Indian J Med Res 112, August 2000, pp 37-46

# Production & characterization of monoclonal antibodies to *Mycobacterium tuberculosis*

K.S. Senthil Kumar, Alamelu Raja, K.R. Uma Devi & R.S. Paranjape*

*Department of Immunology, Tuberculosis Research Centre (ICMR), Chennai*

Accepted June 26, 2000

*Background & objectives:* **Monoclonal antibodies (MAbs) against** *Mycobacterium tuberculosis* **H37Rv culture filtrate (CF) were raised by immunizing BALB/c mice and characterization was done. Attempts have been directed towards identifying mycobacterial antigens in biological fluids by employing polyclonal and monoclonal antibodies specific for** *M. tuberculosis.* **Immunohistologic studies, using MAbs for the localization of whole or fragmented bacilli in the biopsy specimens were also carried out.**

*Methods:* **Intrasplenic IS and intraperitoneal IP routes of immunization, were compared. The MAbs were characterized for their isotype, binding specificity, nature of binding epitope, reactivity in immunoassays** *etc.*

*Results:* **IS and IF' routes of immunization, were compared and IP was found superior. Ten MAbs designated TRC l-10 were produced. Of these, 7 MAbs, TRC 1-7 reacted with the 30/31 kDa doublet (antigen 85 complex), TRC 8 with 12 kDa in addition to 30/31 kDa and TRC 9 and 10 with the 24 and 12 kDa antigens respectively. Six MAbs were classified as broadly cross reactive and 2 showed limited cross reactivity. TRC 8 and 10 showed species specificity. Employing TRC 8 in sandwich ELISA, antigen was detected in sera from 17 of 25 pulmonary tuberculosis patients and 3 of 20 controls. TRC 8 was found to be useful in detecting antigens specifically in** *M. tuberculosis* **and** *M. leprae* **infected tissues, by immunoperoxidase staining.**

*Interpretation & conclusion:* **TRC 8 was found to be restricted in its reactivity to** *M. tuberculosis* **complex and** *M, leprae.* **TRC 8 may prove useful in immuno-diagnosis of tuberculosis.**

Key **words** Enzyme linked immunosorbent assay (ELISA) - immuno-peroxidase staining - intrasplenic immunization (IS) - *Mycobacterium tuberculosis* - monoclonal antibodies (MAbs) - sandwich ELISA

Tuberculosis remains one of the major health problems in the world, with an estimated 8-10 million new cases and 2-3 million deaths occurring each year[1]. In India, there are about 0.5 million deaths annually due to this disease[2]. The recent increase in the number of cases in developing countries, with the spread of HIV infection and the global emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* has had a major impact on the current situation of tuberculosis.

Since the treatment of tuberculosis is highly effective, tuberculosis case finding and case holding should be regarded as the main key to the control and eventual elimination of this disease. Therefore, the need for diagnosis of tuberculosis is of great public health concern.

Although a presumptive diagnosis of tuberculosis can be made on the basis of the patient's history' clinical and radiological findings, and the presence

*Present address:* *National AIDS Research Institute (ICMR), 73, G Block, MIDC, Bhosari, Pune 411026

of acid fast bacilli in specimens from the patient, the definitive diagnosis of tuberculosis depends on the culture of mycobacteria, which is time consuming, cumbersome, and sometimes false negative. Therefore, attempts have been made to develop alternative tests for tuberculosis.

Among the serological tools, the enzyme linked immunosorbent assay (ELISA) has been more frequently used for detecting antibodies and mycobacterial antigens from clinical specimens because of its high sensitivity, simplicity, reproducibility and versatility in screening a large number of specimens. Purified antigens and the use of monoclonal antibodies (MAbs) have begun to overcome the problem posed by the broad cross-reactivity of crude extracts from *M. tuberculosis*, previously encountered in ELISA.

In the present study, efforts to produce and characterize the MAbs against *M. tuberculosis* have been detailed. Attempts have been directed towards identifying mycobacterial antigens in biological fluids by employing polyclonal and monoclonal antibodies specific for *M. tuberculosis*. Immunohistologic studies, using MAbs for the localization of whole or fragmented bacilli in the biopsy specimens were also carried out.

## Material & Methods

*Study subjects :*

*(i)* Animals– Female BALB/c mice, 3 wk old were used for immunization for comparison of immunization schedules. Groups of 5 mice were used for each schedule of immunization and 9 different schedules were compared. Five mice were used for MAb production.

Three rabbits were used for polyclonal antibody production. *M. tuberculosis* infected guinea-pigs (N=10) were included for immunohistochemistry studies.

*(ii)* Patients– Sera were obtained from bacteriologically (smear and culture) proven cases of pulmonary tuberculosis (N=25) and normal, healthy volunteers (N=20) for ELISA. Skin sections from leprosy patients (N=10) and lymph node biopsy specimens from patients of Hodgkin's disease (N=10) were obtained, which form a part of routine specimen collection for an ongoing histopathology study at TRC.

*Antigens:*

*(i)* Culture filtrate antigen (CF)– *M. tuberculosis* H37Rv CF was prepared from filtrates of 6 wk cultures in Sauton's liquid medium, by 90 per cent ammonium sulphate saturation- Similarly, other strains of *M. tuberculosis* such as H37Ra, *M. tuberculosis* SILV (south Indian low virulent strain) and other mycobacterial species *M. bovis* BCG, *M. kansasii*, *M. gordonae*, *M. vaccae*, *M. smegmatis*, *M. diernhoferi*, *M. fortuitum* and *M. thermoresistible* were grown in Sauton's medium and harvested after complete pellicle formation which varied from 2-6 wk depending on the species.

Antigen 85 A, B and C were purified in our laboratory. Antigen 6 and recombinant 85 C were kind gifts from Prof. Thomas Daniel, Cleveland, USA and Prof. Patrick Brennan, Fort Collins, USA, respectively.

*(ii)* Cytosol antigen– *M. tuberculosis* H37Rv bacilli were suspended in the breaking buffer (20m *M* Tris with 8.5% NaCl, pH 7.4) containing the protease inhibitors and sonicated. The sonicate was then passed through the French pressure cell (SLM Instruments, Inc., USA), with a pressure of 2000 psi for 5 cycles and subjected to centrifugation at 10,000 rpm for 10 min at 4°C. The pellet contained the cell wall material. The supernatant was collected and ultracentrifuged at 45,000 rpm for 1 h. This step separated the cell membrane in the pellet and the cytosolic antigens in the supernatant.

*(iii)* Nitro-cellulose paper (NCP) bound CF antigens– CF antigen (100 μg/cm$^2$) was spotted onto NCP and air dried. The NCP was cut, dissolved in dimethyl sulphoxide (DMSO) (Sigma, USA) and precipitated with carbonate buffer, 0.06 *M,* pH 9.6. The suspensions was spun at 3000 rpm for 10 min. The pellet was resuspended and washed with sterile phosphate buffered saline (PBS).

*Escherichia coli* antigen was obtained by sonication and centrifugation.

The protein concentration of the antigen was measured by the method of Lowry *et al* [3].

*Polyclonal antibody production in rabbits*

Polyclonal antiserum against CF was raised in rabbits. Three mg of CF antigen was mixed with incomplete Freund's adjuvant (IFA) to form a fine emulsion and injected at multiple sites (by subcutaneous route) along the back of the animal. At intervals of 2 wk, the immunization was repeated. Blood was collected after 3 days of each immunization by ear vein puncture. The antibody response was tested by ELISA and immunoblot. Boosters at regular intervals were continued till hyperimmune serum was obtained.

The pooled hyperimmune rabbit serum was used in immunoblot and found to recognize all the major bands. Immunoglobulin G (IgG) was purified from the pooled serum using Protein-A column (Pharmacia, Sweden). This was used as the source of polyclonal antibody in antigen detection assays.

The IgG fraction was further purified to get monospecific antibody against 30/31kDa, by elution from immunoblots, which was used in immunoperoxidase staining. Briefly, the CF antigen was resolved and electrotransferred to NCP. Two vertical strips from either end of the NCP were cut and developed with the polyclonal IgG. The stained bands were aligned with the remaining untreated NCP, and bands in the 30/31 kDa region was cut. This strip was incubated with the polyclonal IgG (1:100 dil., overnight). The strip was washed and eluted with 0.1 *M* glycine-HCl, pH 2.5. The procedure was repeated a number of times to collect sufficient quantity of anti 30/31 kDa monospecific antibody[4].

*Production of MAbs* : Monoclonal antibodies were produced by immunizing the mice with soluble CF antigens or NCP bound antigens.

*Immunization of mice :*

*(i)* Intra-peritoneal (IP)– Two hundred microlitres of an emulsion containing equal parts of CF (25 or 50 µg) antigen and IFA or NCP bound antigen was injected intraperitonially into 6 wk-old female BALB/c mice (bred at the National Institute of Nutrition, Hyderabad). Secondary immunizations were carried out. with the same antigen every 3rd wk. A minimum of 4 such injections were given. Blood was obtained from the retrobulbar sinus on day 0 (negative control), 1 wk after the first immunization and 2 days after subsequent immunizations. The animals were then left unstimulated for 4 wk and a booster dose of 100 µg of CF without adjuvant was administered 3 days before the fusion.

*(ii)* Intrasplenic (IS)– The animals were anaesthetized by IP injection of 0.6 ml of 10 mg/ml Nembutol (Sigma, USA). Atropin (0.04 ml of 0.06 mg/ml) (Sigma, USA) was given intraperitoneally before surgery. The spleen was exposed and the NCP bound antigen was injected using a fine needle (26G needle). The spleen was replaced in the peritoneal cavity and the incisions were closed[5].

*Fusion protocol* : The mouse was killed by cervical dislocation. The spleen was removed aseptically, washed thoroughly and teased, so as to release the cells. The splenic cells were fused with the SP2/0 myeloma cells as per standard protocol[6] and the cells were distributed into 96 well microtitre plates (200 µl/well). After periodic changes of hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidine (HT) (Sigma, USA) media, they were maintained in a medium containing 20 per cent Dulbecco's modified Eagle's medium (DMEM) (Sigma,USA). Usually, hybridomas started growing by the 2nd wk. When the medium became yellow, the supernatants were screened for the presence of antibodies by ELISA. The antibody positive cells were cloned by limiting dilution method. Wells showing positive results were recloned until monoclonality was achieved.

Bulk production of MAbs was achieved by growing the cells in large flasks. An increased yield was obtained by producing the ascitic fluid in the peritoneum of BALB/c mice.

*Immunoglobulin sub-class typing :* The immunoglobulin (Ig) subclass of the MAbs produced were determined by Ouchterlony immunodiffusion technique, using the subclass typing kit (The Binding Site, Birmingham).

*Enzyme linked immunosorbent assay (ELISA)* : ELISAs of different configurations have been used for screening the MAb supernatant, to study the nature of the reacting epitope, for antigen detection *etc.* Some of the basic steps are common to all these ELISAs and are described here: Polystyrene microtiter plates (NUNC Maxisorp, USA) were coated with appropriate concentrations of CF antigen or affinity purified rabbit IgG, in carbonate-bicarbonate buffer, 0.06 *M*, pH 9.6 at 4°C overnight. For blocking of additional reactive sites, 1 per cent BSA in PBST (PBS containing 0.1% Tween 20) was added and incubated for 1 h at 37°C. The wells were washed five times with PBST, between incubations. Anti immunoglobulin coupled with horse radish peroxidase (HRP) (Jackson Laboratories, USA) conjugates were used. The enzymatic reaction was developed by adding 100 µl of orthophenylene diamine (OPD) and hydrogen peroxide and the reaction was stopped by adding 50 µl of 8N $H_2SO_4$. The coloured product was read at 492 nm (Spectromax 250, Molecular Devices Corporation, California).

*Screening for antibodies in hybridoma supernatants by ELISA* : One hundred microlitre of supernatants from the growing clones were added to antigen coated wells and allowed to bind at 4°C overnight. Plates were washed and incubated with 100 µl of rabbit antimouse Ig-HRP at 1:4000 dilution for 1 h at 37°C and the colour reaction was developed.

Culture supernatant from the myeloma cell line SP2/0 as a negative control, supernatant from a known positive clone as a positive control and PBST as blank, were used.

*Reactivity of MAbs to sodium periodate treated CF :* The method described by Woodward *et al* [7], permits the identification of MAbs recognizing carbohydrate-containing epitopes associated with glycoproteins and glycolipids.

CF coated and blocked plates were rinsed with 50 mM sodium acetate buffer (pH 4.5). Sets of wells were then exposed to varying concentrations of sodium periodate (0-200 mM) in 50 mM sodium acetate buffer, pH 4.5 for 1 h at room temperature (RT) in the dark. Following a brief rinse with 50 mM sodium acetate, the plates were incubated with 50 mM sodium borohydride in PBS for 30 min at RT. Following washes with PBST, the plates were exposed to MAbs, for 1 h at RT and colour was developed.

*Double antibody sandwich ELISA for the detection of antigen* : For quantitation of antigen in sera, the plates were coated with affinity purified anti-rabbit CF. After blocking, 100 µl of the sample or CF antigens of known concentrations were added in duplicate and incubated at 37°C, for 2 h. After washing, MAbs in appropriate dilutions were added and left for 2 h at 37°C followed by incubation with anti-mouse Ig-HRP at 37°C for 1 h. The colour was developed with the substrate. In all the plates, control wells containing buffer without antigen was run. Antigen concentration in sera was calculated from the standard graph plotted with known antigen concentrations.

*Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting* : SDS-PAGE was performed in slab gels by the method of Laemmli[8]. A 12.5 per cent acrylamide-bis-acrylamide gel (BioRad, USA) with 0.4 per cent SDS in 0.375 *M* Tris HCl, pH 8.8 was used to separate the antigens. The stacking gel was 4 per cent acrylamide-bis-acrylamide with 0.4 per cent SDS in 0.125M Tris HCl, *pH* 6.8. The gel thickness was 1.5 mm. The samples (usually 50 µg of Ag/cm length of the gel) were applied under reducing conditions by boiling the samples in sample buffer for 5 min at 96°C. Gels were run in vertical slab gel apparatus (Bio-Rad, USA).

After electrophoresis, the gels were stained with 0.2 per cent Coomassie brilliant blue stain for 1 h at room temperature with shaking and destained with successive changes of destainer consisting of 45 per cent methanol and 10 per cent acetic acid till the background became clear.

*Immunoblotting* : Resolved antigens were electrophoretically transferred to NCP (0.45 µm pore size) by the method of Towbin *et al* [9], using a transblot apparatus (Bio-Rad, USA). The gels were soaked in transfer buffer (0.025 *M* Tris, pH 8.3 containing 0.192 *M* glycine and 20% methanol) for

30 min and transfer was carried out at 0.65A for 1 h. Reactive sites of the NCP were blocked by incubating with 5 per cent skimmed milk powder in PBS with 0.3 per cent Tween 20 (PBST). After washing, the strips were incubated overnight at 4°C with MAb supernatant or ascitic fluid diluted appropriately. Antibodies were detected with peroxidase conjugated goat anti mouse immunoglobulins in the dilution of 1:1000 (Dakopatts, Denmark). After repeated washes, the strips were developed using 3,3'-diamino benzidine (DAB) as the substrate.

SP2/0 culture supernatant was included as a negative control, polyclonal mouse or rabbit antiserum was used as a positive control and for the conjugate control, PBST alone was used.

*Immunoperoxidase staining* : Skin sections were obtained from guinea-pigs sensitised with *M. tuberculosis* H37Rv ( $10^3$ bacilli) intradermally. Skin lesions from leprosy patients and lymph node specimens from patients of Hodgkin's disease were also tested for the detection of antigen using the MAbs produced. Skin sections were first deparaffinized and blocked with normal swine serum for 10 min at room temperature. They were then incubated with MAbs for 1 h at 37°C followed by incubation with HRP-anti mouse IgG at 1:500 dilution for 1 h. The substrate DAB was added for colour development and the sections were counter stained with haematoxylin and mounted on the slides after being washed and dried.

## Results

*Standardization of immunization protocol for the production of MAbs* : The efficient route of immunization and the schedule was standardized by comparing the antibody titres obtained by IS and IP routes in ELISA. IP alone, IS alone or combination of IP and IS were given. Totally 9 groups of mice were used. Five mice in each group were immunized with NCP bound antigen. It was observed that IP immunization gave a good antibody titre. IS route of immunization with minimal antigen dose did not produce any appreciable antibody titre. While the increased antigen dose by the same route gave an increased antibody titre, the result was no better than that with IP immunization. Of the 2 routes, IP

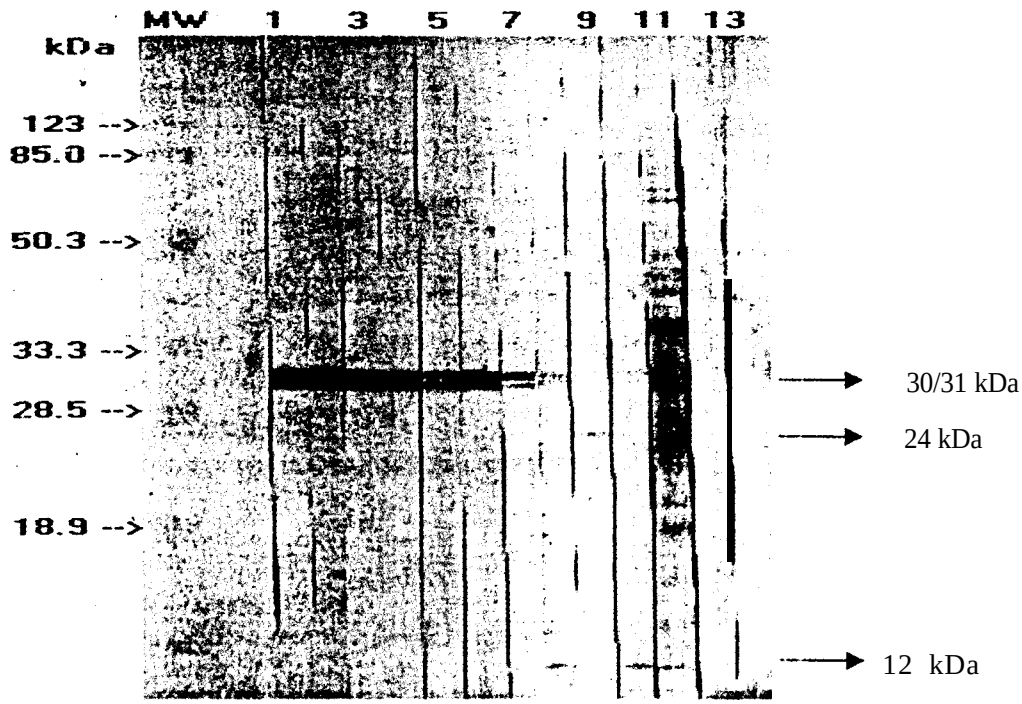


**Fig. 1.** Immunoblot analysis of ten monoclonal antibodies with *M. tuberculosis* H37Rv CF antigen. Lanes 1- 10 : TRC 1 - TRC 10; Lane 11 : Mouse anti-H37Rv CF sera (+ve control), Lane 12 : SP2/0 TC supernatant, Lane 13 : PBST (Blank).

immunization is comparatively a simple procedure and so further immunizations were carried out by this route.

*Production and characterization of MAbs* : Ten MAb producing hybridoma clones designated TRC 1 to TRC 10 were obtained from the fusions performed with splenic cells from BALB/c mice, immunized with H37Rv CF. Their reactivity pattern with CF has been shown in Fig.1. Of the 10, 7 MAbs, TRC 1 to TRC 7, reacted with the 30/31 kDa doublet (Ag 85 Complex); TRC 8 reacted with 12 kDa in addition to 30/31 kDa. TRC 9 and TRC 10 recognized the 24 and 12 kDa antigens respectively.

The Ig subclass was determined by Ouchterlony immunodiffusion technique. Out of 10 MAbs produced, 2 belonged to IgM isotype (TRC 6 & 7), 2 to IgG 2b (TRC 2 & 4), and the remaining 6 were identified as IgG 1 isotype.

The binding capacity of the serially diluted MAbs against varying concentrations of CF, ranging from 5 to 156 µg/ml was established by checker board titration. TRC 3, 6 and 8 exhibited the highest, reactivity (optimal dilution 1: 1000), followed by TRC 1, 7, 2, 4, 9, 5 and 10 in that order (optimal dilution ranging from 1:200 to 1:25). Identical results were obtained when the MAbs were used in a dot-blot assay.

*Nature of the epitopes recognised by MAbs* : Treatment of antigen with 200 mM sodium meta-periodate, led to loss of binding of TRC1 with antigen, as evidenced by ELISA, indicating that this antibody recognised the carbohydrate moiety of the antigen. On the other hand, this treatment did not affect the reactivity of the other MAbs - TRC 2 to TRC10 - suggesting that they recognised the protein epitopes of the antigen (data not shown).

*Inter-species reactivity of the MAbs* : Reactivity of the MAbs with CF of different strains of mycobacteria and *Esch. coli* was investigated by ELISA. Per cent binding of the various MAbs with different strains of mycobacteria was calculated, with reference to absorbance obtained with CF of *M. tuberculosis* H37Rv taken as 100 per cent. The per cent binding of the MAbs is given in the Table.

Based on the reactivity of the MAbs to the atypical mycobacterial species, they were classified as broad (reacting with more than 2 species) or limited cross reactive (with 1 or 2 species) MAbs. TRC 8 and 10 showed reactivity only with H37Ra and south Indian low virulent isolate and to a lesser extent with *M. bovis*. Reactivity was not observed with other atypical mycobacteria and *Esch. coli*. TRC 1 and 2 showed limited cross reactivity with atypical mycobacteria and were also found to react with *Esch. coli*. TRC 3-7 and TRC 9 were categorized under the broadly cross reactive group.

**Table.** Per cent binding of MAbs to various species of mycobacteria

| % binding | H37Ra | SILV* | Cytosol | *M.bovis* | *M.kansasii* | *M.gordonae* | *M.vaccae* | *M. smegmatis* | *M.diernhoferi* | *M.fortuitum* | *M.thermalresistible* | *Esch. coli* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TRC 1 | 95.4 | 109.4 | 26.7 | 73.2 | 10.6 | 35 | 9.9 | 8.7 | 15.3 | 10.1 | 11.1 | 31.8 |
| TRC 2 | 75.7 | 21.5 | 25.6 | 41.4 | 19.1 | 15.3 | 6.2 | 7.4 | 5.3 | 15 | 9.6 | 72 |
| TRC 3 | 105.5 | 70.1 | 35.5 | 95.4 | 68.6 | 65 | 101.9 | 29.5 | 9.2 | 13.4 | 66.4 | 8.4 |
| TRC 4 | 96.5 | 46.7 | 35.8 | 79.2 | 52.9 | 33.9 | 33.5 | 19.2 | 30.3 | 37.6 | 53 | 39 |
| TRC 5 | 93.2 | 91.9 | 72.2 | 80 | 38.1 | 53.3 | 29.6 | 21.2 | 32.6 | 35.1 | 36.9 | 60.2 |
| TRC 6 | 107.6 | 79.3 | 107.4 | 29.6 | 26 | 41.2 | 49.3 | 67.8 | 96 | 94.6 | 56.8 | 114.5 |
| TRC 7 | 132.6 | 133.4 | 57.4 | 70 | 66.4 | 59.3 | 59.5 | 52.1 | 53.6 | 62.1 | 60.5 | 50.4 |
| TRC 8 | 92.7 | 121.8 | 15.1 | 13.4 | 0.65 | 2.7 | 0.12 | 1.5 | 2 | 1.1 | 1.4 | 5.7 |
| TRC 9 | 103.9 | 69.4 | 69.8 | 87 | 52.8 | 63.9 | 59.1 | 56.2 | 60.4 | 85.7 | 58.3 | 23.9 |
| TRC 10 | 81.2 | 123.5 | 13.7 | 10.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.1 |
| Polyclonal | 92.9 | 98.3 | 77.6 | 77.3 | 63.8 | 53.6 | 47.1 | 46 | 62.6 | 57.3 | 72.7 | 59 |

*South Indian low virulent

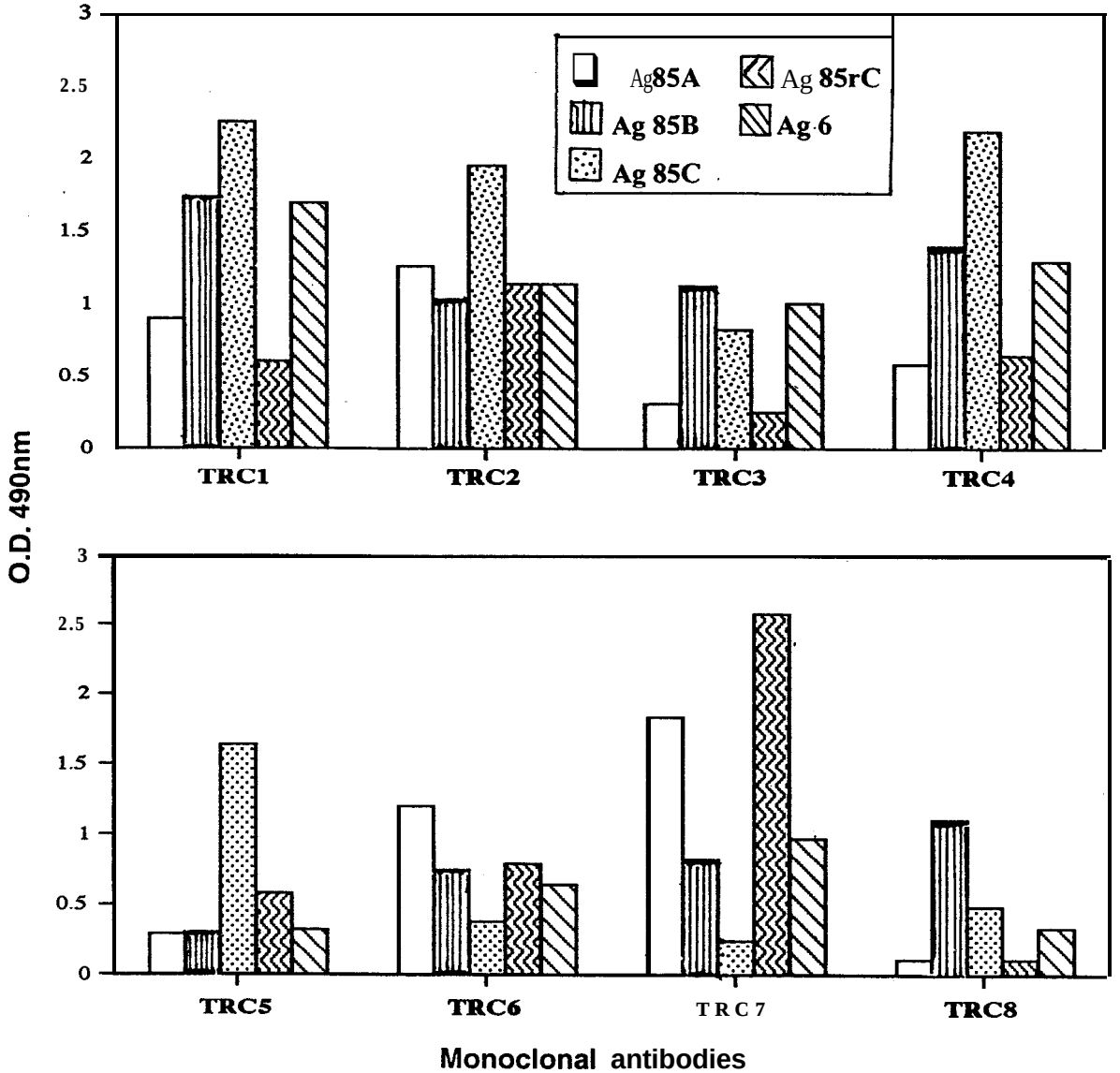


**Fig.** 2. Reactivity patterns of MAbs to Ag 85 complex.

*Reactivity pattern of MAbs to the individual components of the Ag 85 complex :* Out of the eight 30/3 1 reacting antibodies, 4 (TRC 1, 2, 4 and 5) had maximum reactivity with Ag 85 C, as compared to A and B. Two of the MAbs had peak reactivity with Ag 85 B (TRC 3 and TRC 8). Ag 85A and 85rC were the major components recognised by TRC 6 and TRC 7 respectively (Fig. 2). None of MAbs showed reactivity restricted only to one component.

*Antigen detection assay by sandwich ELISA :* An antigen capture assay was developed with hyperimmune rabbit anti H37Rv CF as 'capture' antibody followed by detection with different MAbs. Of the 10 MAbs tested, TRC 4, 6 and 8 were suitable. The lower detection limit corresponded to 1.25 µg/ml for TRC 4 and 156 µg/ml for TRC 6. Antigen detection in serum using TRC 4 and TRC 6 did not permit distinction between patient and control groups. Using TRC 8, the standard curve was obtained, which could detect a minimum of 156 µg/ml (mean+3 SD of 0 conc.). Antigen was detected in 17 of 25 (68%) tuberculosis patients and 3 of 20 (15%) controls.

Antigen detection assay by competitive inhibition ELISA was also standardized using TRC 8. The sensitivity of antigen detection was limited to

1.25 µg/ml of antigen. This assay when used in patients sera did not have enough sensitivity to pick up the antigen.

*Immunoperoxidase staining* : The MAbs were evaluated for their potential to detect mycobacterial antigens from infected tissue, using immuno-peroxidise staining. Skin sections of guinea-pigs sensitized with *M. tuberculosis* H37Rv were used. Sections from leprosy lesions, as well as lymph node specimens from patients of Hodgkin's disease were also included. Of the 10 MAbs tested, 6 were able to detect antigens in biopsy specimens. TRC 8 could detect antigen specifically in *M. tuberculosis* and *M. leprae* infected tissues.

## Discussion

The introduction of hybridoma technology has opened new vistas of research in immunology, diagnosis of infectious diseases, and purification of specific antigens. Many investigators have produced MAbs against mycobacterial antigens[10-16]. The first set of murine MAb raised in BALB/c mice, recognised proteins of 71, 65, 38, 33, 19, 14 and 12 kDa.

A variety of antigen preparations and immunization schedules have been tested, for *M. tuberculosis* antigens. Intraperitoneal injections are the most commonly used method for introducing antigens into mice, because the volume of the immunogen can be larger. Also, particulate antigens can be used. For an antigen, which is only available in minute quantities, intrasplenic immunization with nanograms of antigen immobilised on NCP, results in good immune response[17]. The advantage offered by this procedure is presumably to maximize the number of specific B cells blasts in the spleen by direct local antigen stimulation. Hence a comparative study of intraperitoneal and intrasplenic immunizations was carried out during this study.

The results of the present investigation proved that intrasplenic immunization with 2 µg of NCP bound antigen did not give an appreciable antibody response. Larsson and Nilsson[17] and Nilsson *et al* [s] have used purified single proteins such as BSA and insulin and obtained good response using the IS route, while our complex antigenic mixture gave a suboptimal response.

In the present study, 10 MAbs have been raised against *M. tuberculosis* secreted proteins, CF. The secreted proteins of mycobacterium, are of great current interest since they are available for immune recognition at an early stage of infection and may play a special role in immune mechanisms[18].

The MAbs have been characterized with regard to their isotype, specificity and cross reactivity with *M. tuberculosis* and other mycobacterial species. Of the 10 MAbs produced (TRC 1- 10), 8 were of IgG class and the other 2 were of the IgM type. Majority of the antibodies produced during this study belong to the IgG type, probably because the mice were immunized with soluble antigens mixed with the IFA. Immunization of mice with CFA (with whole mycobacterial cells) preferentially leads to the development of IgM antibodies reactive with polysaccharides[19].

Seven of the MAbs (TRC 1-7) reacted only with 30/31 kDa protein of *M. tuberculosis* H37Rv CF. In addition to binding to the 30/31 kDa antigen, TRC 8 also reacted with the 12 kDa protein antigen of *M. tuberculosis.* The increased number of 30/31 kDa reacting cell lines produced could be due to the immunodominant nature of the 30/31 kDa antigen. This antigen is the most abundant protein (accounting to nearly 15-40% of the total secreted antigen) produced by *M. tuberculosis* growing either extracellularly in broth culture or intracellularly in human mononuclear phagocytes[20,21]. Other workers have observed recognition of 31 and 33 kDa antigens as prominent bands by the human MAbs by Western blot analysis[22]. TRC 1 recognised the carbohydrate moiety while the rest were against the protein moiety of the antigen. Antigen 85 complex has been extensively characterized by many groups of workers and is not reported to be a glycoprotein. Our observation concurs with that of Salata *et al* [23] who have measured a carbohydrate content of 3 µg per 100 µg of protein.

TRC 8 and TRC 10 were found to be reacting only with the members of the *M. tuberculosis* complex. It is interesting to note that even within

this complex, cross reactivity with *M. bovis* was low. However, all the 30/31 kDa reacting MAbs were found to react with all the 3 components, namely antigen 85 A, B and C. This could be explained to be due to the 70-80 per cent sequence homology existing between the Ag 85 complex proteins.

Previous studies, especially those of crossed immunoelectrophoresis, have also provided convincing evidence for the existence of specific epitopes on 30/31 kDa[24]. However, none of the MAbs described so far recognised any species specific epitope on the antigen 85 complex[10,16,23,25,26]. In the present study, TRC 8 has been produced, which recognizes 30/31 kDa (and 12 kDa) of *M. tuberculosis* complex only.

The 30/31 kDa antigen is a suitable candidate for antigen detection assay, due to its early secretion in the culture medium, appearing in the culture as early as day 3[20]. Since it is an early antigen, there is a likelihood of it being detected at an early stage of the disease process. Because of the larger concentration, this antigen can be detected with comparative ease in body fluids. So far, this antigen has not been evaluated for antigen detection. TRC 8, was preliminarily evaluated in a sandwich ELISA for antigen detection where it gave a sensitivity of 68 per cent and specificity of 85 per cent, in a limited number of samples. The lower sensitivity of 68 per cent is explainable by the fact that antigens are not usually present in free form in serum, but complexed with the antibodies. However, the finding 3 of 20 (15%) normal subjects being positive for the antigen is surprising, in the light of specificity of TRC 8. In the present study, it was not possible to follow the positive, normal subjects for probable future break down into disease. This observation underlines the need for repetition of the test with more number of well defined sera, with provision for follow up of the test positives.

A panel of 7 MAbs was evaluated for their potential diagnostic applications in immuno-pathology. The MAb TRC 8 was found to be more specific in reacting with the tuberculous and leprosy lesions and not with Hodgkin's lesion. In the present study, only a few cases from each category were tested with the MAb. It is possible that a trial of a large number of specimens might reveal a more useful staining pattern.

Detection of mycobacterial antigens by immunohistochemistry has been reported to be useful for establishing the mycobacterial etiology of caseating pulmonary granulomas[27] and in lung, lymph node and joint tissue sections of tuberculosis patients[28]. Review of literature pertaining to immuno-peroxidase staining in histopathological sections, reveals that attempts made in this direction are few and far between. Further studies with TRC 8 and 10, using infected material from patients, are worthwhile.

To conclude, TRC 8 sandwich ELISA has been found useful and the test characteristics of the assay match those which have been reported earlier. The sensitivity of the assay developed, will show great improvements, when applied to samples other than serum, where the interference due to antibody is less. Therefore, it is worth evaluating TRC 8 assay in other body fluids, especially CSF. The fact that TRC 8 assay measures the 30/31 kDa antigen, one of the dominant and early secreted antigens adds emphasis to the expected value of the test for early diagnosis. Of more importance is the absence of recognition of mycobacteria other than tuberculosis (MOTT), which allows specific identification of *M. tuberculosis*, particularly in HIV-TB, where disease due to non tuberculous mycobacteria, are more common.

## Acknowledgment

The authors acknowledge with thanks the financial support of Indian Council of Medical Research. New Delhi to Sh. Senthil Kumar Senior Research Fellow and Dr H. Shakila for help in carrying out the immuno-peroxidase technique.

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*Reprint requests :* Dr Alamelu Raja, Assistant Director, Department of Immunology, Tuberculosis Research Centre (ICMR) Mayor V.R. Ramanathan Road, Chetput, Chennai 600031