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Production of Monoclonal Antibodies to Lipoarabinomannan-B and Use in the Detection of Mycobacterial Antigens in Sputum

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Tuberculosis has traditionally been confirmed by AFB staining or culturing Mycobacterium tuberculosis *from clinical specimens. However, because of the low sensitivity of the sputum smear examination and of the delayed report in culturing, the conventional methods for detection of* M. tuberculosis *have not been always satisfactory. In an attempt to develop an alternate tool, this study was initiated to produce monoclonal antibodies (MAb) to lipoarabinomannan B (LAM-B) antigen and to use the antibodies in detecting mycobacteria. In this study, five monoclonal antibodies specific to LAM-B were produced; LAM701 (IgG3), LAM138 (IgM), LAM204 (IgM), LAM302 (IgM), and LAM604 (IgM). A sandwich enzyme-linked immunosorbent assay (ELISA) was developed for detecting LAM-B and other mycobacterial antigens using the monoclonal antibodies. With the MAb LAM701, the minimal detectable concentration was 1.0 ng/ml for LAM-B, and 1.0 μg/ml for* M. tuberculosis *whole cells, respectively. When 14 clinical specimens proven to contain AFB by smear staining or culture were examined, ten (71.4%) were positive by the sandwich ELISA; in contrast, sputum smear examination gave positive results in only six (42.9%) specimens. Meanwhile, none of 25 specimens with no evidence of AFB were positive by the sandwich ELISA using the MAb LAM701. Although further evaluations are required, this study suggests that the monoclonal antibodies to LAM-B may be useful in detecting mycobacteria from clinical specimens.*

Key Words: lipoarabinomannan, *Mycobacterium tuberculosis*, monoclonal antibody, antigen, diagnosis, tuberculosis

Laboratory diagnosis of tuberculosis has relied on the microscopic examination and culture of *Mycobacterium tuberculosis* from clinical specimens. However, the smear examination is not always been satisfactory due to relatively low sensitivity (Boyd and Marr 1975; Kim *et al.* 1984). Also, culturing the organism has been time consuming and expensive (Glassroth *et al.* 1980); moreover, sizable portion of sputum specimens with AFB smear positive failed to grow (Warring and Sutramongkole 1970).

In looking for tools to detect mycobacteria, there have been attempts to use immunoassays to detect mycobacterial antigens in sputum (Straus *et al.* 1981; Yanez *et al.* 1986) and cerebrospinal fluid (CSF) (Kadival *et al.* 1986; Sada *et al.* 1983) specimens. The studies indicated that the sensitivity of the immunoassays was considerably greater than that by microscopic examination. Specificity ranged 95-100% in CSF and 91% in sputum specimens. Since the previous studies used rabbit polyclonal antibodies to mycobacteria, certain cross-reactive binding might be detected, particularly in sputum specimens because of the presence of cross-reacting antigens between mycobacteria and other bacteria (Minden *et al.* 1972a; Minden *et al.* 1972b).

Lipoarabinomannan (LAM)-B is almost identical to arabinomannan which has been known for a long time as a *Mycobacterium* common antigen (Misaki *et al.* 1977). Recently, LAM-B has been fully characterized to consist of mainly arabinose and mannose. It also contains glycerol, a polyol phosphate, lactate, sucinate, palmitate, and 10-methyloctadecanoate (Hunter *et al.* 1986). In an effort to avoid the crossreactivity with other bacterial antigen, we attempted to generate monoclonal antibodies (MAb) to LAM-B and to develop a modified double antibody sandwich ELISA

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using MAb to detect mycobacterial antigens in sputum specimens in this study.

MATERIALS AND METHODS

Mycobacterial antigens

LAM-B purified by anion exchange and gel filtration chromatography in detergent from *M. tuberculosis,* and the whole cells of *M. leprae, M. avium* serovar 9, *M. bovis* BCG, and *M. paratuberculosis* were kindly provided by P.J. Brennan (Colorado State University, Fort Collins, Co.). *M. tuberculosis* H37Rv was obtained from the Korean Institute for Tuberculosis Research and grown in the Sauton medium. The cultured organisms were then heat-killed, harvested by filtration and washed three times with distilled water by centrifugation. Finally, the organisms were lyophilized. *M. tuberculosis* soluble antigen (MTSA) was prepared by thorough sonication of the organisms for 60 minutes using microprobe (3 mm) (Sonics & Materials Inc., Danbury, Ct.), followed by ultracentrifugation at 100,000 *g* for four hours. The supernatant was then collected and the protein concentration was determined by the Lowry method (Keleti and Lederer 1974). Purified protein derivatives (PPD) were purchased from the Statens Seruminstitut (Copenhagen, Denmark).

Monoclonal antibodies

For the production of MAb, BALB/c mice were immunized with *M. leprae* mixed with Freund's incomplete adjuvant (Sigma Chemical Co, St. Louis, Mo.) i.p., and were given booster injections after three weeks. Four days after another booster dose i.v., the spleen was removed and the spleen cells were fused with Sp2/O-Ag-14 mouse myeloma cells using the protocols described by Kohler and Milstein (1975). The culture soup from the well with growing cells was examined for the presence of antibodies to LAM-B as described below. The colonies producing anti-LAM-B antibodies were cloned twice at the density of 0.3 cells/well by limiting dilution. The culture soup from the cloned cells was used in the characterization of the monoclonal antibodies and in detecting mycobacteria from clinical specimens. The isotype of MAb was determined with a commercially available isotyping kit (Hyclone Laboratories, Inc., Logan, Ut.).

Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) described by Voller, *et al.* (1979) was employed with minor modifications as reported previously (Cho *et al.* 1988). Briefly, 50 μl of LAM-B antigen dissolved as 0.2 μg/ml in carbonate buffer, pH 9.6 was added to wells of 96 well U-bottom microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated overnight at 37°C in a moist chamber. The wells were then washed with PBS, pH 7.4, containing 0.05% (v/v) tween 20 (PBST) and blocked by the addition of 100 μl of PBST-0.5% (w/v) BSA (ICN ImmunoBiologicals, Lisle, Il.). After emptying the wells, 50 μl of culture soup was added to the wells and incubated at 37°C for 90 min. This was followed by addition of peroxidase-conjugated goat anti-mouse immunoglobulins (Cappel, Organontecknika Corp., West Chester, Pa.) diluted 1:2,000 in PBST-5% (v/v) normal goat serum (NGS) (Gibco Laboratories, Grand Island, NY.) and of substrate solution, H_2O_2-o-phenylenediamine. The absorbance was read at 490 nm. Each test was performed in duplicate and the absorbance of the wells without antigen was subtracted from those with LAM-B before analysis. The whole cell antigens of various mycobacterial species were used at the concentration of 50 μg/ml in carbonate buffer after a brief sonication for three minutes.

Detection of mycobacterial antigens

A modified double antibody sandwich ELISA was used to detect mycobacterial antigens from sputum specimens, Briefly, 100 μl of rabbit anti-*M. bovis* BCG antibodies (Dakopatts, Glostrup, Denmark) diluted to 50 μg/ml in carbonate buffer was added to each well of 96 well flat-bottom EIA plates (Costar Corp., Cambridge, Ma.), and incubated in a moist chamber at 37°C overnight. After blocking with PBST-5% (v/v) rabbit serum (Gibco Laboratories), 100 μl of sputum specimens as prepared below was added to the wells and incubated at 37°C for two hours. After washing, MAb to LAM-B was reacted, followed by peroxidase-conjugated anti-mouse immunoglobulins and H_2O_2-o-phenylenediamine substrate.

Sputum specimens

Sputum specimens submitted for the microscopic examination and culture of *M. tuberculosis* were processed by the conventional methods (Sommers and Good 1985). Briefly, sputum specimens in 50 ml conical centrifuge tubes were mixed with the same volume of 4% NaOH using vortex for about 30 seconds., followed by centrifugation. The precipitate was resuspended in about 1.0 ml of saline; about two-thirds of the volume was used in smear and inocula-

tion into the Ogawa medium for culture. The residue was then resuspended in about 15 ml of PBS, pH 7.4 and washed by centrifugation at 3000 *g* for 30 minutes. The precipitate was again resuspended in 1.0 ml of PBS and used in the sandwich ELISA for the detection of mycobacteria antigens.

RESULTS

Production and characterization of monoclonal antibodies to LAM-B

Although there were numerous hybrids producing antibodies to LAM-B during seven fusions, only five cell lines producing antibodies highly reactive to the antigen were cloned and maintained for the characterization of MAb. The isotypes of MAb were shown in Table 1; four MAb, LAM038, 204, 302, and 604, were IgM, and one, LAM701, was IgG3. Table 2 shows the reactivity of MAb to LAM-B and several mycobacterial species used in the study; *M. paratuberculosis* gave a moderate or low reactivity. However, all of the mycobacterial antigens including *M. paratuberculosis* were strongly reactive with rabbit antisera to *M. tuberculosis* and *M. leprae*.

Table 1. Isotype of the monoclonal antibodies to LAM-B

MAb	Isotype
LAM038	IgM
LAM204	IgM
LAM302	IgM
LAM603	IgM
LAM701	IgG_3

Sandwich ELISA for the detection of mycobacterial antigens

A modified double antibody sandwich ELISA was developed to detect mycobacterial antigens using the MAb LAM701. With this tool LAM-B was detectable at the concentration of less than 1.0 ng/ml in PBS, pH 7.4 (Fig. 1). *M. tuberculosis* and its soluble antigen (MTSA) were also easily detectable at 1.0 μg/ml level. However, PPD contained a small amount of LAM-B and required about 50 μg/ml to be detected. Since LAM-B is a *Mycobacterium* common antigen and all of MAb were reactive to some mycobacterial species above, the sandwich ELISA was employed to detect

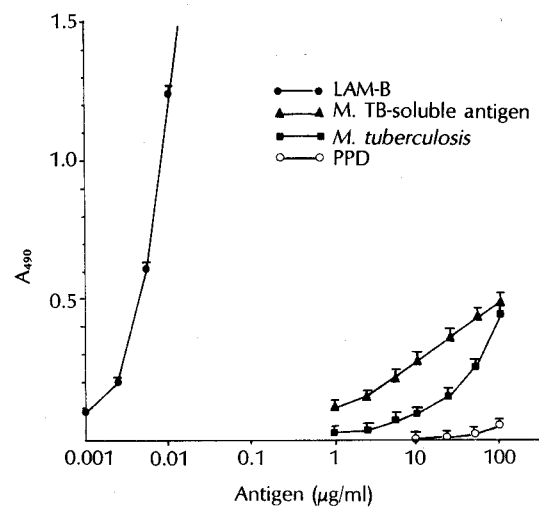

Fig. 1. *Dose response curves of mycobacterial antigen in sandwich ELISA using MAb to LAM-B. Each point indicates the mean absorbance and +/− s.d.*

Table 2. Reactivity of MAb to LAM-B and *Mycobacterium spp.*

Antigens	MAb to LAM-B					Rabbit antisera to	
	038	204	302	604	701	*M. tuberculosis*	*M. leprae*
LAM-B	>2.0*	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
M. tuberculosis	1.09	1.27	1.25	1.59	0.82	>2.0	>2.0
M. bovis BCG	0.89	1.07	0.99	1.44	0.69	>2.0	>2.0
M. leprae	1.12	1.31	1.31	1.55	1.00	>2.0	>2.0
M. phlei	0.83	1.13	1.18	0.89	0.66	>2.0	>2.0
M. avium	0.89	0.99	1.11	1.92	0.66	>2.0	>2.0
M. paratuberculosis	0.18	0.26	0.30	0.99	0.24	>2.0	>2.0

* Numbers indicate absorbances at 490 nm.

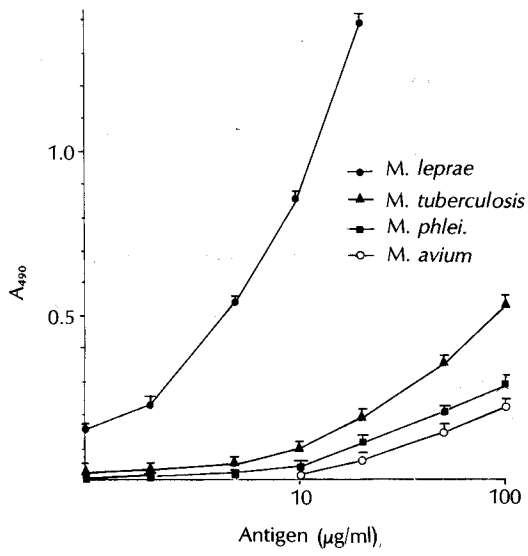

Fig. 2. *Comparison of the dose response curves of various Mycobacterium species in sandwich ELISA using MAb to LAM-B. Each point indicates the mean absorbance and +/− s.d.*

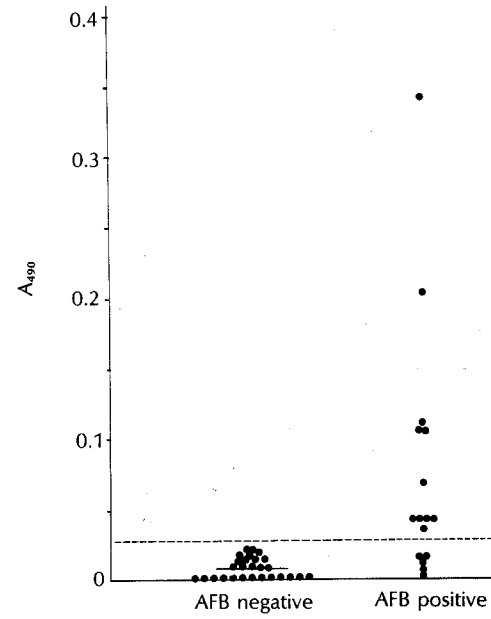

Fig. 3. *Reactivity of sputum specimens in sandwich ELISA using MAb to LAM-B. Each point represents one specimen. Sputum specimens with AFB positive were proven to contain AFB by either microscopy or culture. The dotted line indicates the criteria for the antigen positivity.*

other mycobacteria. As shown in Fig. 2, whole cell antigens of *M. leprae, M. phlei, M. avium* as well as *M. tuberculosis* were detectable with the dose-dependant pattern. Among the mycobacterial species, *M. leprae* had the largest abundant amount of LAM-B, followed by *M. tuberculosis.*

Detection of mycobacterial antigens in sputum specimens

The sandwich ELISA using MAb was also employed to detect mycobacterial antigens in sputum specimens. The results were then compared to those obtained by microscopic examination and culture. The criteria for the antigen positivity was determined by adding three times of s.d. to the mean absorbances of sputum specimens with no evidence of AFB by microscopic examination and culture. The absorbance of 0.030 was used as antigen positive in this study. With this criteria,

Table 3. Detection of mycobacteria from sputum specimens

Subjects	No. of assayed	Positive by.			
		Microscopy		Sandwich ELISA*	
		No.	(%)	No.	(%)
AFB positive**	14	6	(42.9)	10	(71.4)***
AFB negative	25	0		0	

* Criteria for positivity: $A_{490} \geq 0.030$.

** AFB positive by either microscopic examination and/or culture.

*** $x^2=2.3$, $p>0.10$.

none of the 25 specimens with no AFB were positive by the sandwich ELISA (Fig. 3). Out of 14 sputum specimens proven to conain AFB, ten (71.4%) had detectable antigens by the sandwich ELISA; in contrast, only six (42.9%) were smear AFB positive by microscopic examination (Table 3). Although there was no significant difference between the methods, therefore, the sandwich ELISA seemed to be more sensitive in detecting mycobacteria from sputum specimens.

DISCUSSION

Recently, LAM-B has been thoroughly characterized by Hunter, *et al.* (1986), although the presence of the polysaccharide antigen known as arabinomannan has appeared in numerous literature since the late 1940s (McCarter and Watson 1942; Seibert 1949). Hunter *et al.* (1986) produced MAb to LAM-B and used the MAb to differntiate LAM-B which was immunoreactive from LAM-A which was not. The five monoclonal antibodies produced in this study were also strongly reactive to LAM-B; however, it was not known whether or not any of these MAb were reactive to LAM-A. Only LAM-B was known to be reactive to the pooled sera from lepromatous leprosy patients (Hunter *et al.* 1986).

Since LAM-B is a *Mycobacterium* genus common antigen (Misaki *et al.* 1977), it was not surprising to find that all of MAb reacted with whole cell antigens of various mycobacterial species. However, in the sandwich ELISA, *M. leprae* seemed to have the most abundant amount of LAM-B compared to other mycobacteria. This might be due to the fact that *M. leprae* has more LAM-B antigen in its surface because the organism was grown *in vivo* and killed by irradiation before purification from the tissues of armadillo. However, other mycobacteria were grown *in vitro* and killed by autoclave, thus resulting in aggregation of the cells. Therefore, there might be difference in LAM-B content in dried whole cells between mycobacteria used in this study.

In this study, we also showed that the sandwich ELISA using MAb to LAM-B was useful in detecting MTSA and PPD as well as *M. tuberculosis* whole cells. This indicated that metabolic products of mycobacteria or debris of mycobacteria in clinical samples may be also detectable. Although the sample size of sputum specimens used in this study was not large enough to evaluate the sandwich ELISA, the tool gave the sensitivity of 71.4% and specificity of 100% and was much better than smear examination in detecting mycobacteria. These results were comparable to those obtained by Yanez *et al.* (1986) who reported that 12 (60%) of 20 sputum specimens with AFB positive had detectable antigens by a sandwich ELISA using rabbit antisera to *M. bovis* BCG.

Since LAM-B is a common antigen among mycobacteria, the sandwich ELISA using MAb to the antigen may present a problem in detecting non-pathogenic fast-growing mycobacteria as well as pathogenic non-tuberculous mycobacteria, as shown in Fig. 2. However, a recent increase in the incidence of non-tuberculous mycobacteria, especially *M. avium,* among AIDS patients also may support the need of tools detecting all mycobacterial species (Collins 1986). There are also concerns about the complexity of sandwich ELISA; therefore, increased sensitivity by elaborative procedures in sandwich ELISA has to be carefully weighed with simplicity of AFB staining before implementation of such tools in clinical laboratories.

Although further evaluation of the sandwich ELISA has to be done with more specimens; this study does show that use of MAb to LAM-B in detecting mycobacteria from clinical specimens as an alternate tool of microscopic examination looks promising. This is particularly true in the geographic areas where non-tuberculous mycobacteria have been rarely isolated form clinical specimens.

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