INFECTION AND IMMUNITY, May 2005, p. 3083–3095 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.3083–3095.2005

A Monoclonal Antibody That Conveys In Vitro Killing and Partial Protection in Experimental Syphilis Binds a Phosphorylcholine Surface Epitope of *Treponema pallidum*

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Received 19 November 2004/Returned for modification 22 December 2004/Accepted 14 January 2005

Immunization with purified *Treponema pallidum* outer membrane vesicles (OMV) has previously resulted in high-titer complement-dependent serum bactericidal activity. In this study, OMV immunization resulted in the isolation of a monoclonal antibody, M131, with complement-dependent killing activity. Passive immunization of rabbits with M131 administered intravenously conferred significant immunity demonstrated by the failure of syphilitic lesions to appear at 29% of intradermal challenge sites (7/24) and a mean delay of approximately 8 days to lesion appearance at the remaining sites (17/24). M131 not only bound to OMV and to the surfaces of intact motile *T. pallidum* cells but also bound to organisms whose outer membranes were removed, indicating both surface and subsurface locations for the killing target. This target was determined to be a *T. pallidum* lipid. Lipid extracted from *T. pallidum* and made into liposomes bound M131. Reverse-phase high-pressure liquid chromatography separation and fraction collection mass spectrometry (LC-MS+) of *T. pallidum* lipid showed that the target of M131 was phosphorylcholine. M131 binding required both liposome formation and a critical concentration of phospholipid containing phosphorylcholine, suggesting that the epitope has both a conformational and a compositional requirement. M131 did not react with red blood cells, which have phosphorylcholine-containing lipids in their exterior membrane leaflets, or with Venereal Disease Research Laboratory antigen that also contains phosphorylcholine, further indicating the specificity of M131. This is the first physical demonstration of an antigen on the *T. pallidum* surface and indication that such a surface antigen can be a target of immunity.

Treponema pallidum subspecies *pallidum*, the syphilis spirochete, is naturally found only in humans, cannot be maintained in vitro, and is kept in the laboratory by passage in rabbit testes. It is likely that key features of the outer membrane promote lifelong infection of its hosts. Over 20 years ago, it was appreciated that the surface of living *T. pallidum* was poorly antigenic compared to the surfaces of other bacteria. This finding was subsequently related to a remarkably low content of membrane-spanning outer membrane protein (39, 47). The outer membrane does contain a minor amount of lipoproteins also present in far greater abundance in the inner membrane (9) but does not contain lipopolysaccharide (4, 22, 24). Identification of putative outer-membrane-spanning proteins has been controversial. This has been due in part to the lack of established means for demonstrating surface location on a surface that is poorly antigenic. While candidate *T. pallidum* surface proteins have been advanced on the basis of porin activity (7) or homology with the surface proteins of other spirochetes (14), there has been no direct physical evidence that these or any other proteins are surface antigens of *T. pallidum*.

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There are, however, biological indications that surface antigens of *T. pallidum* exist. A strong correlation has been made between the development of infection-derived immunity in rabbits and the appearance of bactericidal antibodies (6, 30). Passive immunization with infection-derived immune serum confers partial to complete protective immunity in experimental animals (1, 2, 5, 38, 44, 45, 50). The target(s) of the bactericidal antibodies has not been identified, although the presumption has been that such a target(s) is on the surface of the spirochete.

Substantiating the view that there is a surface target of bactericidal antibody, we found that immunization of mice with outer membrane vesicles (OMV) isolated from *T. pallidum* induced a serum bactericidal activity 30 times greater than that found in immune rabbit serum (IRS) (8). In contrast, attempts to induce killing antibodies through immunization with recombinant *T. pallidum* proteins or with dead spirochetes have produced no more than weak bactericidal activity. In this report, we describe the immunization of mice with OMV, resulting in the isolation of a monoclonal antibody (MAb) with potent bactericidal activity. This monoclonal antibody binds to a phosphorylcholine epitope on the *T. pallidum* surface and conveys partial protection in experimental rabbit syphilis following passive immunization. This is the first direct physical evidence of an antigen on the *T. pallidum* surface and an indication that such a surface antigen can be a target of immunity.

MATERIALS AND METHODS

Source of *T. pallidum*. *T. pallidum* subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously (34). IRS was acquired from rabbits infected intratesticularly for a period of at least 6 months and shown to be immune to challenge reinfection. Normal rabbit serum (NRS) was acquired from animals with nonreactive Venereal Disease Research Laboratory (VDRL) serology (36). All experiments using animals in this study were approved by the University of California at Los Angeles (UCLA) Animal Research Committee.

***T. pallidum* outer membrane preparation.** OMV were prepared from *T. pallidum* using the following modifications of the previously described procedure (9). This modified procedure results in a 10 to 20% greater yield in OMV recovered (data not shown). A treponemal suspension (approximately 2×10^{11} organisms) treated with 0.1 M citrate buffer, pH 3.0, for 30 min was disrupted by three passages through a French pressure cell (Thermo Spectronic, Rochester, NY) set at 12,000 lb/in^2. The disrupted treponemal suspension was then layered onto a continuous 5 to 40% (wt/wt) sucrose-PBS gradient and centrifuged for 16 h at 100,000 $\times$ *g*. Banded OMV showed no difference in banding position or in the protein composition from that previously described (9).

Mouse OMV immunization and monoclonal antibody isolation. Each of four 12-week-old BALB/c mice (Charles River, Wilmington, MA) was immunized subcutaneously with 100 μl of OMV (5×10^{10} *T. pallidum* equivalents) mixed with an equal volume of Titermax adjuvant (Sigma Chemicals, St. Louis, MO). At 2 and 4 months, the mice were boosted subcutaneously with OMV without adjuvant. Mice were tested for complement-dependent bactericidal activity using the *T. pallidum* immobilization (TPI) test (36), and all were found to possess 100% endpoint killing titers greater than 1:1,400. One mouse was chosen for monoclonal antibody production performed by QED Biosciences Inc., San Diego, CA. Initial fusion supernatants were screened for complement-dependent killing activity using the TPI test. One clone, designated M131, was used for mouse ascites generation and the monoclonal antibody isotype, and concentration was determined by radial immunodiffusion.

TPI test. To assay for complement-dependent killing activity against *T. pallidum*, the TPI test was used as described previously (36). Percent motility was determined by randomly counting 50 organisms. Organisms immobilized under similar conditions are dead based on virulence testing using the intradermal injection of rabbits (6).

Passive immunization. Rabbits with nonreactive VDRL serology were divided into four groups, each containing three rabbits, and were each given three passive intravenous immunizations at 18 h before challenge, on the day of challenge, and at 72 h postchallenge. Animals in each group received at each time point either 10 ml of heat-inactivated (56°C/30 min) NRS (H-NRS), 10 ml of heat-inactivated IRS, 10 ml of mouse ascites containing 100 mg of an irrelevant control immunoglobulin M kappa [IgM(κ)] monoclonal antibody (QED Biosciences, Inc.), or 10 ml of mouse ascites containing 100 mg of M131. For preparation of the challenge inoculum, *T. pallidum* was extracted from infected rabbit testes and resuspended into H-NRS to a concentration of 10^4 organisms/ml. Each animal was challenged intradermally on its shaved back with 100 μl of suspension at eight sites per rabbit (10^3 organisms/site). The animals were observed daily for lesion appearance and development, and two representative sites from each animal were biopsied at 20 and 25 days postchallenge for quantitation of treponemes by real-time PCR. The animals were further observed for a total of 60 days following challenge and then sacrificed, and their popliteal lymph nodes were surgically removed for the detection of treponemes by both real-time PCR and infectivity testing. For infectivity testing, popliteal lymph nodes were triturated with 1 ml of H-NRS followed by intratesticular inoculation into rabbits with nonreactive VDRL serology (36). Blood from these animals was acquired 1 month after inoculation and tested for TPI reactivity.

Real-time PCR. Real-time PCR was performed as previously described (17) on syphilitic dermal lesions from the passively immunized test and control animals. Real-time PCR was also performed on a separate set of four control animals challenged intradermally at each of eight sites with 10^3 organisms. Samples from these control animals were acquired from days 10 through 55 postchallenge. Genomic DNA acquired from biopsied tissue samples and *T. pallidum* was prepared using the Easy-DNA kit from Invitrogen, Carlsbad, CA. Primers and probes were selected from the *flaA* gene of *T. pallidum* (GenBank accession no. M63142). The *flaA* forward primer was from base 121 to base 141 (5′-GGAGG TATGACGCATAATCGG-3′) and the reverse primer from base 202 to base 180 (5′-ATGCCTTCTGCTCGTCAGTGTAC-3′). The probe corresponded to base 146 to base 171 (5′-CCGTTCTGGACTATGCTTCTCTGGCG-3′). The collagenase-1 precursor gene (*MMP-1*) (exon 2) was selected for rabbit tissue quantitation (GenBank accession no. M17820). The forward primer for *MMP-1* was from base 4220 to base 4237 (5′-CCGTCTACCCTGGGTGCC-3′). The reverse primer was from base 4274 to base 4296 (5′-ATGGATTTCCTTGCTTGATTC TG-3′). The probe corresponded to base 4243 to base 4270 (5′-TGTGCAGAC CACAGGAGCACTTGACAAC-3′). The probes were labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with the *N*,*N*′,*N*′-tetramethyl-6-carboxytetramethylrhodamine (TAMRA). Primers and probes were purchased from QIAGEN, Valencia, CA. One hundred nanograms of DNA from infected rabbit tissue was used per reaction in triplicate. Taqman universal PCR master mixture (Applied Biosystems, Foster City, CA) was used for all reactions. Each reaction (25 μl) contained both primers at a concentration of 900 nM and 250 nM for the probe. A standard curve was plotted for each primer-probe set with cycle threshold (C_T) values obtained from the amplification of known quantities of DNA isolated from *T. pallidum* and rabbit liver (Seegene, Seoul, Korea). Values for the *T. pallidum* standard curve were obtained in the presence of 100 ng of rabbit DNA. The copy number of each sample was determined by plotting the C_T value versus the log of the copy numbers included in each standard curve. Control reactions without template were included for each assay for both primer sets.

Extraction of *T. pallidum* lipid. Total lipid from 10^{10} *T. pallidum* cells was isolated using the procedure described by Hossain et al. for *Borrelia burgdorferi* (26). *T. pallidum* used for lipid extraction was first purified from contaminating host tissue by Percoll density gradient centrifugation as previously described (23). Isolated *T. pallidum* lipid was resuspended into either 0.5 ml of 5% octylglucoside in phosphate-buffered saline (PBS) for liposome generation, as described below, or 80% methanol for lipid analysis.

***T. pallidum* lipid analysis.** Dried lipid was redissolved in column equilibration buffer (methanol-water [80:20, vol/vol] containing 5 mM ammonium acetate) and injected (250 μl/injection) onto a silica-based reverse-phase high-performance liquid chromatography (RP-HPLC) column (Betasil C_8, 250 by 10 mm, 5-mm, 100-Å pore size; Keystone Scientific, Bellefonte, PA) equilibrated in the above-mentioned buffer and eluted (4 ml/min) with a linear gradient of increasing methanol concentration (0/80, 60/100, min/percent methanol). Absorbance (210 nm) was recorded, and aliquots (1 ml) of the collected fractions (1 min) were removed for immunoreactivity as described below. The remaining 3 ml of each fraction was dried and resuspended in 100 μl chloroform for mass spectrometry. Aliquots of the redissolved RP-HPLC fractions, diluted in chloroform-methanol (1:1, vol/vol) containing 0.5% formic acid, were injected (20 μl/injection) into a stream of the same solvent flowing (20 μl/min) into an Ionspray source connected to a triple-quadrupole mass spectrometer (API III+; PE Sciex). Positive-ion mass spectra were recorded by scanning from *m*/*z* 450 to 950 (orifice 65 V, 0.3-Da step size, 4 s/scan). Parent ion spectra of the *m*/*z* 184.1 fragment ion were recorded by scanning from *m*/*z* 50 to 800 under tandem mass spectrometry conditions with argon collision gas (collision-activated dissociation gas thickness instrumental setting at 110). Instrument-supplied software was used to average all the spectra from each sample injected.

Liposome preparation. *T. pallidum*-extracted lipids and natural phospholipids (Avanti Polar Lipids, Alabaster, AL) including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylserine (PS), phosphatidylinositol, sphingomyelin, and cardiolipin were made into liposomes by the following method. *T. pallidum* lipid resuspended in 0.5 ml of 5% octylglucoside and 2 mg of dried phospholipids resuspended in 0.5 ml of 5% octylglucoside were loaded into dialysis cassettes having a 3,500-molecular-weight cutoff (Pierce, Rockford, IL). Cassettes were dialyzed against 4 liters of PBS for 24 h at 4°C for liposome formation. Liposomes generated by this method have a uniform size ranging from 100 to 300 μm as determined by electron microscopy.

Dot blot immunoassays. Dot blot immunoassays using 5-μl spots on nitrocellulose (Schleicher & Schuell Biosciences, Inc., Keene, NH) were used for the detection of M131 binding to OMV (derived from 5×10^7 organisms), intact *T. pallidum* (10^7 organisms), *T. pallidum* lipid made into liposomes (10^8 equivalents), and *T. pallidum* (10^7 organisms) treated with Triton X-100, as previously described (37), in order to remove the outer membrane. In other experiments, nitrocellulose strips were spotted with 5-μl suspensions containing 5 μg of the above-described phospholipids in 5% octylglucoside or in liposomal form, both of which bound to the nitrocellulose strips as detected by 1% naphthol blue-black staining (Sigma). Liposomes were also prepared containing varying molar percentages of phosphatidylchloline together with phosphatidylserine or phosphatidylethanolamine. Nitrocellulose strips were incubated with M131 and TEPC-183 (Sigma) at a concentration of 1 μg/ml. In some experiments, IRS and NRS, diluted 1:100, were reacted against phosphatidylcholine liposomes. Bound antibody was detected using horseradish peroxidase (Amersham, Piscataway, NJ)-conjugated anti-rabbit Ig or horseradish peroxidase (ICN/Cappel, Aurora, OH)-conjugated goat anti-mouse μ-chain, followed by enhanced chemiluminescence

(ECL) with ECL+ Plus (Amersham). Visualization was performed using a Fluorchem 8000 imager (Alpha Innotech Corporation, San Leandro, CA).

PK treatments. Proteinase K (PK) treatments of *T. pallidum* OMV and Triton X-100-treated *T. pallidum* were performed as follows. To 50-μl suspensions of OMV (5×10^8 organism equivalents) or Triton X-100-treated *T. pallidum* (10^8 organisms), PK (Roche Diagnostics, Indianapolis, IN) was added to reach a final concentration of 100 μg/ml. The suspensions were then incubated overnight at 56°C and then centrifuged at 25,000 × *g* for 1 h. The pelleted material was washed one time in PBS containing 10 mM EDTA, recentrifuged, and then resuspended into 50 μl of PBS.

IEM. Whole-mount immunoelectron microscopy (IEM) of Triton X-100-treated *T. pallidum* (10^7 organisms/ml), Triton X-100- and PK-treated *T. pallidum* (10^7 organisms/ml), and intact motile organisms (5×10^7 organisms/ml) was performed as previously described (19). For motile intact organisms, freshly extracted *T. pallidum* (5×10^7 organisms/ml) was incubated for 16 h under TPI conditions in the absence of complement. M131 or the TEPC-183 MAbs were used at 30 μg/ml. Bound MAb was detected using μ-chain-specific anti-mouse IgM conjugated to 10-nm colloidal gold particles (Sigma). All grids were examined in an electron microscope (JEOL 100 CX, Peabody, MA) at an 80-kV accelerating voltage.

Indirect immunofluorescence of *T. pallidum* encapsulated in gel microdroplets. Motile *T. pallidum* cell suspensions were encapsulated in agarose gel microdroplets for indirect immunofluorescence as previously described (16). Some of the encapsulated organisms were exposed to concentrations (0.02%, 0.06%, or 0.15%) of Triton X-100 for 30 min. Rat anti-*T. pallidum* flagellar sheath protein serum (anti-FlaA) and M131 (15 mg/ml) were diluted 1:100 and added directly to small aliquots of beads (0.2 ml). Samples were incubated for 2 h with gentle mixing in a 34°C water bath. Bound antibody was detected by incubation with 2 μg of goat anti-mouse Alexa 546 conjugate and 2 μg goat anti-rat Alexa 488 conjugate (Molecular Probes, Eugene, OR) for 2 h at 34°C. The beads were viewed on glass slides with a Nikon E600 fluorescence microscope equipped with 15× oculars, a dark-field condenser, and both fluorescein and rhodamine filters. Images were captured using a SPOT Real-Time Slider digital imager (Spot Diagnostics, Sterling Heights, MI). Three separate images were captured for each spirochete shown in Fig. 4: (i) dark-field, (ii) red fluorescence, and (iii) green fluorescence.

Incubation of RBCs with M131. A 1% suspension of human red blood cells (RBCs) was incubated at room temperature for 1 h with NRS or rabbit anti-human red blood cells (US Biological), each diluted to a final concentration of 1:1,000, and M131 or TEPC-183, each diluted to a final concentration of 1 μg/ml. For some experiments, red blood cells were treated with PK (50 μg/ml) before incubation with the antisera and MAbs. Following incubation, the cells were washed in PBS and then diluted to a final concentration of 0.1% (vol/vol) before spotting a 5-μl sample onto nitrocellulose membranes. Antibody binding of MAbs and antisera was detected as described above.

Incubation of M131 with the VDRL antigen. M131 was tested for reactivity against the VDRL antigen by using the Macro-vue rapid plasma regain circle card test (Becton Dickinson Microbiological Systems, Sparks, MD). M131 and TEPC-183 were assayed for macroagglutination at various concentrations ranging from 0.1 μg/ml to 30 μg/ml. NRS and IRS were also tested as negative and positive controls, respectively.

Statistical analysis. Significant differences of mean lesion incubation periods were compared by two-tailed *t*-test analysis.

RESULTS

Isolation of a killing monoclonal antibody against *T. pallidum*. Out of 400 hybridoma supernatants tested, 1 was found to possess 100% TPI killing activity. This clone, designated M131 and determined to be an IgM isotype, was subsequently used for ascites production and analysis. When quantitatively tested by the TPI test, M131 showed 100% and 90% complement-dependent killing at concentrations of 30 μg/ml and 1 μg/ml, respectively (data not shown). An irrelevant IgM monoclonal antibody, TEPC-183, tested at these same concentrations did not show any TPI activity.

Passive immunization of rabbits with M131 confers significant partial protection. As shown in Table 1, each group containing three animals passively immunized with controls of NRS or control MAb, followed by intradermal challenge using 1,000 organisms per site, developed typical syphilitic lesions at all sites (24/24 for each group) within 11 to 15 days after challenge, with mean times to lesion appearance of 11.0 ± 0 and 13.2 ± 0.23 days, respectively. This observation was consistent with the expected time to lesion appearance (11 to 14 days) in naive animals by using a challenge inoculum of this dosage (27). The lesions on the NRS- and control-MAb-immunized animals developed maximally at 20 to 25 days postchallenge, as shown in Fig. 1, ulcerated between 25 and 35 days postchallenge, and then began to heal after 45 days postchallenge.

TABLE 1. Syphilitic lesion development following intradermal challenge of passively immunized rabbits[a]

Immunization medium	No. of lesions/ no. of sites	Range[c] (days)	Mean ± SE	
			Days	Lesion diam[d] (mm)
NRS	24/24	11	11 ± 0	12.8 ± 0.52
IRS	24/24	12–17	13.2 ± 0.37	3.7 ± 0.36
C MAb[b]	24/24	12–15	13.2 ± 0.23	12.3 ± 0.36
M131	17/24	18–28	21.1 ± 0.75[e]	3.5 ± 0.33

[a] Groups of three rabbits were passively immunized with the indicated sera or MAb at 18 h before challenge, the day of challenge, and 72 h postchallenge. Each animal was challenged intradermally with 10^3 *T. pallidum* cells at each of eight sites.
[b] C MAb, control MAb.
[c] Range represents the day to first detection of erythematous and indurated lesions.
[d] Mean lesion diameter was determined at 25 days postchallenge.
[e] $P < 0.001$, compared with the results of the control MAb and NRS.

Three animals that were passively immunized with IRS followed by challenge also developed lesions at all sites (24/24). All lesions appeared within 12 to 17 days postchallenge with a mean time to lesion appearance of 13.2 ± 0.37 days, which was similar to that for the controls. While lesions in this IRS group were not delayed in appearance compared to previous IRS passive immunization studies (5, 38, 45), the lesions were dramatically smaller in size, less indurated, and less erythematous than controls, as shown at day 20 postchallenge (Fig. 1 and Table 1).

By comparison, the three animals passively immunized with M131 showed partial protective immunity (Table 1). Out of 24 intradermal challenge sites, 7 sites (29%) failed to show lesion appearance throughout the entire 60-day postchallenge observation period. This absence of lesion appearance (seven out of eight sites) was exclusively associated with one animal in this group (Fig. 1). Lesions that developed at the remaining 17 sites (17/24) were all observed to be significantly delayed in appearance, showing a range of 18 to 28 days with a mean time to lesion appearance of 21.1 ± 0.75 days. Further, all of these lesions were observed to be atypical in development and, at day 20 postchallenge, were similar in appearance to the atypical IRS lesions (Fig. 1). These atypical lesions in the M131 passively immunized animals appeared significantly smaller, less erythematous, and less indurated than the control lesions (Fig. 1 and Table 1).

While M131 passive immunization resulted in either the absence of lesions or lesion delays, no alteration in disseminated infection was observed in these animals, as demonstrated by

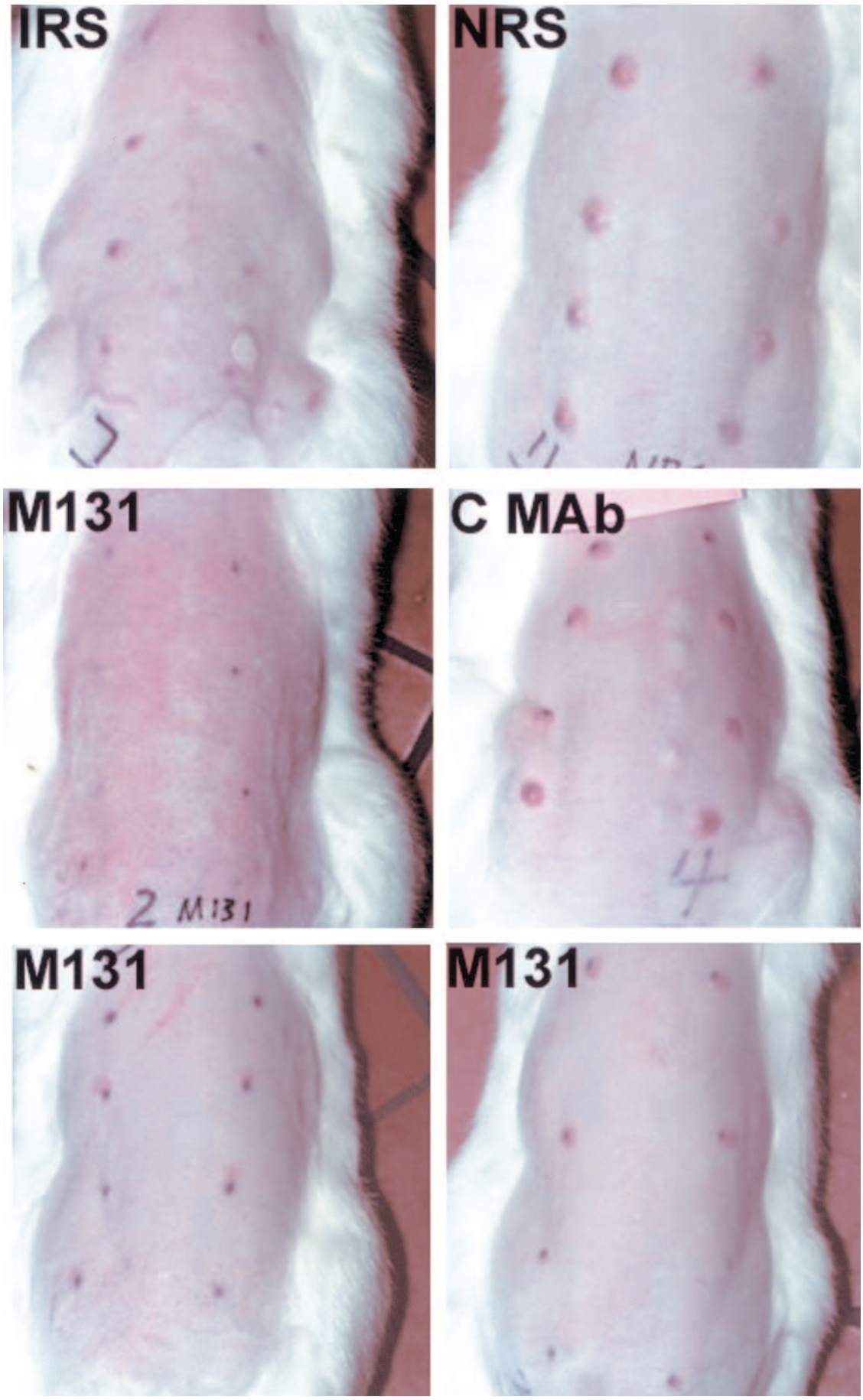


TABLE 2. Quantitation of the relative numbers of *T. pallidum* cells in lesions by using real-time PCR[a]

Passive immunization medium	Avg. no. of *T. pallidum* DNA copies/μg rabbit DNA ± SE	
	Day 20	Day 25
NRS	$4.55 \times 10^6 \pm 5.9 \times 10^5$	$3.93 \times 10^6 \pm 1.1 \times 10^6$
IRS	$3.18 \times 10^5 \pm 1.0 \times 10^5$	$1.09 \times 10^6 \pm 4.5 \times 10^6$
C MAb	$2.16 \times 10^6 \pm 3.7 \times 10^5$	$4.67 \times 10^6 \pm 1.3 \times 10^6$
M131[b]	$7.14 \times 10^5 \pm 2.2 \times 10^5$	$5.03 \times 10^6 \pm 1.3 \times 10^6$

[a] Two representative sites from each animal were biopsied at days 20 and 25 postchallenge for quantitation of *T. pallidum* DNA copies by real-time PCR. Each sample was assayed three times, and the results for each passively immunized group are presented as the average number of *T. pallidum* DNA copies per μg of rabbit DNA ± the standard error.

[b] Sites on the M131 passively immunized animals where no lesions appeared showed fewer than 300 *T. pallidum* DNA copies per μg of rabbit DNA. These values were not included in the DNA copy numbers for the M131 passively immunized animals that developed atypical lesions presented in this table.

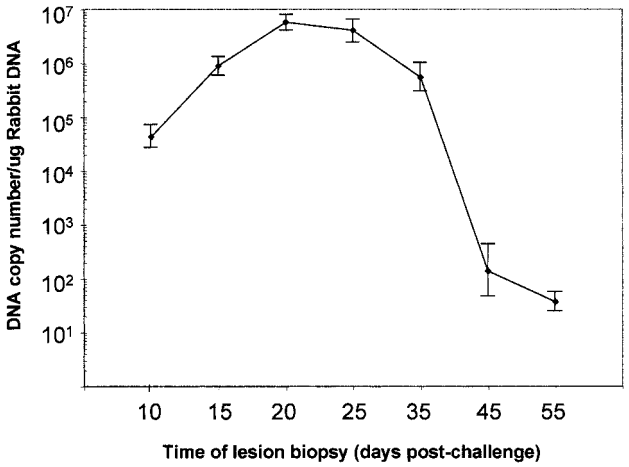


FIG. 2. *T. pallidum* DNA copy number over the course of normal lesion appearance and development. A total of four animals were intradermally challenged at each of eight sites with 10^3 treponemes/site. A representative site from each animal was biopsied at the indicated days postchallenge. Each sample was assayed three times, and the results are presented as the average number of *T. pallidum* DNA copies per μg of rabbit DNA ± standard error indicated by the vertical bars.

the presence of infectious lymph nodes, by infectivity testing (data not shown), and by positive real-time PCR (data not shown).

Real-time PCR analysis of syphilitic lesions. A total of two representative lesion biopsy samples per animal taken at days 20 and 25 postchallenge were obtained for real-time PCR analysis, and each biopsy sample was tested three times using real-time PCR for the average number of *T. pallidum* DNA copies per μg of rabbit DNA. An additional group of four control animals was similarly challenged intradermally and analyzed for *T. pallidum* DNA copy number over the course of normal lesion appearance and development (days 10 through 55). As shown in Fig. 2, control lesions at 10 days postchallenge contained approximately 3×10^4 *T. pallidum* DNA copies per μg of rabbit DNA. At day 15 postchallenge, *T. pallidum* DNA copy numbers increased to approximately 10^6. Maximum *T. pallidum* DNA copy numbers of approximately 5×10^6 were seen at day 20, after which the DNA copy number began to decline. At day 20 postchallenge, the atypical lesions on the animals passively immunized with M131 or IRS had *T. pallidum* DNA copy numbers (7.14×10^5 and 3.18×10^5 copies, respectively) that approximated those in lesions from the control-challenged animals at days 10 through 15 (Table 2 and Fig. 2). Further, these atypical lesions at day 20 postchallenge, compared to 20 day postchallenge lesions from control animals that received control MAb or NRS, showed 3- and 14-fold decreases, respectively, in DNA copy numbers (Table 2). However, at day 25, the DNA copy numbers in the M131 passively immunized animals increased and were similar to those in controls. Biopsy samples at day 25 on the animal passively immunized with M131 at sites where no lesions occurred showed a 4-log decrease in *T. pallidum* DNA copy number compared to those for control lesion sites.

Cellular localization of the antigenic target for M131. In order to demonstrate a surface antigen, living organisms were either incubated with M131 under TPI conditions in the absence of complement for IEM analysis or encapsulated in gel microdroplets for indirect immunofluorescence (16). At the end of the incubation for IEM, all organisms were observed to be actively motile, indicating their structural integrity. IEM showed colloidal gold surface labeling with M131 but not with the TEPC-183 control MAb (Fig. 3). Similarly, the gel microdroplet assay using a double-labeling procedure showed surface binding of M131 but not antibody directed against the periplasmic flagella (Fig. 4), indicating that the treponemes were structurally intact under these conditions. Interestingly, the surface binding of M131 detected by immunofluorescence was in a beaded pattern, possibly reflecting antibody aggregation. M131 binding was also detected by dot blot analysis using whole-organism preparations or purified OMV (Fig. 5A). To determine if the target antigen for M131 was also interior to the outer membrane, organisms used for IEM and within gel microdroplets were first treated with the detergent Triton X-100 to remove the outer membrane before M131 incubation. This treatment resulted in greater M131 binding as determined by IEM, the gel microdroplet assay, and dot blot analysis (Fig. 3, 4, and 5A, respectively), indicating that the M131 target is also an abundant subsurface antigen.

The M131 target antigen is not a protein. In order to establish whether the M131 target antigen was a protein, PK was incubated with *T. pallidum* OMV and Triton X-100-treated organisms before M131 incubation. Following PK treatment, no protein was detectable by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-sepa-

FIG. 1. Intradermal challenge of passively immunized rabbits. Rabbits were passively immunized with NRS, IRS, a control irrelevant monoclonal antibody (C MAb), and M131 MAb at 18 h before challenge, the day of challenge, and at 72 h postchallenge. All animals were challenged using 10^3 *T. pallidum* cells per intradermal site. Photographs of all animals shown were taken at 20 days postchallenge. The location on the backs of the animals where the challenge inoculum was injected is indicated by a small black pen mark. Representative animals are shown for NRS, IRS, and C MAb, while all three M131 passively immunized animals are shown.

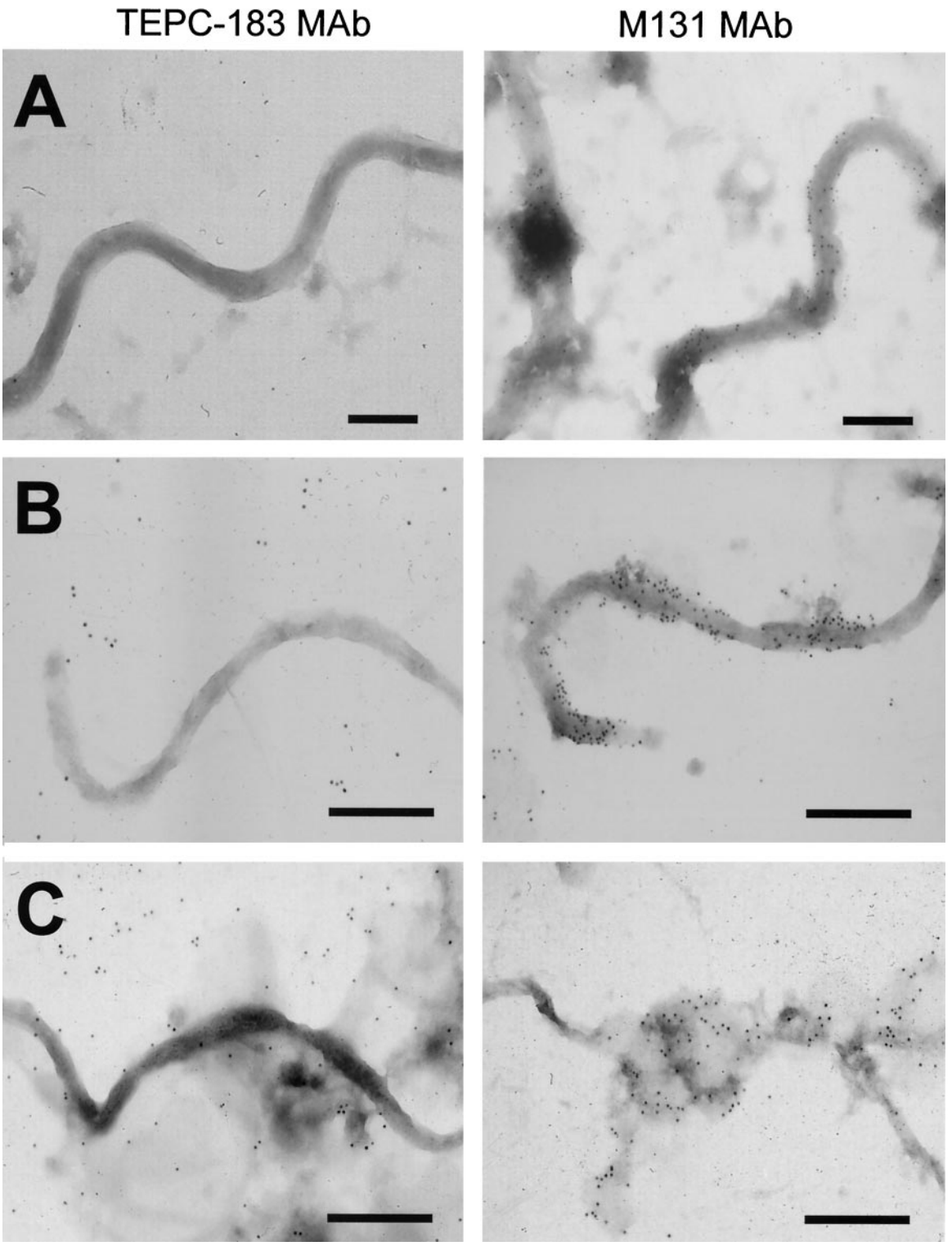


FIG. 3. Immunoelectron microscopy following M131 MAb or an irrelevant MAb (TEPC-183) incubation with living *T. pallidum* (A), Triton X-100-treated *T. pallidum* (B), and Triton X-100- and PK-treated *T. pallidum* (C). Antibody binding was detected using anti-mouse IgM conjugated to 10-nm colloidal gold particles. Bar in each micrograph indicates 0.5 μm.

rated samples (data not shown). As shown in Fig. 5B, both PK-treated organisms and PK-treated OMV showed M131 reactivity comparable to that of the non-PK-treated samples by dot blot analysis. This was further corroborated by IEM, where colloidal gold deposition following M131 incubation was demonstrated on membranous material from PK-treated organisms (Fig. 3). These findings showed that the *T. pallidum* target antigen of M131 is not a protein.

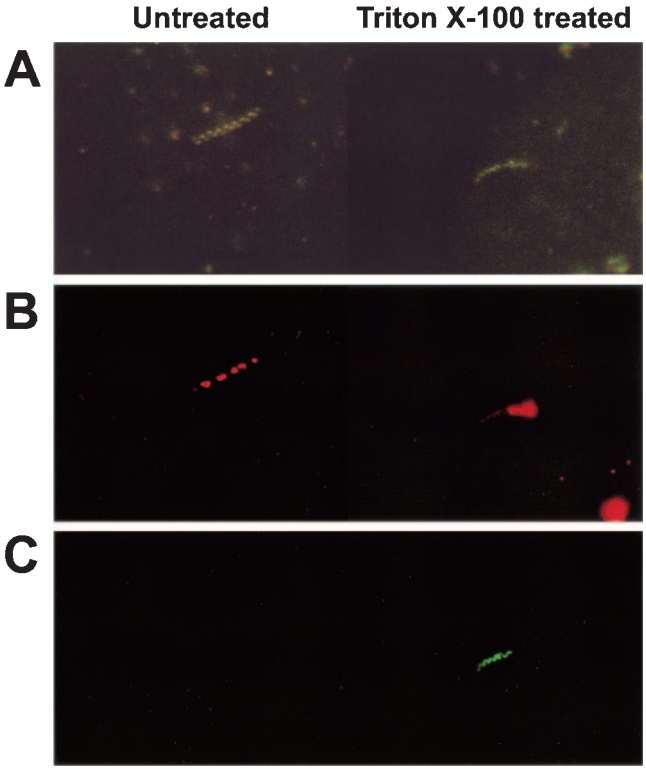


FIG. 4. Double-label indirect immunofluorescence of *T. pallidum* encapsulated in agarose gel microdroplets. Living *T. pallidum* cells encapsulated in agarose microdroplets were incubated with M131 and anti-*T. pallidum* periplasmic flagellar sheath protein prior to double-label immunofluorescence detection using specific rhodamine- and fluorescein-labeled conjugates. *T. pallidum* in microdroplets were also tested following treatment within the droplet by using 0.15% Triton X-100 in order to solubilize the *T. pallidum* outer membrane. The same representative organisms are shown, indicated by darkfield microscopy (A), detection of M131 binding (rhodamine) (B), and detection of anti-flagellar antibody binding (fluorescein) (C).

Demonstration that the M131 target resides in the *T. pallidum* lipid fraction. Since the above results showed that M131 reactivity is not associated with protein, we analyzed extracted *T. pallidum* lipid for M131 reactivity. *T. pallidum* lipid was tested in liposomal form for reactivity with M131. As shown in Fig. 5C, a dot blotted sample of liposomes constructed with *T. pallidum* lipid bound M131 but not the TEPC-183 control MAb. By comparison, when *T. pallidum* lipids were not in liposomal form, little to no reactivity was detected using M131 (data not shown).

***T. pallidum* phosphorylcholine is the basis of M131 reactivity.** In order to identify the specific lipid bound by M131, *T. pallidum* lipid was separated by RP-HPLC into fractions that were then generated into liposomes for testing with M131 (Fig. 6). As shown in Fig. 6A and B, 14 out of 70 fractions were positive for reactivity with M131. All reactive fractions, but not nonreactive fractions, were found to contain a predominant amount of phosphorylcholine-containing lipid by mass spectrometry. Mass spectrometry analysis of a representative positive fraction is shown in Fig. 6C and D. Since it has been reported that *T. pallidum* possesses several types of phospholipids, including phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, and cardiolipin (4, 33), we tested natural forms of these phospholipids for reactivity with M131. As shown in Fig. 6E, only liposomes made with phosphatidylcholine or sphingomyelin reacted with M131. It is pertinent to note that these two phospholipids, which are very different in terms of their fatty acid structures, both have a phosphorylcholine polar head group, consistent with the mass spectrometry result that the target epitope of M131 is phosphorylcholine. M131 reactivity was also dependent upon a liposomal form of either phosphatidylcholine (PC) or sphingomyelin (data not shown), as described above for *T. pallidum* lipid liposomes.

To address whether the phosphorylcholine composition of liposomes relates to the nature of the M131 binding epitope, we formed liposomes with mixtures of PC, which again possesses a phosphorylcholine head group, and PE, or with PC and PS in the molar proportions indicated in Fig. 7. The ability to bind M131 was lost when PC-PE liposomes contained less than 60% PC and when PC-PS liposomes contained less than 90% PC. This effect was not the result of simple dilution, since liposomes made with 90% PC and 10% PS did not lose reactivity with M131 when diluted as indicated in Fig. 7. These findings demonstrate that the epitope defined by M131 is dependent upon the phosphorylcholine concentration in a membranous environment and show that specific membrane

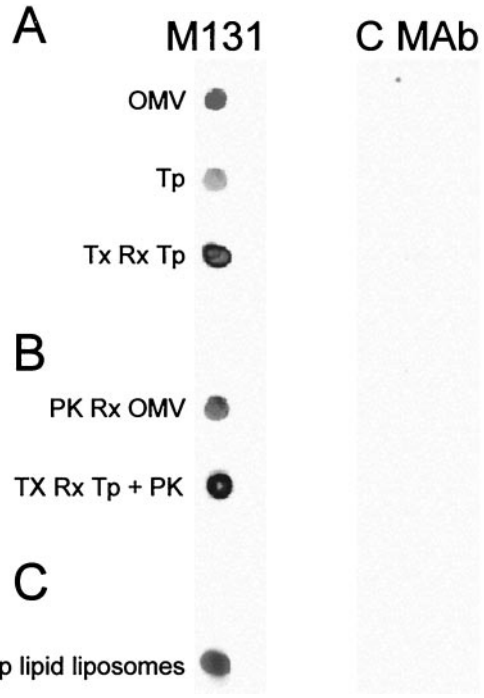
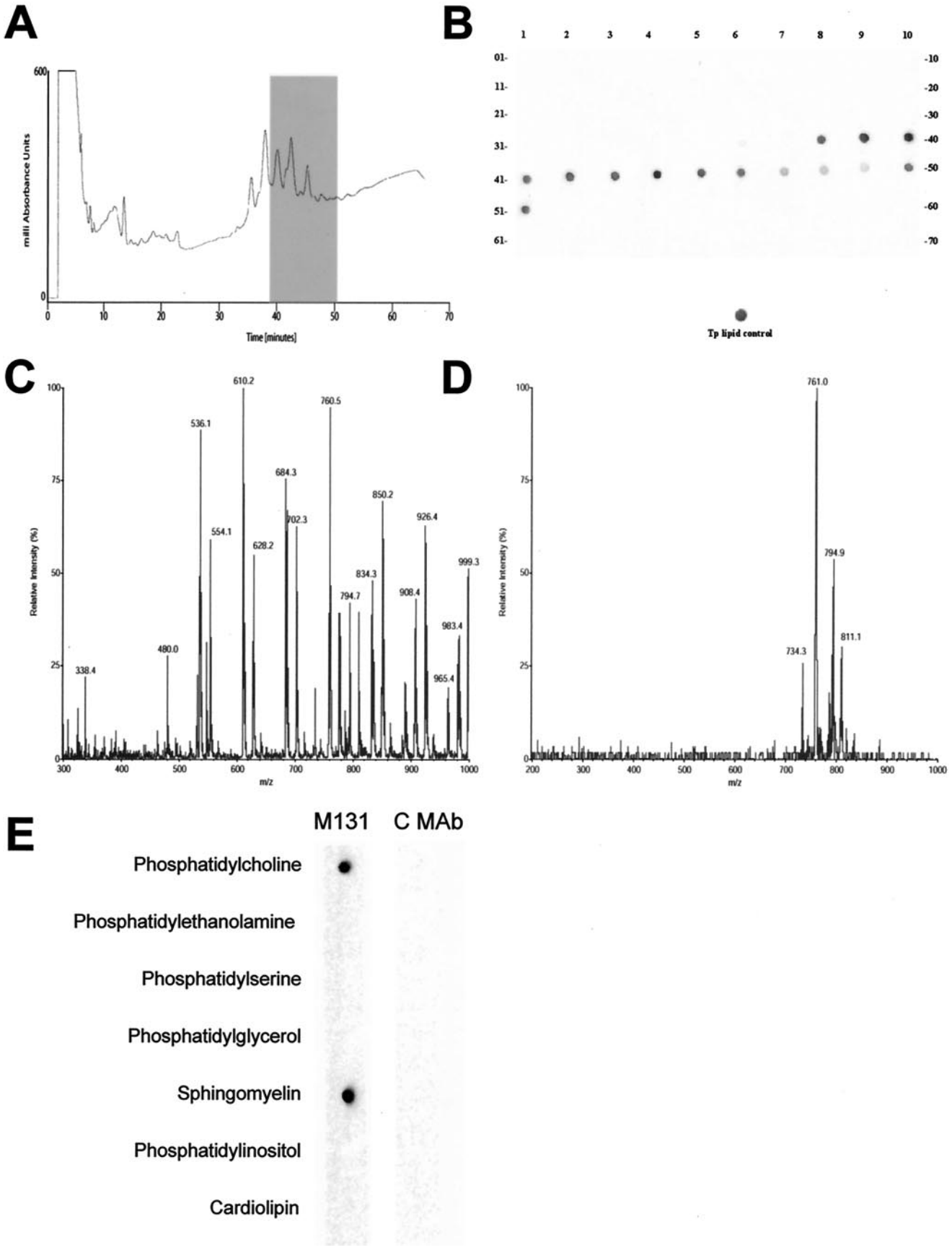


FIG. 5. Immuno dot blots of *T. pallidum* antigen preparations probed with M131 MAb and TEPC-183 MAb (C MAb). (A) Totals of 5×10^8 equivalents of *T. pallidum* OMV, 5×10^7 equivalents of intact *T. pallidum* organisms (Tp), and 5×10^7 equivalents of Triton X-100-treated *T. pallidum* (Tx Rx Tp). (B) Totals of 5×10^8 equivalents of *T. pallidum* OMV treated with PK (PK Rx OMV) and 5×10^7 equivalents of Triton X-100-treated *T. pallidum* additionally treated with PK (Tx Rx Tp + PK). (C) Liposomes made from 5×10^7 equivalents of methanol-chloroform-extracted *T. pallidum* lipid (Tp lipid liposomes).



phospholipids have different effects on the generation of this epitope.

The *T. pallidum* phosphorylcholine epitope defined by M131 is distinct from a phosphorylcholine epitope identified in other bacterial pathogens. Studies have shown that phosphorylcholine is an important surface component for several bacterial pathogens including *Haemophilus influenzae* (49), *Streptococcus pneumoniae* (18, 41, 42), *Pseudomonas aeruginosa* (48), and *Neisseria* spp. (43, 48). For several of these organisms, the phosphorylcholine epitope was identified using the monoclonal antibody TEPC-15. In order to determine whether *T. pallidum* also possesses this common epitope and whether M131 would bind to the common phosphorylcholine epitope, TEPC-15 and M131 were used to probe dot blots of phosphatidylcholine liposomes and phosphorylcholine conjugated to keyhole limpet hemocyanin (KLH; Biosearch Technologies, Novato, CA). TEPC-15 did not react with the phosphatidylcholine liposomes that reacted with M131, and M131 did not react with phosphorylcholine-KLH that reacted with TEPC-15 (data not shown). We have also found that M131 does not react with extracts of *P. aeruginosa* that possess the phosphorylcholine-decorated 43-kDa outer membrane protein, nor does TEPC-15 react with *T. pallidum* (data not shown). M131 also does not react with *Escherichia coli*, *Leptospira interrogans*, and "*Treponema phagedenis*" biotype Reiter (data not shown).

Lack of M131 binding to red blood cells and VDRL antigen. Phosphatidylcholine, which again possesses phosphorylcholine, is a major lipid constituent of RBC membranes and can constitute as much as 23.4% of the phospholipids in the exterior membrane leaflet (28). Phosphatidylcholine is known to be a major phospholipid component in the membranes of most mammalian cells (20). To determine whether M131 would react with the RBC membrane, RBCs were incubated in solution with M131 and analyzed for antibody binding by dot blot assay. As shown in Fig. 8, an RBC-specific antibody, but not M131, bound RBCs under these conditions. RBCs pretreated with PK to digest and remove potentially inhibiting surface proteins also failed to react with M131 (data not shown).

VDRL antigen contains cardiolipin, lecithin, and cholesterol and has been used for decades as a nontreponemal serodiagnostic screening test for the detection of syphilitic infection. Since lecithin contains approximately 23% phosphatidylcholine, we tested whether M131 would react with VDRL antigen by using the rapid plasma regain circle card test. M131 used at various dilutions failed to show any reactivity (data not shown). We also tested M131 for reactivity to VDRL antigen that was directly bound to nitrocellulose. The results showed that only IRS reacted, but not M131, TEPC-183 MAb, or NRS (data not shown).

Syphilitic infection results in antibody with reactivity against phosphatidylcholine liposomes. Because M131 reacts with liposomes made with phosphatidylcholine, we tested whether antibody develops during experimental syphilis that would also react with these liposomes. As shown in Fig. 9, all five IRS specimens tested, but not the five different preinfection NRS specimens, showed reactivity against phosphatidylcholine liposomes by dot blot analysis.

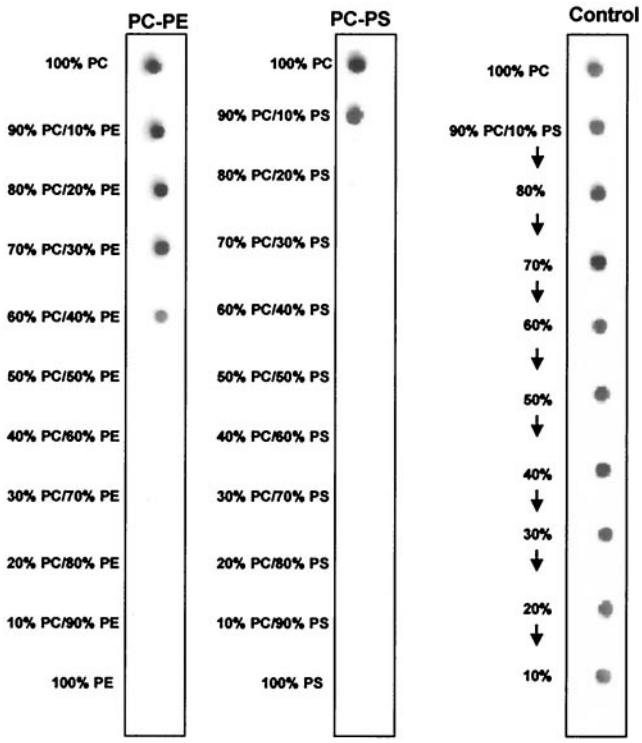


FIG. 7. Liposomes made with the indicated molar percentages of either PC and PE or phosphatidylcholine and PS were spotted on nitrocellulose membrane strips and probed with M131. A dilution control using the 90% phosphatidylcholine- and 10% phosphatidylserine-generated liposomes was diluted as indicated with PBS and also probed with M131.

DISCUSSION

The passive immunization findings presented in this study are best considered in the context of previous passive protection studies in rabbits. Turner et al. (45), Perine et al. (38), and Bishop and Miller (5) demonstrated lesion delays ranging from 3 to 6 days following either a prechallenge passive IRS immunization or the cessation of daily administration of IRS. The meaning of lesion delays in the rabbit after injection of *T. pallidum* was considered by Turner et al. Studies determining the relationship between *T. pallidum* inoculum size and incubation time (32, 46) estimated a generation time of 30 to 33 h for *T. pallidum.* A 10-fold difference in inoculum size resulted in a 4-day difference in the incubation period (46). In another

FIG. 6. *T. pallidum* lipid analysis demonstrating that phosphorylcholine is the target antigen of M131 reactivity. (A) UV trace at 210 nm of a lipid extract from *T. pallidum* eluting during RP-HPLC. Shaded region (minutes 38 to 51) indicates fractions immunoreactive with M131. (B) M131 MAb reactivity against spotted fractions shown in panel A. (C) Electrospray mass spectrum of a representative fraction (46 min) from the RP-HPLC shown in panel A. (D) Electrospray parent ion mass spectrum showing all the ions that give an *m*/*z* 184 fragment ion (corresponding to phosphorylcholine-containing lipids) from the same RPLC fraction used for panel C. (E) M131 MAb and TEPC-183 MAb (C MAb) reactivity against spotted samples of liposomes generated from the indicated phospholipids.

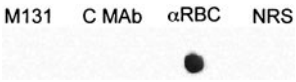


FIG. 8. Human RBCs were tested for reactivity against M131. A 1% suspension of RBCs was incubated with either M131, TEPC-183 MAb (C MAb), rabbit anti-human RBCs (αRBC), or NRS. After incubation, suspensions were washed, diluted to 0.1% RBCs, and spotted on nitrocellulose membrane strips. Antibody-antigen binding was detected using specific horseradish peroxidase-conjugated secondary antibodies and ECL.

study, a 3-day delay in lesion appearance was interpreted to represent a 10-fold reduction in the numbers of *T. pallidum* cells inoculated (45).

Knowing that M131 had potent in vitro bactericidal activity, we chose to conduct passive immunization experiments with M131 because it provided a means of assessing the protective potential of its target epitope without having it available as an immunogen. We were aware that administration of a mouse monoclonal antibody into rabbits would result in an antibody response that might clear M131 from blood and tissues. However, we reasoned that M131 administered to rabbits the day before challenge and immediately following challenge would still be available to reduce treponemal numbers before the development of anti-M131 antibodies.

Rabbits that received IRS developed lesions at a mean time of 13.2 days, as did rabbits that received the control monoclonal antibody. Rabbits that received NRS developed lesions at all sites at day 11. These times to lesion appearance were all within the range expected following an injection of 10^3 *T. pallidum* (5, 46). Although the administration of IRS resulted in no significant delay in time to lesion appearance, the lesions in this group remained atypical compared to those in controls in that they were much smaller, flatter, less erythematous, and nonulcerative. In this context, the findings in the group passively immunized with M131 were striking. At 17 out of 24 sites, lesions developed with an approximately 8-day delay compared with the control MAb group. At the remaining seven sites on one of these animals, no lesions ever developed. These findings represent a higher level of protection than that achieved with IRS in the previously reported experiments using the rabbit model (5, 38, 45).

The following considerations provide a hypothesis of how lesions developed at some sites but not others in the group that received M131. Given that 10^3 *T. pallidum* cells were injected at each site, the 8-day delay observed to lesion appearance is consistent with killing 99% of the organisms injected at a site. Thus, lesions appeared in the time frame expected if 10 organisms had been injected into that site. It has been reported that when one to five treponemes are injected intradermally, lesions may not develop (27). Also pertinent to our findings, Magnuson et al. (32) reported that when intradermal sites were each inoculated with 20 treponemes, lesions appeared at only 70% of the sites. Therefore, the failure of lesions to appear at 29% of the injection sites (7/24) in animals passively immunized with M131 is again consistent with a 99% killing of the 1,000-organism challenge inoculum that was used per site.

In an effort to relate lesion appearance to numbers of treponemes, we used real-time PCR to quantitate *T. pallidum* DNA copy numbers. Real-time PCR of control lesions over a 55-day period showed that at day 10 postchallenge, there were approximately 3×10^4 *T. pallidum* DNA copies per μg of rabbit DNA. Maximal copy numbers of approximately 5×10^6 were seen at day 20. This information is relevant to interpreting the significance of delays in lesion appearance conferred by passive immunization with M131. At day 20 postchallenge, which is approximately the mean time to lesion appearance in the group that received M131 (day 21), the numbers of *T. pallidum* DNA copies approximated those in the control animals at days 10 through 15. Further, these atypical lesions at day 20 postchallenge, compared to 20-day-postchallenge lesions from control animals that received control MAb or NRS, showed a three- to sixfold decrease in DNA copy numbers. However, by day 25 postchallenge, the DNA copy numbers in these atypical lesions were similar to those in the controls, even though these lesions were significantly smaller in appearance. These considerations do not take lesion volume into account, and these atypical smaller lesions may contain fewer total spirochetes than the normal typical larger lesions. Given this measurable decrease in the DNA copy number at 20 days postchallenge in animals passively immunized with M131, it is reasonable to infer that these differences might be even greater at earlier time points during lesion development. In support of this idea, we found that at sites where lesions did not develop in the rabbit passively immunized with M131, a greater than 4-log difference in DNA copy number was detected.

While lesion development and the numbers of treponemes in these developing lesions were clearly affected by passive immunization with M131, we did not observe an alteration in disseminated infection as determined by real-time PCR analysis and infectivity testing of popliteal lymph nodes. One explanation for the lack of protection from disseminated infection might be the requirement for a prolonged presence of killing antibody, whereas in our study, passive immunizations were administered only before, the day of, and shortly after the time of challenge.

The binding of M131 to the surfaces of *T. pallidum* cells was demonstrated by IEM, by indirect immunofluorescence of organisms in gel microdroplets, and by dot blot analysis of whole intact organisms. Treponemes incubated in the absence of complement with M131 for IEM were observed to be actively motile at the end of the incubation, confirming that organisms maintained structural integrity and that M131 binding was to surface outer membrane targets. Treponemes encapsulated in

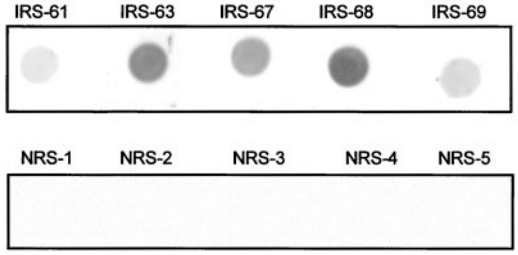


FIG. 9. Reactivity of IRS against phosphatidylcholine liposomes. Phosphatidylcholine liposomes were spotted on nitrocellulose membrane strips and incubated with different samples of either IRS or pre infection NRS diluted 1:100. Antibody-antigen binding was detected using horseradish peroxidase-conjugated secondary antibodies and ECL.

gel agarose microdroplets, a sensitive technique that preserves organism integrity (16), also showed specific M131 surface binding that was observed to be in a beaded pattern, suggesting antibody aggregation. Surface antibody aggregation has been proposed to be an important factor in the activation of complement and killing of *T. pallidum* (30). Treponemes whose outer membranes were removed by Triton X-100 treatment showed greater M131 binding by IEM, by the gel microdroplet assay, and by dot blot analysis, indicating that the target for M131 was both surface and prominently subsurface located on *T. pallidum*.

In our initial attempts to identify the target of M131, we speculated that it was one of the *T. pallidum* lipoproteins previously reported to be both outer membrane and subsurface located (9). However, the findings presented in this study showed that the target for M131 is not a protein but rather phosphorylcholine. The phospholipids phosphatidylcholine and sphingomyelin, both of which possess a phosphorylcholine polar head group, reacted with M131. In contrast, none of the other phospholipid species associated with *T. pallidum* (4, 33, 40), which do not possess a phosphorylcholine head group, were reactive with M131. The suggestion that phosphorylcholine is the specific target of M131 is further supported by the RP-HPLC fractionation of total *T. pallidum* lipid, which shows that all reactive fractions contain a phosphorylcholine-containing lipid. Of further interest was the finding that M131 binding to phosphatidylcholine, sphingomyelin, or *T. pallidum*-extracted lipid requires a liposomal form, suggesting that conformation or possibly membrane packing of these phospholipid polar head groups is required to generate the M131 binding epitope. This was further indicated by experiments showing that liposomes made in combination with phosphatidylcholine and either phosphatidylethanolamine or phosphatidylserine, two phospholipids that do not react with M131, require phosphatidylcholine concentrations of at least 60% and 90%, respectively, in order to maintain M131 reactivity. This suggests that the composition and perhaps distribution of *T. pallidum* phosphorylcholine are critical factors in generating this epitope.

Phosphatidylcholine has been previously shown to be the predominant phospholipid species in *T. pallidum* membranes and in the membranes of other members of the genus *Treponema* (4, 31, 33). Both *T. pallidum* and *Treponema denticola* possess a *licCA* fusion gene, suggesting that both utilize a CDP-choline pathway for the biosynthesis of phosphatidylcholine (22, 29). However, our finding that "*T. phagedenis*" biotype Reiter did not react with M131 further demonstrates the specificity of this epitope and indicates that the mere presence of a phosphorylcholine-containing phospholipid, like phosphatidylcholine, in a biological membrane is by itself not sufficient to generate the M131 binding epitope. It is pertinent to note that "*T. phagedenis*" and the other cultivatable treponemes, but not *T. pallidum*, possess lipopolysaccharide in their outer membranes. It is possible that lipopolysaccharide may inhibit the formation of this epitope in the outer membranes of these other treponeme species.

Phosphorylcholine has been shown to be an important pathogenesis-related surface molecule for several bacterial pathogens including *Haemophilus influenzae* (49), *Streptococcus pneumoniae* (18, 41, 42), *Pseudomonas aeruginosa* (48), and *Neisseria* spp. (43, 48). With several of these pathogens, a common phosphorylcholine epitope has been demonstrated using monoclonal antibodies, including MAb TEPC-15 that is specific for phosphorylchloline (48). In our studies, however, TEPC-15 did not react with *T. pallidum* or to phosphatidycholine liposomes and M131 did not react with *P. aeruginosa* or to phosphorylcholine-conjugated KLH, indicating that *T. pallidum* does not possess this common phosphorylchloline epitope and that the *T. pallidum* epitope defined by M131 is not common to these pathogens that possess phosphorylcholine.

VDRL antigen has been used for many decades as a serological screening test for the diagnosis of syphilitic infection. VDRL antigen contains lecithin, which is composed of approximately 23% phosphatidylcholine. Baker-Zander et al. (3) previously showed that immunization of rabbits with VDRL antigen elicited partial protection evidenced by both delays and the absence of lesions following challenge, similar to the findings presented here. However, as shown in this study, M131 did not react with VDRL antigen. Further, we have tested the anti-VDRL serum from the Baker-Zander study (kindly provided by Sheila Lukehart) and have found no bactericidal activity against *T. pallidum*. Taken together, these results suggest that the immunological target for the partial protection demonstrated in the Baker-Zander study is different from the target defined by M131.

We have also tested whether M131 would react with other biological membranes that contain phosphatidylcholine. Red blood cells are known to contain as much as 23.4% phosphatidylcholine in the exterior membrane leaflet (28). However, red blood cells also did not react with M131. These findings again suggest that the target of M131 is unique and possibly conferred by the phosphorylcholine composition and conformation in the membranes of *T. pallidum*.

We have previously reported that IRS binding to the surface of *T. pallidum*, evidenced by killing activity, cannot be demonstrated by immunoelectron microscopy (21). However, the binding of M131 to the *T. pallidum* surface was easily detected by this technique, indicating that the titer of antibodies in IRS to this phosphorylcholine epitope must not be high. Nonetheless, the reaction of phosphatidylcholine liposomes with all IRS specimens tested suggests that the phosphorylcholine epitope that M131 recognizes may contribute to the protective immunity that develops during syphilitic infection, although this hypothesis must be directly tested.

In an effort to elicit protective immunity, past studies have been conducted that have examined the course of experimental syphilis in the rabbit after active immunization with individual *T. pallidum* proteins. Immunizations with endoflagella (15), 4D (10), glycerophosphodiester phosphodiesterase (11), Tp92 (12), the 15-kDa lipoprotein (13), and recently, the *T. pallidum* repeat protein K (TprK) (14, 35) have been conducted. Challenge results of these studies have shown the development of atypical lesions at most sites, appearing in either the time frame expected for control lesions or in an accelerated time frame, but delays in lesion appearance were not observed. While several of these *T. pallidum* proteins have been suggested to be surface exposed, based in part upon antibodies that were either opsonophagocytic (11, 12, 14) or had some killing activity (10, 15), none of these proteins have been physically visualized on the surface of the spirochete by using these antibodies. Indeed, the microdroplet assay failed to demon-

strate surface exposure of the recently studied TprK protein (25), a member of a *T. pallidum* orthologous gene family that has been suggested to be surface exposed, based in part upon opsonophagocytic activity (14). By comparison, binding of M131 to the surfaces of structurally intact *T. pallidum* cells, as demonstrated by both the microdroplet assay and immunoelectron microscopy, represents the first physical demonstration of a *T. pallidum* surface antigen. The ability of the M131-defined epitope to serve as a protective immunogen is currently under study in our laboratory.

ACKNOWLEDGMENTS

We thank Xiao-Yang Wu for his excellent technical assistance in these studies.

This work was supported by U.S. Public Health Service grants AI21352 and AI-12601 to M. A. Lovett. Funds from the UCLA Jonsson Comprehensive Cancer Center (P30-A16042) were used toward the purchase of the matrix-assisted laser desorption ionization–time of flight mass spectrometer used. The authors have no conflicting financial interests.

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Editor: D. L. Burns