during *T. pallidum* infection to identify components of *T. pallidum* that are antigenic and/or immunogenic. Baseman and Hayes (25) and Alderete and Baseman (26), using polyclonal antisera from rabbits or humans, identified 11 outer membrane proteins of *T. pallidum*, three of which, with the apparent molecular weights of 72,000–89,000 (72–89 K), 28–29.5 K, and 25.5–26 K, were relatively immunogenic. Using serum from hyperimmunized rabbits, Lukehart et al. (27), detected six antigens with molecular weights ranging from 30 to 48 K and two additional antigens at 12 and 14 K. Hanff and colleagues (28) used human serum from syphilitic patients to detect 14 *T. pallidum*-specific antigens, ranging from 15 to 94 K in molecular weight. In a further study with human syphilitic serum, Hanff et al. (29) suggested that the major antigenic proteins of *T. pallidum* are comprised of four polypeptides of 30–45 K and two smaller molecular weight polypeptides of 15–16 K. In an extension of these results, Moskophidis and colleagues (30) used human serum and detected similar proteins reported by Hanff et al. (28, 29), in addition to proteins with molecular weights of 37, 38, 59 K, and 69 K.

The use of polyvalent antisera in conventional antigen survey studies detected immunogenic *T. pallidum* components. However, the relative importance of these antigens has been difficult to assess. Alternatively, monoclonal antibodies against *T. pallidum* provide a unique opportunity to use antibodies of predefined specificity for use in in vitro studies, similar to those performed in the past, as well as for use within in vivo experiments. Functional studies using monoclonal antibodies potentially allow the assessment of biological relevance to prospective immunogens, and possibly also to the polyclonal antibodies normally elicited against them. On this basis, a defined working hypothesis relative to the biological importance of an immunogen can be formulated and tested in further studies. Subsequent in vitro techniques can then be used to characterize the immunogen and to investigate its potential role in the immune response of the host.

Using this novel approach, a monoclonal antibody directed against an abundant, immunodominant, 47,000 mol wt, surface-exposed immunogen of *T. pallidum* has been shown to possess reactivities in the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP test)¹ and the *Treponema pallidum* immobilization (TPI) test, as well as to block significantly the attachment of *T. pallidum* to host cells in vitro. Moreover, this monoclonal antibody was capable of neutralizing *T. pallidum* in the in vitro–in vivo neutralization test of Bishop

¹ Abbreviations used in this paper: Abs, ABS, absorbed with *T. phagedenis* biotype Reiter; DOC, desoxycholate; IRS, immune rabbit serum; LPO, lactoperoxidase; β -ME, 2-ME, beta-mercaptoethanol; MET, methionine; MHA-TP, microhemagglutination assay for *T. pallidum* antibodies; mAb, monoclonal antibody; NET, TNE, 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.2; NRS, serum from VDRL nonreactive normal adult rabbits; NRT, normal rabbit testicular tissue; PBI, phosphate buffer with 50 mM NaI; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tp, *Treponema pallidum*; TPI, *T. pallidum* immobilization; VDRL, Venereal Disease Research Laboratory.

and Miller (16). These results serve to establish the 47,000 mol wt surface antigen as the first functionally defined, major surface immunogen of *T. pallidum*.

Materials and Methods

Treponemal Strains. *T. pallidum* subspecies *pallidum* (Nichols strain) was propagated using standard intratesticular inoculation of New Zealand White rabbits with $2-5 \times 10^7$ *T. pallidum* per testicle as previously described (31).

T. phagedenis biotype Reiter (Reiter) was cultivated in vitro through maintenance in BBL thioglycollate medium 135C supplemented with either 10% heat-inactivated (56°C, 30 min) bovine or hemolyzed rabbit serum (Pel-Freez Biologicals, Rogers, AR). The bacteria were passaged monthly and processed as described previously (31).

Animals. Adult male New Zealand White rabbits, obtained from Hickory Hill Farms, Flint, TX, were used both for maintenance of *T. pallidum* and for neutralization assays. The animals were examined for the absence of *T. paraluiscuniculi* infection both serologically (Venereal Disease Research Laboratory [VDRL]) (32) and clinically. Animals were housed at 17–20°C and were given antibiotic-free food and water ad libitum.

Antibodies, Sera, and Antisera. Monoclonal antibodies directed specifically against *T. pallidum* were obtained, maintained, and characterized as described previously (31). Serum from VDRL nonreactive normal adult rabbits (NRS) was collected and in some cases heated at 56°C for 30 min to inactivate complement. Immune rabbit serum (IRS) was obtained from rabbits intratesticularly infected with $2-5 \times 10^7$ *T. pallidum* (Nichols) per testicle; immune serum was then collected from these rabbits 3–12 mo after a firm orchitis in each testicle (20). The rabbits were shown to be “chancere immune” when challenged intradermally with 1×10^5 *T. pallidum*. Normal mouse serum was obtained from uninoculated BALB/c mice, and in some cases was heated at 56°C for 30 min. Mouse anti-*T. pallidum* serum was obtained after the immunization of adult BALB/c mice as described previously (31). IgG from normal and immune rabbit sera was purified using a modification of established methods (24, 33). Affinity-purified goat anti-rabbit IgG, goat anti-mouse IgM (mu chain specific), sheep anti-mouse IgG, and goat anti-mouse IgM were obtained from Cappel Laboratories (Cochranville, PA). Affinity-purified rabbit IgM was obtained from Pel-Freez Biologicals.

Purification of *T. pallidum*. *T. pallidum* for analytical studies was purified using Percoll (Pharmacia, Piscataway, NJ) density gradient centrifugation (34, 35). After Percoll purification, *T. pallidum* cells were washed free of residual Percoll by centrifugation (13,500 g) several times in phosphate-buffered saline (PBS) (31) until the treponemes formed a dense pellet at the bottom of a microcentrifuge tube, rather than layering on top of contaminating Percoll particles.

Radiolabeling of Treponemes and Monoclonal Antibodies. Initially (method 1) (36, 37), Percoll-purified *T. pallidum* ($5-10 \times 10^8$) were suspended in 200 μ l of PBS. A 50 μ l vol of 0.03% H₂O₂ (freshly prepared using 5 μ l of a 30% stock solution of H₂O₂ in 4.995 ml of PBS), 20 μ l of Na-I¹²⁵ (2 mCi; New England Nuclear, Boston, MA), and 50 μ l of lactoperoxidase (LPO) (Sigma Chemical Co., St. Louis, MO) were combined with treponemes, mixed well, and allowed to incubate at room temperature for 4 min. 50 μ l each of 0.03% H₂O₂ and LPO were added at 4 and 8 min after initiation of the reaction. At 12 min after time zero, 50 μ l of H₂O₂ was again added and allowed to incubate for 4 additional minutes, the reaction was stopped by addition of phosphate buffer containing 50 mM NaI (PBI). Labeled cells were washed by multiple centrifugations (13,000 g) in PBI. The cells were resuspended in PBS for use in assays. More recently, the LPO labeling procedure reported by Alderete and Baseman (26) for *T. pallidum* has also been used successfully (method 2). Labeling of monoclonal antibodies with ¹²⁵I using chloramine T was performed as described (38).

Radioimmunoprecipitation. Radioimmunoprecipitation (RIP) was adapted from Hansen et al. (36). $\sim 1 \times 10^7$ cpm of I¹²⁵-labeled treponemes (1–3 counts per treponeme) were solubilized at 37°C in 5 ml of solubilization buffer composed of 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, 0.2% (vol/vol) sodium desoxycholate,

and 0.1% sodium dodecyl sulfate, pH 7.6. The partially solubilized (~70–80%) suspension was centrifuged at 30,000 *g* (16°C) for 1 h, and the supernatant containing solubilized *T. pallidum* antigens was removed from the pellet. To this supernatant was added either 10–20 μ g of monoclonal antibody, 200 μ l of PBS, normal rabbit serum, immune rabbit serum, normal mouse serum, or mouse anti-*T. pallidum* serum, and the mixture was rocked for 1 h at 25°C. A 200- μ l suspension of prewashed protein A-bearing *Staphylococcus aureus* (Cowan I strain), prepared by the method of Kessler (39), was subsequently added to the suspension, which was rocked for 1 h at 4°C. The samples were centrifuged at 3,000 *g* for 20 min, and the supernatant was discarded. The antigen-antibody-*Staphylococcus* pellet was then washed five times by centrifugation at 13,000 *g* in fresh solubilization buffer. The final pellet was suspended in digestion buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, pH 6.8), heated to 100°C for 5 min, and cooled to room temperature. The supernatant was removed and collected after centrifugation. Beta-mercaptoethanol to a final concentration of 5% was then added to each sample, and the samples were heated to 100°C for 5 min and allowed to cool before use in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Radioimmunoprecipitated samples were subjected to discontinuous SDS-PAGE (4% acrylamide stacking gel and 10% acrylamide running gel) (40). Dried gels were exposed to Fuji x-ray film with an enhancing screen (Kodak Cronex Lightening Plus) for autoradiography.

Radioimmunoassays. Radioimmunoassays (RIA) were performed as described previously (31) using various antibodies and preparations as antigens.

Immunoblots. Immunoblots were performed as previously described (41–43).

Serological Tests for Syphilis. Mouse anti-*T. pallidum* monoclonal antibodies, either affinity purified (44) or found within hybridoma clone supernatants (31), were tested for their ability to immobilize *T. pallidum* (Nichols) in the *T. pallidum* immobilization (TPI) test. The TPI assay was carried out, with minor modifications, as described (45). Penicillinase was incorporated into the test procedure due to the possibility of residual penicillin in the hybridoma clone supernatants (31).

The MHA-TP (Sera-Tek; Ames Division, Miles Laboratories, Elkhart, IN) was performed on both respective hybridoma clone supernatants and affinity-purified anti-*T. pallidum* monoclonal antibodies, according to the methods described by the manufacturer (46).

In Vitro–In Vivo Neutralization Test. The in vitro–in vivo neutralization test of Bishop and Miller was performed essentially as described previously (16). Briefly, 1×10^4 *T. pallidum* per ml, rabbit serum as a source of complement, and antibody (either monoclonal or polyclonal) were incubated for 16 h at 34°C under an atmosphere of 95% N₂ plus 5% CO₂. After incubation, 0.1 ml portions of reaction mixtures were injected intradermally into clipped rabbit backs. The sites were observed daily for lesion development; the delay and nature of lesion development was observed at the test sites and compared with both positive (*T. pallidum* plus complement plus immune rabbit serum) and negative (*T. pallidum* plus complement plus normal rabbit serum, or *T. pallidum* plus antibody) control sites. Percent neutralization of the virulent organisms was extrapolated from previous data examining the *T. pallidum* dose response and subsequent time delay for lesion development after intradermal injection (7, 47, 48).

Results

Spectrum of *T. pallidum* Immunogens. Solubilized ¹²⁵I-labeled antigens of *T. pallidum* were immunoprecipitated and subjected to SDS-PAGE analysis. Fig. 1 shows that *T. pallidum* antigens radioimmunoprecipitated in this manner ranged in molecular weight from approximately 13 to 47 K, with the major immunogens at 36 and 47 K. The 47-K mol wt antigen appeared to be highly dominant in the profile. To determine those immunogens specific for pathogenic treponemes, similar RIP analyses using IRS (200 μ l) preabsorbed with solubilized Reiter (3×10^9) cells were employed. In Fig. 2, lanes 1, 3, and 5 serve as negative controls,

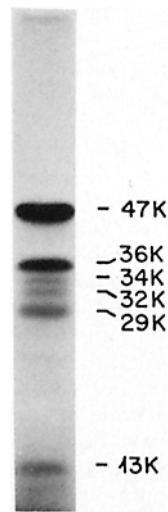


FIGURE 1. Radioimmunoprecipitation and SDS-PAGE of ^{125}I -labeled, solubilized *T. pallidum*. Anti-*T. pallidum* immune rabbit serum (IRS) was used as the precipitating antibody source. Precipitable products were detected by autoradiography.

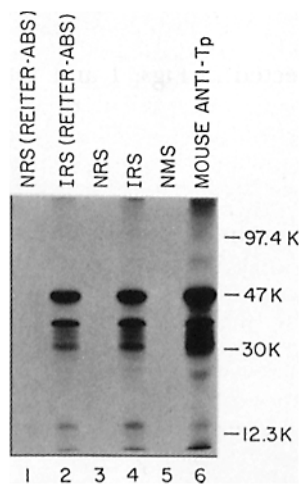


FIGURE 2. Radioimmunoprecipitation and SDS-PAGE of ^{125}I -labeled, solubilized *T. pallidum*. Precipitable products were detected by autoradiography. Lanes 1, 3, and 5 employ normal rabbit serum (NRS) absorbed with *T. phagedenis* biotype Reiter (Reiter-ABS), normal rabbit serum (NRS), and normal mouse serum (NMS), respectively, as controls. Lanes 2 and 4 used anti-*T. pallidum* immune rabbit serum (IRS) absorbed with the Reiter treponeme (Reiter-ABS) and anti-*T. pallidum* immune rabbit serum (IRS) as antibody sources, respectively. Lane 6 employed mouse anti-*T. pallidum* (Tp) serum.

showing that all sera from either normal rabbits or normal mice did not immunoprecipitate detectable antigens of *T. pallidum*. Lanes 4 and 6, using serum from infected immune rabbits or immunized mice, respectively, showed similar immunogenicity of the antigens in rabbits and mice. Furthermore, lane 2 shows that preabsorption of IRS with Reiter did not alter the antigenic pattern obtained (as seen in lane 4) when untreated IRS was used. These results suggest that the

six major antigens of *T. pallidum* detected by radioimmunoprecipitation with IRS are not found in similar configuration in the representative nonpathogen.

Reiter cells surface labeled with ^{125}I were used in similar RIP experiments. As shown in lanes 1 and 2 of Fig. 3, NRS or NRS absorbed with the Reiter treponeme (NRS-ABS), respectively, revealed the faint presence of apparent immunoprecipitable products similar to those observed when using PBS; these results are most likely due to a degree of nonspecific binding. Lanes 3 and 4 of Fig. 3 show the results of anti-*T. pallidum* IRS and anti-*T. pallidum* IRS absorbed with Reiter (IRS-ABS). Lane 4 shows results using Reiter-absorbed immune rabbit serum, and demonstrates the absence of antigens previously detected when using untreated immune rabbit serum (lane 3). The antigens in lane 3 are thus Reiter antigens, detectable using IRS from rabbits infected with *T. pallidum* and are cross-reactive with antibodies generated during *T. pallidum* infection. Note that lanes 3 and 4 lack any antigen with a molecular weight of 47 K, indicating that the 47 K antigen is not detectable in the Reiter treponeme using this technique. Furthermore, the overall antigenic profile observed (Fig. 3, lane 3) through the immunoprecipitation of ^{125}I -labeled Reiter with immune rabbit serum is dissimilar from that observed when the same IRS is used to immunoprecipitate ^{125}I -*T. pallidum* (Figs. 1 and 2).

Screening of a Monoclonal Antibody Panel. A monoclonal antibody (mAb) panel reported by Robertson et al. (31), was screened for reactivity in an RIP with the immunogens previously detected in Figs. 1 and 2 using polyvalent IRS. Fig. 4 shows the results obtained using one particular mAb (11E3), a murine monoclonal antibody of the subclass IgG2a. Lane 1 (Fig. 4) displays the reproducible immunogenic pattern obtained using polyvalent IRS. Lane 2 (Fig. 4) shows that the mAb (11E3) efficiently immunoprecipitated the 47 K mol wt antigen normally detected when IRS was used as the antibody source in RIP analysis. The relative

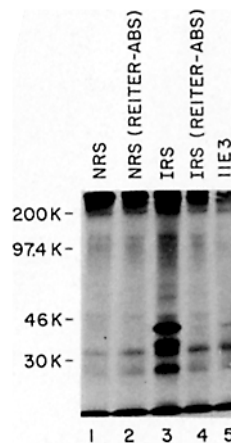


FIGURE 3. Radioimmunoprecipitation and SDS-PAGE of ^{125}I -labeled, solubilized *Treponema phagedenis* biotype Reiter cells. Precipitable products were detected by autoradiography. In lanes 1 and 2, NRS and *T. phagedenis* biotype Reiter-absorbed NRS (Reiter-ABS) were used as negative serum controls. Lanes 3 and 4 employed anti-*T. pallidum* IRS and anti-*T. pallidum* IRS absorbed with the Reiter treponeme (Reiter-ABS). Lane 5 used anti-47-K immunogen (of *T. pallidum*) mAb 11E3 as precipitating antibody.

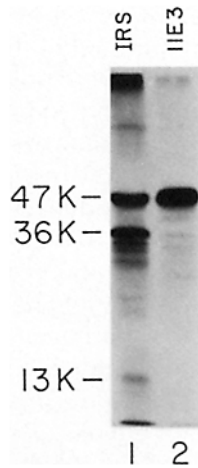


FIGURE 4. Radioimmunoprecipitation and SDS-PAGE of ^{125}I -labeled, solubilized, *T. pallidum*. Either immune rabbit serum (*IRS*) (lane 1) or mAb 11E3 (lane 2) were used as precipitating antibody sources. 11E3, monoclonal antibody 11E3.

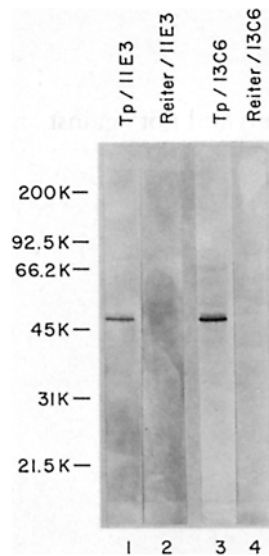


FIGURE 5. Immunoblots of sonicated *T. pallidum* and *T. phagedenis* biotype Reiter using anti-47 K mol wt immunogen (of *T. pallidum*) mAb 11E3 and 13C6. Solubilized antigens of *T. pallidum* or *T. phagedenis* biotype Reiter were detected after SDS-PAGE and protein transfer by incubating nitrocellulose strips with mAb 11E3 (lanes 1 and 2) or 13C6 (lanes 3 and 4) before immunologic detection with horseradish peroxidase-conjugated goat anti-mouse IgG. *Tp*, *T. pallidum*; 11E3, mAb 11E3; 13C6, monoclonal antibody 13C6; *Reiter*, *T. phagedenis* biotype Reiter.

intensity of the 47 K band immunoprecipitated by the mAb suggests that this antigen is found abundantly in *T. pallidum*, is very efficiently labeled, and/or that the mAb possesses high binding affinity for the antigen. Fig. 5 shows the results of immunoblotting using the same mAb reacted against either *T. pallidum* or the Reiter treponeme as antigens. In lanes 1 and 3, where *T. pallidum* was

probed with mAb 11E3 or an additional mAb directed against the 47 K mol wt antigen (13C6), mAb 11E3 reacted with the 47 K antigen of *T. pallidum*, but not with any similarly detectable antigen(s) of Reiter (lanes 2 and 4).

To ascertain that the 47 K mol wt antigen was the same antigen observed when using polyvalent antisera (IRS) in either RIP or immunoblots, a variation of the RIP assay was performed. In Fig. 6, lanes 2 and 3 are negative controls, demonstrating a lack of nonspecific binding. Lane 5 shows the normal immunogenic profile when polyvalent immune rabbit serum is used in the RIP. In Fig. 6, lane 6, the 47 K mol wt antigen of *T. pallidum* is detected using mAb 11E3. In lane 7, labeled *T. pallidum* cells were solubilized and preabsorbed twice with mAb 11E3, followed by incubation with staphylococcal protein A-bearing cells and centrifugation to remove the mAb-antigen complexes. The remaining solubilized antigen in the supernatant was then incubated with polyvalent IRS as the final immunoprecipitating antibody source. As is evident in lane 7, preincubation with mAb effectively removed the 47 K mol wt antigen, resulting in an absence of that component when IRS was subsequently employed.

Screening of Anti-47-K mAb for Reactivity with Rabbit IgG or IgM. Growth of *T. pallidum* in vivo results in binding of host anti-*T. pallidum* antibodies (49) and other proteins (50) to the organism. For this reason, and because the 47 K mol wt antigen is near the molecular weight for rabbit immunoglobulin heavy chain (~50 K) (51), mAb was tested to ensure that its reactivity was specifically directed against *T. pallidum* immunogens and not against anti-*T. pallidum* antibodies that may be bound to *T. pallidum*. RIA were used to quantitate the reactivity of ¹²⁵I-labeled mAb 11E3 with several sources of other antibodies bound to assay wells as test antigens. Because mAb 11E3 is of the mouse subclass IgG2a, goat anti-rabbit IgG and goat anti-mouse IgM (mu chain specific) antibodies served as

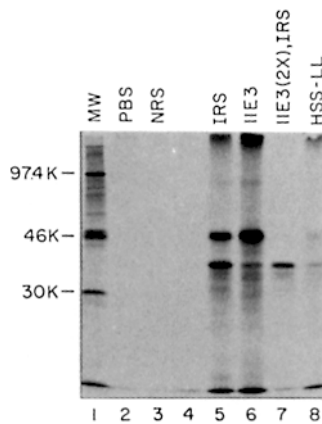


FIGURE 6. Radioimmunoprecipitation and SDS-PAGE of ¹²⁵I-labeled, solubilized *T. pallidum*. Lanes 2 and 3 were controls, employing phosphate-buffered saline (PBS) and normal rabbit serum (NRS), respectively, as sham antibody sources. Lane 4 employed anti-*T. pallidum* immune rabbit serum (IRS). Lane 5 utilized anti-*T. pallidum* mAb 11E3. Lane 6 displays the immunoprecipitable products obtained following preabsorption of *T. pallidum* antigens with mAb 11E3 (twice), followed by detection of the remaining antigen with immune rabbit serum (IRS). Lane 1 shows molecular weight standards. Lane 8 used late latent human syphilitic serum (HSS-LL) as precipitating antibody.

negative control antigens in the RIA, resulting in background binding levels of mAb in the RIA. A positive control antigen included sheep anti-mouse IgG, which showed high specific binding to mAb 11E3 when ^{125}I -labeled mAb 11E3 was used as a probe. Test antigens also included IgG purified from NRS, IgG from IRS, and normal rabbit testicular tissue (1, 2, 49, 50). As presented in Table I, sheep anti-mouse IgG (the positive control) showed ^{125}I -labeled monoclonal antibody 11E3 counts bound to be about 20-fold higher than those of the negative controls or other test antigens, indicating that the test antigens and the negative controls both showed background levels of reactivity in RIA. Sheep anti-mouse IgG reacted strongly with mAb 11E3, as expected. These results indicated that mAb 11E3 has no detectable reactivity with IgG from both normal and immune rabbit serum, and lacks reactivity with normal rabbit testicular tissue. Table II shows similar experiments using goat anti-mouse IgM as a negative control antigen, and sheep anti-mouse IgG again as the positive control antigen (with ^{125}I -labeled monoclonal antibody 11E3 as the probe). This assay investigated the possible reactivity between mAb 11E3 and pooled rabbit IgM. The reactivity of rabbit IgM test antigen was equivalent to RIA reactivity observed for the negative control, indicating a lack of reactivity of mAb 11E3 with rabbit IgM.

Serological Test for Syphilis. mAb 11E3 proved to be reactive in both the

TABLE I
*Reactivity of ^{125}I -Labeled Anti-*T. pallidum* Monoclonal Antibody 11E3 with Selected Antigens by Radioimmunoassay*

Antigens*	^{125}I bound† <i>cpm</i>
Controls:	
(-) Goat anti-rabbit IgG	4,154 ± 698
(-) Goat anti-mouse IgM (μ chain specific)	4,475 ± 512
(+) Sheep anti-mouse IgG	84,507 ± 6,040
Tests:	
NRS IgG	4,617 ± 704
IRS IgG	4,974 ± 616
NRT (testicular)	4,122 ± 444

* Abbreviations: NRS, normal rabbit serum; IRS, immune rabbit serum; NRT, normal rabbit testicular tissue.

† Mean ± SD; $n = 12$.

TABLE II
*Reactivity of ^{125}I -Labeled Anti-*T. pallidum* Monoclonal Antibody 11E3 with Selected Antigens by Radioimmunoassay*

Antigens	^{125}I bound* <i>cpm</i>
Controls:	
(-) Goat anti-mouse IgM (μ chain specific)	1,303 ± 273
(+) Sheep anti-mouse IgG	79,303 ± 5,057
Test:	
Rabbit IgM	1,664 ± 547

* Mean ± SD; $n = 12$.

TABLE III
In Vitro-In Vivo Neutralization of T. pallidum by Anti-47,000-d Immunogen Monoclonal Antibody 11E3

Experiment	Assay tube designation	Contents of <i>in vitro</i> assay tube*	No. of rabbit sites injected [‡]	Average time to lesion development [§]	Delay from control lesions	Neutralization (killing) [¶]
				<i>d</i>	<i>d</i>	%
1	Negative control	NRS	4	12	NA	0
	Positive control	IRS + NRS	4	0	∞ [¶]	100
	Experimental	200 µg mAb + NRS	4	27	15	99
2	Negative control	NRS	4	15	NA	0
	Positive control	IRS + NRS	4	0	∞	100
	Experimental	200 µg mAb + NRS	4	0	∞	100

* All tubes contained 1×10^4 viable *T. pallidum* per ml.

[‡] One site injected intradermally on each of four different rabbits.

[§] Lesion delays never varied more than ± 1 d.

[¶] (7, 47, 48).

[¶] No lesions observed after 77 d.

NA, not applicable.

MHA-TP and TPI tests. These results suggest a relative abundance of the antigen, and a surface-exposed location on *T. pallidum*, respectively.

In Vitro-In Vivo Neutralization Test. Table III provides data obtained from two independent neutralization experiments. Anti-47 K immunogen monoclonal antibody caused a significant delay in or absence of lesion development in comparison with appropriate controls, equivalent to either a 99% or 100% neutralization activity for each experiment, respectively (7, 47, 48).

Discussion

Functionally defined mAb directed against a major 47 K mol wt surface-exposed immunogen of *T. pallidum* were used to assign biological relevance to a potentially significant immunogen of the organism. The anti-47 K monoclonal antibody possessed biological activities as assessed by both the TPI test (45) and the *in vitro-in vivo* neutralization test (16). Furthermore, recent preliminary studies have shown the mAb to be capable of significantly blocking (~50%) the attachment of *T. pallidum* to tissue culture host cells (Dr. Thomas J. Fitzgerald, unpublished data, personal communication). Although this study and the work of others (25-30) have previously defined several antigens of the organism, strategies to determine or assign biological relevance to any one particular antigen have thus far been lacking.

Experiments described here focused upon the nature of the 47 K mol wt immunogen of *T. pallidum*. Antigens of similar molecular weights have been described previously (25-30), providing support for findings presented in this study. Lukehart et al. (27) detected an antigen of 48 K mol wt using serum from

hyperimmunized rabbits, while Hanff and colleagues (28, 29) reported an antigen of ~45 K using serum from human syphilitic patients. This implies that the presence of the 47 K mol wt antigen is a specific result of the detection methods employed, and not merely an artifact of this particular study.

Several other antigens of *T. pallidum* have been defined using serum from infected rabbits and immunized mice. Previous investigators (25–30) identified antigens of comparable molecular weights to all of the six antigens defined in this study; however, some exceptions should be noted. Baseman and Hayes (25) and Alderete and Baseman (26) used a different combination of purification methods and intrinsic labeling techniques than most other investigators. This may explain their apparently dissimilar antigenic profile for *T. pallidum*. However, Moskophidis et al. (30) recently employed labeling methods similar to those of Baseman et al. (25), and yet obtained both similar and additional antigenic profiles to Hanff et al. (28, 29); it should be noted that the additional proteins are of molecular weights >50 K. Baseman et al. (25) also detected proteins in this molecular weight range. The fact that Moskophidis et al. (30) and Baseman et al. (25) used similar analytical systems and detected additional higher molecular weight proteins than other investigators surveyed, may indicate that the labeling methods used by Moskophidis et al. (30) and Baseman et al. (25) preferentially detected higher molecular weight antigens. Moskophidis et al. (30) also detected antigens of molecular weights between 30 K and 50 K, suggesting that either the method of detection and/or purification used by Baseman et al. (25) may be the source(s) of difference in antigenic profiles. Definitive conclusions about the variation among antigenic profiles cannot be drawn at this time, but the overall results of most investigators appear to provide a rather consistent antigenic profile for *T. pallidum*.

The anti-*T. pallidum* mAb was found to be directed against the 47 K mol wt immunogen present in the antigenic profile obtained using polyvalent IRS reacted with ¹²⁵I-labeled, solubilized *T. pallidum* antigens. Studies to substantiate the specificity of this mAb for *T. pallidum* and the *T. pallidum* origin of the 47 K mol wt immunogen were accomplished using the host-indigenous nonpathogen, *T. phagedenis* biotype Reiter (the Reiter treponeme). The use of the Reiter treponeme as antigen in immunoprecipitations or immunoblots demonstrated the lack of reactivity of the mAb with a Reiter protein. Furthermore, the use of various antisera absorbed exhaustively with the Reiter treponeme failed to detect a 47 K mol wt protein in immunoprecipitations using anti-*T. pallidum* immune rabbit serum as an antibody source. The fact that immunoblot analysis using sonicated treponemes also failed to detect Reiter reactivity with the mAb is especially important; both intracellular and extracellular antigens are detected using this method. Consequently, the lack of mAb reactivity in the immunoblots indicates that the 47 K mol wt molecule is not found within the Reiter treponeme. The combined evidence suggests that the 47 K mol wt antigen is not found in nonpathogenic treponemes. Several other investigators have employed the Reiter treponeme as a strategy to screen for treponemal class antigens (27, 30, 31, 52), thus providing a consistent approach for surveying specific antigenic profiles of *T. pallidum* for cross-reactive treponemal activity.

Results from this laboratory reported elsewhere (43) demonstrate that anti-47

K immunogen monoclonal antibody is capable of reacting with two other virulent treponemes, *T. pallidum* subspecies *pertenue* and *T. pallidum* subspecies *endemicum* (Bosnia A). This cross-reactivity was demonstrated with RIA, immunoblot analyses, and immuno-electron microscopy. These results, combined with those of this study, substantiate the specificity of the 47 K mol wt antigen for pathogenic treponemes.

Additional specificity studies on mAb reactivity with *T. pallidum* involved screening of the mAb to examine possible binding affinity for rabbit immunoglobulins (49). Neither rabbit IgG nor rabbit IgM bound to anti-47 K mAb, demonstrating the specificity for *T. pallidum* antigens. mAb was also nonreactive with rabbit testicular tissue and rabbit tissue antigens, as shown here and in results previously reported (31). All assays employed thus far have demonstrated reactivity of the mAb specifically with *Treponema pallidum*.

The 47 K mol wt protein has been shown to have several characteristics that suggest its importance as a general or structural protein of *T. pallidum*, rather than specifically as an immunogen in the infected host. These characteristics include the localization of the 47 K protein as an abundant, surface-exposed immunogenic protein of *Treponema pallidum*; preliminary studies have also shown the 47 K immunogen to be sensitive to the activities of proteinase K and thermolysin (data not shown). The fact that lactoperoxidase labeling of *T. pallidum* results in the appearance of the characteristic antigenic profile further serves to establish the proteinaceous nature and surface localization of the antigen on *T. pallidum* cells. Other studies from this laboratory (43) have demonstrated that mAb against the 47 K mol wt antigen can bind to intact treponemes, supporting the conclusion that the 47 K protein is indeed surface exposed. The density of the band observed at 47 K upon radioimmunoprecipitation and SDS-PAGE analysis is also indicative of the abundance of the protein on the cell surface. Corroboration of the abundance of this protein on the surface of the cell can be found by mAb reactivity in the MHA-TP test. The antigen is so abundant that *T. pallidum*-coated sheep erythrocytes agglutinate even using anti-47 K mAb, which are believed to cross-link antigens poorly due to their monospecificity.

Preliminary studies in this laboratory with serum from human syphilitic patients also have revealed the presence of an antigen corresponding to the 47 K antigen discussed above, further substantiating the ability of the 47 K mol wt antigen to elicit a humoral immune response in the human host. Results of one human syphilitic serum trial using human late latent serum in radioimmunoprecipitation can be visualized in lane 8 of Fig. 6.

A convincing argument for the importance of the role of the 47 K mol wt immunogen in pathogenesis was demonstrated through biological activities of the respective mAb directed against it. The ability of the mAb to immobilize *T. pallidum* in the TPI test provided initial evidence for significant biological activity of the mAb. Further evidence for the biological activity of the anti-47 K mAb is the fact that it is capable of significant blockage of *T. pallidum* attachment to host cells in tissue culture (12, 13; Dr. Thomas J. Fitzgerald, personal communication); in preliminary studies, the anti-47 K antibody repeatedly blocked 50% of *T. pallidum* from attaching to host cells.

A striking correlation of mAb reactivity with importance to in vivo biological activity was provided using the in vitro–in vivo neutralization test of Bishop and Miller (16). mAb was capable of causing delays in or absence of experimental lesion development equivalent to 99 and 100% neutralization, respectively, as judged by the criteria of Turner and Hollander (7), Magnuson et al. (47), and Fitzgerald (48). Although the ability of mAb to neutralize *T. pallidum* was unequivocal in these experiments, results of other neutralization assays (not shown) indicated that prolonged storage of the mAb at 4°C or the use of mAb at high final assay concentrations (>200 µg/ml) abrogated its neutralizing activity. mAb were concentrated using several different methods, including vacuum dialysis and membrane filtration, none of which were successful in maintaining the treponemicidal characteristic of the mAb in neutralization. Reasons for the loss of the neutralizing effect by these technical manipulations remain unclear; further experiments are in progress to determine why such losses in activity occur. In any event, it has been demonstrated that mAb stored at –70°C and used at a final assay concentration of 200 µg/ml in the neutralization test exhibits significant neutralizing activity.

The 47 K mol wt immunogen, with the potential of possessing important biological relevance, provides the best candidate to date for induction of a protective immune state to *Treponema pallidum* in previously unexposed, susceptible hosts. Use of the anti-47 K mol wt antibody to evaluate its efficacy in passive protection studies and as an opsonin for macrophage phagocytosis is currently underway. Of equal significance is the potential utilization of the mAb in affinity purification (53) to obtain purified “native” 47 K mol wt immunogen; for the first time, a method is provided to obtain purified protein antigen from *T. pallidum* that is free of contamination with host tissue. Induction of active immunity with this purified “native” immunogen provides among the most hopeful treponemal vaccine candidate to date. Based on the combined biochemical and biological data presented here, it is proposed that the 47 K mol wt immunogen represents a major surface immunogen of *T. pallidum*. Results of this study, therefore, provide a rationale for further definitive studies on the role of the *T. pallidum* immunogen in the pathogenesis of syphilis and as a potential vaccine candidate.

Summary

Radioimmunoprecipitation (RIP) analyses performed on ¹²⁵I-surface-labeled *Treponema pallidum* cells using various immune sera revealed the presence of six major surface antigens (immunogens) with apparent molecular weights of 47 K, 36 K, 34 K, 32 K, 29 K, and 13 K. Among these, the 47 K surface antigen was most abundant. Radioimmunoprecipitation assays using ¹²⁵I-labeled *T. phagedenis* biotype Reiter or immunoblot analyses using the same strain, failed to reveal the presence of the 47 K mol wt antigen in the representative nonpathogenic treponeme. Preabsorption of anti-*T. pallidum* immune rabbit serum (IRS) with the Reiter organism did not remove anti-*T. pallidum* antibodies from immune serum that reacted with the 47 K mol wt immunogen or other immunogens of *T. pallidum* present in the characteristic antigenic profile. Monoclonal antibodies (mAb) directed specifically against the 47 K mol wt immunogen of *T. pallidum*

also failed to react with an analogous 47 K mol wt component in *Treponema phagedenis* biotype Reiter, further suggesting the unique presence of this antigen in pathogenic treponemes. The presence of the 47 K mol wt surface immunogen in pathogenic treponemes other than *T. pallidum* subspecies *pallidum* was also observed (43). Anti-47 K immunogen mAb was nonreactive against rabbit IgG or IgM.

mAb directed specifically against the 47 K mol wt immunogen of *T. pallidum* was examined for strategic functional activities. It was found to be reactive in the microhemagglutination assay for *T. pallidum* antibodies, the *T. pallidum* immobilization test, and was found to be capable of significant blockage of attachment of virulent *T. pallidum* to host cells in tissue culture. Additional significant biological activity for the anti-47 K mol wt immunogen mAb was revealed through results of the in vitro-in vivo neutralization test of Bishop and Miller, in which a 99% or 100% neutralizing activity was demonstrated. The combined data of this study suggest that the 47 K mol wt immunogen of *T. pallidum* represents an abundant, immunodominant, surface-exposed immunogen possessing potential biological importance in the pathogenesis and immunology of *T. pallidum* infection. These studies serve to establish the first functionally defined immunogen for *T. pallidum*, which may represent the major immunogen of the organism.

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