Use of an antigen-capture assay for characterisation of monoclonal antibodies to mycobacterial lipoarabinomannan

SAROJ R. KHANOLKAR, D. B. YOUNG*, P. J. BRENNAN†, T. M. BUCHANAN§ and K. P. W. J. McADAM||

Department of Clinical Tropical Medicine, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, *MRC Tuberculosis and Related Infections Unit, Hammersmith Hospital, London, †Department of Microbiology, Colorado State University, Fort Collins, CO, USA, §Immunology Research Laboratory, University of Washington, Seattle, WA, USA, and ||The National Hansen's Disease Center, Carville, LA, USA

Summary. Monoclonal antibodies directed to six separate antigen molecules of *Mycobacterium leprae* have been tested in an antigen-capture assay based on combined use of polyclonal ("capture") and monoclonal ("detector") antibody reagents. This approach provides a potentially versatile, sensitive and specific assay for detection and relative quantitation of *M. leprae* antigens. Characterisation of monoclonal antibodies to mycobacterial lipoarabinomannan (LAM-B) by the antigen-capture assay indicates that some of the antigenic determinants present on LAM-B from *M. leprae* may be either absent altogether or present at much lower concentrations on the corresponding LAM-B structure from *M. tuberculosis*.

Introduction

Assays for the detection of mycobacterial antigens in tissues and body fluids from infected individuals have a potential role in the diagnosis of disease and in monitoring the effectiveness of chemotherapy. In the case of leprosy, it is important that such assays distinguish between specific antigens of *Mycobacterium leprae* and the many cross-reactive antigens shared by related mycobacteria, such as *M. tuberculosis*, which may often be present at high frequencies in leprosy endemic areas. The availability of mouse monoclonal antibodies (MAbs) directed to individual antigenic determinants of *M. leprae* (Gillis and Buchanan, 1982; Ivanyi *et al.*, 1983; Engers *et al.*, 1985) provides an opportunity for the development of novel antigen detection assays which would have the attractions of being potentially species-specific and of being suitable for monitoring the presence of antigens of defined molecular nature.

Two reports of the use of MAbs for detection of mycobacterial antigens in clinical samples have been published. Chandramuki *et al.* (1985) used a MAb (ML34) directed to a common carbohydrate determinant of mycobacteria to detect antigen in cerebrospinal fluid from patients with tuberculous

meningitis and Young *et al.* (1985*b*) used a MAb (PG2.B8F) in conjunction with immunoblotting to detect the species-specific phenolic glycolipid antigen in sera from patients with lepromatous leprosy. Antigen-capture (or "tandem") assays with MAbs have been used to detect antigens from other infectious agents (Voller, 1980) and development of such assays has been described with the mycobacterial ML34 MAb (Praputpittaya and Ivanyi, 1985), and also for the 28-Kda protein of *M. leprae* (Young *et al.*, 1985*a*) and the 38-Kda protein of *M. tuberculosis* (Young *et al.*, 1986). These assays involve binding of antigen to a solid-phase support through interaction with a "capture" antibody, followed by detection with a second antibody directed to a separate epitope on the same molecule, and can be employed either for an antigen with repeating epitopes (e.g., the ML34 assay) or for an antigen with two spatially separate epitopes, as in the case of the 28-Kda protein. The format of such assays is attractive for detection of antigens in biological samples because the "capture" step allows for an initial partial purification or concentration of the specific antigen. In this paper we report the development of an antigen-capture assay with polyclonal antibody directed to cross-reactive mycobacterial determinants for the "capture" step followed by detection with MAbs directed to *M. leprae* determinants. It can be predicted that this

strategy should be successful for antigens which contain a mixture of cross reactive and species-specific determinants, as has been shown for the 65-Kda and 28-Kda proteins of *M. leprae* (Gillis *et al.*, 1985; Young *et al.*, 1985a).

The antigen-capture assay has been used for analysis of MAb-defined determinants of the mycobacterial lipoarabinomannan-B antigen. Hunter *et al.* (1986) have recently shown that the previously described arabinomannan antigen of mycobacteria is a partially hydrolysed form of a more complex lipoarabinomannan structure (LAM-B) which contains inositol 1-phosphate, lactate, succinate and fatty acids in addition to arabinose and mannan. The results reported here suggest the presence of species-specific determinants associated with the native LAM-B molecule.

Materials and methods

Mycobacterial antigens

Soluble extract from *M. leprae* was obtained from the World Health Organization Leprosy programme through Dr R. J. W. Rees, Clinical Research Centre, London. *M. tuberculosis* H37Rv was grown for 8 weeks as a surface pellicle on Sauton's medium and a soluble extract was prepared as described previously (Young *et al.*, 1986). The protein concentration in mycobacterial extracts was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (BSA) as standard. LAM-B was purified from *M. leprae* and *M. tuberculosis* as described previously (Hunter *et al.*, 1986).

Monoclonal antibodies

The following MAbs were used in the study: IIIIE9 (*M. leprae* 65-Kda protein; Gillis and Buchanan, 1982), L12 (*M. leprae* 65-Kda protein; Britton *et al.*, 1985), SA1.B11H and SA1.D2D (*M. leprae* 28-Kda protein; Young *et al.*, 1985a), L5 (*M. leprae* 18-Kda protein; Britton *et al.*, 1985), and MLO4 and MLO6 (*M. leprae* 35-Kda and 12-Kda proteins; Ivanyi *et al.*, 1983). MAbs SA1.C7F and SA1.C11H were derived from a fusion with spleens from mice immunised with a soluble extract from *M. leprae* as described previously (Young *et al.*, 1985a). Both antibodies belonged to the IgG₁ subclass and recognised subtilisin-resistant determinants. All MAbs were obtained in the form of ascitic fluid and were used in assays at dilutions ranging from 1 in 1000 to 1 in 20 000.

Antigen-capture assay

Antibody from a rabbit hyperimmunised with *M. bovis* BCG (Dako Ltd, High Wycombe, Bucks) was diluted 1 in 5000 in carbonate-bicarbonate buffer, pH 9.6, and coated overnight at 0–4°C to Immulon II microtitration

plates (Dynatech Laboratories, Inc., Alexandria, VA). Plates were washed with phosphate-buffered saline, pH 7.2, containing Tween 20 0.05% v/v (PBS-T) and then incubated for 1 h at room temperature (RT) with BSA 1% w/v in PBS-T (BSA-PBS-T) to block non-specific binding. The BSA was discarded and antigens (mycobacterial extracts or LAM-B) were diluted in 1% BSA-PBS-T and added to the microtitration plate. After incubation for 1 h at RT, plates were washed three times with PBS-T and MAbs diluted in 1% BSA-PBS-T, were added for 1 h at RT. Plates were washed again and peroxidase-conjugated affinity-purified goat anti-mouse IgG antibody (Bio Rad, Richmond, CA) diluted 1 in 3000 in BSA-PBS-T was added. After 1 h at RT, plates were washed with PBS-T and colour was developed with tetramethylbenzidine (dihydrochloride) 0.1 mg/ml in citrate buffer, pH 5, containing hydrogen peroxide 0.1% v/v. Reactions were stopped by adding sulphuric acid and absorbance was read at 450 nm. Separate control wells without polyclonal antibody, antigen, or MAbs, respectively, were included in each experiment.

Direct binding assay with human sera

A direct binding assay was performed by diluting LAM-B at different concentrations in carbonate-bicarbonate buffer, pH 9.6 and coating overnight at 0–4°C to Immulon II microtitration plates. After washing with PBS-T and blocking as described for the antigen-capture assay, human serum samples (diluted 1 in 300 in BSA-PBS-T) were added to the plates and incubated for 1 h at RT. Plates were washed with PBS-T and peroxidase-conjugated affinity-purified goat anti-human IgG antibody (Sigma Chemical Co., St Louis, MO) was added for 1 h at RT. Plates were washed again and colour was developed as described above. Results were corrected for any background binding of human serum antibody to uncoated wells of the microtitration plate. Human serum samples consisted of pooled sera from at least six separate donors with lepromatous leprosy, tuberculosis, or without mycobacterial disease.

Results

Antigen-capture ELISA

An antigen-capture assay was developed with hyperimmune rabbit anti-*M. bovis* BCG as "capture" antibody followed by detection with a variety of MAbs directed to *M. leprae*. The peroxidase-conjugated affinity-purified anti-mouse antibody used in the assay showed negligible binding to rabbit immunoglobulin and provided a convenient and versatile detection system which avoided the necessity of labelling individual mouse MAbs. Table I shows the results of the antigen-capture ELISA with MAbs IIIIE9, L12, MLO4, SA1.B11H,

SA1.D2D, L5, MLO6, SA1.C11H, and SA1.C7F directed towards 65-Kda, 35-Kda, 28-Kda, 18-Kda, 12-Kda protein and LAM-B individual antigen molecules of *M. leprae*. The highest absorbance readings were obtained with antibodies directed to the 65-kda protein and to the LAM-B carbohydrate antigen. The assay was routinely performed in the presence of an excess of BSA (10 mg/ml) and was thus independent of the purity of the mycobacterial antigen extract used. Preliminary results (data not shown) indicated that normal human serum could be added to the assay with little adverse effect except for a general increase in the background binding of the peroxidase-conjugated antibody reagent.

Monoclonal antibodies to LAM-B

Two of the MAbs listed in table 1 (SA1.C11H and SA1.C7F) were originally characterised as binding to alkali-sensitive, subtilisin-resistant determinants and were subsequently found to recognise the LAM-B antigen purified from *M. leprae*. The high absorbance readings observed with these antibodies in the antigen-capture assay are consistent with the presence of repeating epitopes on the carbohydrate antigen, and addition of purified *M. leprae* LAM-B in the antigen-capture assay showed that sub-nanogram amounts of LAM-B could readily be detected by these MAbs (fig. 1A). SA1.C11H gave consistently higher absorbance readings than SA1.C7F indicating either that the former antibody possessed a higher affinity for the

Table I. Screening of MAbs in antigen-capture assay

MAb	Antigen recognised	Absorbance at 450 nm with <i>M. leprae</i> soluble extract ($\mu\text{g/ml}$)				
		10	2	0.4	0.08	0.01
III E9	65-Kda protein	1.17	0.94	0.61	0.26	0.07
L12	65-Kda protein	1.15	0.91	0.63	0.23	0.07
MLO4	35-Kda protein	0.24	0.20	0.12	0.09	0.08
SA1.B11H	28-Kda protein	0.22	0.19	0.06	0.08	0.05
SA1.D2D	28-Kda protein	0.30	0.28	0.14	0.10	0.06
L5	18-Kda protein	0.18	0.15	0.13	0	0
MLO6	12-Kda protein	0.30	0.23	0.20	0.12	0.12
SA1.C11H	LAM-B	1.03	0.74	0.32	0.10	0.03
SA1.C7F	LAM-B	0.65	0.32	0.16	0.02	0.02

Different MAbs were used in the antigen-capture assay with *M. leprae* soluble extract as described in the text.

antigen, or that its epitope was present at a higher frequency than that of SA1.C7F on the *M. leprae* LAM-B molecule. When LAM-B from *M. tuberculosis* was used in the assay (fig. 1B), a markedly higher concentration was required to produce absorbance values comparable to that of the *M. leprae* antigen with SA1.C11H (see table II) while no significant recognition of *M. tuberculosis* LAM-B by SA1.C7F was found even at high antigen concentrations. Analogous results were obtained when comparing extracts from *M. leprae* or *M. tuberculosis* in the antigen-capture assay with SA1.C11H showing preferential binding to the *M.*

Fig. 1. Antigen-capture assay with purified LAM-B. LAM-B from *M. leprae* or *M. tuberculosis* was added at various concentrations to the antigen-capture assay described in the text and antigen detection was with MAb SA1.C11H (○) or MAb SA1.C7F (●).

Table II. Quantitative comparison of LAM-B preparations from *M. leprae* and *M. tuberculosis*

Antibody	Antigen concentration (ng/ml) required for A_{450} 0.2	
	<i>M. leprae</i> LAM-B	<i>M. tuberculosis</i> LAM-B
MAb SA1.C11H	1.2	1200
MAb SA1.C7F	20	> 10 000
Pooled leprosy sera	0.3	3
Pooled tuberculosis sera	1.5	3.5

LAM-B purified from *M. leprae* or from *M. tuberculosis* was added at different concentrations to the antigen-capture and the direct binding assay as described in the text. The antigen concentration required to produce an absorbance reading of 0.2 was determined for each of the antibody preparations.

leprae extract and SA1.C7F showing no significant binding to *M. tuberculosis* (figs. 2A and 2B).

Recognition of LAM-B by human sera

Because of the apparent species-specificity of antigenic determinants on LAM-B, the purified preparations from *M. leprae* and *M. tuberculosis* were each analysed for recognition by antibodies in sera from patients with leprosy or tuberculosis. As shown in fig. 3, pooled sera from leprosy patients or tuberculosis patients bound strongly to LAM-B prepared from either mycobacterial species while a

serum pool from uninfected individuals showed only a low degree of binding. Some quantitative differences between the two samples were observed with a lower concentration of the *M. leprae* LAM-B producing absorbance values of 0.2 compared to the *M. tuberculosis* LAM-B, particularly in the case of the lepromatous serum pool (table II).

Discussion

Monoclonal antibodies directed to several antigenic determinants of *M. leprae* were found to function well in an antigen-capture ELISA employing a polyclonal anti-mycobacterial antibody for the "capture" step. This indicates that these antibodies recognise either repeating epitopes (as in the LAM-B molecule) or *M. leprae*-specific epitopes on antigens which also possess separate cross-reactive epitopes. Thus the format of combined use of polyclonal and monoclonal antibodies in such assays appears potentially broadly applicable for monitoring several individual *M. leprae* antigens. While the use of a peroxidase-conjugated secondary antibody for screening a wide variety of MAbs was a convenient aspect of the study described here, further development of such tests with material from human clinical samples has indicated that use of directly conjugated MAbs may be necessary to avoid background binding of the anti-mouse secondary antibody to components in the biological extracts. The antigen capture ELISA provides a highly sensitive assay for antigen

Fig. 2. Antigen-capture assay with mycobacterial extracts. Soluble extract from *M. leprae* or *M. tuberculosis* was added at various concentrations to the antigen-capture assay described in the text and antigen detection was with MAb SA1.C11H (○) or MAb SA1.C7F (●).

Fig. 3. Recognition of LAM-B by antibodies in human sera. Various concentrations of LAM-B from *M. leprae* or *M. tuberculosis* were coated to microtitration plates for the direct binding assay described in the text. Assays were then developed with pooled human serum samples (1 in 300 dilution) from lepromatous leprosy patients (●—●), tuberculosis patients (○—○) or uninfected individuals (▲).

detection, as was seen with the SA1.C11H assay in which 60 pg of *M. leprae* LAM-B was sufficient to produce an absorbance reading of 0.1. The fact that the antigen-capture assay was not influenced by the presence of other proteins in the test sample, suggests that it may be useful not only for detection but also for relative quantitation of particular determinants in different antigen preparations.

Analysis of purified LAM-B in the antigen-capture ELISA with two MAb's demonstrated a marked difference between the antigens purified from *M. leprae* and *M. tuberculosis*. MAb SA1.C11H showed a distinct preference for the *M. leprae* LAM-B whereas SA1.C7F bound well to *M. leprae* LAM-B but did not bind to *M. tuberculosis* LAM-B in the antigen-capture assay. Since it is known that the determinant recognised by each of these antibodies is susceptible to mild alkaline hydrolysis, it can be postulated that the difference in binding has resulted from some partial degradation of the *M. tuberculosis* LAM-B during its purification. Arguments opposed to this explanation are: the observed recognition of both antigens to an almost equal extent by pooled tuberculosis serum; the lack of quantitative differences in the chemical analysis of the two preparations; and the fact that an analogous discrimination between the two mycobacterial species is observed when unfractionated extracts are used in the antigen-capture

assay. It seems likely, therefore, that both of the MAb's recognise determinants which occur more frequently on the *M. leprae* LAM-B than on the *M. tuberculosis* LAM-B and, particularly in the case of SA1.C7F, may be useful in specifically detecting the *M. leprae* antigen. Whether this difference in antigenic configuration represents a genotypic difference between *M. leprae* and *M. tuberculosis*, or is a reflection of the different growth conditions (*in vivo* and *in vitro*) of the two strains, remains to be answered. The chemical composition of LAM-B could allow for a multiplicity of antigenic determinants involving complex succinyl and lactyl modification of sugar residues and further detailed structural analysis of LAM-B will be required to understand fully the origin of the MAb-defined differences in antigenicity. The possible occurrence of species-specific epitopes on complex carbohydrates of mycobacteria is an intriguing possibility with potential biological significance in addition to its importance from the point of view of antigen detection and species identification.

This research was supported by the THELEP component of the UNDT/World Bank/WHO special programme for Research and Training in Tropical Diseases. We thank Drs W. J. Britton and J. Ivanyi for kindly providing the monoclonal antibodies for this study, Dr R. J. W. Rees for helpful discussion, and Susan Dickinson for preparing the manuscript.

REFERENCES

- Britton W J, Hellqvist L, Basten A, Raison R L 1985 *Mycobacterium leprae* antigens involved in human immune responses. I. Identification of four antigens by monoclonal antibodies. *Journal of Immunology* **135**: 4171–4177.
- Chandramuki A, Allen P R J, Keen M, Ivanyi J 1985 Detection of mycobacterial antigen and antibodies in the cerebrospinal fluid of patients with tuberculous meningitis. *Journal of Medical Microbiology* **20**: 239–247.
- Engers H D et al. 1985 Results of a World Health Organization-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. *Infection and Immunity* **48**: 603–605.
- Gillis T P, Buchanan T M 1982 Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. *Infection and Immunity* **37**: 172–178.
- Gillis T P, Miller R A, Young D B, Khanolkar S R, Buchanan T M 1985 Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. *Infection and Immunity* **49**: 371–377.
- Hunter S W, Gaylord H, Brennan P J 1986 Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *Journal of Biological Chemistry* **261**: 12345–12351.
- Ivanyi J, Sinha S, Aston R, Cussell D, Keen M, Sengupta U 1983 Definition of species specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. *Clinical and Experimental Immunology* **52**: 528–536.
- Lowry O H, Rosebrough N J, Farr A L, Randall R J 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.
- Praputpittaya K, Ivanyi J 1985 Detection of an antigen (MY4) common to *M. tuberculosis* and *M. leprae* by “tandem” immunoassay. *Journal of Immunological Methods* **79**: 149–157.
- Voller A 1980 The use of solid phase isotopic and non-isotopic immunoassays in parasitic diseases with special reference to malaria. *International Journal of Nuclear Medicine and Biology* **7**: 157.
- Young D B, Harnisch J P, Knight J, Buchanan T M 1985a Detection of phenolic glycolipid I in sera from patients with lepromatous leprosy. *Journal of Infectious Diseases* **152**: 1078–1081.
- Young D B, Fohn M J, Khanolkar S R, Buchanan T M 1985b Monoclonal antibodies to a 28,000 mol. wt protein antigen of *Mycobacterium leprae*. *Clinical and Experimental Immunology* **60**: 546–552.
- Young D, Kent L, Rees A, Lamb J, Ivanyi J 1986 Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. *Infection and Immunity* **54**: 177–183.