Br. J. exp. Path. (1989) 70, 215–225

The 45 kilodalton molecule of *Mycobacterium tuberculosis* identified by immunoblotting and monoclonal antibodies as antigenic in patients with tuberculosis

A.R.M. Coates, H. Nicolai, M.J. Pallen,* A. Guy,† S.D. Chaparas‡ and D.A. Mitchison†

*Department of Medical Microbiology, The London Hospital Medical College, London; *Department of Medical Microbiology, St Bartholomew's Hospital Medical College, London; †Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, London and ‡Mycobacteria and Cellular Immunology Laboratory, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20205, USA*

Received for publication 1 June 1988
Accepted for publication 27 November 1988

Summary. The object of this study was to discover new *M. tuberculosis* antigens which are recognized by patients with tuberculosis, because effective serodiagnostic tests are likely to require combinations of different antigens. In our early experiments using immunoblotting, the findings suggested that human sera from smear-negative tuberculosis patients bound to an antigen in the 45 kDa region. Subsequently, estimates of molecular weight in the immunoblots confirmed that the murine monoclonal antibody (MAB) HGT-6 and sera from patients both recognized the same 45 kDa molecule. An antibody–antibody competition assay between MAB HGT-6 and sera from smear-positive tuberculosis patients yielded a positive result in 23 out of 43 sera from patients, but in only four out of 23 from controls. This is further evidence that the 45 kDa antigen is recognized by tuberculous patients. We analysed whether a combination of the 45 kDa antigen results and those of known antigens might better discriminate between minimal smear-negative disease and healthy controls than could tests with single antigens. There is no clinically useful laboratory test for smear-negative tuberculosis. In immunoblotting, combining the results with the 65, 45, 38 and 10 kDa antigens gave the best discrimination. This suggests that future serodiagnostic tests for minimal disease, such as the antibody–antibody competition assay, should contain a MAB against the 45 kDa antigen and possibly also against the 10 kDa antigen.

Keywords: *Mycobacterium tuberculosis*, monoclonal antibodies, antigens, immunoblotting, serodiagnosis, vaccine

Tuberculosis causes about 20 million new cases in the world each year, and is responsible for two to three million deaths. It continues to be one of the most important infectious diseases, particularly in developing countries.

Correspondence: A.R.M. Coates, Department of Medical Microbiology, The London Hospital College, Turner Street, London E12 AD, UK.

Available evidence (Hewitt *et al.* 1982; Kaplan & Chase 1980; Reggiardo *et al.* 1981; Coates 1984) suggests that no single antigen will provide a satisfactory serological test for tuberculosis; rather a combination of antigens is required. The same situation may also apply to vaccine development. Our objective was to identify new antigens which are recognized by tuberculous patients. This could lay the foundations for new diagnostic serological tests and vaccine development. At least 60 *M. tuberculosis* complex antigens are visualized by two-dimensional crossed immunoelectrophoresis (Closs *et al.* 1980) using hyperimmune rabbit sera, but the number of antigens to which antibodies can be readily detected in serum of patients may be more restricted. For instance, in one report (Kaplan & Chase 1980) each tuberculosis patient recognized a unique subset of antigens within a principal group of nine but failed to produce precipitating antibodies to the other antigens.

Methods for the identification of antigens which are recognized by tuberculous patients are: purification of the antigen followed by testing of sera against that molecule (Daniel *et al.* 1979; Nassau & Nelstrop 1976; Reggiardo & Middlebrook 1975 a, b) development of monoclonal antibodies (MABs) which are then used as probes in an antibody–antibody competition assay with human sera (Coates *et al.* 1981; Hewitt *et al.* 1982; Ivanyi *et al.* 1983), and gel separation techniques such as crossed immunoelectrophoresis (Closs *et al.* 1980; Kaplan & Chase 1980) or immunoblotting (Chakrabarty *et al.* 1982; Klatser *et al.* 1984). MABs have been particularly successful in this regard and the first group (the 'TB' series) which was described against mycobacteria (Coates *et al.* 1981) has resulted in the identification of four important *M. tuberculosis* antigens (reviewed by Engers *et al.* 86): 65 kDa (binds to MAB TB78), 38 kDa (MAB TB71 and TB72), 19 kDa (MAB TB23) and 14 kDa (MAB TB68). The binding of all these MABs is inhibited by sera from tuberculous patients (Hewitt *et al.* 1982; Ivanyi *et al.* 1983) and so may be useful for the development of serological tests.

We sought to find out which of the known 60 antigens might be added to the antigens of the 'TB series' in order to improve serological tests for tuberculosis. We have provided preliminary evidence that a combination of tests which includes the use of the MAB against the 45 kDa (Kadival & Chapars 1987) antigen might improve such tests.

Materials and Methods

Preparation of M. tuberculosis sonicates

M. tuberculosis strain H37Rv was grown as surface pellicles on Sauton's medium, which contained potassium dihydrogen orthophosphate (0.5 g), magnesium sulphate (0.5 g), citric acid (2 g), ferric ammonium citrate (0.05 g), glycerol (60 g) and asparagine (4 g) in 1 l of distilled water. The pH was adjusted to 7.4 using 1 M sodium hydroxide. The organisms were harvested and sonicated at 0°C for 1 min followed by 1 min pause over 30 min with a Branson sonicator. The sonicate was centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant was removed and filtered through a 0.22 μm membrane to remove debris and remaining organisms. Protein estimations were determined according to a modification of Lowry's method (Ohnishi & Barr 1978). This material was stored in aliquots at −70°C and is referred to as the sonicate.

Patients' sera

Serum was obtained from 34 Chinese patients with pulmonary tuberculosis in Hong Kong. All patients had radiographic evidence of active disease. When examined by fluorescence microscopy, no acid-fast bacilli were seen in sputum from these patients, but *M. tuberculosis* was cultured from them all. Serum was also taken from 36 healthy Chinese controls in Hong Kong at the same time. A further set of sera was taken from 43 smear-positive tuberculosis patients

in Hong Kong. All sera were stored at −70°C.

Monoclonal antibodies

The MABs included: the TB series (Coates *et al.* 1981) TB68, TB77, TB71, TB78, TB23 and TB73, HGT-6 (Kadival & Chaparas 1987), F29-F29 (Kolk *et al.* 1984) and SA12 (Minden *et al.* 1984). The MAB TB68 binds to 14 kDa, TB23 to 19 kDa (see Fig. 3), TB77 to 30 kDa (see Fig. 1), TB71 to 38 kDa, TB78 to 65 kDa (Ivanyi *et al.* 1985), TB73 to a 90 kDa multi-band (see Fig. 3), HGT-6 to a twin band in the 45 kDa region (Kadival & Chaparas 1987), F29–F29 to the 45 kDa region (Kolk *et al.* 1984) and SA12 to a 10 kDa molecule (Minden *et al.* 1984).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The *M. tuberculosis* sonicates were analysed by SDS-PAGE using the method of Laemmli (1970) with some modifications (Hames 1981). A Bio-rad slab gel electrophoresis system (Bio-rad Laboratories, California) was used with 12.5% polyacrylamide gels containing 0.1% SDS. The sonicate was diluted in loading buffer (11% v/v glycerol, 1% w/v SDS, 0.01% w/v bromophenol blue, 26% v/v 0.5 M Tris-HCl buffer, pH 6.8), to which 5% v/v beta-mercapto-ethanol was added so that the quantity of protein was 20 μg per track. The samples were heated to 100°C for 3 min before loading. Some gels were stained for protein using Coomassie Brilliant Blue.

Transfer of proteins from gel to nitrocellulose filter paper

SDS-PAGE separated proteins were transferred onto BA85 nitrocellulose membrane filters (Schleicher & Schuell, Dassell, FR Germany) by capillary blotting, using a method commonly used in Southern transfer (Maniatis *et al.* 1982). Blotting was performed overnight using a non-denaturing buffer (150 mM glycine, 20 mM Tris, 20% methanol, pH 8.3.

Immunodetection of antibody binding to proteins on nitrocellulose paper

Immunodetection was performed using the Amersham biotin–streptavidin system (Amersham International PLC, Amersham, UK). The nitrocellulose paper was first incubated for 1 h with 3% bovine serum albumin (BSA) (3 g of BSA in 100 ml of phosphate-buffered saline, pH 7.5 (PBS)) and then with streptavidin, both of which reduced background. Thorough washing was then performed in 0.1% Tween 20 in PBS. The paper was cut vertically into strips each of which contained the proteins blotted from one PAGE track. Human sera from tuberculosis patients or healthy controls (1 in 50 dilution in 1% BSA) or monoclonal antibodies (diluted 1:1000 in 1% BSA) were then incubated with each of the strips for 1 h. The strips were washed five times in PBS and 2% Tween and then incubated with biotin-conjugated anti-human whole immunoglobulin or anti-murine whole immunoglobulin or anti-human IgG (Amersham International PLC, Amersham, UK) diluted 1 in 500 in 1% BSA. The strips were washed again five times and then incubated for 30 min in a solution containing streptavidin-conjugated horse-radish peroxidase (HRP) (Amersham International PLC, Amersham, UK), diluted 1 in 300 in PBS. After three more washes in PBS the strips were incubated for 5 min in the substrate solution which contained 0.03% hydrogen peroxide and 0.4 g/l 4-chloronaphthol in PBS. The strips were then washed and dried. Each serum was tested three times using these methods.

Antibody–antibody competition assay

To each of the wells of a microtitre tray (Nunc Immunoplate 1F) was added 50 μl of *Mycobacterium tuberculosis* sonicate (at

100 mg protein/l in PBS 0.1 M, pH 7.2 (Oxoid)) and allowed to dry at 37°C overnight. Wells were then blocked by an overnight incubation at 4°C with 1% BSA w/v (Sigma) in PBS with 0.1% v/v Tween-20 (BDH), 150 μl/well. The plates were then washed twice with PBS/Tween 0.1% v/v. All subsequent reactions were with 50 μl per well.

The antigen contained biotin which bound to one or more of the test reagents, particularly streptavidin. This raised background optical density and so was blocked using streptavidin (Sigma) at 2·5 mg/l, in PBS/Tween diluent, for 10 min at room temperature. Wells were washed twice and incubated similarly with biotin (Sigma) at 100 mg/l in the same diluent. A further two washes followed.

Serum, either undiluted or 1:2 dilution in PBS/Tween with 0.1% BSA, was added to test wells in triplicate and allowed to bind for 1.5 h at 37°C in a humidity chamber and the plates were then washed four times.

MAB was then added at 50% maximal binding diluted in PBS/Tween/0.1% BSA, and allowed 1.5 h at 37°C to bind. Plates were then washed four times.

The amount of bound MAB was determined using Amersham biotin–strepatividin products with their biotinylated anti-murine second layer antisera, HRP-linked preformed complexes, and ABTS (Sigma) as the chromogen, as recommended in the kit literature.

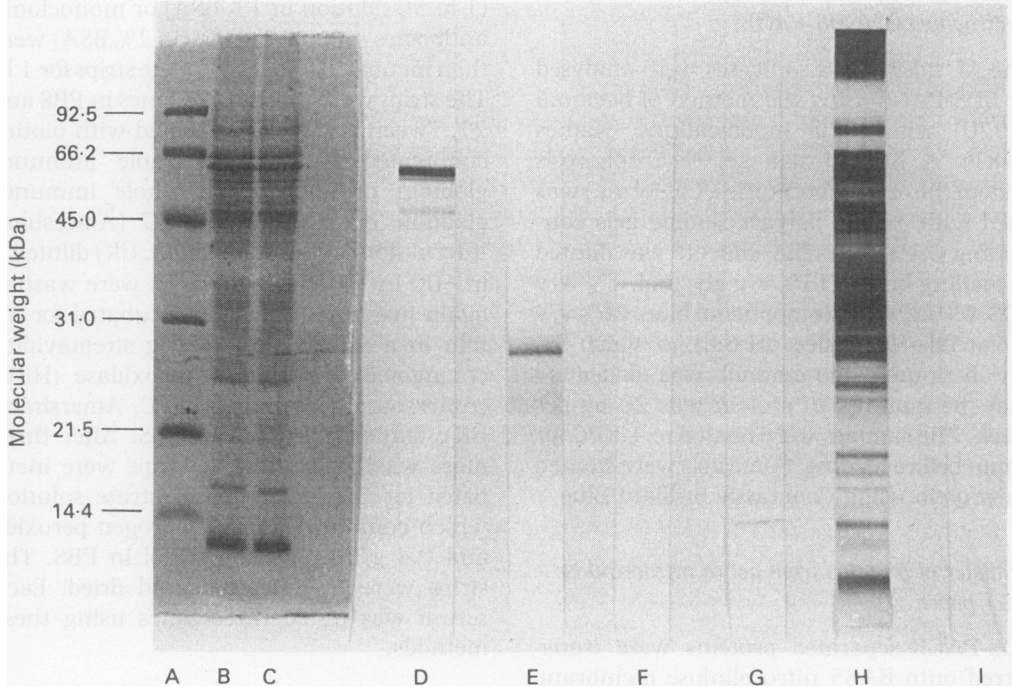

Fig. 1. Binding of monoclonal antibodies to *M. tuberculosis* antigens. Tracks A–C represent an SDS-polyacrylamide gel containing electrophoretically-separated proteins stained with Coomassie Brilliant Blue: A, molecular weight markers; B and C, *M. tuberculosis* sonicate. Lanes D–I are SDS-PAGE-separated *M. tuberculosis* proteins that have been blotted onto strips of nitrocellulose paper: D incubated with MAB TB78; E with MAB TB77; F with MAB TB71: G with MAB TB68; H with rabbit anti-*M. tuberculosis* polyclonal serum; I with PBS instead of first antibody.

At a 1 in 300 dilution in PBS/Tween/0.1% BSA diluent, the second layer antibody was given 1.5 h incubation and the complexes 30 min, both at 37°C. Each incubation step was followed by four washes. Substrate was incubated for 10 min at RT before stopping with Na azide. Absorbance was read at 414 nm.

Control wells were incubated without serum (diluent used as a substitute) as high control (100% binding), and without MAB as a low control (0% binding) for each serum sample tested. Results are expressed as the % MAB that binds when serum is present.

Results

Monoclonal antibody immune detection of SDS-PAGE separated M. tuberculosis proteins

Figure 1 shows the binding of MABs to *M. tuberculosis* antigens. The MAB TB68 bound to 14 kDa, TB77 to 30 kDa, TB71 to 38 kDa and TB78 to 65 kDa molecules. The outermost two strips from each nitrocellulose paper containing proteins from one gel were incubated with a mixture of these MABs. This provided an accurate molecular weight marker system.

Binding of human sera to SDS-PAGE separated antigens

Figure 2 illustrates the binding of two sera from smear-negative tuberculosis patients and a single serum from a healthy control. The panel on the left (lane E) shows results with a patient's serum which produced a single band at 30 kDa and a double band at 45 kDa, while the panel on the right (lane F) is with a patient serum showing bands at 30, 38, 45 and 65 kDa. Considering all sera, when binding of the sera occurred it was to one or more of the six antigens with molecular weights of 10, 14, 30, 38, 45 and 65 kDa. There were considerable differences in the antibody responses of individuals. Some sera bound to no antigens while others reacted with all six. A summary of the results (Table 1) shows that similar proportions of the sera from the smear-negative tuberculosis patients and the healthy controls bound to the 14, 30, 38 and 45 kDa antigens while positive reactions to the 65 kDa antigen occurred more often in patients (14 of 34) than in the controls (seven of 36). The only sera to bind with the 10 kDa antigen were from four tuberculosis patients.

Analysis of combinations of results of individual antigens

Discrimination between sera from smear-negative tuberculous patients and healthy controls was obtained with combinations of results of individual antigens. The combination of results which yielded the best discrimination was binding either to all three of the 38, 45 and 65 kDa antigens, or to the 10 kDa molecule (Table 2). Of the sera in this combination, 13 of 34 from the tuberculous patients, but none of the 36 control sera, bound to the antigens. Only one serum bound to all of the 65, 45, 38, 14 and 10 kDa

Table 1. Binding of sera from patients with smear-negative tuberculosis and healthy controls to *M. tuberculosis* antigens

	Antigens (kDa)						One or more antigens (excluding positive for 30 kDa antigen)
	10	14	30	38	45	65	
Smear-negative tuberculosis (34)	4	14	30	14	13	14	21
Healthy controls (36)	0	18	34	15	13	7	27

antigens, but a spectrum of combinations was observed with other sera.

Confirmation that the 45 kDa antigen is recognized by sera from tuberculosis patients and by the anti-45 kDa monoclonal antibody, HGT-6

Figure 3 shows binding of one serum from a smear-negative tuberculosis patient to the 45 kDa antigen (lane B). The 45 kDa antigen band is in the same position as the band recognized by the MAB HGT-6 in lane A. A band at about 65 kDa was seen in all immunoblots including the controls (lanes F and G) which contain second layer antibody but no first layer serum or MAB. This represents binding of the second layer antibody to 65 kDa molecule since no band was seen if no second layer antibody was used (lane H).

Careful comparison of the twin bands to which MAB HGT-6 bound with MAB-defined antigens of TB series revealed that both bands may be in excess of 45 kDa, in the regions of 47 and 49 kDa respectively. We detected a separate, distinct antigen at about 45 kDa with the MAB F29-F29 (lane C). Interestingly, several sera from smear-negative tuberculosis patients bound to an antigen in the F29-F29 band region, one of which is shown in lane D. Thus with SDS-PAGE it appears that there are three antigenic molecules in the region of 45 kDa, the two slightly larger ones bind to MAB HGT-6 and the smaller one to MAB F29-F29. In this

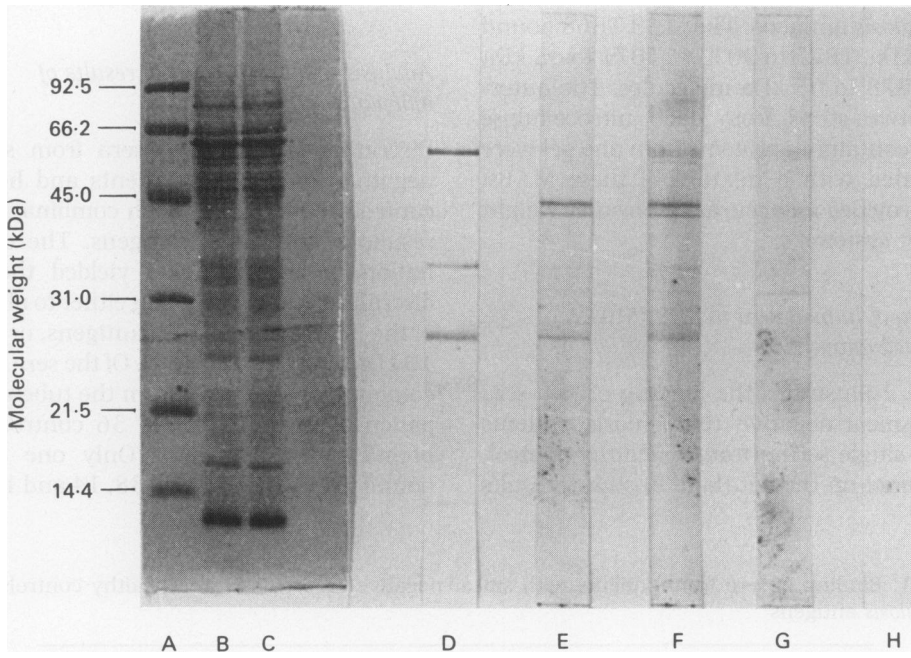

Fig. 2. Binding of two sera from smear-negative tuberculosis patients and a single serum from a healthy control to *M. tuberculosis* antigens. Lanes A–C represent an SDS-polyacrylamide gel which contains electrophoretically-separated proteins stained with Coomassie Brilliant Blue: A, molecular weight markers; B and C, *M. tuberculosis* sonicate. Lanes D–H are SDS-PAGE-separated *M. tuberculosis* proteins that have been blotted onto strips of nitrocellulose paper: D incubated with MABs TB78, TB77, TB71 and TB68; E and F with sera from two smear-negative tuberculosis patients; G with serum from a healthy control; H with PBS instead of first antibody.

Table 2. Analysis of combinations of results for the binding of human sera to individual *M. tuberculosis* antigens

	Antigens (kDa)									
	All	65,45, 38,14	(65,45,38) or 10 alone	65,45, 38	65,38, 14	65,45, 14	65,38	65,45	65,14	14,45
Smear-negative tuberculosis (34)	1	6	13	10	7	6	11	10	8	8
Healthy controls (36)	0	0	0	0	2	2	2	2	4	9

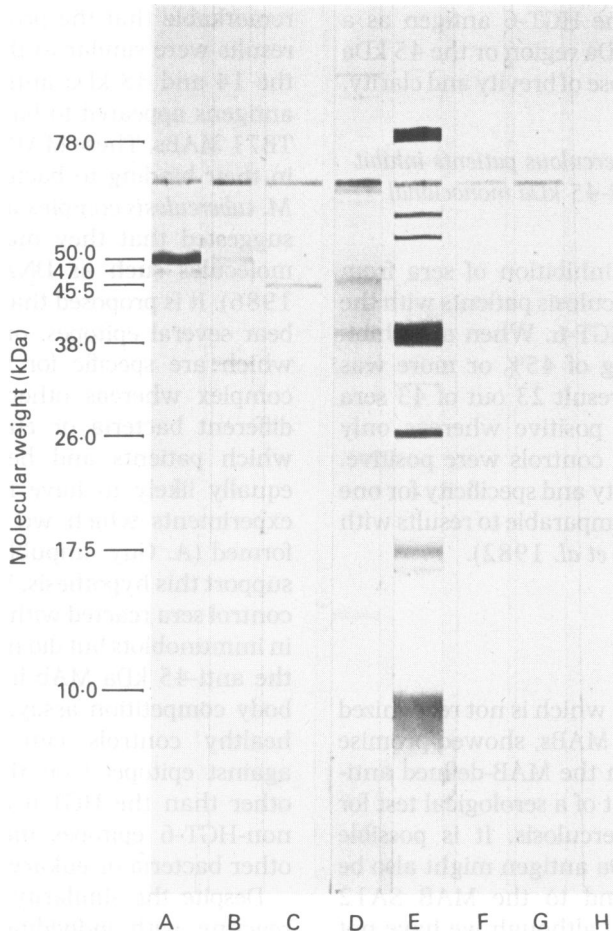

Fig. 3. Immunoblot analysis of SDS-PAGE separated *M. tuberculosis* sonicate and blotted onto nitrocellulose paper. Lane A incubated with MAB HGT-6; B and D with sera from two smear-negative tuberculosis patients; C with MAB F29-F29; E incubated with MABs TB71, TB77, TB23, TB73 and SA12; F incubated with serum from a healthy human control; G with normal mouse serum; H with no second layer antibody.

Table 3. Results of antibody–antibody competition enzyme immunoassay. Human sera compete with a monoclonal antibody (HGT-6) for the 45 kDa antigen of *M. tuberculosis* H37Rv

	Number of sera tested	Diagnosis positive*	Diagnosis negative	Percentage correct diagnosis
Serum from smear positive patients	43	23	20	54
Serum from healthy laboratory control individuals	23	4	19	83

* Where an inhibition of MAB binding of 45% or more is a positive result.

paper we refer to the HGT-6 antigen as a molecule in the 45 kDa region or the 45 kDa antigen for the purpose of brevity and clarity.

Human sera from tuberculous patients inhibit the binding of the anti-45 kDa monoclonal antibody, HGT-6

Table 3 shows the inhibition of sera from smear-positive tuberculosis patients with the anti-45 kDa MAB HGT-6. When an inhibition of MAB binding of 45% or more was taken as a positive result 23 out of 43 sera from patients were positive whereas only four out of 23 from controls were positive. This level of sensitivity and specificity for one MAB on its own is comparable to results with other MABs (Hewitt *et al.* 1982).

Discussion

The 45 kDa antigen, which is not recognized by the 'TB' series of MABs, showed promise in combination with the MAB-defined antigens as a component of a serological test for smear-negative tuberculosis. It is possible that a further 10 kDa antigen might also be useful and may bind to the MAB SA12 (Minden *et al.* 1984) although we have not as yet confirmed this experimentally.

The proportions of sera binding to each of the antigens with molecular mass of 14–65 kDa were similar for the tuberculosis patients and the healthy controls. It is remarkable that the proportions of positive results were similar in these two groups for the 14 and 38 kDa antigens because these antigens appeared to bind to the TB68 and TB71 MABs. These MABs are highly specific in their binding to bacterial extracts of the *M. tuberculosis* complex although it has been suggested that they may cross-react with molecules such as DNA (Shoenfeld *et al.* 1986). It is proposed that antigen molecules bear several epitopes, only one or some of which are specific for the *M. tuberculosis* complex whereas others are shared with different bacteria or eukaryotic molecules which patients and healthy controls are equally likely to have met. The results of experiments which we have recently performed (A. Guy, unpublished observations) support this hypothesis. We found that three control sera reacted with the 45 kDa antigen in immunoblots but did not inhibit binding of the anti-45 kDa MAb in the antibody–anti body competition assay. This suggests that healthy controls can produce antibody against epitope(s) on the 45 kDa antigen other than the HGT-6 determinant. These non-HGT-6 epitopes may be shared with other bacteria or eukaryotic molecules.

Despite the similarity in the proportions reacting with individual antigens, certain combinations of binding occurred more frequently in patients than controls. Thus 10 of the 34 sera from the tuberculosis patients bound to all three of the antigens of 65, 45 and 38 kDa, whereas none of the sera from

the healthy controls had this binding pattern. Furthermore, the 10 kDa antigen bound to an additional four tuberculous sera but to none of the controls. While this binding pattern distinguished best between sera from our patients and controls, it will be appreciated that there is a large number of combinations of results forming different patterns of results, which were examined to see whether any of them separated the tuberculosis from the control sera. There is no recognized statistical method for excluding the possibility that the apparent value of any particular combination, such as the one proposed here, might have arisen by chance. Positive evidence supporting the proposal could be obtained only by repeating the work with a different set of sera. Nevertheless, it is clear from the work reported here, and from previous studies with smear-positive cases (Coates 1984), that patients with tuberculosis recognize more antigens than do controls. Furthermore, it seems that binding patterns including the 45 kDa antigen are likely to improve discrimination. Possible explanations for these observations are that increased exposure to *M. tuberculosis* as occurs in the disease (1) might lead to higher antibody levels against the same epitopes that healthy controls recognize and (2) might, in addition, cause patients to 'see' more epitopes and more antigens, including those specific for mycobacteria than do controls.

When we consider the application of our results to the future development of serological tests for tuberculosis, it is evident that one of the greatest needs is for a satisfactory test for smear-negative pulmonary disease. Direct microscopy fails to diagnose smear-negative disease which accounts for a substantial proportion of cases of tuberculosis (Mitchison 1968; Hong Kong Chest Service, Tuberculosis Research Centre, Madras & British Medical Research Council 1984). So far, studies of the serological diagnosis of smear-negative patients using haemagglutination with glycolipid antigens and antigen 5 ELISAs (D.A. Mitchison, unpublished observations) have shown a low sensitivity, although studies using smear-positive patients typically yield a higher sensitivity of 70–80% (Grange 1984; Reggiardo & Middlebrook 1975b; Benjamin & Daniel 1982; Hewitt *et al.* 1982). Furthermore, immunoblotting is unsuitable for routine serodiagnosis, although there are good prospects for the antibody–antibody competition assay (Hewitt *et al.* 1982), particularly using a combination of different MABs. The fact that the best binding patterns included binding to the 45 kDa antigen suggests that we should explore as many MABs as possible against that molecule in an antibody–antibody competition assay. We used the anti-45 kDa MAB HGT-6 in the antibody–antibody competition assay and found that 53% of smear-positive tuberculous patients' sera inhibited the binding of the MAB. This level of sensitivity for one MAB is comparable to that obtained with other MABs (Hewitt *et al.* 1982; Ivanyi *et al.* 1983). The specificity of the MAB HGT-6 was 83% and this is rather lower than some MABs. This data confirmed that the 45 kDa antigen is recognized by tuberculosis patients. Further work is continuing with the MABs F29-F29 and SA12.

The prospects for the use of the 45 kDa antigen in vaccine development are uncertain as yet. The major 65, 38, 19 and 14 kDa antigens which were first defined by MABs all stimulate both antibody responses and T cell mediated immunity in man (Andrew *et al.* 1984; Matthews *et al.* 1985; Emmrich *et al.* 1986; Y. Nitzan & A.R.M. Coates, unpublished observations). T cells are thought to be an important component of protective immunity against tuberculosis (Andrew *et al.* 1984; Lefford *et al.* 1973). The 45 kDa antigen stimulates an IgG antibody response. This was shown by the inclusion of specific antihuman IgG as the second layer reagent of an immunoblot (A. Guy, unpublished observation). Such a response is usually associated with a T cell-mediated response against the same antigen (Brenner & Munro 1981) and so the 45 kDa antigen may contain T epitopes.

Acknowledgements

We wish to thank the European Economic Community for financial support. We are also most grateful to Dr A.H.S. Kolk for the supply of MAB F29-F29, to Dr P. Minden for providing MAB SA12, and to Mrs P. Edwards for typing the manuscript.

References

ANDREW P.W., REES A.D., SCOGING A., DOBSON N., MATTHEW R., WHITTAL J.T., COATES A.R.M. & LOWRIE D.B. (1984) Secretion of macrophage-activating factor distinct from interferon-γ by human T cell clones. *Eur. J. Immunol.* **14**, 962–974.

BENJAMIN R.G. & DANIEL T.M. (1982) Serodiagnosis of tuberculosis using the Enzyme-linked Immunoadsorbent assay (ELISA) of antibody to antigen 5. *Am. Rev. Resp. Dis.* **126**, 1013–1016.

BRENNER M.K. & MUNRO A.J. (1981) Human anti-tetanus antibody response *in vitro*: autologous and allogeneic T cells provide help by different routes. *Clin. Exp. Immunol.* **46**, 171–177.

CHAKRABARTY A.K., MAIRE M.A. & LAMBER P.H. (1982) SDS-PAGE analysis of *M. leprae* protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos. *Clin. Exp. immunol.* **49**, 523–531.

CLOSS O., HARBOE M., AXELSEN N.H., BUNCH-CHRISTENSEN K. & MAGNUSSON M. (1980) The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunoelectrophoresis: a reference system. *Scand. J. Immunol.* **12**, 249–263.

COATES A.R.M. (1984) Monoclonal antibodies against *Mycobacterium tuberculosis*. MD thesis, University of London.

COATES A.R.M., HEWITT J., ALLEN B.W., IVANYI J. & MITCHISON D.A. (1981) Antigenic diversity of *Mycobacterium tuberculosis* and *Mycobacterium bovis* detected by means of monoclonal antibodies. *Lancet* **ii**: 167–169.

DANIEL T.M., ELLNER J.J., TODD L.S., MCCOY D.W., PAYNE V.D.N., ANDERSON P.A. & BHE F.T. (1979) Immunobiology and species distribution of Mycobacterium tuberculosis antigen 5. *Infect. Immun.* **24**, 77–82.

EMMRICH F., THOLE J., VAN EMBDEN J. & KAUFMANN S.H.E. (1986) A recombinant 64 kilodalton protein of *Mycobacterium bovis* Bacillus Calmette-Guerin specifically stimulates human T4 clones reactive to mycobacterial antigens. *J. Exp. Med.* **163**, 1024–1029.

ENGERS H.D., HOUBA V., BENNEDSEN J. & BUCHANAN T.M. (1986) Results of a WHO-sponsored Workshop to characterise antigens recognised by mycobacterium-specific monoclonal antibodies. *Infect. Immun.* **51**, 718–720.

GRANGE J.M. (1984) The humoral immune response in tuberculosis: its nature, biological role and diagnostic usefulness. *Adv. Tuberculosis Res.* **21**, 1–78.

HAMES B.D. (1981) Introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins–a Practical Approach*. Eds D. Rickwood & B.D. Hames. Oxford: Oxford IRL Press. pp. 1–91.

HEWITT J., COATES A.R.M., MITCHISON D.A. & IVANYI J. (1982) The use of murine monoclonal antibodies without purification of antigen in the serodiagnosis of tuberculosis. *J. Immunol. Methods* **55**, 205–11.

HONG KONG CHEST SERVICE, TUBERCULOSIS RESEARCH CENTRE, MADRAS & BRITISH MEDICAL RESEARCH COUNCIL (1984) A controlled trial of 2-month, 3-month, and 12-month regimens of chemotherapy for sputum-smear-negative pulmonary tuberculosis. *Am. Rev. respir. Dis.* **130**, 23–28.

IVANYI J., KRAMBOVITIS E. & KEEN M. (1983) Evaluation of a monoclonal antibody (TB72) based serological test for tuberculosis. *Clin. Exp. Immunol.* **54**, 593–599.

IVANYI J., MORRIS J.A. & KEEN M. (1985) Studies with monoclonal antibodies to mycobacteria. In *Monoclonal Antibodies Against Bacteria Volume 1*. Eds A.J.L. Macario & E.C. Macario. New York, London: Academic Press. pp. 59–90.

KADIVAL G.V. & CHAPARAS S.D. (1987) Production, characterization, and species specificity of five monoclonal antibodies to *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **25**, 76–80.

KAPLAN M.H. & CHASE M.W. (1980) Antibodies to mycobacteria in human tuberculosis. II. Response to nine defined mycobacterial antigens with evidence for an antibody common to tuberculosis and lepromatous leprosy. *J. Infect. Dis.* **142**, 835–843.

KLATSER P.R., VAN RENS M.M. & EGGELTE T.A. (1984) Immunochemical characterisation of *Mycobacterium leprae* antigens by the SDS-polyacrylamide gel electrophoresis immunoperoxidase technique (SGIP) using patient's sera. *Clin. Exp. Immunol.* **56**, 537–544.

KOLK A.H.J., HO M.L., KLATZER P.R., EGGELTE T.A., SJOUKJE K. DE JOUGE S. & VAN LEEUWEN J. (1984)

Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis, M. bovis (BCG)* and *M. Leprae. Clin. Exp. Immunol.* **58**, 511–521.

LAEMMLI U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LEFFORD M.J., MCGREGOR D.D. & MACKANESS G.B. (1973) Immune response to *Mycobacterium tuberculosis* in rats. *Infect. Immun.* **8**, 182–189.

MANIATIS T., FRITISCH E.F. & SAMBROOK J. (Eds) (1982) In *Molecular Cloning. A Laboratory Manual.* New York: Cold Spring Harbor Laboratories.

MATTHEWS R., SCOGING A. & REES A.D. (1985) Mycobacterial antigen-specific human T cell clones secreting macrophage activating factors. *Immunology* **54**, 17–23.

MINDEN P., KELLEHER P.J., FREED J.H., NIELSEN L.D., BRENNAN P.J., MCPHERSON L. & MCCLATCHY J.K. (1984) Immunological evaluation of a component isolated from *Mycobacterium bovis BCG* with a monoclonal antibody to *M. bovis BCG. Infect. Immun.* **46**, 516–525.

MITCHISON D.A. (1968) Examination of sputum by smear and culture in case-finding. *Bull. Int. Union Tuberc.* **41**, 139–149.

NASSAU E. NELSTROP A.E. (1976) Specific tubercle antigen. *Tubercle* **57**, 197–201.

OHNISHI S.T. & BARR J. (1978) A simplified method for quantitating protein using the biuret and phenol reagents. *Analyt. Biochem.* **86**, 193–200.

REGGIARDO Z., ABER V.R., MITCHISON D.A. & DEVI S. (1981) Hemagglutination test for tuberculosis with glycolipid antigens. *Am. Rev. Respir. Dis.* **124**, 21–25.

REGGIARDO Z. & MIDDLEBROOK G. (1975 a) Serologically active glycolipid families from *Mycobacterium bovis BCG.* II Serologic studies on human sera. *Am. J. Epidemiol.* **100**, 477–486.

REGGIARDO Z. & MIDDLEBROOK G. (1975 b) Serologically active glycolipid families from *Mycobacterium bovis BCG.* I. Extraction, purification and immunologic studies. *Am. J. Epidemiol.* **100**, 469–476.

SHOENFELD Y., VILNER Y., COATES A.R.M., RAUCH J., LAVIE G., SHAUL D. & PINKHAS J. (1986) Monoclonal anti-tuberculosis antibodies react with DNA, and monoclonal anti-DNA autoantibodies react with *Mycobacterium tuberculosis. Clin. Exp. Immunol.* **66**, 255–261.