Modification of the *Mycobacterium bovis* Extracellular Protein MPB70 with Fluorescein for Rapid Detection of Specific Serum Antibodies by Fluorescence Polarization

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The principle of fluorescence polarization described by Perrin (F. Perrin, J. Phys. Radium 7:390–401, 1926) was applied to the development of a novel assay that used fluorescein-labeled *Mycobacterium bovis* secretory protein MPB70 for rapid detection of anti-MPB70 antibodies in selected sera from three *M. bovis*-infected species (elk, llama, and bison). Labeling of purified MPB70 with fluorescein isothiocyanate resulted in the incorporation of 0.96 ± 0.08 (mean ± standard deviation; n = 3) fluorescein group per MPB70 molecule. The labeled protein fluoresced strongly with an emission maximum at 518 nm when excited with light of a wavelength near 493 nm, and its immunoreactivity with anti-MPB70 monoclonal antibody 4C3/17 was not altered by modification with fluorescein. The fluorescence polarization assay protocol was optimized for analysis of serum samples by incorporating into the assay buffer 0.05% lithium dodecyl sulfate, which prevents the occurrence of some nonspecific interactions. Sera from *M. bovis*-infected animals, selected on the basis of exhibiting the presence of anti-MPB70 antibodies, as detected by enzyme-linked immunosorbent assay (ELISA), reacted with fluorescein-labeled MPB70, resulting in an increase in polarization of up to 330 milli-polarization units, in contrast to the values for noninfected sera (167 to 178 mP), which were close to that obtained in the absence of specific antibodies (164.7 ± 3.3 mP; n = 6). These results demonstrated the feasibility of using fluorescein-labeled MPB70 to detect anti-MPB70 antibodies by fluorescence polarization and suggested that the assay described here can be an alternative to ELISA or other antibody assay systems. The advantages of this original methodology and its general applicability to the diagnosis of infectious diseases are discussed.

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*Mycobacterium bovis* causes infection (tuberculosis) in a variety of farm and wild animals and also in humans (35). This organism, along with *M. tuberculosis*, *M. africanum*, and *M. microti*, is one of the taxonomically closely related members of the *M. tuberculosis* complex. Numerous attempts have been made to find a species-specific antigen with high sensitivity for diagnosis of *M. bovis* infection. The protein antigen MPB70 is secreted from *M. bovis* cells following cleavage of a 30-amino-acid signal peptide which directs the active transport of the protein across the cytoplasmic membrane (33). The protein forms a major component of *M. bovis* culture filtrate, accounting for up to 10% of the protein excreted by some *M. bovis* bacillus Calmette-Guérin (BCG) strains (14, 25), and possibly as much as 23% (2). The function of this protein is unknown. The protein was first purified to homogeneity from culture filtrates of *M. bovis* (25) and later was isolated from culture filtrates of *M. bovis* AN-5 by Firis et al. (10, 11). Also, the gene encoding MPB70 in *M. bovis* has been cloned, sequenced (28, 33), and expressed in *Escherichia coli* (18). The molecular mass of MPB70 was estimated to be between 18 and 23 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1, 10, 11, 24, 25), 15 kDa by sedimentation equilibrium analysis (25), and 16 kDa as deduced from the gene-derived amino acid sequence (28, 33). The MPB70 protein exists in a glycosylated or a nonglycosylated form (10).

The MPB70 protein is considered to be a highly species-specific immunodominant antigen that contains at least three separate *M. bovis*-specific epitopes (37), although some cross-reactivity with *Nocardia asteroides* (13, 14) has been reported. The protein is an active component of purified protein derivative tuberculin (15) and is able to elicit a delayed-type hypersensitivity response (14, 16, 24, 25) and to stimulate T- and B-lymphocyte responses (9, 11, 12, 36) in *M. bovis*-infected animals. Because of its high species specificity and its immunodominant properties that lead to stimulation of antibody production in infected animals, MPB70 has been incorporated into an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-MPB70 antibodies, which serve as an indicator of *M. bovis* infection (15, 28). A simple assay that can rapidly detect anti-MPB70 antibodies in serum would be of considerable value in diagnosis of the disease. The objective of this study was to develop such an assay based on the concept of fluorescence polarization.

The theory of fluorescence polarization was first described in 1926 by Perrin (27). When fluorescent molecules in solution are excited by a plane-polarized light beam, they emit fluorescence back into the same polarized plane, provided that the molecules remain stationary. However, if the excited molecules rotate or tumble while in the excited state, then fluorescence is emitted into a plane different from the plane used for excitation. The degree of fluorescence polarization, *P*, is defined as follows: $P = (I_v - I_h)/(I_v + I_h)$, where $I_v$ and $I_h$ are the intensities of the vertically and horizontally polarized compo-
temperature for 2 h with murine monoclonal antibody 4C3/17 at a 1:5,000 concentration of MPB70 in this sample was determined by the colorimetric Fluorescein labeling of MPB70 from M. bovis BCG. MPB70 was purified to homogeneity from culture filtrates of M. bovis BCG Tokyo by chromatofocusing, lectin affinity chromatography, and hydrophobic interaction chromatography (32). Lyophilized MPB70 (1 mg) was dissolved in 0.5 ml of 0.15 M Na2HPO4-NaOH (pH 9.5), and then incubated at 37°C for 1 h. Following the incubation, the reaction mixture was immediately applied, at a rate of 0.5 ml/min, to a column of Sephadex G-25 (1 by 23 cm) preequilibrated with 0.1 M Na2HPO4-NaHPO4 buffer (pH 7.0) containing 0.04% NaN3. The absorbance was monitored at 492 nm, and 0.5 ml fractions were collected. The elution profile showed two well-separated peaks. The first peak contained fluorescein-labeled MPB70. Fractions of this peak were pooled and stored at 4°C.

The degree of incorporation of fluorescein groups into MPB70 was determined by independent measurements of the concentrations of FITC and protein in a given sample. The FITC concentration was estimated spectrophotometrically, taking the molar absorption coefficient to be 7.45 × 10^4 M^−1 cm^−1 (4). The concentration of MPB70 in this sample was determined by the colorimetric method using a biotinylated monoclonal antibody assay kit (Pierce) and bovine serum albumin (BSA) as the standard.

**SDS-PAGE and Western blotting (immunoblotting).** SDS-PAGE was carried out by the method of Laemmli (21) with 4% stacking gels and 12% resolving gels, using a Bio-Rad mini-mill apparatus. The separated proteins were either stained with Coomassie blue or electroblotted onto a nitrocellulose membrane by using a Bio-Rad Trans-Blot SD semidry transfer cell. The nitrocellulose membrane was blocked with 3% skim milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 1.5 h and then incubated at room temperature for 2 h with murine monoclonal antibody 4C3/17 at a 1:5,000 dilution in PBS-T containing 3% BSA. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G at a 1:1,000 dilution in PBS-T containing 3% BSA, with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (23).

**Fluorescence labeling of MPB70 from M. bovis BCG.** MPB70 from M. bovis BCG was purified to homogeneity from culture filtrates of M. bovis BCG Tokyo by chromatofocusing, lectin affinity chromatography, and hydrophobic interaction chromatography (32). Lyophilized MPB70 (1 mg) was dissolved in 0.5 ml of 0.15 M Na2HPO4-NaOH (pH 9.5), and then incubated at 37°C for 1 h. Following the incubation, the reaction mixture was immediately applied, at a rate of 0.5 ml/min, to a column of Sephadex G-25 (1 by 23 cm) preequilibrated with 0.1 M Na2HPO4-NaHPO4 buffer (pH 7.0) containing 0.04% NaN3. The absorbance was monitored at 492 nm, and 0.5 ml fractions were collected. The elution profile showed two well-separated peaks. The first peak contained fluorescein-labeled MPB70. Fractions of this peak were pooled and stored at 4°C.

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Research, Inc., Round Lake, Ill.), the characteristics and use of which have been described previously (19). Blank fluorescence polarization was determined prior to adding fluorescein-labeled MPB70. The observed polarization is expressed in terms of milli-polarization units (mP).

**Spectroscopic methods.** A Pharmacia LKB spectrophotometer ( Ultrospec Plus) was used to measure absorbance. An SLM-Aminco Model 8000C spectrofluorometer equipped with an IBM microcomputer and a circulating water bath was used for determination of fluorescence excitation and emission spectra. Excitation and emission bandpasses were 8 nm. Excitation and emission wavelengths were 490 and 520 nm, respectively. Spectra were smoothed by using the SLM data manipulation software.

**RESULTS**

**Characterization of fluorescein-labeled MPB70.** Reaction between MPB70 and FITC resulted in a covalent attachment of fluorescein groups to MPB70, as indicated by the absorption spectrum of fluorescein-labeled MPB70, which shows an absorbance maximum at 493 nm resembling that of FITC (Fig. 1a). The labeled protein exhibits a fluorescence spectrum similar to that of fluorescein, with excitation and emission maxima at 493 nm and 518 nm, respectively (Fig. 1b). Incorporation of FITC into MPB70 was confirmed by SDS-PAGE (Fig. 2). The samples containing fluorescein-labeled MPB70 migrated to a position corresponding to the unmodified MPB70 on the Coomassie blue-stained gel (Fig. 2a) and displayed corresponding fluorescent bands in the photograph of the gel taken under UV illumination prior to staining (Fig. 2b). Under the reaction conditions employed (FITC-to-MPB70 mole ratio of 8:1, 37°C for 1 h), the degree of modification (mean ± standard deviation) was quantitated to be 0.96 ± 0.08 mol of FITC per mol of MPB70 (n = 3). At the concentration used in FPA, fluorescein-labeled MPB70 showed a fluorescence polarization value of 164.7 ± 3.3 mP (n = 6) in PBS.

To determine whether modification with FITC altered the structural integrity of the 4C3/17-defined epitope, fluorescein-labeled MPB70 was visualized by Western blotting (Fig. 3). The reaction between 4C3/17 and fluorescein-labeled MPB70 yielded a protein band on nitrocellulose (Fig. 3a) which has a corresponding fluorescent band in the photograph of the gel taken under UV illumination prior to electrophoresis (Fig. 3b).

**Effect of LDS on FPA.** The detergent LDS was added to the FPA buffer to prevent possible nonspecific interactions between fluorescein-labeled MPB70 and other serum components. To establish an optimal concentration of LDS for use in the assay, fluorescence polarization was investigated as a function of LDS concentration after mixing the labeled MPB70 with serum from either an infected or a noninfected elk. The results are shown in Fig. 4a. For the serum from an infected animal (1:10 dilution), fluorescence polarization increased slightly in response to the increase in LDS concentration up to 0.3% and decreased dramatically afterwards. For the same serum at a dilution of 1:50, fluorescence polarization increased sharply when the LDS concentration was increased from 0 to...
0.06% and decreased rapidly beyond this concentration range. As a negative control, fluorescence polarization for the serum from a noninfected elk was also examined as a function of LDS concentration. Polarization for this serum at a dilution of 1:10 decreased slightly, from 160 to 150 mP, when the LDS concentration was increased from 0 to 0.12% and then increased up to 170 mP at LDS concentrations higher than 0.12%. The increase of LDS concentration from 0 to 1% also slightly increased fluorescence polarization (from 160 mP to a plateau value of about 180 mP) for the serum from the noninfected elk at a 1:50 dilution. The optimal concentration of LDS for use in the assay buffer was determined to be the one that gives the greatest polarization difference between the sera from the infected and noninfected elks (Fig. 4b). As shown in Fig. 4b, the optimal concentration of LDS was about 0.05% for a serum dilution of 1:10 or 1:50 and a higher LDS concentration (0.1%) may be used with a lower dilution of serum (1:10).

**Detection of serum antibodies to MPB70 by fluorescence polarization.** Nine sera from each of three species (elk, llama, and bison), six from infected animals and three from noninfected animals (determined by cultural isolation of *M. bovis* from tissues) (Table 1), were selected for the presence or absence of anti-MPB70 antibodies by ELISA with purified MPB70 on the solid phase. The ELISA results are shown in Table 1. Six sera from noninfected representatives of each species were chosen, each with an *A*405 value similar to that obtained with PBS (<0.09), indicating the absence of anti-MPB70 antibodies; three sera from infected representatives of each species were chosen, each with an *A*405 value 2- to 30-fold greater than 0.09, indicating the presence of anti-MPB70 antibodies. These sera were further analyzed by fluorescence polarization with fluorescein-labeled MPB70 being used as a probe. As shown in Table 1, all the sera from noninfected animals gave polarization values between 167 to 178 mP, which are similar to the value (mean ± standard deviation) of 164.7 ± 3.3 (*n* = 6) in the absence of specific antibodies (i.e., in PBS). However, the reaction between fluorescein-labeled MPB70 and the sera from infected animals resulted in higher values of fluorescence polarization, ranging from 203 to 330 mP (Table 1). These results were in good agreement with the ELISA data and demonstrated the feasibility of using fluorescein-labeled MPB70 to detect anti-MPB70 antibodies by fluorescence polarization.

**DISCUSSION**

This report described a novel immunoassay for antibody detection in *M. bovis*-infected animals based on the principle of fluorescence polarization described by Perrin (27). Fluorescence polarization has been widely applied to the study of molecular interactions in a variety of biological systems (6) and to the determination of the levels of numerous compounds, including drugs and hormones (19, 30). Recently it has been applied to the detection of antibodies to *B. abortus* (26). However, no reports are available on its use in the detection of specific antibodies in animals with mycobacterial infection, although some preliminary work in this laboratory has shown that antibodies to mycobacteria can be detected with nonspecific fluorescein-labeled arabinomannan (31). In this study, the nonglycosylated form of MPB70 was covalently conjugated with fluorescein groups for use as a probe to detect anti-MPB70 antibodies, using selected sera from three *M. bovis*-infected species (elk, llama, and bison) as a model. Labeling of MPB70 did not interfere with its immunoreactivity with anti-MPB70 monoclonal antibody 4C3/17. This result was expected,
because FITC, an amine-reactive reagent, is known to covalently bind preferentially to the amine groups of lysine residues at an alkaline pH (5) and all three lysine residues (Lys-79, Lys-88, and Lys-134) on MPB70 were distant from the monoclonal-antibody-reactive epitopes (28). *M. bovis* MPB70 is a major immunodominant secretory antigen with unique species specificity (13, 14, 37), and its nonglycosylated form has the least cross-reactivity (9). In this study, we developed an FPA using fluorescein-labeled nonglycosylated MPB70, which may be specific for the detection of *M. bovis* infection. One primary concern when designing an FPA is the size of the fluorescently labeled antigen; it must be much smaller than the antibody molecule in order to maximize polarization shifts and therefore increase the detection sensitivity. Numerous studies have suggested that fluorescence polarization technology may be applicable to antigens whose molecular mass is less than 40 kDa (3, 17, 20, 34, 38), although acceptable assays may be developed with a labeled molecule of up to 60 kDa (19). According to these studies, the antigen MPB70 (15 to 23 kDa) is suitable for use as the fluorescently labeled molecule in an FPA.

In this study, 27 animals of three species were classified as infected (3 per species) or noninfected (6 per species) on the basis of whether *M. bovis* could be cultured from tissues. This microbiological procedure is generally considered to be the “gold standard,” i.e., the definitive test for the confirmation of *M. bovis* infection (22, 36). The MPB70-based ELISA was performed to select *M. bovis*-infected animals with anti-MPB70 antibodies and noninfected animals lacking these antibodies (Table 1). The FPA described here was also able to detect anti-MPB70 antibodies in the sera of infected animals, but not in the sera of noninfected animals, as revealed by the fact that (i) the polarization values for the sera of infected animals were greater than those for the sera of noninfected animals and (ii) the values for the latter sera were very close to that in the absence of specific antibodies (i.e., in PBS). The detergent LDS at the optimal concentration was included in the assay buffer, and presumably this would prevent some of the nonspecific binding of other serum components to the fluorescein-labeled antigen. The FPA results are in good agreement with those obtained by ELISA, demonstrating the feasibility of the former assay as an alternative to ELISA or other antibody assay systems. It may be expected that the assay described in this study offers the same or at least similar specificity and sensitivity for detection of *M. bovis*-infected animals as the MPB70-based ELISA because both assays are essentially designed to target the same antibodies. However, further studies using a large number of *M. bovis*-infected and noninfected animals are needed to demonstrate this, and they are in progress. The FPA has several advantages over the ELISA and other antibody assay systems, including the following: (i) polarization measurements are carried out in a single-phase reaction mixture (homogeneous assay); (ii) no separation and washing steps are necessary; (iii) measurements are simple and fast, and the results can be obtained within a few minutes (2 min in the present study); and (iv) it is very economical and may be performed outside the laboratory. Thus, the FPA may be useful in epidemiological surveys. Work by Harboe et al. (15) showed that the formation of anti-MPB70 antibodies was highly specific for infection with *M. bovis*. However, contrasting results have been reported by other researchers (9, 12, 36), who showed that the specificity and sensitivity of detecting *M. bovis* infection were low in the MPB70-based ELISA tests. Further studies are necessary to determine the value of MPB70 as an immunodiagnostic reagent. Certainly, this study demonstrates the usefulness of fluorescein-labeled MPB70 as a probe in an immunoassay, based on the theory of fluorescence polarization, for rapid detection of specific serum antibodies in *M. bovis*-infected animals. The specificity of this assay should be very attractive in a field situation in which the diagnostic results must be obtained in a very short period of time. Also, the concept of this novel immunoassay should have a general applicability to the diagnosis of other infectious diseases, especially those in which the causative agents normally induce an antibody response in the host and the demonstration of such a response is an indicator of infection.

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**REFERENCES**


