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Monoclonal antibodies to a 28,000 mol. wt protein antigen of *Mycobacterium leprae*

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SUMMARY

Monoclonal antibodies (MoAb) have been used to analyse a protein antigen from *Mycobacterium leprae* with a subunit mol. wt of 28,000 daltons. Three different patterns of species specificity were observed with two antibodies being specific for *M. leprae*, two partially specific, and one broadly cross-reactive amongst all mycobacteria. Competitive binding and sandwich assays demonstrated that the specific and partially specific antibodies recognized closely related regions of the molecule while the cross-reactive antibody recognized a spatially separate epitope on the same polypeptide chain. Identification of specific and cross-reactive epitopes on a single antigenic molecule may be of considerable importance for understanding the functioning of the cell-mediated immune system during leprosy infection and the use of MoAb for such analyses is discussed.

Keywords monoclonal antibodies *Mycobacterium leprae* protein antigens

INTRODUCTION

Monoclonal antibodies (MoAb) are useful reagents for the identification and study of the antigens of human pathogens such as *Mycobacterium leprae*. Gillis & Buchanan (1982) and Ivanyi *et al.* (1983) have used murine MoAb to demonstrate species specific and cross-reactive antigens from these bacilli while Young *et al.* (1984) have demonstrated the surface location of the major phenolic glycolipid of *M. leprae* using MoAb. Sinha *et al.* (1983) have developed a serological test for detection of specific antibodies to *M. leprae* based on competitive inhibition of a murine MoAb by human sera. In addition to the identification of antigens involved in the humoral immune response to *M. leprae*, MoAb may be of use in identifying the antigens involved in stimulation or suppression of the cell-mediated immune response to these bacilli and may aid in the design of future vaccines for the control of leprosy (Bloom & Godal, 1983).

Previous studies of *M. leprae* proteins using MoAb have identified antigens with subunit mol. wt of 12,000 daltons (Ivanyi *et al.*, 1983) and 68,000 daltons (Gillis & Buchanan, 1982). Additional MoAb recognized a protein antigen which was destroyed during SDS gel electrophoresis (Ivanyi *et al.*, 1983) and an epitope present on several protein bands with mol. wt ranging from 35,000 to 75,000 daltons (Ivanyi *et al.*, 1983). In this paper we describe the use of MoAb to characterize a previously undescribed *M. leprae* protein antigen with subunit mol. wt of 28,000 daltons. The

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presence of species specific and cross-reactive epitopes on the same polypeptide chain is demonstrated.

MATERIALS AND METHODS

Antigen preparation. Mycobacterium leprae purified from infected armadillo tissue was supplied by Dr R.J.W. Rees, National Institute for Medical Research, London, UK, through the WHO/IMMLEP Program. Lyophilized bacilli (30 mg) were resuspended in 5 ml of phosphate-buffered saline pH 7·2 (PBS) and disrupted using a Model W-225 R Sonicator (Heat Systems-Ultrasonics Inc, Plainview, New York, USA) operating at full power for 8 min with an enclosed probe and ice water cooling. Sonicated bacilli were centrifuged for 30 min at 27,000*g* and the supernatant passed through a 0·22 μm filter (Millipore). The resulting filtrate had a protein concentration of 1 mg/ml and is referred to as the 'sonicate antigen'. Other mycobacteria were harvested from the surface of Middlebrook's 7H11 agar (DIFCO), washed with PBS, sonicated and filtered as described for *M. leprae*. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Production of MoAb. BALB/c mice were inoculated with *M. leprae* sonicate antigen by an i.p. injection of 50 μg of protein emulsified with Freund's incomplete adjuvant, and three subsequent weekly i.p. injections of antigen alone. A final i.v. injection was delivered 3 days prior to sacrifice for fusion experiments.

Spleen cells were fused with NSI/1 myeloma cells in the presence of polyethylene glycol and cloned cell lines were established as described previously (Gillis & Buchanan, 1982; Young *et al.*, 1984). The five MoAb described in this paper were generated from a single fusion using spleen cells derived from two immunized mice. From an initial screening of 384 fusion wells, 20 stable clones were established—five of which were found to produce antibodies to the 28,000 mol. wt protein.

Screening of antibodies by enzyme linked immunosorption assay (ELISA). For initial screening of fusion wells, *M. leprae* sonicate antigen was prepared in 10 mM TrisHCl pH 8 at a concentration of 5 μg/ml and allowed to adsorb overnight at 37°C to Linbro polystyrene microtitre plates (Flow Laboratories Inc., McLean, Virginia, USA). Plates were washed with PBS and incubated for 1 h at 37°C with bovine serum albumin (BSA) in PBS (5% wt/vol.) to block non-specific binding. Culture supernatants were diluted two-fold in PBS and added to wells after removal of BSA. Plates were incubated for 1 h at 37°C, washed with PBS, and then incubated with peroxidase conjugated affinity purified goat anti-mouse immunoglobulin (IgG + IgM) (Pel Freez Biologicals, Rogers, Arkansas, USA) for 1 h. After further washing with PBS colour development was accomplished using *o*-phenylenediamine (0·1 mg/ml) in 0·1 M citrate buffer (pH 5) with 0·003% H_2O_2. Absorbance was measured at 492 nm after stopping reactions with H_2SO_4.

For subsequent analysis of antibodies to the 28,000 mol. wt protein, sonicate antigen was coated to plates in 0·1 M citrate buffer (pH 5) rather than Tris-HCl (pH 8) since these conditions were found to result in enhanced binding of this particular antigen. Tween 20 (0·05% vol./vol.) was added to the PBS buffer used for assay of antibodies to the 28,000 mol. wt protein.

Purification of antibodies. Positive cell lines were injected into the peritoneal cavity of pristane primed mice to produce antibody rich ascites fluid (Gillis & Buchanan, 1982). Antibodies were partially purified from ascites fluid by precipitation for one hour at 0–4°C in the presence of 35% saturated ammonium sulphate. Pellets were washed with 40% saturated ammonium sulphate, then resuspended and dialysed against PBS. Antibody preparations were analysed for purity by electrophoresis using the Beckman Microzonal System (Beckman Instruments, Berkeley, California, USA).

Radiolabelling of antibodies. Purified MoAb and affinity purified goat anti-mouse immunoglobulin (IgA + IgG + IgM) (Cappel Laboratories, Cochranville, Pennsylvania, USA) were labelled with ^{125}I using chloramine-T (Greenwood, Hunter & Glover, 1963). The specific activity of radiolabelled antibodies ranged from $1–5 \times 10^6$ ct/min/μg protein. For radioimmunoassay, sonicate antigen was coated to Immulon II Removawell Strips (Dynatech Laboratories Inc., Alexandria, Virginia) in citrate buffer. Wells were washed with 0·05% (vol./vol.) Tween 20 in PBS (PBST), blocked with BSA, and incubated with labelled MoAb ($1·5 \times 10^5$ ct/min per well) diluted in PBST

with 1% (wt/vol.) BSA for 1 h at 37°C. Wells were then washed with PBST and counted using a Packard Gamma Counter. Alternatively, wells were incubated first with unlabelled MoAb and bound antibody was detected using radiolabelled secondary antibody.

Species specificity of MoAb. **Adsorbed antigen** Sonicate antigens from different mycobacteria were coated to Immulon II Removawell Strips at a protein concentration of 5 μg/ml in citrate buffer pH 5. After washing, wells were blocked with BSA and then ascites fluid (diluted 1:2,500 in PBST with 1% [wt/vol.] BSA) was added for a 1 h incubation. After washing with PBST, bound MoAb was detected using radiolabelled affinity purified goat anti-mouse immunoglobulin ($1{\cdot}5 \times 10^5$ ct/min per well).

Dot blot A dot blot procedure was developed based on the method described by Hawkes, Niday & Gordon (1982). Sonicate antigens from different mycobacteria were spotted onto a strip of nitrocellulose membrane (type GS, 0·22 μm, Millipore Corp., Bedford, Massachusetts, USA) at a concentration of 1 μg protein in 5-μl citrate buffer. Strips were left overnight at room temperature then washed with PBST. After 30 min incubation with 5% (wt/vol.) BSA in PBST, strips were incubated for 90 min at room temperature with ascites fluid diluted 1:2,500 in PBST containing 1% (wt/vol.) BSA. Strips were washed for 45 min in four changes of PBST and then incubated with radiolabelled affinity purified goat anti-mouse immunoglobulin (10^6 ct/min per strip) for 1 h at room temperature. After thorough washing with PBST, strips were exposed with X-ray film overnight to form autoradiographs. Similar results were obtained using a peroxidase conjugated secondary antibody followed by detection with 3,3′-diaminobenzidine as substrate.

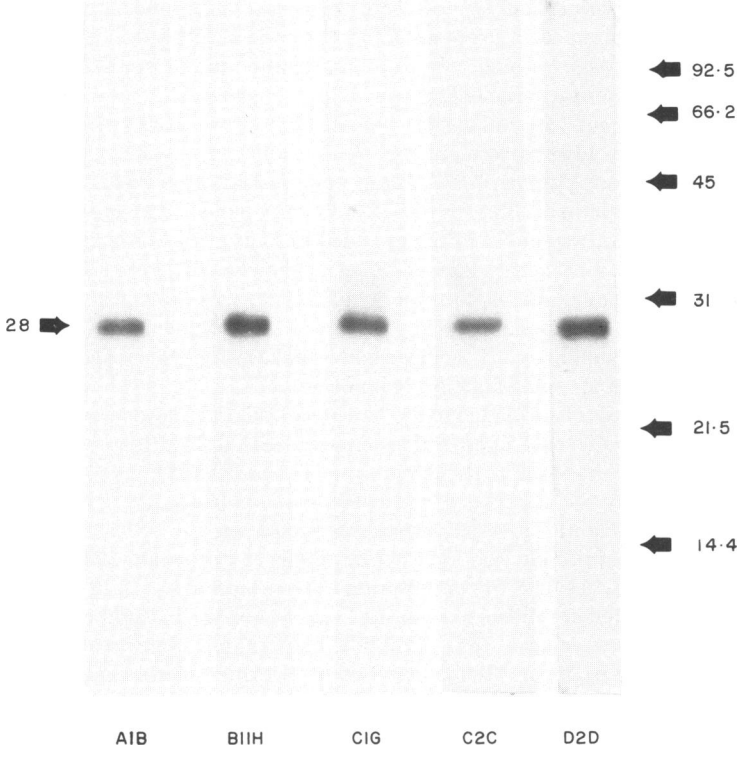

Fig. 1. Western blot analysis showing reactivity of MoAb with *M. leprae* sonicate antigen. *M. leprae* sonicate antigen (10 μg protein per lane) was electrophoresed on a 12·5% (wt/vol.) polyacrylamide gel in the presence of SDS. Proteins were transferred onto nitrocellulose and reacted with MoAb as described in the text. The figure shows an autoradiograph prepared from the nitrocellulose strips after detection with ^{125}I-labelled secondary antibody. The arrows on the right hand side of the figure indicate the positions of standard proteins with mol. wt (in kD) as shown.

Competition assay. The ability of each MoAb to compete for binding with a different radiolabelled MoAb was tested by first incubating antigen coated wells with ascites fluid (diluted 1:500 in PBST with 1% [wt/vol.] BSA) for 1 h at 37°C. Radiolabelled MoAb ($1{\cdot}5 \times 10^5$ ct/min per well in a 10 μl volume) was then added and the incubation continued for a further 1 h. Wells were then washed and counted.

Sandwich assay. Ammonium sulphate precipitated antibodies were suspended in 0·1 M bicarbonate buffer pH 9 (10 μg protein/ml) and allowed to coat to Immulon II Removawell Strips during a 2 h incubation at 37°C. Wells were washed with PBST and then incubated with 5% (wt/vol.) BSA in PBST for 30 min at 37°C. The BSA was replaced by *M. leprae* sonicate antigen (5 μg protein/ml in PBST with 1% [wt/vol.] BSA) for a further 1 h incubation. After washing with PBST, radiolabelled MoAb was added and allowed to react at 37°C for 1 h. Wells were then washed with PBST and counted. Controls included wells with no primary (binding) antibody, and wells with no added antigen.

Western blot assay. Sonicate antigen (10 μg protein/lane) was subjected to SDS polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970) and the separated proteins were transferred to a nitrocellulose sheet by overnight electrophoresis at 7 V as described by Towbin, Staehelin & Gordon (1979). Western blots were washed with PBS containing 0·2% (vol./vol.) Triton X-100 and then processed as described above for the 'dot blot' method except that Triton X-100 replaced Tween 20 in the buffers.

RESULTS

Western blot analysis demonstrated five MoAb reactive with an *M. leprae* antigen of mol. wt 28,000 (Fig. 1). All of the antibodies belonged to the IgG1 class and none bound to the surface of intact *M. leprae* as judged by immunofluorescence or whole organism ELISA (Young *et al.*, 1984).

Table 1. Reactivity of MoAb with sonicate antigens from different mycobacteria

Mycobacterium	Strain No.	Reactivity of MoAb*				
		SA1.A1B	SA1.B11H	SA1.C1G	SA1.C2C	SA1.D2D
M. bovis	ATCC 19015	0·03	0·04	0	0·02	1·37
M. chelonei	ATCC 14472	0	0·01	0	0·01	0·15
M. flavescens	ATCC 14474	0	0·01	0·01	0·01	0·63
M. gastri	ATCC 15754	0	0	0	0	1·03
M. intracellulare	TMC 1403	0·02	0·02	0·01	0·01	0·73
M. kansasii	TMC 1203	0·03	0·01	0·01	0·01	0·19
M. marinum	ATCC 927	0	0	0	0·01	0·15
M. nonchromogenicum	ATCC 19530	0·01	0·02	0·02	0·01	0·27
M. peregrinum	ATCC 14467	0·02	0	0·01	0·01	0·28
M. phlei	ATCC 11758	0·02	0	0·03	0·04	0·20
M. scrofulaceum	ATCC 19981	0·01	0	0	0·01	0·33
M. smegmatis	ATCC 19420	0·02	0·01	0·01	0·01	0·44
M. terrae	ATCC 15755	0·01	0	0	0·01	0·28
M. triviale	ATCC 23290	0·01	0·01	0·10	0·11	0·25
M. tuberculosis H37Ra	ATCC 25177	0·04	0·01	0·03	0·03	0·28
M. ulcerans	ATCC 19423	0·02	0·01	0·03	0·02	0·54
M. leprae		0·88	1·00	0·90	0·86	0·95

* Expressed as ct/min. $\times 10^{-4}$.

Assays were carried out as described in the text with ascites fluid diluted 1:2,500 and detection by ^{125}I-labelled secondary antibody.

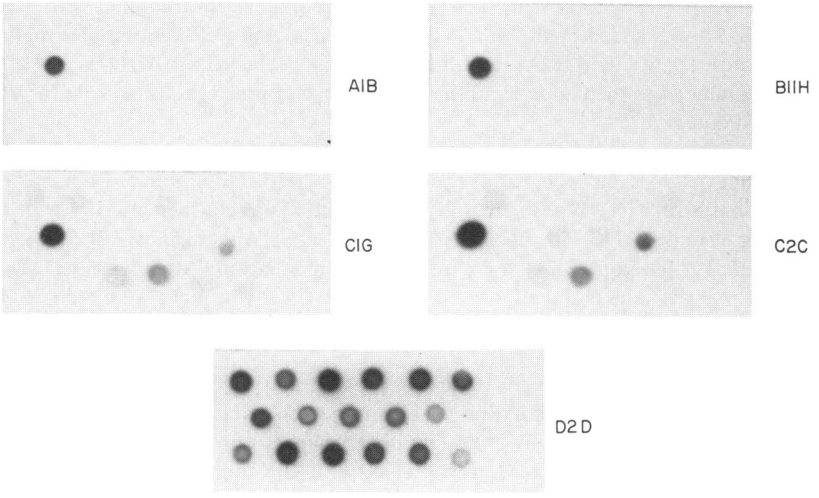

Fig. 2. Dot blot analysis to show species specificity of MoAb. Sonicate antigens (1 μg protein each) from different mycobacteria were spotted onto nitrocellulose strips and reacted with MoAb as described in the text. The figure shows an autoradiograph prepared from the strips after detection with ^{125}I-labelled secondary antibody. Mycobacteria (top row, left to right): *M. bovis*, *M. chelonei*, *M. flavescens*, *M. gastri*, *M. intracellulare*, *M. kansasii*; (middle row): *M. leprae*, *M. marinum*, *M. nonchromogenicum*, *M. peregrinum*, *M. phlei*; (bottom row): *M. scrofulaceum*, *M. smegmatis*, *M. terrae*, *M. triviale*, *M. tuberculosis*, *M. ulcerans*.

Pre-incubation of antigen coated wells with subtilisin (50 μg/ml, 2 h at 37°C) reduced antibody binding by at least 90%, indicating the protein nature of the antigen.

Species specificity of MoAb

Binding of MoAb to sonicate antigens from mycobacteria other than *M. leprae* was tested using antigens adsorbed to plastic (Table 1) or bound to nitrocellulose (Fig. 2). Both assays revealed a considerable extent of species cross-reactivity with MoAb SA1.D2D, while SA1.A1B and SA1.B11H appeared specific for *M. leprae* having a very faint interaction only with *M. tuberculosis* in the dot blot assay (Fig. 2). Antibodies SA1.C1G and SA1.C2C showed the same pattern of partial

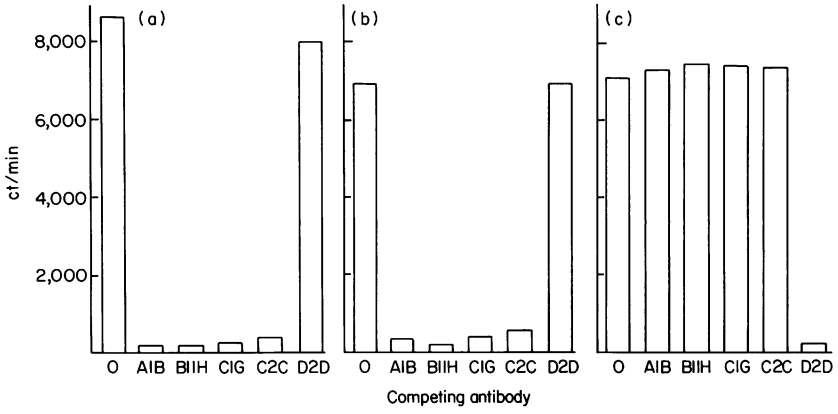

Fig. 3. Competition assay. Antigen coated wells were incubated first with competing antibody (ascites fluid diluted 1:500) and then ^{125}I-labelled test antibody was added. Bound radioactivity was determined after washing. ^{125}I-labelled antibody: (a) SA1.A1B, (b) SA1.C2C, (c) SA1.D2D.

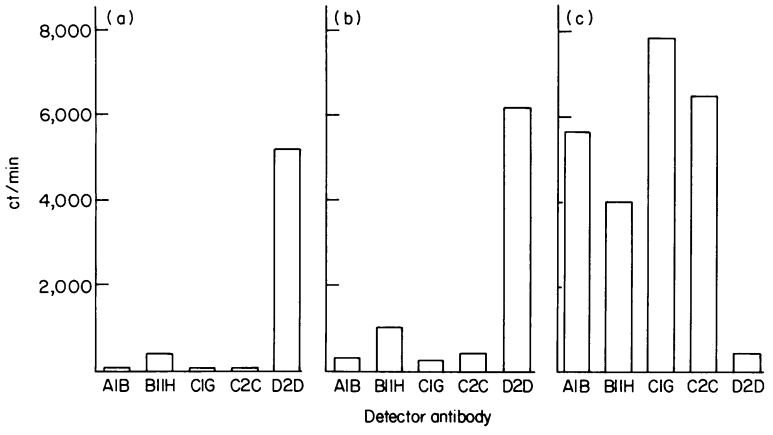

Fig. 4. Sandwich assay. Ammonium sulphate purified antibody was adsorbed to microtitre wells and used to immobilize antigen from *M. leprae*. The ability of ^{125}I-labelled antibodies to react with the immobilized antigen was then tested. Binding antibody: (a) SA1.A1B, (b) SA1.C2C, (c) SA1.D2D.

cross-reactivity having a slight interaction with *M. triviale* in the adsorbed antigen assay (Table 1) and a more pronounced binding to *M. triviale* and also to *M. phlei* in the dot blot assay (Fig. 2).

Competition assay

In order to determine the relationship between epitopes recognized by different MoAb, competition experiments were carried out by assaying the ability of each unlabelled antibody to compete for binding with a radiolabelled test antibody. The results with three labelled antibodies are shown in Fig. 3. The cross-reactive antibody (SA1.D2D) did not compete for binding with any of the other antibodies, while each of the specific and partially specific antibodies competed for binding with the remaining three antibodies. No competition effect between SA1.D2D and the other antibodies was observed even when very high concentrations of unlabelled antibody (ascites fluid diluted 1:25) were used.

Sandwich assay

In order to obtain further data concerning the spatial relationship between epitopes recognized by different MoAb, a sandwich assay was developed in which one antibody was bound to the solid phase and used to capture the antigen, with a second (radiolabelled) MoAb being used for detection. When SA1.D2D was used as the initial binding antibody, each of the remaining four antibodies was able to recognize the immobilized antigen. With SA1.A1B or SA1.C2C as binder, however, only SA1.D2D fully functioned as detector, and the next highest level of detection was at only 16% of the SA1.D2D signal (Fig. 4). These results were consistent with those of the competition assay in demonstrating reaction of SA1.D2D with a region of the molecule which is distinct from that recognized by the other antibodies. Radiolabelled MoAb recognizing unrelated *M. leprae* antigens showed no reaction when used as detector antibodies in this assay.

DISCUSSION

The five MoAb described here reacted with a protein antigen of *Mycobacterium leprae* having a subunit mol. wt of 28,000 daltons. This protein is distinct from those recognized by previously described MoAb to *M. leprae* (Gillis & Buchanan, 1982; Ivanyi *et al.*, 1983).

The dot blot assay with antigen bound to a nitrocellulose support was found to be a useful and sensitive method for screening the species specificity of MoAb to mycobacterial proteins. Antibodies SA1.C1G and SA1.C2C which showed only marginal cross-reactivity with *M. triviale* in

a radioimmunoassay using microtiter wells (Table 1) showed a more pronounced pattern of cross-reactivity in the dot blot assay (Fig. 2).

When using a mixed antigen preparation such as a crude bacterial sonicate, it is possible that a reduction in the relative amount of a particular protein could result in its being adsorbed to microtitre wells in amounts insufficient for detection. MoAb could, therefore, show an apparent species specificity due to strain-dependent differences in the relative concentrations of particular proteins. This is clearly not the case here since the presence of a cross-reactive epitope on the same antigen molecule allowed its detection in all of the strains tested.

Competition assays demonstrated that two *M. leprae* specific antibodies (SA1.A1B and SA1.B11H) and two partially specific antibodies (SA1.C1G and SA1.C2C) reacted with closely associated regions of the 28,000 dalton molecule while a broadly cross-reactive antibody (SA1.D2D) reacted with a spatially separate site. Competition between MoAb with different species specificity patterns could be the result of a close proximity, or overlap, between two different epitopes. Alternatively, the antibodies could bind to the same epitope but have a difference in specificity such that, for example, alteration of a single amino acid may be sufficient to destroy recognition by the specific antibody, but may be tolerated by the cross-reactive antibody.

The results of the competition assays could indicate that the two groups of antibodies recognized two separate proteins having similar mol. wt. In order to exclude this possibility sandwich assays were carried out in which the antigen molecule is immobilized by binding to one MoAb and the ability of a second MoAb to recognize the bound molecule is then tested. This assay confirmed the results of the competition assays and demonstrated that the cross-reactive and specific epitopes were indeed associated with the same molecule. We are currently investigating the possible use of this type of sandwich assay for detection and quantitation of *M. leprae* antigens in infected tissue samples.

The 28,000 mol. wt protein may prove representative of many *M. leprae* protein antigens in having a mixture of specific and cross-reactive epitopes. It is possible that different sets of T cells respond to the different portions of the molecule and the type of approach described here using MoAb to identify different epitopes on the same molecule may therefore be crucial in understanding the overall interaction of the cell-mediated immune system with the protein antigens of *M. leprae*.

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