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PRELIMINARY REPORT

MONOCLONAL ANTIBODIES AGAINST BETA-ANTIGEN OF *MYCOBACTERIUM TUBERCULOSIS* AND THEIR INTERSPECIES REACTIVITIES

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Beta-antigen is one of the major proteins of *Mycobacterium tuberculosis*. We purified this antigen from the unheated culture filtrate of *Mycobacterium tuberculosis* Aoyama B and obtained nine monoclonal antibodies against the beta-antigen. Nine monoclonal antibodies were divided into two groups according to their patterns on Western blotting. The result indicated the existence of two or more determinant groups against these monoclonal antibodies on the beta-antigen molecule. The interspecies reactivity of monoclonal antibodies among twenty-one species of *Mycobacteria* was also examined by dot blotting analysis. Two monoclonal antibodies, designated 4G5E10 and 5F3F2, showed a specificity restricted to the *Mycobacterium tuberculosis* complex, could be used for serodiagnosis of *Mycobacterium tuberculosis* infection.

Recent studies have shown that monoclonal antibodies (MAbs) can be used for characterization of mycobacterial antigens (Coates et al., 1981, Gillis et al., 1982, Kolk et al., 1984). The MAbs of *Mycobacterium tuberculosis* (*M. tuberculosis*) have been used for the purification of mycobacterial antigens (Young et al., 1986) and serodiagnosis of tuberculosis (Hewitt et al., 1982, Ivanyi et al. 1983). In a recent comparative survey, 31 MAbs were prepared and their reactivities with the antigens of 23 species of mycobacteria were tested (Engers et al., 1986). None of the MAbs was found to be the specific for a given mycobacterial species or strain, and only six MAbs showed specificity restricted to the *M. tuberculosis* complex, such as *M. tuberculosis*, *M. bovis* BCG, *M. microti* and *M. africanum*. At the moment these MAbs that are specific for the *M. tuberculosis* complex are the only ones available for serodiagnosis of mycobacterial infections. But species specific

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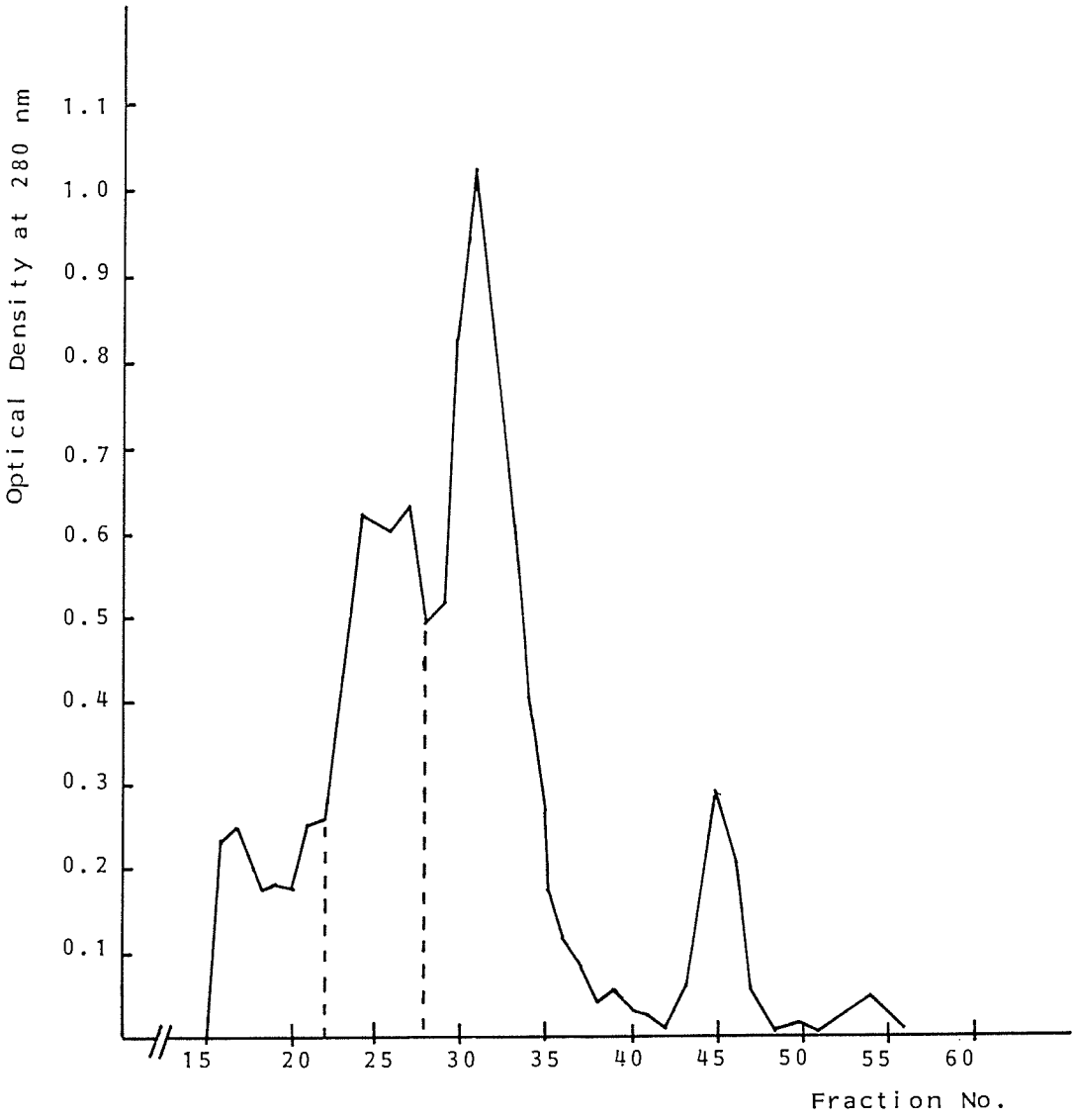

FIGURE 1. Gel filtration of 30-50% ammonium sulfate fraction of the culture filtrate of M. tuberculosis Aoyama B on Sephacryl S-200 Superfine. Column size: 2.0×50.0; bed height: 45.0 cm; eluent: Tris-HCl buffer solution (pH 8.0, 0.1 M) containing NaCl (0.5 M); flow rate: 3.4 ml/h; fraction size: 3.0 ml/tube; sample applied: O.D. 11.8 at 280 nm. Beta antigenic activities were monitored by immunodiffusion with rabbit anti-beta antibody. Beta antigen was eluted in tubes No. 22 to 28.

MAbs would clearly be preferable.

Yoneda and Fukui (1961a, 1961b, 1965) isolated and partially characterized two major protein antigens, alpha and beta, from unheated culture filtrates of *M. tuberculosis* H37RV (Fukui and Yoneda, 1961, Fukui et

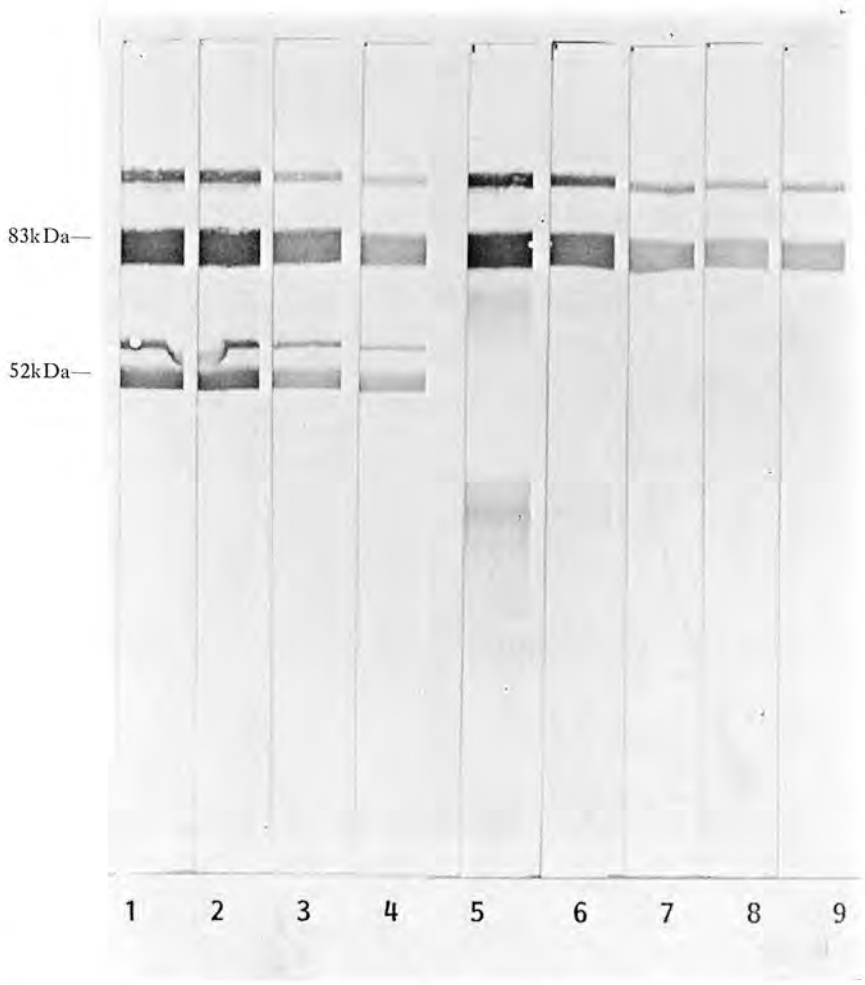

FIGURE 2. Western blotting analysis of beta antigen with nine monoclonal antibodies. Lanes: 1, 1B5G11; 2, 4D8G1; 3, 4G5E10; 4, 3D11G4; 5, 5F3F2; 6, 1G9D4; 7, 1E9D8; 8, 2F5F10; 9, 3F6H5.

al., 1965). The alph-antigen is a cross-reacting antigen that is observed in all slowly growing mycobacteria, while the beta-antigen is found only in the *Mycobacterium tuberculosis* complex. The beta-antigen has not been studied further, but Tasaka et al. reported that the alpha-antigen molecule consists of two parts, one containing the cross-reacting determinant group(s), and the other containing the species-specific determinant group(s) (Tasaka et al., 1983, Tasaka and Matsuo, 1984).

In the study reported here, we purified the beta-antigen from the unheated culture filtrate of *M. tuberculosis* Aoyama B. Then we obtained hybridomas producing Mab against the beta-antigen and examined their interspecies reactivities by immunoblotting analysis.

M. tuberculosis Aoyama B was cultured on Sauton's synthetic medium for 4–6 weeks. The unheated culture filtrate was concen-

trated by ammonium sulfate precipitation and subjected to column chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) (Fig. 1). The beta-antigenicity was examined by immunodiffusion with anti-beta rabbit serum. Antibeta rabbit serum and reference beta-antigens were provided by Dr. Yoneda and kept in our laboratory. Fig. 1 shows the elution pattern of the material from a Sephacryl S-200 column. The fractions containing beta-antigen were collected, concentrated and subjected to preparative SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed using 7.5% separating gel in non-reducing conditions. After electrophoresis, the gel was cut into 5 mm-width sections, and the material in each section was tested for beta-antigenicity. On preparative PAGE, the beta-antigenicity is as detected in a single, slightly thick band staining with Coomassie Brilliant blue R. The material in this band was extracted from the gel, concentrated and used to immunize mice for production of hybridomas.

BALB/c mice were immunized with beta antigen by intraperitoneal injection of 100 μg of protein emulsified with Freund's incomplete adjuvant and boosted with three subsequent, weekly intraperitoneal injections of 10 μg of beta antigen in saline. Animals were killed 3 days after the last booster injection. MAbs were produced as described by Kolk et al., (1984). Supernatants from growing clones were screened by enzyme-linked immunosorbent assay (ELISA).

Western blotting analysis was also performed as described by Towbin et al. (1979). The discontinuous system described by Laemli (1970) was used in SDS-PAGE; the separation gel contained 10% (w/v) acrylamide, and the stacking gel 4.5% acrylamide.

Nine MAbs against beta-antigen were obtained. They were divided into two groups according to their patterns on Western blotting (Fig. 2) Four MAbs reacted with three distinct bands of 110 kilodaltons (kDa), 83kDa and 52kDa and a faint band of 56kDa, while the other five MAbs reacted with two distinct bands of 110kDa and 83kDa. The beta-antigen used for immunization of mice had been highly purified by preparative PAGE. In the hybridoma procedure, limiting dilution (Mishell and Shiigi, 1980) was used to obtain monoclonal cells. Limiting dilution was repeated at least twice, and the wells were carefully examined by microscopy to confirm the presence of only one cell in one well. Therefore, the antibodies were considered to be monoclonal. Cell fusion experiments to obtain hybridomas were repeated several times. In this way MAbs were obtained in five serial experiments. These MAbs showed the same or very similar patterns on Western blotting analysis with purified beta antigen. This result indicates that there are at least two determinant groups on the beta antigen molecule and that the MAbs recognize one or both these determinants.

The interspecies reactivity of MAbs was examined by dot immunoblotting analysis. Twenty-one species of Mycobacteria were cultured using three kinds of media: modified Sauton medium, Middlebrook 7H9 medium, and Ogawa's egg yolk medium. The cells were harvested and sonicated for 15 min in a cold-water bath. The sonicates were centrifuged for 1 h at 27,000 rpm, and the resulting supernatants were diluted to a final protein concentration of 1 mg/ml. A sample of 5 μl of each sonicate was spotted onto a strip of nitrocellulose membrane (Schleicher und Schuell Inc., Keene, N. H.) and allowed to air dry. Each strip was then treated with 5% skim milk (Bacto skim milk, dehydrated; Difco Detroit, Mi.) in PBS for 1 h at 37 C with constant shaking, and then incubated at 37 C with 1: 200 dilution of ascites for 1 h at room temperature. The strips were washed for 5 min in PBS with gentle shaking, and then for 15 min in PBS containing 0.05% Tween-20 (PBS-T). After a second washing for 5 min in PBS-T, they were incubated with

TABLE 1. *Interspecies reactivity of the MAbs characterized by immunoblotting*

	Reactivities of MAbs[a]								
	1B5G11	4D8G1	4G5E10	3D11G4	5F3F2	1G9D4	1E9D8	2F5F10	2F6H5
M. tuberculosis H37Rv	+	+	+	+	+	+	+	+	±
M. tuberculosis H37Ra	+	+	+	±	+	+	±	±	±
M. tuberculosis Aoyama B	+	+	+	+	+	+	+	+	+
M. bovis BCG Yoken	+	+	+	±	+	+	±	+	+
M. kansasii ATCC 12478	+	+	−	−	−	±	−	−	−
M. gastri ATCC 15754	±	−	−	−	−	−	−	−	−
M. marinum ATCC 927	−	−	−	−	−	±	−	−	−
M. scrofulaceum ATCC 19981	−	−	−	−	−	−	−	−	−
M. gordonae ATCC 14470	−	−	−	−	−	−	−	−	−
M. szulgai NCTC 10831	+	+	−	±	−	+	+	+	−
M. avium ATCC 19075	+	+	−	−	−	+	−	±	−
M. intracellulare ATCC 13950	−	−	−	−	−	−	−	−	−
M. nonchromogenicum ATCC 19530	−	−	−	−	−	±	−	−	−
M. terrae ATCC 15755	−	−	−	−	−	−	−	−	−
M. triviale ATCC 23292	−	−	−	−	−	−	−	−	−
M. fortuitum ATCC 6841	−	−	−	−	−	−	−	−	−
M. chelonel ATCC 19977	−	−	−	−	−	−	−	−	−
M. chitae ATCC 19627	−	−	−	−	−	−	−	−	−
M. smegmatis ATCC 607	±	+	−	−	−	+	±	−	−
M. lepraemurium ATCC 35779	+	+	−	±	−	+	±	+	±
M. leprae	−	−	−	−	−	−	−	−	−

[a] +, ± and − indicate strong, weak and no reaction, respectively.

peroxidase conjugated goat anti-mouse IgG (Cappel Laboratories, Downington, Pa.) diluted 1: 500 with PBS-1% skim milk for 1 h at room temperature. The strips were then drained, and washed with PBS-T once for 5 min, and three times for 10 min, and then with PBS for 5 min. The washed strips were treated with color development solution (4-Cl-1-naphthol, Bio-Rad Laboratories, Richmond, Ca.) for 45 min. They were then washed with distilled water several times, air dried and stored in the dark. Dot immunoblotting reactions were graded as negative (−), weakly positive (±) and strongly positive (+).

The MAbs raised against beta-antigen were examined with sonic extracts of 21 mycobacterial species. Results on interspecies specificities are summarized in table 1. Fig. 3 shows the results of immunoblotting analysis by MAbs, 5F3F2 as a representative example. None of the nine MAbs against beta-antigen was specific for a given mycobacterial species. Two MAbs, 4G5E10 and 5F3F2, showed a specificity restrictedly to the *M. tuberculosis* complex. Although, Yoneda and Fukui reported that beta-antigen was restricted to the *M. tuberculosis* complex, some MAbs reacted strongly with *M. avium*, *M. szulgai*, *M. kansasii*, *M. smegmatis* and *M. lepraemurium*. Yoneda and his coworkers used polycolnal antibodies and immunodiffusion to examine

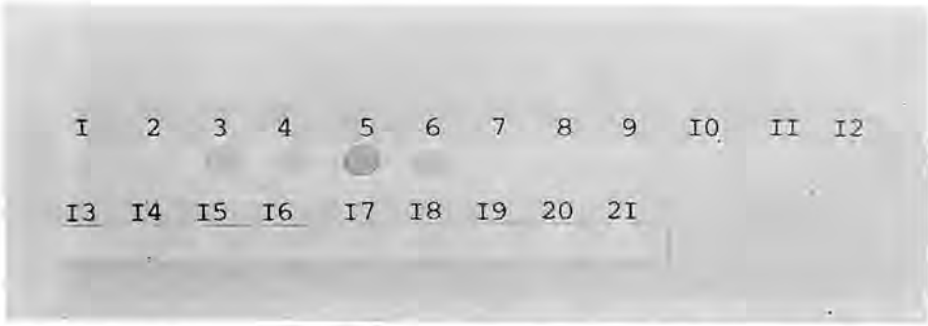

FIGURE 3. Dot-blotting immunoassay of 5F3F2 monoclonal antibody witn sonicates of 21 species mycobacterial 1, M. leprae; 2, M. lepraemurium; 3, M. tuberculosis H37Rv; 4, M. tuberculosis H37Ra; 5, M. tuberculosis Aoyama B; 6, M. bovis BCG; 7, M. kansassi; 8, M. gastri; 9, M. nonchromogenicum; 10, M. terrae; 11, M. triviale; 12, M. gordonae; 13, M. scrofulaceum; 14, M. intracellulare; 15, M. avium; 16, M. marinum; 17, M. smegmatis; 18, M. fortuitum; 19, M. chelonei; 20, M. chitae; 21, Negative control.

the distribution of beta-antigen in Mycobacteria, whereas we used monoclonal antibodies and immunoblotting for this purpose. We do not have any definite explanation for the discrepancy in results. Possibly some of these MAbs could distinguish two mycobacteria that are very similar, but very difficult to distinguish. For example using the MAbs, 1B5G11, 4D8G1 and 1E9D8, *M. szulgai* may be distinguished from *M. gordonae*, and *M. avium* from *M. intracellulare*.

The MAbs 4G5E10 and 5F3F2 which show specificity for the *M. tuberculosis* complex, could be used for serodiagnosis of *M. tuberculosis* infection. As we mentioned in the Introduction only six MAbs can be regarded as *M. tuberculosis* complex specific. These six MAbs reacted with proteins of two molecular sizes, 14kDa and 28kDa, which are completely different in size from beta-antigen. This indicates that we could develop new *M. tuberculosis* complex specific MAbs.

Further studies on the characterization and molecular structure of the beta-antigen of *M. tuberculosis*, especially antigenic analysis using the MAbs reported here and conformational studies on beta-antigen, should be useful in development of methods for serodiagnosis of tuberculosis.

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