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# Specific Immunofluorescent Staining of Pathogenic Treponemes with a Monoclonal Antibody

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**Two hybrid cell lines which produced mouse monoclonal antibody to the DAL-1 street strain of *Treponema pallidum* subsp. *pallidum* were established. These monoclonal antibodies strongly reacted with *T. pallidum* subsp. *pallidum* (Nichols strain, DAL-1, and two other street strains, strains MN-1 and MN-3) and *T. pallidum* subsp. *pertenue* by indirect microimmunofluorescent antibody and enzyme-linked immunosorbent assay techniques, but they did not react with normal rabbit testicular tissue. These monoclonal antibodies did not react with nonpathogenic treponemes, such as *T. phagedenis* Reiter, *T. denticola* MRB, *T. refringens* Noguchi, or other spirochetes, such as *Borrelia burgdorferi* and *Leptospira interrogans* serovar pomona in microimmunofluorescent antibody smear slides or in Western blots (immunoblots). While unlabeled antibodies are useful for investigating the antigenic structures of *T. pallidum*, we labeled these monoclonal antibodies with fluorescein isothiocyanate and used them for diagnosing syphilis by direct staining of lesion exudate or *T. pallidum* subsp. *pallidum* in formalin-fixed tissues from patients suspected of having syphilis. Both monoclonal antibodies were directed against antigens of *T. pallidum* subsp. *pallidum* with a molecular weight of 37,000 as determined by the Western blotting technique.**

Syphilis is a chronic, complex sexually transmitted disease of humans caused by *Treponema pallidum* subsp. *pallidum*. The diagnosis of early syphilis is based on clinical presentation, serologic testing, and when possible, the demonstration by microscopy of *T. pallidum* subsp. *pallidum* in lesion exudates or lesion biopsy or autopsy tissue specimens. Unfortunately, each of these means of diagnosis is imperfect because (i) the clinical manifestations of syphilis may be confused with other genital ulcer diseases and a variety of diseases that cause rashes or lesions; (ii) serologic tests for syphilis may be nonreactive in up to 30% of patients with primary syphilis at the time of initial presentation (32); (iii) the serologic tests also have been reported to fail to detect at least one case of secondary syphilis in a patient with a human immunodeficiency virus infection (11); (iv) dark-field microscopy, although rapid and inexpensive, is often not available outside of sexually transmitted disease clinics or dermatology clinics and requires the expertise of a microscopist to differentiate *T. pallidum* subsp. *pallidum* from the cultivatable spirochetes common to the oral and rectal mucosae; and (v) the direct fluorescent antibody to *T. pallidum* procedure, while requiring less microscopic expertise, requires a labeled conjugate that must be absorbed to remove cross-reactivity with nonpathogenic treponemes, and the conjugate cannot be used in the direct staining of tissues (15), presumably because of the low specific titer. Thus, we need a rapid, sensitive, and specific procedure for identifying *T. pallidum* subsp. *pallidum* in smears and tissue sections.

Monoclonal antibody technology provides a means for producing specific antibodies without the necessity for a highly purified antigen and a complicated absorption procedure. Production of a monoclonal antibody that defines an immunodominant surface-exposed antigen with a molecular weight of 47,000 was reported by and characterized by Robertson et al. (30), Jones et al. (17), and Marchitto et al. (23–25). Other investigators (1, 2, 22, 28, 31, 34, 35, 37) have reported monoclonal antibodies for predominant *T. pallidum* subsp. *pallidum* antigens of 48, 47, 46, 44, 37, and 12 kDa. *T. pallidum* subsp. *pallidum* Nichols, which was isolated in 1912, was used in the previous studies to develop *T. pallidum*-specific monoclonal antibodies. We used a recent street isolate, DAL-1 (38) (courtesy of Michael Norgard), to establish two hybrid cell lines that produce a mouse monoclonal antibody to *T. pallidum* subsp. *pallidum* antigen of 37 kDa.

We describe for the first time monoclonal antibodies that are specific for *T. pallidum* that can be labeled with fluorescein isothiocyanate and that can be used to stain infected human tissues. In addition, we describe the use of this monoclonal antibody in the examination of chancre smears.

## MATERIALS AND METHODS

**Treponemal strain and antigens.** New Zealand White rabbits (weights, 6 to 8 lb [2.7 to 3.6 kg]) that were nonreactive by the Venereal Disease Research Laboratory test were inoculated intratesticularly with *T. pallidum* subsp. *pallidum* DAL-1, MN-1, or MN-3; *T. pallidum* subsp. *pallidum* Nichols; or *T. pallidum* subsp. *pertenue* Gauthier by using 0.5 ml (per testicle) of a suspension containing $10^7$ treponemes per ml of passage medium (0.85% [wt/vol] sodium chloride plus an equal volume of heat-inactivated [58 to 60°C, 3 h] normal rabbit serum). To harvest the treponemes, minced tissue of each testis was placed in 10 ml of extraction medium (0.01 M phosphate-buffered saline [PBS; pH 7.2] containing 0.075 M sodium citrate) and extracted for 15 to 20 min. Gross tissue debris was removed from the treponemal suspension by centrifugation at 500 × *g* for 10 min at 22 to 25°C. Treponemes purified by use of Percoll density gradients (10) (Pharmacia Fine Chemicals, Piscataway, N.J.) were used for immunization of mice and

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as a source of antigen for immunofluorescent, enzyme-linked immunosorbent assay (ELISA), and Western blot (immunoblot) techniques. The fluorescent treponemal antibody absorption (FTA-ABS) test antigen was obtained from the Biological Products Branch, Centers for Disease Control, Atlanta, Ga. *T. pallidum* subsp. *pallidum*-infected rabbit testicular tissues were also used for making tissue impression smears and were fixed in 10% neutral buffered formalin before being embedded in paraffin blocks for tissue study applications.

*T. phagedenis* Reiter, *T. denticola* MRB, and *T. refringens* Noguchi were grown in NIH thioglycolate broth (GIBCO Laboratories, Detroit, Mich.) containing 10% (vol/vol) heat-inactivated normal rabbit serum. Four-day-old cultures incubated at 37°C were sedimented by centrifugation at 12,000 × *g* for 10 min and washed three times in PBS. Antigen suspensions with protein concentrations adjusted to approximately 400 μg/ml were used for ELISA and electrophoretic analysis.

*Borrelia burgdorferi* was grown at 33°C for 5 to 7 days in a modified Barbour-Stoener-Kelly medium (4, 33), sedimented by centrifugation at 12,000 × *g* for 10 min, and washed three times in PBS. *B. burgdorferi*-infected hamster kidney, bladder, and spleen were received from R. C. Johnson, University of Minnesota, Minneapolis, in 10% buffered formalin for embedding in paraffin blocks.

*Leptospira interrogans* serovar pomona was grown at 25 to 30°C (6) for 7 days in Polysorbate Liquid Medium-5 (Armour Pharmaceuticals, Kankakee, Ill.), sedimented by centrifugation at 12,000 × *g* for 10 min, and then washed three times in PBS. *L. interrogans*-infected hamster kidneys were received from the National Veterinary Service Laboratories, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, in 10% buffered formalin and were processed as described above for the other tissues.

**Normal rabbit testicular extracts.** Normal rabbit testicular extracts were prepared by placing finely minced testicular tissue from three rabbits in a flask containing 100 ml of extraction medium. The flask containing the rabbit testicular tissues and medium was shaken on the rocker arm of a Burrell (Swedesboro, N.J.) wrist-action shaker at room temperature for 1 h, and then the gross tissue debris was removed by centrifugation at 210 × *g* for 1 min and 500 × *g* for 10 min. Supernatant from each of the steps was spun at 30,000 × *g* for 1 h, and the pellet was resuspended with 25 ml of supernatant for concentration.

**Monoclonal antibody production and immunoglobulin G (IgG) subtyping and quantitation.** Adult male BALB/c mice (4 to 5 weeks old) were immunized with Percoll-purified antigen (strain DAL-1) by intraperitoneal injection of $2.9 \times 10^8$ cells in a 1:1 solution of PBS-Freund's incomplete adjuvant. Three additional injections of *T. pallidum* subsp. *pallidum* in incomplete adjuvant were given on days 15, 29, and 66, with a final booster of $2.9 \times 10^8$ cells given intravenously without adjuvant on day 70.

SP 2/0 plasmacytoma cells and spleen cells from the immunized mice were fused by using a modification of the general procedures of Kennett et al. (19) and Galfre and Milstein (7). Briefly, spleen cells ($2 \times 10^7$) were mixed with SP 2/0 at a 4:1 ratio and fused in the presence of polyethylene glycol (molecular weight, 1,500) at a constant temperature of 37°C. The final cell pellet was resuspended in 2× hypoxanthine-aminopterin-thymidine medium and distributed into 96-well plates that were already prepared with a supportive mouse thymocyte suspension. Culture supernatants were tested in the screening assays described below, and selected cultures were cloned twice by limiting dilution.

Monoclonal antibody isotypes were identified by immunofluorescence in the microimmunofluorescent antibody (MIFA) procedure described below with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse subclass-specific antibody (Fisher Scientific, Orangeburg, N.Y.) and with a commercial isotyping kit (Behring Diagnostic, La Jolla, Calif.). Immunoglobulin secretion was measured by radial immunodiffusion.

**MIFA screening assay.** Culture supernatants were screened by an MIFA technique (13) initially by using *T. pallidum* subsp. *pallidum* DAL-1 and *T. phagedenis* Reiter. Antigens were placed on microscope slides with a Hunt size 104 pinpoint. Slides were air dried and fixed in acetone. Supernatants were placed on antigen slides and incubated for 30 min at 37°C. After appropriate washing in PBS, fluorescein-labeled anti-mouse globulin was added and incubation was repeated. Supernatants that continued to develop specific antibody were further screened with *T. pallidum* subsp. *pertenue*, normal testicular tissue, *T. denticola*, *T. refringens*, *L. interrogans* serovar pomona, and *B. burgdorferi*. When they developed, ascitic fluids were tested in a similar manner.

**ELISA screening.** Potentially useful clones were also selected by testing culture supernatants in an ELISA. We used either 50 μl of whole-cell antigen ($4 \times 10^8$ Nichols or Reiter strains per ml) or 50 μl of normal rabbit testicular extract in PBS as the antigen for this test. The PBS suspensions were dried onto the surface of a 96-well polyvinyl chloride plate (Dynatech Laboratories, Inc., Chantilly, Va.) overnight at 37°C. Then, the antigen was resuspended in 100% ethanol, and the alcohol was evaporated at 37°C for 2 h. Next, the plates were blocked for 2 h at 37°C with 1% fetal calf serum (FCS) in 0.02 M PBS (pH 7.2). All subsequent serum dilutions and wash steps were performed with the same buffer but included 0.25 M NaCl and 0.2% Tween 20. Horseradish peroxidase goat anti-mouse conjugate and the substrate *o*-phenylenediamine were used. Optical density readings of less than 0.100 at 492 nm were considered negative; those over 0.200 were considered positive (MR580 Microplate reader; Dynatech Laboratories, Inc., Chantilly, Va.).

**SDS-PAGE and Western blot.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting were done with the Bio-Rad Mini-Protean II and Mini-Blot systems (Bio-Rad Laboratories, Richmond, Calif.), respectively, by using the methods of Laemmli (20) and Towbin et al. (36), respectively. Briefly, antigens and molecular weight markers (Pharmacia) were diluted 1:1 with treatment buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.005% bromophenol blue), heated in a 100°C water bath, cooled, and loaded onto 10 to 20% SDS gradient gels (Daiichi Pure Chemicals Company, Tokyo, Japan) or 11 to 17% SDS gradient gels poured in our laboratory and were then electrophoresed at 200 V for 55 min. Gels were next blotted onto 0.22-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) at 100 V for 1 h, blocked with Tris-buffered saline (TBS) (25 mM Tris-HCl [pH 7.5], 0.25 M NaCl) with 1% FCS for 2 h, and incubated for 2 h with 1:100 dilutions of antisera in TBS–1% FCS with 0.05% Tween 20. After being washed with TBS-FCS-Tween 20, the membranes were incubated for 1 h with either horseradish peroxidase anti-mouse or anti-rabbit conjugates (Bio-Rad) and were developed with the substrate 4-chloro-1-naphthol (Bio-Rad). All incubations

and washes were done at room temperature. Portions of some membranes were stained with colloidal gold (Enprotech, Hyde Park, Mass.) after they were washed with TBS–0.3% Tween 20.

**Ascites production and immunoglobulin separation.** Mice that had been primed with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) intraperitoneally 1 to 2 weeks earlier received intraperitoneal injections of $10^6$ hybrid cells. After 10 days, the swelled abdomens of the mice were tapped with an 18-gauge needle, and the ascitic fluid was drained into a sterile polyethylene container. This fluid was allowed to sit at room temperature for 2 h and then overnight at 4°C before being subjected to two spins at 500 × *g* for 10 to 20 min to remove the clot and cells. This was followed by a 30-min centrifugation at 30,000 × *g*, after which any surface film or precipitate was discarded. The ascitic fluids were filtered and mixed 50/50 with 1 M NaCl and 0.05 M borate buffer (pH 8.5) before they were applied to a protein A column that was equilibrated with the same buffer. After unwanted proteins were washed through, immunoglobulins were eluted with an acid buffer (pH 3.0) into an equal volume of 1 M Tris buffer (pH 9.0). All fractions were monitored at 280 nm for protein.

**Evaluation of ascitic fluids.** Mouse ascitic fluids were diluted in PBS starting at a 1:5 dilution and going through to a 1:12,800 dilution, and then each dilution was tested on *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *phagedenis* antigen slides. FITC-labeled $F(ab')_2$ fragment to anti-mouse IgG or anti-mouse whole globulin was used as the indicator system.

**FITC labeling of monoclonal antibody.** Ascites immunoglobulin fractions separated with a protein A column were pooled and dialyzed against PBS and concentrated with an Amicon stir cell. FITC was added by standard procedures. Globulin was labeled with a fluorescein/protein ratio of 12 μg/mg by standard procedures (26).

**Evaluation of FITC-labeled monoclonal antibody for smears.** FITC-labeled mouse anti-*T. pallidum* globulin was diluted 1:5 through 1:12,800 in PBS (pH 7.2) containing 2% Tween 80. Each dilution was tested for staining and specificity against the homologous *T. pallidum* subsp. *pallidum* strain, the *T. pallidum* subsp. *pallidum* Nichols FTA-ABS test antigen, *T. pallidum* subsp. *pertenue* Gauthier, impression smears of *T. pallidum* subsp. *pallidum* Nichols-infected rabbit testes, *T. phagedenis* Reiter, *T. denticola* MRB, *T. refringens* Noguchi, *L. interrogans* serovar pomona, and *B. burgdorferi*. After an initial evaluation of acetone-fixed antigens that were stained for 30 min at 37°C (5), staining times were varied from 5 to 30 min at 37°C. Antigen smears were fixed in acetone for 10 min (5), 10% methanol for 30 s (5), or 100% methanol for 10 s. Storage temperatures for antigen smears before staining were compared with and without 100% methanol fixation by using impression smears of *T. pallidum* subsp. *pallidum*-infected rabbit testes and FTA-ABS test antigen. Undiluted FITC-labeled globulin stored frozen at −70°C and FITC-labeled globulin diluted 1:2 in glycerol and stored at −20°C were compared for over an 11-month period. Mounting medium buffered with 0.2 M $Na_2HPO_4$ (16) was compared with mounting medium buffered with PBS (pH 7.2). Duplicate antigen smears were used to evaluate the intensity of staining.

To determine whether the monoclonal antibodies were produced against a surface antigen, freshly extracted treponemes with and without 1% Triton X-114 (Sigma Chemical Company, St. Louis, Mo.) (29) were combined with a 1:10 dilution of the monoclonal antibody in PBS or with PBS alone. The suspensions were incubated for 30 min at 2 to 8°C and were then washed in PBS by centrifuging the suspensions in a Beckman microcentrifuge for 1 to 2 min; the sediment was resuspended in PBS and recentrifuged. The sedimented treponemes were then resuspended in a 1:100 dilution of an FITC-labeled $F(ab')^2$ fragment of anti-mouse IgG and were reincubated and washed as described above. The final treponeme sediments were examined in wet mounts for fluorescing treponemes. FTA-ABS test antigen treated in the same manner was used as a control.

**Evaluation of FITC-labeled monoclonal antibody in the direct fluorescent-antibody tissue test for *T. pallidum*.** FITC-labeled monoclonal antibody was evaluated in the direct fluorescent-antibody tissue test for *T. pallidum* as described previously (12). Formalin-fixed paraffin blocks submitted to the Treponemal Pathogenesis and Immunobiology Branch, Division of Sexually Transmitted Diseases Laboratory Research, Centers for Disease Control, were cut at a thickness of approximately 3 μm and processed as described previously (15). The deparaffinized tissue sections were stained with the FITC-labeled monoclonal antibody or the FITC-labeled anti-*T. pallidum* conjugate in current use. The conjugates were diluted in PBS (pH 7.2) containing 2% Tween 80 and a 1:20,000 dilution of Evans blue. Slides were pretreated with 1% $NH_4OH$–0.25% trypsin or received no pretreatment prior to staining. *T. pallidum* subsp. *pallidum*-infected human gastric mucosal tissue was received from John F. Turner, Jr. (Roanoke Memorial Hospital, Roanoke, Va.), and *T. pallidum* subsp. *pallidum*-infected human brain tissue was received from Ronald Woosley (Oklahoma State Health Department, Lawton, Okla.).

## RESULTS

**Isolation of hybrid cell clones producing antibodies to *T. pallidum* subsp. *pallidum*.** Of 376 primary fusion wells, 81 wells with hybrid cell clones were screened by MIFA. We detected nine wells that reacted with *T. pallidum* subsp. *pallidum* DAL-1 by MIFA but that did not react with *T. phagedenis*. None of the nine wells with hybrid cell clones contained an anti-rabbit testicular tissue antibody or an anti-*T. phagedenis* antibody in the supernatant, as determined by screening by ELISA. On the basis of the results of the MIFA assay and ELISA, three wells were selected for cloning by limiting dilution and further characterization. Following a second screening by the MIFA assay and ELISA, two hybrid cell clones were picked and propagated for the production of monoclonal antibodies.

**Specificity of monoclonal antibodies in the MIFA assay.** The specificities of the ascitic fluids with monoclonal antibody LLP/73 in the MIFA assay are demonstrated in Table 1. Essentially no difference between monoclonal antibodies LLP/73 and LLO/2 was detected. The monoclonal antibodies were specific by the MIFA assay for the pathogenic *Treponema* spp. The two monoclonal antibodies were identified by both the immunofluorescence and ELISA procedures as being of the IgG1 isotype.

**Cross-reactivities and molecular specificities of anti-*T. pallidum* subsp. *pallidum* monoclonal antibodies.** Monoclonal antibodies LLO/2 and LLP/73 reacted with the 37-kDa molecule of *T. pallidum* subsp. *pallidum* DAL-1, as determined by the Western blotting technique. In Fig. 1, the 37-kDa molecule is demonstrated with monoclonal antibody LLP/73 when the monoclonal antibody and human syphilitic serum were reacted with the DAL-1 strain. Both monoclonal antibodies LLP/73 and LLO/2 reacted with a 37-kDa mole-

TABLE 1. Specificity of ascites monoclonal antibody LLP/73 in an indirect immunofluorescent test

| Antigen | Specificity at the following reciprocal dilutions: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 50 | 100 | 200 | 400 | 800 | 1,600 | 3,200 | 6,400 | 12,800 |
| *T. pallidum* subsp. *pallidum* | | | | | | | | | | |
| DAL-1 | 4+ | 4+ | 4+ | 4+ | 3+ | 2+ | 2+ | 2+ | 2+ | 1+ |
| Nichols, FTA 89-0070 | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 1+ | 1+ | ± | − |
| *T. pallidum* subsp. *pertenue* Gauthier | 4+ | 4+ | 4+ | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ |
| *T. phagedenis* Reiter | − | − | − | − | − | − | − | − | − | − |
| *T. refringens* Noguchi | − | − | − | − | − | − | − | − | − | − |
| *T. denticola* MRB | ± | − | − | − | − | − | − | − | − | − |
| *B. burgdorferi* | − | − | − | − | − | − | − | − | − | − |
| *L. interrogans* serovar pomona | − | − | − | − | − | − | − | − | − | − |

cule of *T. pallidum* subsp. *pallidum* Nichols and *T. pallidum* subsp. *pertenue* Gauthier but did not react with *T. phagedenis* or normal rabbit testicular tissue (Fig. 2 with the monoclonal antibody LLP/73). In similar gels, neither monoclonal antibody reacted with *T. refringens*, *T. denticola*, *B. burgdorferi*, *L. interrogans*, or *Escherichia coli*; but both monoclonal antibodies reacted with two additional street strains, MN-1 and MN-3.

**Serology of the monoclonal antibody.** The ascitic fluids were reactive in the FTA-ABS test, nonreactive in the microhemagglutination assay for antibodies to *T. pallidum*, and nonreactive in the rapid plasma reagin card test.

**Staining characteristics of monoclonal antibodies in the direct fluorescent-antibody test for *T. pallidum*.** FITC-labeled monoclonal antibodies LLO/2 and LLP/73 were tested on acetone-fixed smears of the homologous strains *T. pallidum* subsp. *pallidum* DAL-1 and Nichols, *T. phagedenis* Reiter, *T. denticola* MRB, *T. refringens* Noguchi, *B. burgdorferi*, and *L. interrogans* serovar pomona. The homologous 2+ staining endpoint ranged from 1:12,800 to 1:51,200, with no staining at a 1:5 dilution for the other spirochetes tested. The staining demonstrated over 11 months later was essentially the same when conjugate that had been frozen at −70°C or diluted 1:2 in glycerol and stored at −10 to −20°C was used. The intensity of staining of the treponemes was influenced by the method of fixation, the incubation time at 37°C, the microscopic magnification, and the pH of the mounting medium. Treponemal staining on acetone- and 10% methanol-fixed slides was not as brilliant as staining of antigens that were fixed with 100% methanol (Table 2). The specificity for pathogenic treponemes was not altered when fixation with 100% methanol was examined with other spirochetes. Increased microscopic magnification and the use of oil objectives enhanced the clarity and degree of staining (Table 3). Ample staining was observed when the antigen and antibody reaction times were reduced from 30 to 10 min, provided that oil objectives were used. Staining of *T. pallidum*-infected rabbit testicular impression smears after fixation with acetone, 10% methanol, or 100% methanol showed an increase in the number of spirochetes that adhered to the slide when methanol was used. Staining intensity was enhanced with the 0.2 M $Na_2HPO_4$-buffered mounting medium.

Impression smears of *T. pallidum* subsp. *pallidum*-infected rabbit testes, unfixed or fixed in 100% methanol, were stored at 25°C or at 2 to 8°C for 14 days. *T. pallidum* subsp. *pallidum* on slides that were held for up to 14 days at either temperature with and without fixation stained with approximately the same 3+ to 4+ intensities. *T. pallidum* subsp.

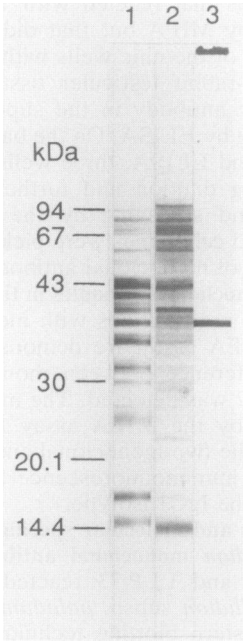


FIG. 1. Western blot analysis of 11 to 17% gradient PAGE of *T. pallidum* subsp. *pallidum* DAL-1. Lane 1, incubation with human syphilitic serum; lane 2, colloidal gold stain of protein on membrane; lane 3, incubation with monoclonal antibody LLP/73.

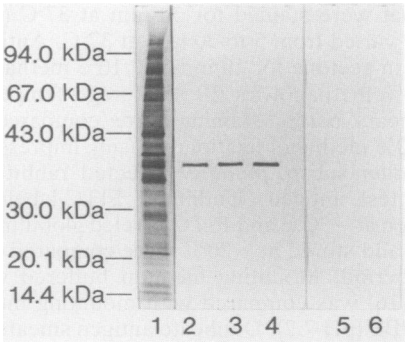


FIG. 2. Western blot of 10 to 20% gradient PAGE. Lane 1, gold stain of *T. pallidum* subsp. *pallidum* Nichols; lanes 2 to 6, monoclonal antibody LLP/73 reacted with *T. pallidum* subsp. *pallidum* Nichols, *T. pallidum* subsp. *pallidum* DAL-1, *T. pallidum* subsp. *pertenue* Gauthier, normal rabbit testicular tissue, and *T. phagedenis*, respectively.

TABLE 2. Effect of antigen fixation on staining when using FITC-labeled *T. pallidum* monoclonal antibody

| Antigen | Fixative | Titer |
|---|---|---|
| *T. pallidum* subsp. *pallidum* DAL-1 | Acetone | 51,200 |
| | 10% Methanol | 51,200 |
| | 100% Methanol | ≥51,200 |
| *T. pallidum* subsp. *pallidum* Nichols | Acetone | 1,600 |
| | 10% Methanol | 800 |
| | 100% Methanol | 51,200 |
| *T. pallidum* subsp. *pertenue* Gauthier | Acetone | 1,600 |
| | 10% Methanol | 800 |
| | 100% Methanol | ≥51,200 |

*pallidum* in chancre smears from 23 patients who were dark-field positive for syphilis also stained well.

The fresh suspension of treponemes did not stain in wet mounts with monoclonal antibody LLP/73 alone; only in the suspension containing LLP/73 plus 1% Triton X-114 could immunofluorescent antibody staining be detected. Staining intensity was a bright 3+ to 4+ fluorescence after adding Triton X-114. The FTA-ABS test antigen stained in both suspensions containing LLP/73. One percent Triton X-114 was not needed to obtain 4+ staining, and the addition of 1% Triton X-114 did not interfere with staining.

**Staining characteristics of monoclonal antibodies in the direct fluorescent-antibody tissue test for *T. pallidum*.** Titration of LLP/73 on *T. pallidum*-infected rabbit and human tissues indicated that although a 2+ staining intensity was obtained on both tissues through a 1:2,560 dilution, maximum fluorescence (3+ to 4+) was obtained at a 1:160 or a 1:320 dilution. The 1:160 dilution was selected for evaluating diagnostic tissues. Examples of the stainings that were obtained in two diagnostic samples from patients with syphilis are shown in Fig. 3. Staining could be accomplished following trypsin or $NH_4OH$ pretreatment of mounted sections.

The specificities of the FITC-labeled monoclonal antibodies (LLP/73 and LLO/2) were demonstrated by our failure to stain hamster tissues infected with *B. burgdorferi* and *L. interrogans* with a 1:40 dilution of the conjugates. In addition, we were unable to detect staining of any organisms in a human fetal tissue sample known to contain a pathogenic *Borrelia* isolate with the FITC-labeled LLP/73. The titer obtained when the LLP/73 conjugate was tested on rabbit testicular sections infected with *T. pallidum* subsp. *pertenue* was approximately the same as the titer obtained when *T. pallidum* subsp. *pallidum* was tested. Thus, conjugate dilution could not be used as a means to separate *T. pallidum* subsp. *pallidum* from *T. pallidum* subsp. *pertenue*.

## DISCUSSION

In this report, we described the production of monoclonal antibodies against strain DAL-1, a recently isolated street strain of *T. pallidum* subsp. *pallidum*, and the application of the FITC-labeled antibody to the identification of *T. pallidum* subsp. *pallidum* in smears and tissue sections. The molecular specificity of the two monoclonal antibodies (LLO/2 and LLP/73) was 37 kDa. According to Western blot results, LLO/2 and LLP/73 reacted with analogous 37-kDa antigen components of the *T. pallidum* subsp. *pallidum* Nichols strain and *T. pallidum* subsp. *pertenue* but not with the other spirochetes tested. By two-dimensional gel electrophoresis with a nonequilibrium pH gradient, which has the advantage of resolving very basic proteins that are not stained on standard isoelectric focusing gels, a prominent 37-kDa polypeptide as well as a lesser band at 80 kDa were detected (28a). The possibility of shared epitopes will be discussed in a review by S. J. Norris (28b). Studies of the humoral response to *T. pallidum* subsp. *pallidum* (3, 9, 21) found that an antigen of approximately 37 kDa contains common and specific determinants. The finding that our monoclonal antibody to the 37-kDa antigen was specific for *T. pallidum* subsp. *pallidum* tends to support the theory of Lukehart et al. (22) that the common and specific determinants may be located on separate polypeptides that comigrate by SDS-PAGE. Bailey et al. (2) produced four monoclonal antibodies against the 37-kDa molecule by inoculating mice with purified axial filaments. Two of these monoclonal

TABLE 3. Effect of incubation time and microscopic magnification on treponemal staining with monoclonal antibody LLP/73

| Time and magnification | Staining intensity at the following reciprocal dilutions: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 100 | 200 | 400 | 800 | 1,600 | 3,200 | 6,400 | 12,800 | 25,000 | 51,200 |
| 5 min | | | | | | | | | | |
| ×40, high dry | 2+ | 2+ | 1+ | 1+ | 1+ | 1+ | ± | ± | − | − |
| ×63, oil | 3+ | 3+ | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | ± | − |
| ×100, oil | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | 1+ | 1+ | − | − |
| 10 min | | | | | | | | | | |
| ×40, high dry | 2+ | 2+ | 2+ | 2+ | 1+ | 1+ | 1+ | − | − | − |
| ×63, oil | 3+ | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | 1+ | 1+ | ± |
| ×100, oil | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 2+ | 2+ | 1+ | 1+ |
| 20 min | | | | | | | | | | |
| ×40, high dry | 3+ | 2+ | 2+ | 2+ | 2+ | 2+ | 1+ | ± | ± | ± |
| ×63, oil | 4+ | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ | 1+ | ± |
| ×100, oil | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ | 1+ | 1+ |
| 30 min | | | | | | | | | | |
| ×40, high dry | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 2+ | 1+ | 1+ | ± |
| ×63, oil | 4+ | 4+ | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ | 1+ |
| ×100, oil | 4+ | 4+ | 3+ | 3+ | 3+ | 2+ | 2+ | 2+ | 1+ | 1+ |

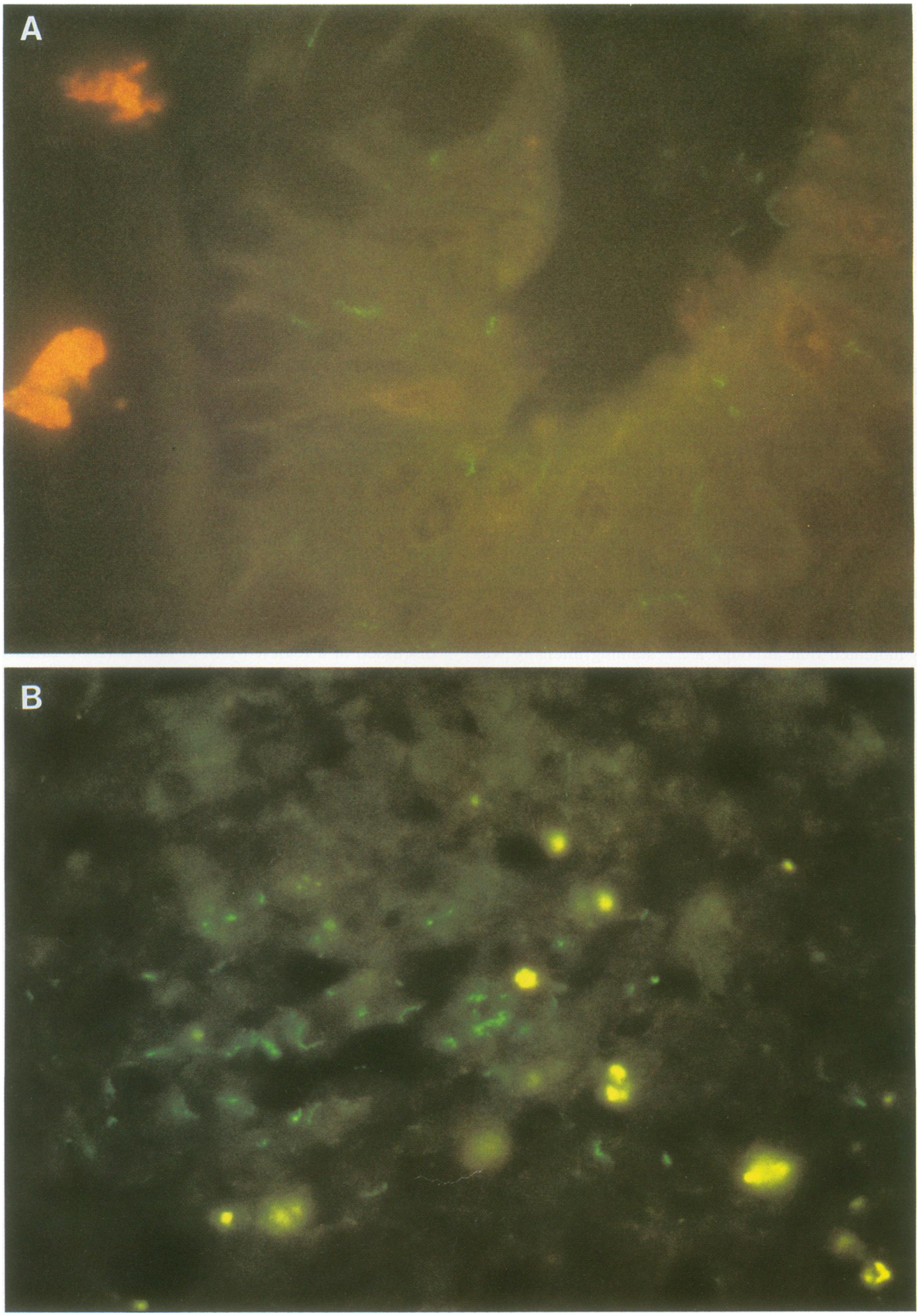


FIG. 3. Direct immunofluorescent staining of *T. pallidum* with FITC-labeled monoclonal antibody LLP/73 in human gastric tissue (A) and human brain tissue (B). Magnification, ×220.

antibodies were specific for *T. pallidum* subsp. *pallidum*, and two also reacted with a 39.5-kDa molecule of *T. phagedenis*. Monoclonal antibody to the 37-kDa molecule was not obtained when mice were inoculated with unfractionated treponemes. We obtained monoclonal antibodies to the 37-kDa molecule after inoculating mice with intact Percoll-purified treponemes. We have not detected the common determinant by MIFA, the direct fluorescent-antibody test, or SDS-PAGE. We do not know whether the DAL-1 strain has more pathogen-specific determinants for the 37-kDa antigen than the Nichols strain does, but we produced a specific 37-kDa antibody that is applicable to the diagnosis of syphilis.

Because this antibody did not stimulate reactivity in the rapid plasma reagin card test and the microhemagglutination assay for antibodies to *T. pallidum* currently used in syphilis serology, the possibility of isolating this antigen for future vaccine studies should be considered, since immunization would not interfere with routine laboratory tests. However, yet to be considered in future studies is the development of protective immunity when this antigen is used.

Since the monoclonal antibodies were produced for immunofluorescence applications, the selection of suitable clones was based first on the results of an MIFA test and was later confirmed by an ELISA. Ascitic fluids remained reactive in the FTA-ABS test after absorption; however, absorption was not necessary because antibody was specific without sorbent. Clones were screened by the MIFA test for their ability to specifically identify *T. pallidum* subsp. *pallidum*. Reactivity by the immunofluorescence assay might support the supposition that antigens of *T. pallidum* subsp. *pallidum* to which the monoclonal antibodies LLO/2 and LLP/73 are directed are localized on the surface of the microorganisms, since antigen staining was affected by the method of fixation; however, in wet mounts, freshly isolated spirochetes did not react until 1% Triton X-114 was added. Antigens of 37 kDa (Nichols) have previously been reported (17, 27) to be localized on the surface of *T. pallidum* subsp. *pallidum*; however, we do not know whether our monoclonal antibodies produced with the DAL-1 strain detect the same antigen on the surface of treponemes.

Antigen slides stored at −20°C or room temperature, with and without the prefixing of smears before storage, stained equally well. Since chancre smears may be sent to a reference laboratory for testing, storage conditions that may affect the results needed evaluation. The improved staining with defined optics was not unexpected and has been emphasized in earlier reports (8, 14). The addition of 0.2 M $Na_2HPO_4$ to buffer the mounting medium enhanced staining because of the high alkaline pH (16).

Because the staining time in the direct fluorescent-antibody to *T. pallidum* test with monoclonal antibody can be reduced to 10 to 15 min without a reduction in staining intensity, as reported in an earlier study in which a broad-spectrum conjugate was used (18), wider use of the test as a replacement for the dark-field examination at the clinic level may be stimulated. In addition, the FITC-labeled monoclonal antibody LLP/73 appears to have a long shelf-life, since stable antibody activity was maintained for over 11 months.

A major advantage of using a monoclonal antibody for the detection of *T. pallidum* subsp. *pallidum* in lesion exudates or tissues taken from the lesion is the specificity of the antibody for a pathogen-specific determinant without complicated and inconsistent procedures such as absorption. The FITC-labeled monoclonal antibodies LLO/2 and LLP/73 yielded brilliant specific identification of *T. pallidum* in biopsy specimens. Although brilliant direct immunofluorescent staining without specificity is available, this is the first report of specific direct staining of *T. pallidum* in tissues. As shown in Fig. 3, *T. pallidum* subsp. *pallidum* was stained intensely in infected human brain and stomach membrane tissues as well as a variety of other tissues submitted for diagnostic testing (data not shown). The direct staining procedure is less complex than the indirect fluorescent-antibody or immunoperoxidase methods; however, the monoclonal antibodies could also be used in those techniques. Specific staining is especially beneficial when testing tissues from which other spirochetes could be isolated and would speed the diagnosis of syphilis.

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