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Monoclonal Antibodies to Surface Antigens of *Mycobacterium tuberculosis* and Their Use in a Modified Enzyme-Linked Immunosorbent Spot Assay for Detection of Mycobacteria

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Three monoclonal antibodies (MAbs) were generated from splenocytes of a BALB/c mouse immunized with heat-killed *Mycobacterium tuberculosis*. All three MAbs bound to surface epitopes of *M. tuberculosis* as shown by whole-cell enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, and immunoelectron microscopy. One immunoglobulin M (IgM) MAb bound to lipoarabinomannan, the second IgM MAb bound to mycolyl-arabinogalactan-peptidoglycan complex, and the third MAb, an IgG3, bound to a surface epitope of an uncertain nature. The MAbs demonstrated different cross-reactivity patterns with other mycobacteria. Two of the MAbs were used to develop a modified ELISA spot assay for the detection of mycobacteria.

The worldwide incidence of tuberculosis was estimated by the World Health Organization to be 8.8 million in 1995 and is expected to rise to 10.2 million by the year 2000 (3). Human immunodeficiency virus-related cases of tuberculosis are growing steadily (3). Major problems concerning tuberculosis are the need for rapid laboratory diagnosis, the requirement for prolonged treatment, and the absence of a reliable vaccine. Several antibody-based tests for the detection of *Mycobacterium tuberculosis* have been designed by using polyclonal antibodies and monoclonal antibodies (MAbs) (1, 2, 4, 6, 7, 11, 13, 14, 17, 18, 20, 21), but none have acquired a widespread role in the diagnosis of tuberculosis. Problems associated with antibody-based tests are cross-reactivity with other mycobacterial strains (2, 4, 6, 20, 21), the need for a large amount of mycobacteria or mycobacterial antigen to obtain significant results (1, 7, 11, 14, 17), and variable sensitivity. Improvements in antibody-based diagnostic tests for the detection of *M. tuberculosis* are likely to require specific antibody reagents with high affinities for mycobacterial antigens. Several MAbs have been generated against surface components of *M. tuberculosis* (1, 2, 8), but they are often cross-reactive with other strains or cytoplasmic fractions (2, 8). We describe here three MAbs that react strongly with surface epitopes of *M. tuberculosis*. On the basis of the selectivity of one of these MAbs, we designed a modified antibody-capture enzyme-linked immunosorbent (ELISA) spot assay.

MATERIALS AND METHODS

***M. tuberculosis* for immunization and hybridoma testing.** *M. tuberculosis* Erdman was obtained from Trudeau Mycobacterial Culture Collection (TMC), Trudeau Institute, Saranac Lake, N.Y. (strain TMC 107), and was grown in Proskauer-Beck-Trudeau (PBT) medium without Tween at 37°C for 5 weeks. Mycobacterial cells were washed twice in phosphate-buffered-saline (PBS), heat inactivated at 80°C for 2 h, and sonicated (Branson Ultrasonics, Danbury, Conn.) for 3 to 5 s.

Mycobacterial strains for cross-reactivity testing. The mycobacterial strains used in our study originated from the American Type Culture Collection (ATCC), Rockville, Md.; TMC; the Centers for Disease Control and Prevention (CDC), Atlanta, Ga.; P. D'Arcy Hart (PDH), London, England; and the College of American Pathologists (CAP), Northfield, Ill. *M. tuberculosis* TMC 107, *M. bovis* BCG (Pasteur Institute), *M. microti* (PDH), *M. avium* Inderlied 101 (CAP), *M. smegmatis* (CDC), *M. xenopi* ATCC 19250, *M. chitae* ATCC 19627, *M. marinum* ATCC 927, *M. chelonae* (CDC), *M. gastri* ATCC 25028, *M. kansasii* ATCC 12478, *M. vaccae* (CDC), *M. phlei* TMC 1516, *M. fortuitum* ATCC 6841, *M. terrae* ATCC 15755, *M. szulgai* ATCC 35799, *M. gordonae* ATCC 14470, and three clinical strains of *M. tuberculosis* identified by the Clinical Microbiology Laboratory, Montefiore Medical Center, Bronx, N.Y., were grown on Lowenstein-Jensen (LJ) slants. Several bacterial species were obtained as well: *Streptococcus pneumoniae*, *Escherichia coli*, *Corynebacterium pseudodiphtheriticum*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* (quality control strains from the Clinical Microbiology Laboratory, Montefiore Medical Center) and *Nocardia asteroides* (clinical isolate; Mycology Laboratory, Montefiore Medical Center). Cells were obtained from the surface of the medium with a sterile loop, suspended in PBS with 0.1 mM sodium azide, sonicated briefly as described above to break the clumps (when needed), and heat treated at 80°C for 2 h.

Whole-cell ELISA for *M. tuberculosis*. A 50-μl suspension of 1×10^7 to 2×10^7 *M. tuberculosis* cells suspended in PBS (pH 7.2) was placed in microtiter ELISA plate wells, and the plates were incubated at room temperature for 2 h. Prior to use in the ELISA the *M. tuberculosis* suspension was briefly sonicated as described above. The plates were blocked with 1% bovine serum albumin (BSA) and 0.05% horse serum in PBS, and stored at 4°C before use. The plates were washed three times with 0.05% Tween 20 in PBS. Hybridoma cell supernatants containing MAbs were added to each well, and the plates were incubated for 1 to 1.5 h at 37°C or overnight at 4°C. The plates were then washed three times, 1 μg of goat anti-mouse alkaline phosphatase-conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) per ml was added to each well, and the plates were incubated for 1 to 1.5 h at 37°C. After washing five times, a solution of 1 mg of *p*-nitrophenylphosphate (Southern Biotechnology Associates, Inc.) per ml in substrate buffer (0.001 M $MgCl_2$, 0.05 M Na_2CO_3 [pH 9.8]) was added (50 μl per well), and the A_{405} was measured in a Ceres 900 HDi reader (Bio-Tek Instruments Inc. Winooski, Vt.). The optical densities of three microtiter wells were averaged. Negative controls consisted of wells in which PBS was substituted for *M. tuberculosis.*

Immunization. BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with approximately 2×10^9 cells of *M. tuberculosis* Erdman in an emulsion with incomplete Freund's adjuvant (0.2 ml per mouse). The mice were boosted every 12 to 18 days for 7 weeks with 4.4×10^7 to 1×10^9 organisms. Some booster injections included incomplete Freund's adjuvant. Serum was examined for antibodies to *M. tuberculosis* by whole-cell ELISA, and the mouse with the highest rise in titer was boosted 4 days prior to fusion with 10^9 organisms in incomplete Freund's adjuvant.

Fusion. Spleen cells were harvested on day 50, fused with NSO cells, a murine myeloma fusion partner, at a ratio of 4:1, and suspended in medium containing hypoxanthine-aminopterin-thymide. A total of 12 plates were seeded with hybridomas and incubated at 37°C with 10% CO_2. Hybridoma supernatants were screened for antibody production by whole-cell ELISA.

Indirect immunofluorescence. The indirect immunofluorescence method was

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adapted from the method of Jones et al. (5). Approximately 10^7 heat-killed *M. tuberculosis* cells were placed on a poly-L-lysine-coated glass microscope slide (Poly-Prep slides; Sigma Diagnostics, St. Louis, Mo.) and were fixed by heating at 65°C for 2 h. Primary antibody was added at concentrations of 10, 1, 0.1, 0.01, and 0.001 μg/ml, and the slides were incubated for 30 min at room temperature. The slides were then washed with distilled water and incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin M (IgM) or IgG (Southern Biotechnology Associates, Inc.) at a concentration of 10 μg/ml for 30 min at room temperature in the dark. The slides were washed again with distilled water and sealed with mounting medium (1.4 g of glycine, 0.07 g of NaOH, 1.7 g of NaCl, and 0.1 g of sodium azide in 100 ml of distilled water [pH 8.6]) with 1% *n*-propyl gallate. As a positive control, separate slides with *M. tuberculosis* cells were acid-fast stained (Bacto Tb Carbolfuchsin KF, Bacto Tb Decolorizer, and Bacto Tb Methylene Blue; Difco Laboratories, Detroit, Mich.) prior to indirect immunofluorescence (with the MAbs at a concentration of 10 μg/ml). Negative controls consisted of *Cryptococcus neoformans* ATCC 24067 cells incubated with anti-*M. tuberculosis* antibodies and *M. tuberculosis* cells incubated with anticryptococcal MAbs of the same isotype. An additional negative control consisted of incubation of *M. tuberculosis* with FITC-labeled antibodies.

Immunoelectron microscopy. A small pellet of heat-killed *M. tuberculosis* was incubated in a microcentrifuge tube with 10 μg of MAb per ml in 1% BSA in PBS for 1 h at room temperature in a slow shaking motion. The cells were washed twice with PBS and incubated with gold-labelled goat anti-mouse IgM plus IgG (Amersham Life Science, Buckinghamshire, England) diluted 1:30 in 1% BSA in PBS under the same conditions described above. The cells were then washed and fixed in Trump's fixative solution (4% paraformaldehyde and 1% gluteraldehyde in 0.1 M phosphate buffer [pH 7.3]) overnight. Postfixation was done with 2% osmium for 1 h. Afterward the cells were washed in 0.1 M phosphate buffer (pH 7.3) and dehydrated by incubation in solutions with increasing ethanol concentrations (10 min each in 50, 70, 80, and 95% ethanol and then two 15-min dehydrations in 100% ethanol) and two 10-min dehydrations in acetonitrile. The cell pellet was then infiltrated with 1:1 acetonitrile–araldite-Epon overnight; this was followed by two changes of aralide-Epon and overnight incubation at room temperature. The blocks were polymerized for 2 days at 65°C. Thick sections were stained with toluidine blue, and thin sections were stained with 3% uranyl acetate in 30% ethanol for 15 min and with lead citrate for 2 min. The sections were examined in a JEOL 100CX or 100S electron microscope. Negative immunoelectron microscopy controls included samples incubated with irrelevant isotype-matched anticryptococcal MAbs and secondary gold-labelled reagents.

Epitope chemical analysis ELISAs. To investigate the nature of the epitopes recognized by the MAbs several ELISAs were used. Sodium *meta*-periodate treatment was used to determine whether the epitopes were carbohydrates. The sodium *meta*-periodate ELISA protocol was adapted from the method of Udaykumar and Saxena (16). A 50-μl volume containing 1×10^7 to 2×10^7 *M. tuberculosis* cells suspended in PBS was incubated in microtiter polystyrene plate wells for 2 h at room temperature. After the *M. tuberculosis* organisms attached to the plate, the supernatant was removed and 50 μl of 0.1 M sodium *meta*-periodate (Sigma Chemical Co. St. Louis, Mo.) in 0.1 M acetate buffer (pH 4.5) was added to each well. Control wells had buffer only. The plates were incubated for 2 h at 4°C in the dark, washed five times with 0.05% Tween 20 in PBS, and blocked with 200 μl of 1% BSA in PBS. The plates were then used in the ELISA to determine the level of antibody binding to periodate-treated *M. tuberculosis*. A similar ELISA procedure was done with proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) instead of sodium *meta*-periodate to investigate whether the epitope was susceptible to proteolytic digestion. Briefly, the plates were incubated with 100 μl of proteinase K at a concentration of 1 mg/ml in PBS or with PBS alone (as a control) at room temperature for 20 h and the plates were used as described above.

ELISA was used to determine MAb binding to mycobacterial fractions. Total lipid fraction (TLF), lipoarabinomannan (LAM), lipomannan (LM), mycolylarabinogalactan-peptidoglycan complex (mAGP; with protein contamination of 34 ng/mg), and phosphatidylinositol mannoside (PIM) were kindly supplied by P. J. Brennan and J. T. Belisle (Department of Microbiology, Colorado State University, Fort Collins). All fractions except the LM fraction were prepared from *M. tuberculosis* Erdman; the LM fraction was prepared from a fast-growing mycobacterium species.

For the TLF ELISA a modification of protocols described previously (1) was used. TLF was suspended in 100% ethanol, added to polystyrene microtiter plates, serially diluted starting at a concentration of 1 mg/ml, and air dried overnight. The plates were then blocked with a solution of 1% BSA in PBS with 0.05% horse serum for 1.5 h at 37°C and used to study MAb binding to the TLF by ELISA. Wells incubated with 100% ethanol without lipid antigen served as negative controls.

For the mycobacterial carbohydrate fraction ELISAs, a suspension of 100 μl of mycobacterial antigens dissolved in carbonate buffer (pH 9.6) was placed in microtiter ELISA plate wells and the plates were incubated overnight at 4°C. The concentrations of the LAM and mAGP antigens were 10 μg/ml and 1 mg/ml, respectively. LM and PIM were placed in polystyrene plates and serially diluted starting at 50 μg/ml. The plates were then blocked with 3% BSA in PBS for 1.5 h at 37°C. After washing, 50 μl of MAb solution (serial dilutions starting at 10 μg/ml for the LAM and mAGP ELISAs and a fixed concentration of 10 μg/ml for the PIM and LM ELISAs) was added and the ELISA procedure was followed as described above. For comparative LAM versus LM ELISA, 100 μl of antigen solution at 1 μg/ml was suspended in carbonate buffer (pH 9.6) and the mixture was placed in microtiter ELISA plates. A 50-μl volume of a relevant MAb (MAb 5c11) was serially diluted across a microtiter plate starting at 10 μg/ml, and the procedure was followed as described above. Wells containing 100 μl of carbonate buffer without antigen served as a control.

Western blot (immunoblot) analysis. *M. tuberculosis* Erdman cells were suspended in RIPA buffer (50 mM Tris Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), frozen at −70°C, thawed, sonicated for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis before and after reduction with β-mercaptoethanol in 12% gels. The gels were blotted onto nitrocellulose sheets, and nonspecific binding sites were blocked with 3% gelatin in Tris-buffered saline (TBS; pH 7.5; Bio-Rad Laboratories, Hercules, Calif.). The blots were incubated overnight with either 10 or 50 μg of MAb per ml diluted with 1% gelatin in 0.05% Tween in TBS at room temperature. After primary antibody incubation the blots were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody solution (Bio-Rad Laboratories) diluted 1:30 in 1% gelatin (Bio-Rad Laboratories). The blots were developed with color development reagents (Bio-Rad Laboratories) until the appearance of a brown color. The positive control was an IgG1 MAb to the 70-kDa heat shock protein of *M. tuberculosis.*

Binding to other mycobacterial and bacterial strains. Comparative binding to other mycobacterial strains was done by whole-cell ELISA and indirect immunofluorescence. Cells of mycobacterial and nonmycobacterial strains were suspended in PBS with 0.01 M sodium azide and washed twice. For whole-cell ELISA mycobacterial and bacterial cells were resuspended to a turbidity of a no. 1 McFarland standard, placed in microtiter polystyrene plates, and incubated overnight at 4°C. The plates were blocked with 1% BSA in PBS with 0.05% horse serum 1.5 h at 37°C. After washing, 50 μl of the MAb solution at 5 μg/ml was added, and the ELISA procedure was followed as described above.

For comparative indirect immunofluorescence the cells of mycobacterial strains were suspended in PBS with 0.01 M sodium azide and were washed twice with PBS. In addition to the standard strains, three clinical isolates of *M. tuberculosis* grown on LJ slants were tested. A 50-μl volume of the mycobacterial suspensions was placed on poly-L-lysine-coated glass microscope slides (Poly-Prep slides; Sigma Diagnostics) and fixed by heating at 65°C for 2 h. The immunofluorescence protocol was performed as described above by using primary antibody at a concentration of 5 μg/ml and secondary FITC-labelled antibody at a dilution of 1:100 (10 μg of anti-IgM per ml and 5 μg of anti-IgG per ml). Negative control tests consisted of incubating the various mycobacterial strains with FITC-labelled antibodies. The presence of mycobacteria on the slides was verified by acid-fast staining (performed on a separate slide).

Capture ELISA spot assay. The capture spot ELISA was adapted from the assay of Spira and Scharff (15) for the purpose of capturing and visualizing a single mycobacterium. A slide chamber (developed by Nunc, Inc. [Naperville, Ill.], as a research and development product) was used. The slide chamber combines the qualities of an ELISA plate and a microscope slide. The slide chamber is made of a Maxi-sorp-treated polystyrene slide to which removable 800-μl-volume chambers are attached. The chambers were coated with the first capture antibody by incubating a 200-μl solution of 10 μg of unlabeled goat anti-mouse IgM-specific antibody per ml at 37°C for 1.5 h. The chambers were then blocked for nonspecific binding by adding 400 μl of 2% BSA in PBS and incubating at 37°C for 1.5 h. The chambers were washed three times with 0.05% Tween 20 in PBS. MAb 5c11 (IgM) at a concentration of 10 μg/ml in PBS was then added, and the mixture was incubated at 37°C for 1.5 h; this was followed by washing with 0.05% Tween 20 in PBS. A sample preparation, consisting of heat-killed *M. tuberculosis* Erdman mixed with mouse serum, was incubated overnight at 4°C. The chambers were washed three times, and 5 μg of MAb 9d8 (IgG3) per ml was added. The chambers were incubated for 1 h at 37°C, washed as described above, and incubated with 1 μg of goat anti-mouse biotin-labeled IgG3 (Southern Biotechnology Associates, Inc.) per ml for 1 h at 37°C. After washing, a 200-μl volume of Vectastain ABC-AP (standard kit; VECTOR Laboratories, Burlingame, Calif.) was added and the chambers were incubated at room temperature for 30 min. After five wash cycles, staining was performed by adding 200 μl of 1 mg of bromo-4-chloro-3-indolyl phosphate (BCIP; Amersco, Solon, Ohio) per ml diluted in AMP buffer (95.8 ml of 2-amino-2-methyl-1-propanol, 0.1 ml of Triton X-405, 0.2 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of double-distilled water [ph 8.6]) (Sigma Chemical Co.). After 1 h the chambers were washed with distilled water and air dried. The chamber walls were removed, a coverslip was placed on the slide, and the slide was observed under a light microscope at ×100 magnification for mycobacteria which were stained blue.

RESULTS

Isolation of hybridomas producing anti-*M. tuberculosis* antibodies. A single fusion was performed with the spleen from the mouse that raised the highest antibody titer against whole-cell *M. tuberculosis* (titer, 2,187-fold over the background titer prior to the last boosting). A total of 1,152 wells were seeded with fused NSO myeloma splenocytes, and their supernatants

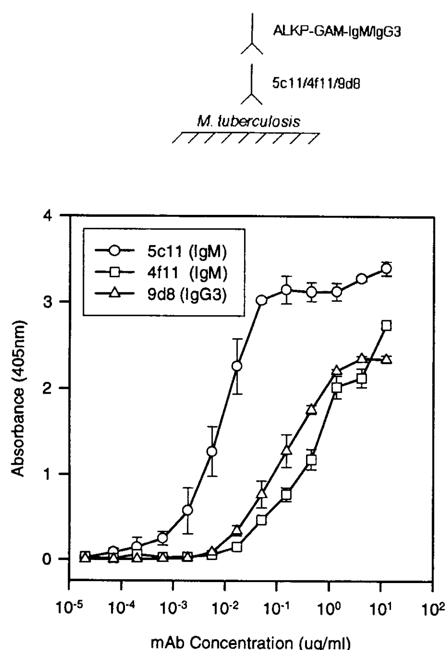

FIG. 1. Binding of MAbs 5c11, 4f11, and 9d8 to *M. tuberculosis* in whole-cell ELISA at various concentrations. The diagram indicates the ELISA configuration. Points represent averages of three measurements; error bars represent one standard deviation. The experiment was done twice, with similar results. ALKP, alkaline phosphatase; GAM, goat anti-mouse.

were screened by whole-cell *M. tuberculosis* ELISA 8 days after fusion. A total of 25 wells had optical densities of >0.4 at 405 nm. After 2 cloning procedures in soft agar three stable clones (clones 5c11, 9d8, and 4f11) were obtained. Isotype determination by ELISA with goat anti-mouse isotype-specific reagents revealed that one clone (clone 9d8) secreted IgG3 and two clones (clones 5c11 and 4f11) secreted IgM. All three MAbs had the kappa light chain.

***M. tuberculosis* whole-cell ELISA.** All three MAbs bound to plates coated with whole *M. tuberculosis* cells by ELISA. None of the MAbs bound to wells without *M. tuberculosis* coated with 1% BSA. Comparative binding of the three MAbs was performed by serially diluting the MAbs. The binding curves show that MAb 5c11 (IgM) required a 10 to 15 times lower concentration than MAb 4f11 (IgM) or MAb 9d8 (IgG3) to achieve the same optical density signal (Fig. 1). This difference was maintained even at very low optical density signals. This suggests either a higher binding affinity for MAb 5c11 or a higher prevalence of MAb 5c11 epitopes on the surface of *M. tuberculosis.*

Indirect immunofluorescence. All three MAbs showed strong indirect immunofluorescence after incubation with whole *M. tuberculosis* cells. The fluorescence intensity was strongest at MAb concentrations of 1 to 10 μg/ml and faded at MAb concentrations of between 0.1 and 0.01 μg/ml (Table 1). A positive control reaction by using acid-fast staining prior to the addition of MAbs (10 μg/ml) and FITC-conjugated antibodies altered slightly the pattern of immunofluorescence but had little or no effect on the fluorescence intensity (Fig. 2).

Immunoelectron microscopy. The binding of each MAb to *M. tuberculosis* was studied by immunoelectron microscopy. The mycobacterial cell wall architecture was easily visualized, but cytoplasmic mycobacterial structures could not be clearly identified because of the prolonged heat killing. Gold particles

TABLE 1. Immunofluorescence endpoints demonstrating signal intensities at various MAb concentrations[a]

MAb	Signal intensity at the indicated MAb concn (μg/ml)				
	10	1	0.1	0.01	0.001
5c11 (IgM)	+++	++	+[b]	−	−
4f11 (IgM)	++	++	+[b]	−	−
9d8 (IgG3)	+++	++	+	−	−

[a] The experiment was performed another time with different concentrations, and the results were comparable to those presented here.
[b] Weak fluorescence.

appeared to concentrate on the surface of the organism at or outside the level of the outer layer for each of the three MAb specimens (Fig. 3). No gold labelling was seen in the negative control samples. Localization of gold particles to cell surface structures is consistent with the results of whole-cell ELISA and immunofluorescence.

Epitope chemical analysis ELISAs. Sodium *meta*-periodate at acid pH causes mild oxidation of carbohydrate hydroxyl groups and opens sugar rings (19). Treatment of whole *M.*

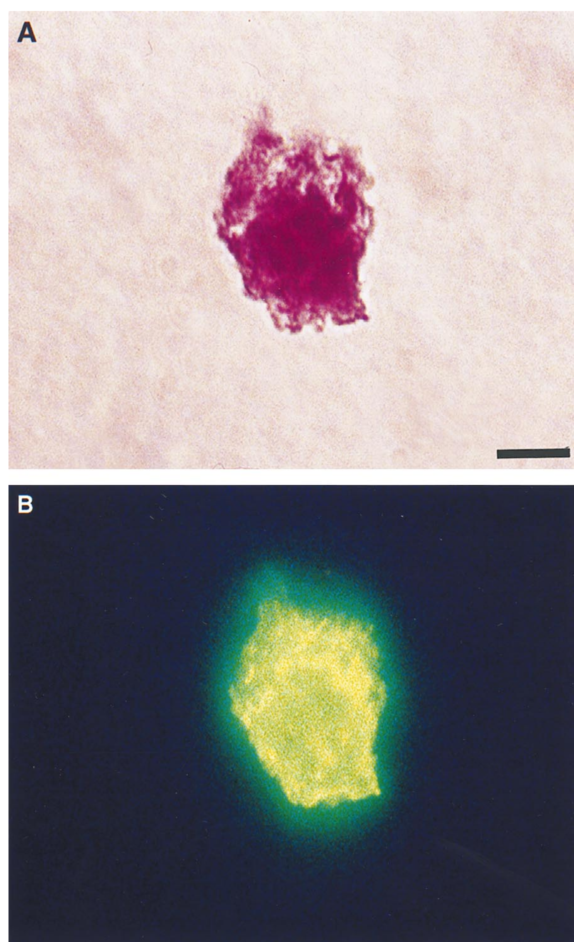

FIG. 2. Double staining of *M. tuberculosis* by acid-fast staining and immunofluorescence (shown here as a clump) with MAb 5c11 at a concentration of 10 μg/ml. (A) Acid-fast staining. (B) Indirect immunofluorescence. The experiment was done four times, with similar results. Immunostaining with MAbs 4f11 and 9d8 produced similar fluorescences (data not shown). Bar, 10 μm. The picture was generated with a Kodak RFS 2035 scanner and Adobe Photoshop, version 3.0, for Macintosh.

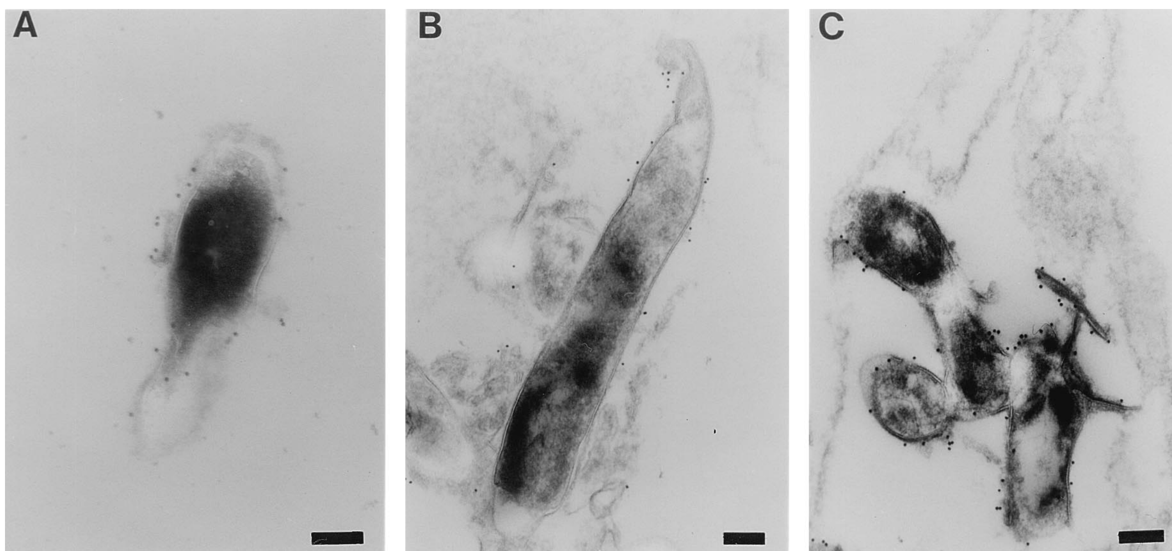

FIG. 3. Immunoelectron microscopy demonstrating the binding of MAbs to *M. tuberculosis*. Gold particles denote secondary antibody binding to the primary MAb. (A) MAb 5c11; (B) MAb 4f11; (C) MAb 9d8. Control experiments with irrelevant isotype-matched MAbs revealed no binding of gold balls to mycobacteria (data not shown). Bars, 0.2 μm.

tuberculosis cells with sodium *meta*-periodate resulted in reduced levels of binding of MAbs 5c11 and 4f11 to whole *M. tuberculosis* cells (Fig. 4), consistent with the presence of carbohydrates in the MAb epitopes. ELISAs performed with specific cell wall carbohydrates revealed that MAbs 5c11 and 4f11 bound to mAGP (Fig. 5B), while only MAb 5c11 bound to LAM (Fig. 5A). MAb 5c11 bound significantly more strongly to LAM than to LM at a MAb concentration of 1 μg/ml (Fig. 5C). Proteinase K treatment of whole *M. tuberculosis* cells reduced the level of binding of MAbs 9d8 and 4f11 but did not affect the binding of MAb 5c11 (Fig. 6). None of the MAbs bound PIM or TLF by ELISA.

Western blot analysis. None of the MAbs reacted with mycobacterial antigens by Western blot analysis, while the control MAb to the *M. tuberculosis* 70-kDa heat shock protein showed a clear band.

Binding to other mycobacterial strains. Two methods were used to compare MAb binding to other mycobacterial strains:

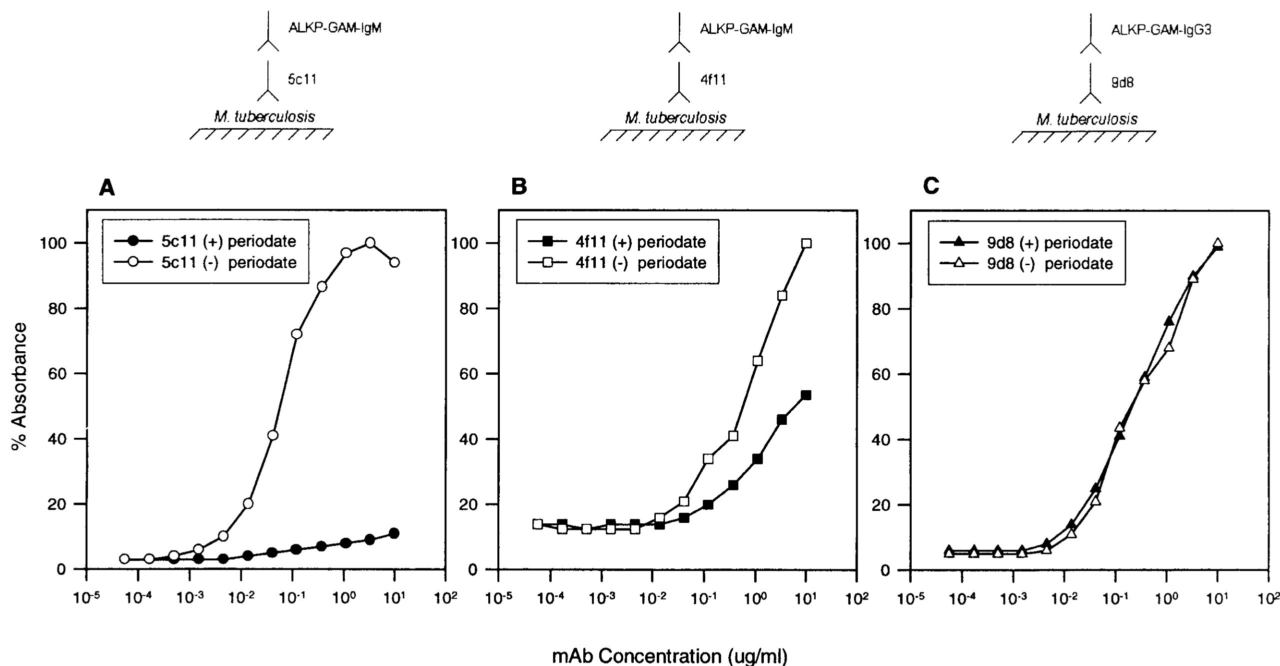

FIG. 4. Binding of MAbs 5c11, 4f11, and 9d8 to *M. tuberculosis* with and without sodium *meta*-periodate treatment, by whole-cell ELISA. Filled symbols correspond to MAb binding to periodate-treated mycobacteria, whereas open symbols correspond to non-periodate-treated mycobacteria. (A) MAb 5c11; (B) MAb 4f11; (C) MAb 9d8. The diagrams indicate the ELISA configurations. The percent absorbance was calculated relative to the maximal absorbance measured. For each point three absorbance measurements were made. ALKP, alkaline phosphatase; GAM, goat anti-mouse.

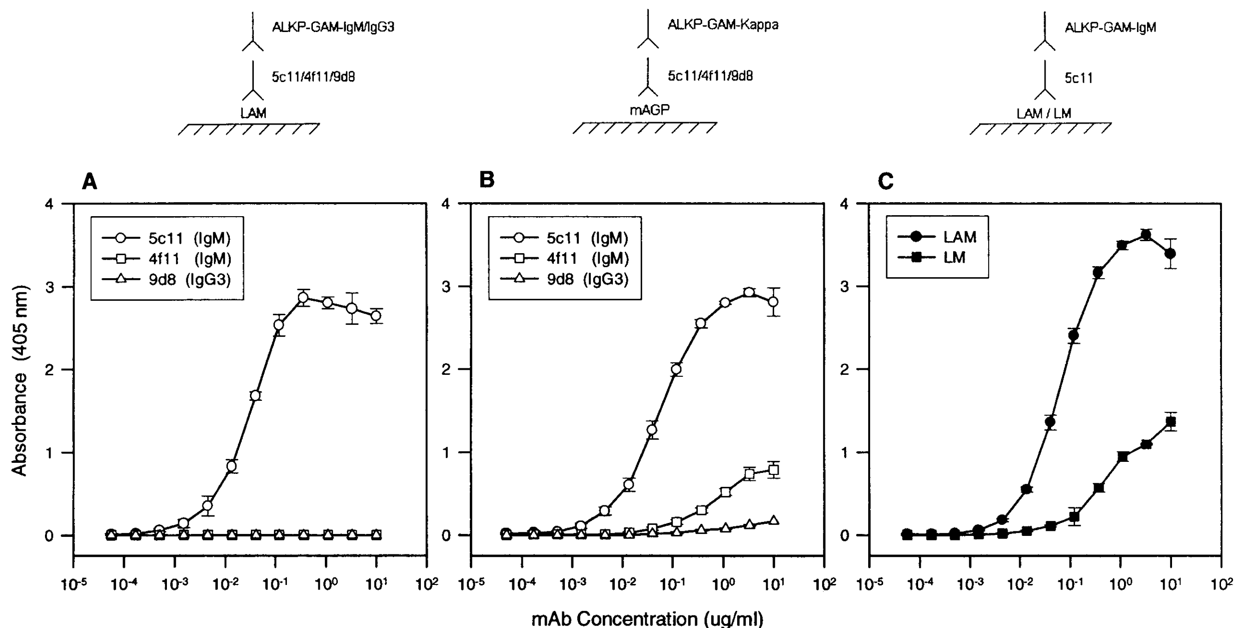

FIG. 5. Binding of MAbs at various concentrations to mycobacterial surface carbohydrates. (A) Binding of MAbs 5c11, 4f11, and 9d8 to LAM. (B) Binding of MAbs 5c11, 4f11, and 9d8 to mAGP. (C) Comparative binding of MAb 5c11 to LAM and LM at an antigen concentration of 1 μg/ml. The diagrams indicate the ELISA configurations. Values represent the averages of three measurements; error bars represent 1 standard deviation. Similar binding experiments were done another time with different concentrations, with comparable results. ALKP, alkaline phosphatase; GAM, goat anti-mouse.

whole-cell ELISA and indirect immunofluorescence. By whole-cell ELISA both IgM MAbs (MAbs 5c11 and 4f11) bound to multiple mycobacterial strains. IgG3 MAb 9d8 was more selective than the other MAbs. In addition to binding to the surface of *M. tuberculosis* MAb 9d8 also bound to *M. gordonae*, *M. gastri*, and *M. kansasii*. Indirect immunofluorescence demonstrated a trend similar to that demonstrated by the ELISA results (Table 2).

Capture ELISA spot assay. A capture spot ELISA was developed by taking advantage of the fact that we had MAbs of different specificities and isotypes. The strong binding of MAb 5c11 to *M. tuberculosis* suggested that it may be feasible to

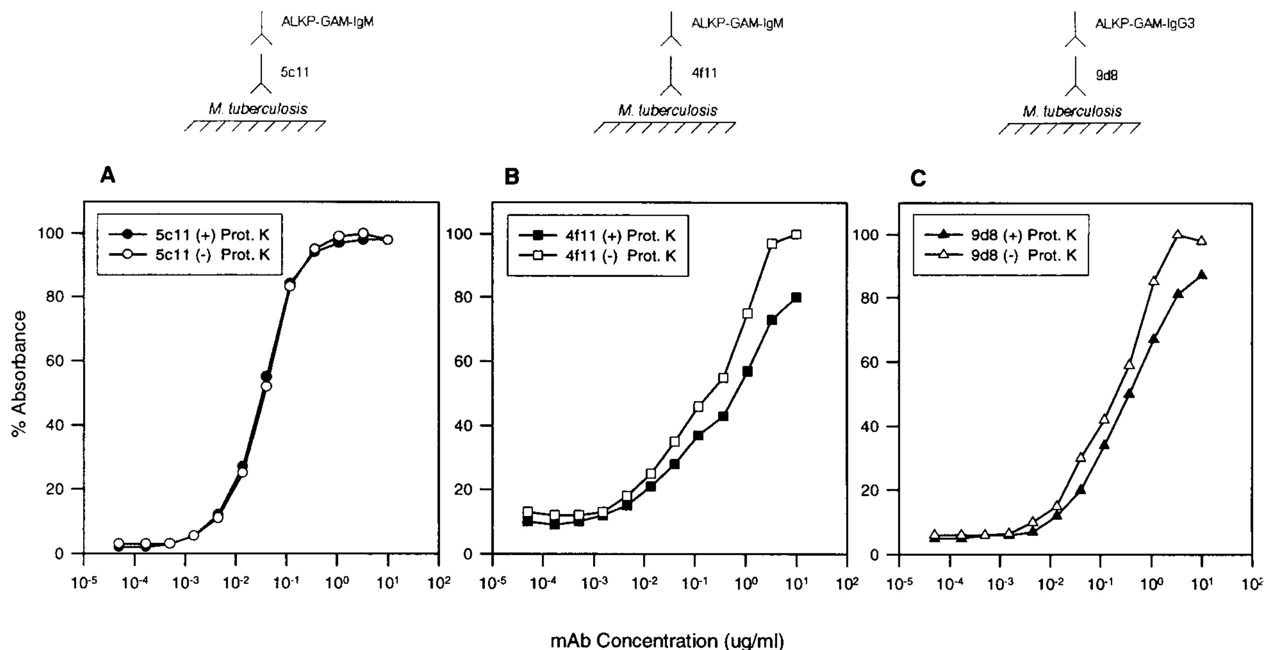

FIG. 6. Binding of MAbs to *M. tuberculosis* with and without pretreatment with proteinase K, by whole-cell ELISA. Filled symbols correspond to MAb binding to proteinase K-treated mycobacteria, whereas open symbols correspond to non-proteinase K-treated mycobacteria. (A) MAb 5c11; (B) MAb 4f11; (C) MAb 9d8. The diagrams indicate the ELISA configurations. The percent absorbance was calculated relative to the maximal absorbance measured. For each point three absorbance measurements were made. ALKP, alkaline phosphatase; GAM, goat anti-mouse.

TABLE 2. Binding of MAbs to various mycobacterial and nonmycobacterial strains

Organism	MAb 5c11 (IgM)		MAb 9d8 (IgG3)		MAb 4f11 (IgM)	
	ELISA[a]	IF[b]	ELISA	IF	ELISA	IF
M. tuberculosis[c]	1.000	+++	1.000	++	1.000	+++
M. tuberculosis[d]	0.490	++[e]	0.237	++[e]	0.393	++[e]
M. bovis BCG	0.614	+++	0.032	–	0.267	+++
M. microti	0.493	++	0.160	–	0.166	++
M. avium	0.175	++	0.013	–	0.070	++
M. smegmatis	0.491	+[f]	0.036	–	0.414	I[g]
M. xenopi	0.603	++	0.035	–	1.397	++
M. chitae	0.243	++	0.039	–	0.125	+
M. marinum	0.207	+	0.055	–	0.091	–
M. chelonae	0.377	++	0.050	–	0.199	+++
M. gastri	0.720	++	0.710	++	0.975	++
M. kansasii	0.535	++	0.485	+	0.606	+
M. vaccae	0.368	++	0.017	–	0.044	+
M. phlei	0.592	++	0.027	–	0.096	++
M. fortuitum	1.054	+	0.096	–	1.237	+
M. terrae	0.092	+	0.105	–	0.318	+
M. szulgai	0.836	++	0.163	–	2.090	++
M. gordonae	0.879	++	0.955	+	1.871	++
S. pneumoniae	0.000	ND[h]	0.000	ND	0.002	ND
E. coli	0.000	ND	0.001	ND	0.017	ND
C. pseudodiphtheriticum	0.002	ND	0.009	ND	0.009	ND
N. asteroides	0.049	ND	0.077	ND	0.109	ND
P. aeruginosa	0.000	ND	0.000	ND	0.000	ND
H. influenzae	0.001	ND	0.017	ND	0.031	ND

[a] ELISA comparison was done by determining the optical density ratio, using *M. tuberculosis* Erdman grown in PBT medium as the reference. The ELISA was performed twice. In the experiment whose results are presented here, we used heat-killed mycobacteria. In the other ELISA experiment we used intact non-*M. tuberculosis* mycobacteria which were not heat killed. Both experiments produced comparable results.

[b] IF, indirect immunofluorescence.

[c] *M. tuberculosis* Erdman grown in PBT medium.

[d] *M. tuberculosis* Erdman grown on LJ medium.

[e] Indirect immunofluorescence with three clinical strains of *M. tuberculosis* grown on LJ medium gave similar results.

[f] Weak fluorescence.

[g] I, indeterminate.

[h] ND, not done.

capture and immobilize mycobacteria. The mycobacteria captured by MAb 5c11 were detected by MAb 9d8 and visualized under a light microscope after staining. MAbs 5c11 and 9d8 recognized different epitopes, suggesting that the capture MAb (MAb 5c11) would not interfere with the binding of the detecting MAb (MAb 9d8). The use of insoluble BCIP stained the immobilized mycobacteria blue, and the use of a slide chamber with removable cell walls allowed a ×100 microscope lens to be used. This assay allowed the capture and visualization of single mycobacteria (Fig. 7).

DISCUSSION

All three MAbs generated in the present study bound to the surface of *M. tuberculosis*, as demonstrated by whole-cell ELISA, indirect immunofluorescence, and immunoelectron microscopy. The results of binding studies with defined mycobacterial fractions suggest that MAbs 5c11 and 4f11 bind to epitopes containing carbohydrates. MAb 5c11 binds to both LAM and LM, but the stronger affinity for LAM relative to that for LM by ELISA suggests that the arabinose moiety may be an important part of the recognized epitope. Both MAbs 5c11 and 4f11 bound to mAGP, which is a fraction of the mycobacterial cell wall left after removing all soluble carbohydrates, proteins, and lipids (9). The strong binding of MAb 5c11 to this complex is consistent with either the presence of LAM in the preparation or binding to arabinose, which is also found in the mAGP complex. mAGP is associated with protein in the mycobacterial cell wall skeleton in a complex called mycolyl-arabinogalactan-peptidoglycan-protein (mAGPP) (9). A small reduction in the level of binding of MAb 4f11 to proteinase K-treated *M. tuberculosis* suggested that the MAb 4f11 epitope is affected by proteinase K digestion. For MAb 9d8 we found no direct evidence for binding to protein, carbohydrate, or lipid antigen. However, treatment of mycobacteria with proteinase K also reduced the level of MAb 9d8 binding, suggesting that the MAb 9d8 epitope either contains or is attached to a protein moiety. No evidence for MAb binding to protein was obtained by Western blot analysis for any of the three MAbs. Hence, we tentatively conclude that MAb 5c11 binds to LAM, MAb 4f11 binds to a cell wall carbohydrate that belongs to the mAGP complex, and MAb 9d8 binds to a cell wall epitope of an uncertain composition which may be associated with protein.

The reactivities of the three MAbs with 17 mycobacterial and 6 nonmycobacterial species were investigated. MAbs 9d8 and 5c11 were the most and least selective, respectively, in their reactivities with different mycobacterial species. The low degree of selectivity of MAb 5c11 can be explained by the fact that most, if not all, mycobacterial strains contain LAM (10). None of the MAbs bound to nonmycobacterial bacterial species, consistent with their specificities for mycobacterial antigens. When interpreting the data in Table 2 it is important to consider that interspecies comparisons are difficult because there are differences in the adherence of mycobacterial species to polystyrene. This is not a problem for intraspecies comparisons of MAb 5c11, 9d8, and 4f11 binding. The ELISA and indirect immunofluorescence binding results parallel each other for the majority of mycobacterial species. For some strains such as *M. avium* indirect immunofluorescence and ELISA reactivities were significantly different. This problem is not understood but may reflect differences in epitope availability for mycobacteria attached to polystyrene or glass. The differences in MAbs 5c11, 9d8, and 4f11 with individual strains are consistent with recognition of different epitopes by each MAb.

Multiple protocols for direct ELISA (7, 14, 17), capture ELISA (1, 2, 4, 6, 12, 13, 18, 20, 21), and DOT ELISA (1, 11) have been described for the detection of mycobacteria and their antigens. Our assay differs from other assays in several aspects. First, our modified ELISA spot assay combines ELISA technology with light microscopy. Second, this is the first assay that applies the ELISA spot assay technique to the detection of microorganisms and allows for the visualization of a single captured mycobacterium. The use of two MAbs avoids the need for polyclonal immunoglobulins, which have the potential disadvantage of lot-to-lot variation, reliance on animal sources, and unwanted cross-reactivities. The use of MAbs to different mycobacterial epitopes prevents competition by the detecting MAb (MAb 9d8) and the capture MAb (MAb 5c11) and should, in theory, increase the sensitivity of the assay. Furthermore, use of the combination of a broadly cross-reactive, high-affinity MAb (MAb 5c11) with a more selective MAb (MAb 9d8) has the potential for offering sensitivity and specificity when analyzing clinical specimens. An additional advantage is the simplicity of the assay in terms of the equipment and personnel required for its use. In addition to the adapted slide chamber, this assay requires only ELISA reagents and a light

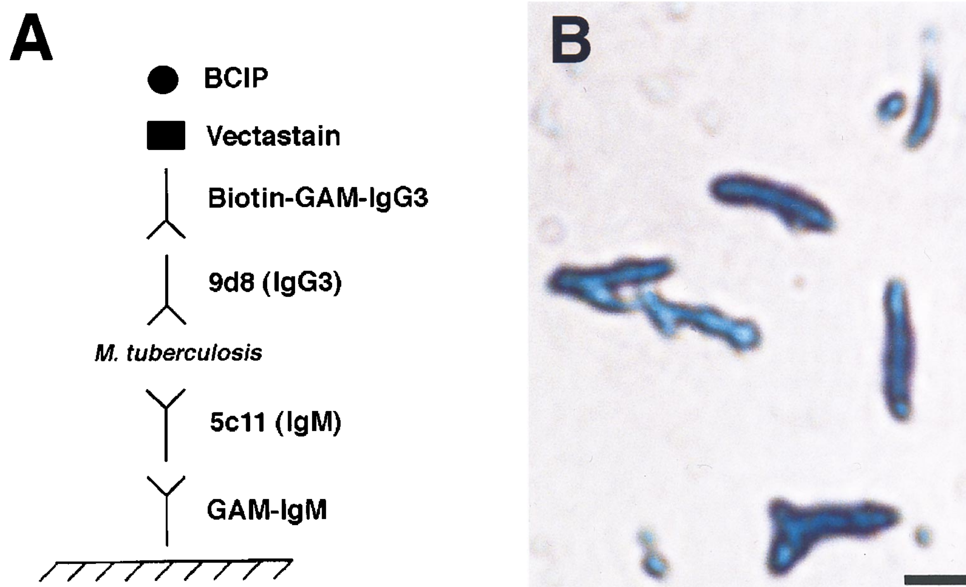

FIG. 7. Modified ELISA spot assay. (A) Graphic representation of the ELISA configuration. (B) Light microscopy image of *M. tuberculosis* captured and detected by the assay. Bar, 5 μm. The assay was performed in various formats four times, with similar results. The picture was generated with a Kodak RFS 2035 scanner and Adobe Photoshop, version 3.0, for Macintosh.

microscope. Completion of the assay required 1.5 days in our laboratory. If it is found to be applicable for use with clinical specimens, this assay may be developed into a useful tool for the detection of *M. tuberculosis*. The simplicity of the assay could be an advantage in laboratories where access to complex and expensive technology or instrumentation is not possible because of cost, as may be the case in many developing countries. Further experiments are required to determine the exact usefulness of this test for detecting *M. tuberculosis* directly in clinical specimens.

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