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The characterization and immunoreactivity of a 70 KD protein common to *Mycobacterium leprae* and *Mycobacterium bovis* (BCG)

W J BRITTON, R J GARSIA, L HELLQVIST, J D WATSON & A BASTEN
Clinical Immunology Research Centre, University of Sydney, Sydney, Australia; and Department of Immunobiology, University of Auckland, Auckland, New Zealand

Summary
A 70 kD protein antigen has been identified in sonicates of *Mycobacterium leprae* and *M. bovis* (Bacillus Calmette-Guerin) (BCG) with murine monoclonal antibodies (Mabs). The antigen was readily radiolabelled and was immunoprecipitated by the Mab, L7, or leprosy sera. On two-dimensional electrophoresis, it had a pI of 5.1. The protein, after purification from BCG, stimulated proliferation and lymphokine secretion in lymphocytes from Mantoux positive subjects and elicited skin test reactivity *in vivo*. The sequence of N-terminal residues of the BCG protein was determined. Two DNA clones encoding the Mab-defined epitopes have been isolated from the genome of *M. leprae*.

Introduction
M. leprae and *M. bovis* (BCG) share a number of common antigens as defined by serological cross-reactivity in crossed immunoelectrophoresis (CIE) (1,2). Such antigens include those with cellular reactivity since subjects vaccinated with BCG but not exposed to leprosy bacilli have comparable *in vitro* lymphocyte proliferative responses to both organisms (3). These shared antigens may be responsible for the variable and poorly understood protection conferred by BCG vaccination against the development of clinical leprosy (4,5). Mabs are powerful tools for defining whether a single antigenic determinant is species-specific or shared by related or distant organisms. In the case of the leprosy bacillus, interest has focussed on Mabs recognizing *M. leprae*-specific determinants (6-10). Protective immunity, however, may depend on the cellular response to both species-specific and shared antigenic determinants. Whilst screening anti-*M. leprae* Mabs for reactivity with a panel of mycobacteria, we noted that one antibody, L7, had greater reactivity with BCG than *M. leprae* (10). This Mab-defined determinant was present on a 70 kD protein and was limited to BCG, *M. leprae, M. tuberculosis* and *M. scrofulaceum*. This report will describe the characterization of this antigen, its purification from BCG sonicate and its cellular and humoral reactivity in man.

Materials and methods
ANTIGENS AND ANTIBODIES
L7, an lgG1 Mab, was derived from a cell fusion of P3-NS1-Ag4-1 mouse myeloma cells with spleen cells from BALB/c/J mice immunized with a soluble sonicate of *M. leprae* ba-

cilli (IMMLEP preparation CD45) (10). Mycobacterial reactivity of L7 hybridoma supernatant was tested in a direct solid-phase ELISA with sonicates of 13 other mycobacterial species including BCG, which had been obtained from the Commonwealth Serum Laboratory (Melbourne, Australia). The antibody was purified from ascites fluid by ammonium sulphate precipitation and Protein A-Sepharose chromatography. Subsequently, a second anti-*M. leprae* Mab, L27 (lgG2b), was derived from a separate cell fusion, and shown by competitive inhibition assay to react with the same epitope on the 70 kD antigen as L7.

The leprosy sera used were from untreated Nepali patients with lepromatous (LL or BL) and tuberculoid (BT or TT) leprosy, untreated smear-positive patients with pulmonary tuberculosis and healthy Mantoux negative residents of Sydney who had not been exposed to leprosy. These sera were tested individually or as a leprosy sera pool (LSP) from 10 BL/LL patients or a tuberculosois sera pool (TBSP) from 10 tuberculosis patients. Rabbit anti-BCG antiserum was obtained from Dako (Copenhagen, Denmark).

CHARACTERIZATION OF THE L7-DEFINED ANTIGEN

The L7-defined antigen was first identified in immunoblots of mycobacterial sonicates which had been separated by 6-20 % gradient SDS-PAGE and probed with the Mab (10). The second antibody used was 125I-labelled sheep anti-mouse immunoglobulin and the immunoblots were developed as autoradiographs. The immunochemical nature of the antigen was identified by treating the *M. leprae* or BCG sonicate with either 10 mg/ml trypsin (Sigma, St. Louis, MO) or 1, 5 and 10 mM sodium periodate (Sigma) prior to SDS-PAGE and immunoblotting (11).

The antigen was also identified in a solid-phase immunoprecipitation assay (12). In this 125I-labelled BCG or *M. leprae* sonicate was incubated overnight with L7 coated to the wells of a polyvinyl chloride microtitre tray. After washing, the bound antigen was eluted with 0.05 M diethylamine, pH 11.5, freezedried and dissolved in SDS-PAGE sample buffer (10) for one dimensional electrophoresis. Additional samples were dissolved in 2 % Nonidet P40 (NP 40), 4.5 M urea for isoelectric focusing (IEF) in 7.5 % polyacrylamide containing 7.6 % Pharmalyte, pH range 4.0-6.5 (Pharmacia, Uppsala, Sweden), 2 % NP40 and 4.5 M urea. This was followed by electrophoresis in the second dimension by 6-20 % SDS-PAGE (13). The gels were then stained with Coomassie Blue G250 and dried onto filter paper before development by autoradiography. The molecular weight markers ranged from 94,0000 to 14.400, and the pH markers for IEF ranged from 2.5 to 6.5 (Pharmacia).

The relative concentration of the L7-defined determinant in different mycobacterial sonicates was assessed with an antigen capture assay (11). For this, varying protein concentrations of the sonicates were incubated in triplicate with L7 coated to the wells of a polyvinyl chloride microtitre tray. After washing, the wells were incubated with 125I-labelled L7, 100, 000 cpm per well, washed and counted in a gamma counter. The localisation of the L7-defined antigen witin the bacillus was examined by testing both *M. leprae* sonicate and a cell wall preparation, *M. leprae* resonicate (10), in the capture assay. The results were compared with those using Mab L5, which identified an *M. leprae*-specific epitope on an 18 kD protein.

PURIFICATION OF 70 KD PROTEIN BY MAB AFFINITY CHROMATOGRAPHY

The 70 kD protein was purified from BCG sonicate by passing the crude sonicate through a column of cyanogen bromide-activated Sepharose 4B (Pharmacia) coupled to L7 (14). Non--specific binding was reduced by first passing the sonicate through a column of normal mouse Ig-Sepharose 4B. After extensive washing with 0.15 M phosphate buffered saline (PBS), pH 7.2, and then 0.65 M sodium chloride, the bound fraction was eluted with 0.05 M diethy-

lamine, pH 11.5, dialysed against 0.015 M PBS and lyophilised. The lyophilised material was redissolved in one-tenth of its original volume of sterile water, filtered through a 0.22 micron filter and the protein concentration measured with Folin's reagent. The antigen eluted at pH 11.5, PBS fraction containing unbound material and original sonicate were analysed by SDS-PAGE followed by protein staining with Coomassie Blue R250 or by immunoblotting with L7. The relative concentration of the L7 determinant in the pH 11.5 eluate and the original sonicate was measured with the antigen capture assay and an antigen inhibition ELISA in which the two samples inhibited the binding of L7 to *M. leprae* sonicate in an ELISA assay (14).

The eluted antigen was also radiolabelled with 125I and analysed by one and two dimensjonal SDS-PAGE with and without immunoprecipitation by LSP *(vide infra).*

The N-terminal residues of the protein were analysed on a Model 890A gas-phase sequencer (Applied Biosystems, Foster City, CA) (14). The derived phenylhydantoins were identified by HPLC.

IMMUNOREACTIVITY OF 70 KD IN MAN

The reactivity of the 70 kD antigen with human B lymphocytes was examined with an immuno precipitation assay (Britton *et al,* submitted). 125I-labelled *M. leprae* or BCG sonicate was reacted with individual or pooled sera and the antigen-antibody complexes immunoprecipitated with heat-killed, formalin-fixed *Staphylococcus aureus.* The precipitated antigens were then released by boiling with SDS-PAGE sample buffer or by incubation with 2 % SDS, 2 % NP40 and 9 M urea for one and two dimensional gel electrophoresis.

The Mab affinity purified 70 kD antigen was tested in a direct ELISA with human sera (14). LSP, TBSP and control sera diluted 1:300 were incubated in wells coated with the antigen at concentrations from 100-0.01 ug/ml. The second antibody was goat anti-human IgG conjugated with alkaline phosphatase (Sigma) and color was developed with nitrophenyl phosphate.

The human T lymphocyte reactivity of the purified 70 kD antigen was assessed in healthy volunteers after ethical approval for its use was obtained from the University of Sydney Human Studies Ethics Committee. The sybjects were skin tested with PPD of *M. tuberculosis* (100 U/ml) and consisted of three groups; those naturally Mantoux positive (7), those Mantoux positive after BCG vaccination (12) and Mantoux negative subjects with no history of BCG vaccination or exposure to tuberculosis (8). The *in vitro* reactivity was tested in a lymphocyte proliferation assay (LPA) in which $2x10^5$ peripheral blood monuclear cells (PBM) were incubated with the 70 kD antigen or the original BCG sonicate in triplicate at final concentrations of 10, 3, 1.0, 0.3 and 0.1 ug/ml for six days at 37° C in 5 % CO_2. Sixteen hours prior to harvesting, 100 ul of supernatatant was removed from triplicate wells and replaced with fresh medium and the wells were pulsed with 1.0 uCi [3H] thymidine incorporation was subsequently measured by liquid scintillation spectroscopy and the nett uptake for each antigen concentration calculated as the mean in test wells minus the mean in control wells cultured without antigen. The response to p70 and BCG sonicate at 1 ug/ml was compared with that to PPD (Statens Seruminstitut, Copenhagen, Denmark) at 1 ug/ml and *M. leprae* sonicate at 5 ug/ml. The 100ul samples from triplicate wells were pooled and stored at -20° C for subsequent measurement of interferon-gamma (IFN-gamma) activity by a cytopathic effect inhibition assay (14, 15) using human reference IFN-gamma as standards (NIH, Bethesda, MD).

For DTH skin testing, the 70 kD antigen was diluted to a concentration of 1 ug/ml in sterile PBS. 100 ul was injected intradermally on the volar aspect of the forearm, and the horizontal and vertical diameters measured at 48 hours.

ISOLATION OF DNA CLONES ENCODING THE 70 KD ANTIGEN

The genomic library of *M. leprae* inserted into bacteriophage lambda gt11 and *Escherichia coli* strain Y1090 were kindly provided by Dr R.A. Young through the IMMLEP program (16). Lytic plaques containing DNA fragments of *M. leprae* were prepared (17) and probed with Mab L7 using immunochemical staining with an avidin-biotin-peroxidase system (Vectastain, Vector, Burlingame, CA). Bacteriophage containing DNA encoding the L7 epitope was isolated from positive plaques. *E. coli* lysates containing the recombinant proteins were prepared (17), separated by SDS-PAGE and probed in immunoblots with L7, L27 or the anti BCG antiserum. The lysates were also radiolabelled with 125I and immunoprecipitated with leprosy, tuberculosis or control sera as described above.

Results

CHARACTERIZATION OF ANTIGEN

Mabs L7 and 127 identified a band of Mr 70 kD in immunoblots of *M. leprae* sonicate, with an additional faint band of 68 kD evident in some preparations. The L-7-reactive band was more dense in immunoblots of *M. tuberculosis* and BCG sonicates, and was distinct from the 65 kD cell wall protein identified in the three species by Mabs L22 and L 28 (18). Treatment of *M. leprae* sonicate with trypsin abolished the binding of L7 to the 70 kD antigen, but L7 reacted weakly with bands of 26 kD and 16 kD, consistent with these being proteolytic fragments of the larger antigen. Oxidation with 5mM and 10mM sodium periodate had no effect on the reactivity of L7 with the 70 kD antigen, whereas it abrogated the binding of Mabs which identified polysachharide determinants in *M. leprae* and BCG sonicates (11).

The 70 kD antigen was readily immunoprecipitated by L7 from radiolabelled *M. leprae,* although it was not visible on SDS-PAGE analysis of unfractionated 125I-labelled *M. leprae.* In the case of BCG, the 70 kD antigen was evident in crude 125I-labelled sonicate, but was greatly concentrated by precipitation with L7. The precipitated antigen from *M. leprae* focused as a single band after IEF, and on two dimensional electrophoresis resolved as a discrete band with a pI value of 5.1.

The antigen capture assay with L7 demonstrated that there was more L7-reactive antigen present in sonicates of BCG and *M. tuberculosis,* than in *M. leprae* (Fig. 1). More antigen was detected with this assay in *M. leprae* sonicate than resonicate, confirming the finding with immunoblotting (10). As cell wall antigens are concentrated in the latter preparation (11), this result suggests the 70 kD antigen is a cytoplasmic protein. A small amount of L7 reactive antigen was present in PPD, and none was detectable in *M. smegmatis* confirming the ELISA results (10). When L5 was used in the assay, the Mab-defined antigen was detected only in *M. leprae* sonicate and resonicate (Fig. 1).

PURIFICATION OF 70 KD PROTEIN FROM BCG SONICATE

The 70 kD protein was successfully purified from BCG sonicate with the L7-Sepharose 4B affinity column. Staining of SDS-PAGE fractionated samples from the column revealed a marked increase in the density of the 70 kD band in the pH 11.5 eluate, compared to the narrow band seen in the original BCG sonicate. There was a second faint band of 68 kD beneath the major band in the pH 11.5 eluate, and both reacted strongly with L7 in immunoblots. This pattern resembled the doublet of L7-reactive bands seen in some immunoblots of *M. leprae* sonicate (10). Weakly reactive bands of 60 kD and 32 were considered proteolytic fragments of the dominant p70.

SDS-PAGE of radiolabelled eluate from the column revealed that the p70 antigen was strongly labelled. On two dimensional electrophoresis the column purified p70 had a pI of 5.35.

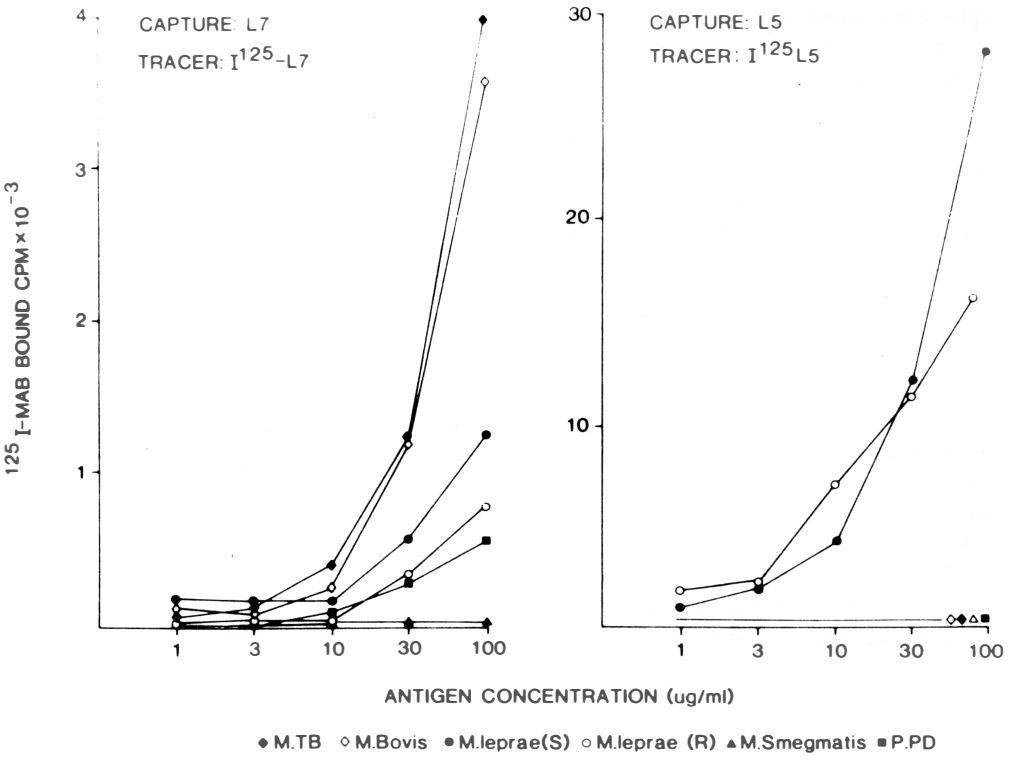

Fig. 1. Antigen capture assay in which samples (as labelled) were tested with L7 (left) or L5 (right) as capture and tracer Mab.

The enrichment of p70 in the column eluate was confirmed by the capture assay. The L7 reactive antigen was 16 fold more concentrated in the pH 11.5 eluate than in the original sonicate when tested at a protein concentration of 1 ug/ml. In the antigen inhibition ELISA, the concentation causing 50 % inhibition of binding of L7 to BCG sonicate was 20 ug/ml for the eluate, compared to 750 ug/ml for the whole sonicate.

The N-terminal residues of the p70 antigen were analysed with a gas-phase sequenator. The first 15 amino acids were:
Ala-Arg-Ala-Val-Gly-Ile-Asp-Leu-Gly-Thr-Asp-Asn-Ser-Val-Val.

REACTIVITY OF 70 KD PROTEIN WITH LEPROSY AND TUBERCULOSIS SERA

Individual and pooled sera were examined for reactivity with radiolabelled *M. leprae* and BCG sonicates. The LSP immunoprecipitated the p70 antigen from both sonicates. Half (8/15) of the individual BL/LL sera precipitated p 70 from *M. leprae*. None of the BT/TT or tuberculosis sera tested reacted with this band. Two dimensional analysis of precipitates of 125I-labelled *M. leprae* sonicate with LSP demonstrated a faint 70 kD band, but the dominant antigens identified with this technique were bands of 12 kD and 36 kD (Britton *et al,* submitted).

The p70 purified from BCG also reacted with patient sera. Two dimensional analysis of immunoprecipitates showed that LSP precipitated the radiolabelled p70. In a direct ELISA,

leprosy sera reacted with the p70 fraction diluted to a coating concentration of 0.01 ug/ml. Tuberculosis sera were less reactive with p70 than the leprosy sera, and only at antigen concentrations higher than 1.0 ug/ml.

CELLULAR REACTIVITY OF 70 KD PROTEIN

The p70 antigen purified from BCG stimulated the proliferation of PBM from Mantoux positive subjects at concentrations of 10-0.3 ug/ml. The response to p70 was 50-70 % of that to whole BCG sonicate at the same concentration in the majority of cases, however in a few subjects the response to the crude and purified antigens were comparable. Among the eight Mantoux negative controls, one had a weak proliferative response to p70 and BCG sonicate at 10 ug/ml. The response to both antigens was greater in subjects with natural immunity to *M. tuberculosis* than in those BCG vaccinated (Table 1).

IFN-gamma was released in response to stimulation with either p70 or BCG sonicate in all the Mantoux positive subjects tested (13/13), but not in the Mantoux negative controls (4/4). At an antigen concentration of 10 ug/ml, the mean IFN-gamma response in BCG vaccinated subjects (7) to p70 was 983 IU/ml (SEM 309) and to BCG sonicate 297 (65) IU/ml, while in those naturally Mantoux positive (6) the response to p70 was 1066 (135) IU/ml and to BCG 693 (212) IU/ml.

The *in vivo* response to p70 was examined in the same three groups of subjects. There was no response in the eight Mantoux negative individuals, including the subject who had a weak proliferative response to p70 at 10 ug/ml. All the Mantoux positive subjects had skin test reactivity at 48 hours greater than 5mm, with the mean response again slightly greater in subjects with natural Mantoux reactivity (Table 1). There was a moderate correlation between the skin test reactivity and proliferative response to p70 at 1 ug/ml (Spearman rank coefficient, Rs, 0.708). A strong correlation was observed between the LPA response to BCG and p70 (Rs, 0.839), compared to a correlation of 0.773 for the p70 and PPD responses and 0.751 for the p70 and *M. leprae* responses.

Table 1. T cell responses to purified p70 and BCG sonicate

Group	Number	*DTH skin test* [mean mm (+/-SEM] p70 (1 ug/ml)	*Proliferative response to:* [nett dpm, mean x10^{-3} (+/-SEM)] p70 (1 ug/ml)	BCG sonicate (1 ug/ml)
1	8	0	1.9 (05)	1.8 (06)
2	12	8.8 (1.6)	24.5 (8.5)*	40.4 (10.8)**
3	7	12.2 (2.6)	42.2 (12.8)**	84.7 (29.4)**

The groups were: 1, naive recipients; 2, Mantoux positive after BCG vaccination; 3, Mantoux positive without vaccination. Significantly different to group 1 (* $p<0.01$; ** $p<0.005$).

DNA CLONES ENCODING THE 70 KD ANTIGEN

Two DNA clones, JKL2 and JKL15, were isolated by probing the genomic library of *M. leprae* with Mab L7. The peptides expressed by both clones reacted with either L7 or L27, but not with Mabs (L22 or L28) which identified the gene encoding the 65 kD cell wall protein (16, 18). Both peptides were secreted as L7-reactive fusion with Mr of 160 kD for the JKL2

clone and 135 kD for JKL15. The fusion proteins also reacted in immunoblots with a polyclonal rabbit anti-BCG antiserum. This anti-serum reacted with the 65 kD protein which was secreted as a free peptide by *M. leprae* DNA clone Y3178, but not with the fusion protein expressed by clone Y3179. The latter encodes the *M. leprae* specific 18 kD protein identified by L5 (16). The fusion proteins from JKL2 were both radiolabelled with 125I and were immunoprecipitated strongly by lepromatous leprosy sera and weakly by som tuberculosis sera.

Discussion

This 70 kD protein is one of the six Mab-defined protein antigens of *M. leprae* for which the genes have been isolated (16). Like the 28 kD and 65 kD antigens (9, 18) it is not limited to *M. leprae,* and is present in at least three other species. The Mab binding patterns suggested that the 12 kD, 18 kD and 36 kD proteins were specific to *M. leprae* (7, 8, 10), however the T cell reactivity of the purified 36 kD antigen suggests it also bears cross-reactive determinants (19). During the IMMTUB Mab Workshop, the gene for a 71 kD protein of *M. tuberculosis* was isolated with Mab 51A which had limited cross-reactivity with other mycobacterial spcies (20). This is likely to be the homologous antigen to p70 in *M. leprae,* for when the two Mabs, L7 and 51A, were tested in CIE, both identified line 63, a major peak in the CIE reference system for BCG (Harboe, personal communication). In addition, L7 identified a previously unrecognized line in the CIE pattern of *M. leprae.*

The T cell reactivity of the 70 kD protein from BCG was clearly established in man. In Mantoux positive subjects it stimulated lymhocyte proliferation and IFN-gamma secretion, and it elicited skin test reactivity. Isolation of the gene for the *M. leprae* protein will permit analysis of T cell reactivity of the equivalent leprosy antigen. The cellular reactivity of the antigen may prove more significant in the human immune response than its humoral reactivity. Although leprosy sera precipitated p70 purified from BCG and reacted with the expression product of *M. leprae* clone JKL2, anti-p70 antibodies were detected in only half of the individual BL/LL sera tested.

Two other Mab-defined protein antigens of BCG possess cellular reactivity. The 10 kD protein purified by Mab affinity chromatography elicited DTH in sensitised guinea pigs (21), and the 64 kD antigen tested as a recombinant protein stimulated human T cell clones from a leprosy patient (22). In the case of *M. leprae* proteins, both the 12 kD and 36 kD antigens stimulated human T cell clones after Mab affinity purification (19), as did the 18 kD protein when expressed in *E. coli* as a recombinant protein (23). In both species, therefore, T cell reactivity appears to be a property of a number of proteins. The relative importance of individual antigens in stimulating protective immunity against mycobacterial infections remains to be established. This will require studies with a panel of antigens in experimental models of infection, and in the case of leprosy detailed analysis of T cell reactivity and precursor cell frequency for each antigen in a spectrum of leprosy patients, naturally immune subjects and recipients of candidate vaccines.

Acknowledgements

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