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Separate Antigenic Determinants on Cell Wall Associated Carbohydrate Antigens of *Mycobacterium leprae* Defined with Monoclonal Antibodies[1]

Warwick J. Britton, Linus Hellqvist, and Antony Basten[2]

The advent of monoclonal antibody (Mab) technology has led to a rapid expansion in the understanding of the antigens of *Mycobacterium leprae*. At least six different protein antigens have been identified with Mabs, and DNA clones encoding the genes for five of them have been isolated ([2, 13, 18, 20, 30, 31]). During the Immunology of Leprosy (IMMLEP) Workshop on these antibodies, a number of others were observed to react with a broad antigenic band labeled "carbohydrate/lipid" as well ([12]). We had developed similar Mabs which in immunoblots bound to crossreactive determinants on two broad bands with apparent molecular weight (M_r) of 30–40 kD and 4.5–6 kD ([2]). Inhibition assays indicated that lepromatous leprosy sera contained a high titer of antibody directed against these Mab-defined determinants. This finding was confirmed by the strong reaction of unabsorbed leprosy sera with the same antigenic bands in immunoblots. Sera absorbed with *M. bovis* and *M. vaccae* still reacted with a triplet of bands of M_r 32–35 kD which was consistent with the presence of species-specific epitopes on those molecules in addition to the Mab-defined crossreactive determinant. The existence of *M. leprae*-specific antigens of similar molecular weight ([3, 19]) has now been confirmed in two other studies.

We have sought to characterize the 30–40 kD and 4.5–6 kD antigens immunochemically, and to examine their localization within the mycobacterium. Using a combination of inhibition ELISA and antibody capture assays, we have demonstrated the relative abundance of the antigens in preparations of *M. bovis* (BCG) and *M. tuberculosis*, as well as *M. leprae*. Experiments with Mab-affinity columns indicated that the low molecular weight (LMW) antigen of 4.5–6 kD is a fragment of the larger 30–40 kD molecule.

MATERIALS AND METHODS

Antigens. *M. leprae* bacilli were provided through the IMMLEP component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases by Dr. R. J. W. Rees. The bacilli were sonicated, as described previously ([2]), for 15 min to yield *M. leprae* sonicate. The insoluble cell wall fraction was sonicated again for 15 min to yield a solubilized preparation of the cell wall, *M. leprae* resonicate leaving a final insoluble pellet, *M. leprae* pellet. *M. bovis* (BCG) was obtained as freeze-dried bacilli from the Commonwealth Serum Laboratories (CSL, Melbourne, Australia). *M. smegmatis* and *M. tuberculosis* H37Rv were grown in Dubos broth (Difco, Detroit, Michigan, U.S.A.) supplemented with albumin and glycerol. The bacilli were then harvested, washed in phosphate buffered saline (PBS) pH 7.2, and sonicated. After measuring the protein content of the sonicate by Folin's reagent, the material was aliquoted and stored at −70°C until used. Purified protein derivative (PPD) was obtained from Statens Seruminstitut, Copenhagen, Denmark.

Monoclonal antibodies (Mabs). A panel of Mabs to *M. leprae* and *M. bovis* (BCG) was produced by fusion of immunized BALB/c spleen cells with NS-1 cell line ([2]). Six of them were found to bind to antigens with M_r of 30–40 kD and 4.5–6 kD. Other

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[2] W. J. Britton, M.B., B.S., B.Sc.(Med.), F.R.A.C.P., M.R.C.P., D.T.M.&H.; L. Hellqvist, Ph.D.; A. Basten, M.B., B.S., Ph.D.(Oxon), F.T.S., F.R.A.C.P., F.R.C.P.A., F.R.C.P., Clinical Immunology Research Centre, University of Sydney, Sydney, Australia 2006.

Mabs bound to antigens of M_r 16–18 kD and 70 kD. L1 (IgM), L9 (IgG3) and L10 (IgG3) reacted with a single epitope on the 30–40 kD molecule. L3 (IgM) and L4 (IgG2a) reacted with the 4.5–6 kD antigen. B6 (IgG3) was derived from a fusion with mice primed to *M. bovis* (BCG). It recognized the same epitope on the LMW antigen as L3 and L4. All six Mabs reacted with 13 of the 14 mycobacterial species tested in enzyme-linked immunosorbent assays (ELISA).

Immunochemical characterization. *M. leprae* sonicate and resonicate were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions as described previously ([2, 21]). After transfer of the antigens to nitrocellulose paper ([30]) (NCP; Biorad, Richmond, California, U.S.A.), the NCP strips were probed with Mab supernatant followed by ^{125}I-labeled sheep anti-mouse Ig ([2]). The NCP strips were dried and exposed to Kodak XRP-1 film (Kodak, Melbourne, Australia) and developed after 2 days at −70°C. The effect of proteolysis on Mab binding was examined by incubating *M. leprae* sonicate (1 mg/ml) with trypsin (Bovine Pancreas Type 1; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) at 10 mg/ml for 1 hr at 37°C ([17]). The samples were then boiled for 5 min in electrophoresis buffer (2% SDS, 10% sucrose, 2% 2-mercaptoethanol, 0.05 M Tris-HCl pH 6.8), and analyzed as described above. The susceptibility of the antigens to periodate oxidation was examined in a similar way by first incubating *M. leprae* sonicate with sodium periodate (Sigma) at a final concentration of 1, 5, and 10 mM for 1 hr at 37°C before boiling in electrophoresis buffer ([28]).

Antigen inhibition ELISA. The inhibitory effect of various mycobacterial sonicates on the binding of L9 or L4 to *M. leprae* or to BCG sonicates was examined by antigen inhibition ELISA. Initially, the titer of hybridoma supernatant causing 75% of the maximum optical density (OD) reading for each Mab was determined and used as the final dilution in the inhibition ELISA. Doubling dilutions of the antigens, *M. leprae* sonicate, *M. leprae* resonicate, BCG or *M. tuberculosis* sonicate, were then made in PBS in a polystyrene microtiter tray (Linbro, Flow Laboratories, Edinburgh, Scotland), the wells of which had been blocked with 3% bovine serum albumin (BSA). An equal volume of hybridoma supernatant, appropriately diluted, was added, and the tray incubated for 1 hr at 37°C in a humidified incubator. Fifty μl of each dilution were then transferred in triplicate to the wells of a polystyrene tray coated with *M. leprae* or BCG sonicate (50 μg/ml). After incubating for 1 hr, the wells were washed three times with PBS-azide, and sheep anti-mouse Ig-alkaline phosphate conjugate (Sigma) diluted 1:1000 in 0.1% BAS-PBS was added for 1 hr. The wells were washed three times with PBS-azide and once with sodium carbonate buffer before adding the enzyme substrate nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.15 M sodium carbonate buffer pH 9.6. The tray was read at 60 min in a Titertek Scanner (Flow Laboratories). The OD of wells coated with BSA alone was subtracted from each reading. Maximum binding occurred with the Mabs supernatant at the same dilution but without inhibiting antigen. The percentage inhibition of this binding by each antigen concentration was calculated and plotted. From this, the antigen concentration causing 50% inhibition of binding for that antigen and the particular Mab was determined.

Monoclonal antibody capture assays. The relative concentration of Mab-defined determinants in *M. leprae, M. leprae* resonicate, and other mycobacteria was examined using a capture assay in which the capture and tracer antibody as well as the antigen could be changed. Briefly, "capture" Mab (50 μl, 100 μg/ml) purified by protein A chromatography was diluted to 100 μg/ml in PBS before incubation in the wells of a polyvinyl chloride (PVC) microtiter tray (Linbro). After 2 hr at 37°C, the wells were washed with PBS-azide and blocked with 5% casein in PBS. After washing with PBS, 50 μl of mycobacterial sonicates diluted in PBS from 100 μg/ml to 10 ng/ml were added in triplicate for an overnight incubation at 4°C. Subsequently, the trays were washed five times with PBS, and the "tracer" Mab, radiolabeled with ^{125}I by the chloramine T method ([15]) (10^5 cpm in 50 μl), was added for 4 hr at 37°C. At the end of this period,

the wells were again washed extensively in PBS before counting in a Riagamma counter (LKB, Stockholm, Sweden). The binding of the tracer Mab to wells coated with BSA and incubated without antigen was subtracted from the triplicates for each antigen concentration. An irrelevant Mab (K-1-21, IgG1, anti-kappa light chain) was also used as the "capture" Mab.

The assay was also used to determine if intact bacilli could be bound by monoclonal antibodies. Whole bacilli, diluted in PBS from 10^9 to 10^5 ml, were used as antigen before washing and incubation with the tracer Mab. The binding of bacilli to L9 or L4 as capture Mab was compared to the binding of bacilli to BSA.

Immunofluorescence of *M. leprae* with monoclonal antibodies. Whole *M. leprae* bacilli (10^7 in 50 μl) were transferred and fixed to template slides (Sigma). The slides were incubated with Mab supernatant or ascites fluid in a moist chamber for 1 hr at 37°C and then washed three times in PBS/azide. Twenty-five μl fluorescein conjugated goat anti-mouse Ig (Silenus, Melbourne, Australia) 1:20 dilution in PBS was added to the slides for 1 hr at 37°C. Following this, they were washed three times, mounted in phosphate buffered glycerol (pH 8.8), and examined under a Zeiss fluorescent microscope. The control preparations consisted of slides incubated with irrelevant IgG1 and IgM Mabs. The binding of lepromatous leprosy sera was compared with that of the Mabs. A pool of sera from 20 untreated lepromatous leprosy patients was used with fluorescein-conjugated goat anti-human Ig (Sigma) at a dilution of 1:50 as a second antibody.

Monoclonal antibody affinity chromatography. Purified Mabs L4 and L9 were conjugated to cyanogen bromide activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at a ratio of 5 mg antibody to 1 ml gel in a coupling buffer of 0.25 M sodium bicarbonate (pH 9.0) containing 0.5 M sodium chloride. After a 2-hr incubation at room temperature, the remaining active groups were blocked with 1 M ethanolamine (pH 8.0), and the gel washed alternately with coupling buffer and acetate buffer (0.1 M, pH 4.0) containing 0.5 M sodium chloride. The gels were equilibrated with PBS and stored at 4°C. *M. leprae* sonicate or *M. bovis* sonicate was recirculated through the L9-CNBr Sepharose column for 2 hr at flow rate 2 ml/min and then through the L4-CNBr Sepharose column for a similar period of time. Both were washed extensively with PBS until a stable base line was achieved. The bound material was eluted with 0.05 M diethylamine (pH 11.5) followed by 1 M propionic acid (pH 2.0). The eluates were dialyzed against ammonium bicarbonate, freeze dried, and dissolved in 1.0 ml PBS. The eluates from both columns were then examined by SDS-PAGE and immunoblots with L9 and L4.

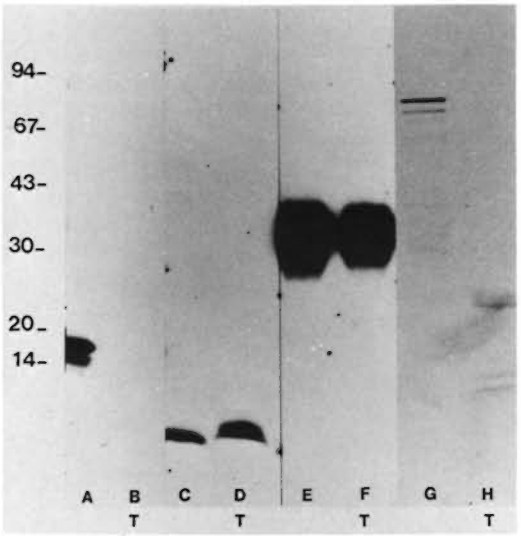

FIG. 1. Immunoblot of *M. leprae* sonicate with (Tracks B, D, F, H) and without prior treatment with trypsin (Tracks A, C, E, G). Antibodies used were: L5 (Tracks A, B); L4 (Tracks C, D); L9 (Tracks E, F) and L7 (Tracks G, H). Position of protein molecular weight markers (in kD) are shown on left hand side.

RESULTS

Immunochemical nature of antigens. Our initial experiments had demonstrated that the 30–40 kD diffuse band defined by L1, L9, and L10 and the low molecular weight (LMW) band of 4.5–6 kD identified by L3, L4, and B6 in *M. leprae* sonicate were present in the samples examined under both reducing and nonreducing conditions. These bands were resistant to treatment with subtilisin after transfer to NCP ([2]). This protease resistance was confirmed by incubat-

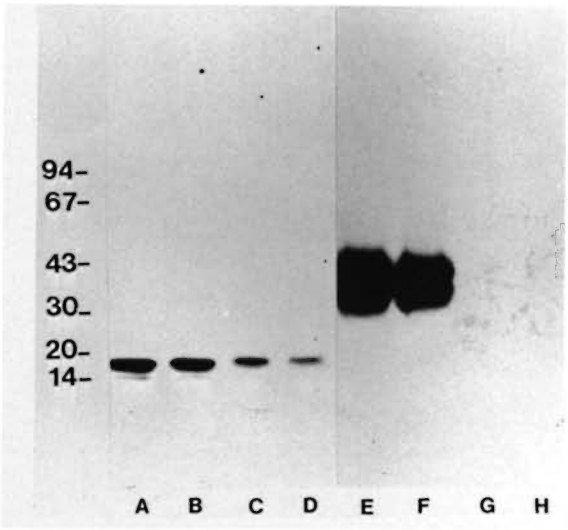

FIG. 2. Immunoblot of *M. leprae* sonicate without (Tracks A, E) and with prior treatment with sodium periodate at a final concentration of 1 mM (Tracks B, F), 5 mM (Tracks C, G) and 10 mM (Tracks D, H). Antibodies used were L5 (Tracks A–D) and L9 (Tracks E–H). Position of protein molecular weight markers (in kD) are shown on left hand side.

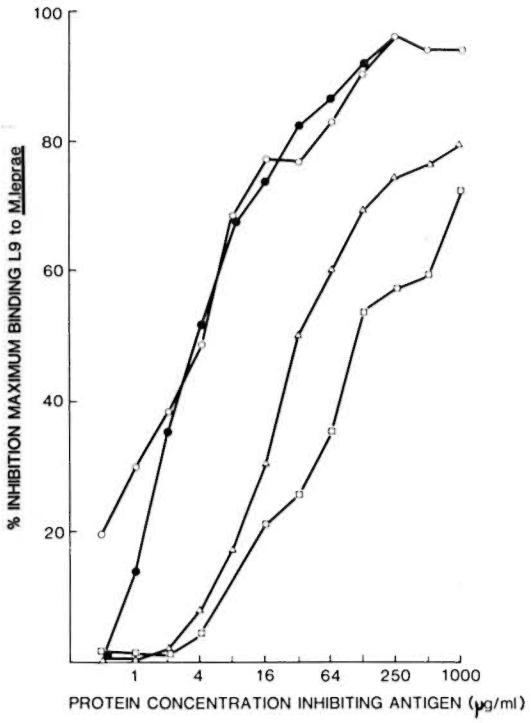

FIG. 3. Inhibition ELISA in which different mycobacterial sonicates inhibited the binding of L9 to *M. leprae* sonicate. Antigens were *M. leprae* sonicate (○), *M. leprae* resonicate (●), *M. bovis* sonicate (△), and *M. tuberculosis* sonicate (□).

ing the *M. leprae* sonicate with trypsin prior to SDS-PAGE. The 30–40 kD and LMW bands were unaffected whereas the 16–18 kD and 70 kD protein bands defined by two other anti-*M. leprae* Mabs, L5 and L7, respectively, were destroyed (Fig. 1). Incubation with sodium periodate also had a differential effect on the antigens. The protease-resistant bands were sensitive at a concentration of 5 mM and above, with complete loss of reactivity in immunoblots indicating that the Mab-defined determinants were predominantly carbohydrate in nature (Fig. 2). In view of this, the figures given for apparent molecular weight as defined by comparison with protein markers should be regarded only as approximations.

By contrast, binding to the determinants defined by L5 and L7 was less affected after exposure to 5 mM and 10 mM sodium periodate, which suggests they are proteins rather than carbohydrates.

Antigen inhibition ELISA. Both L9 and L4 had been shown to react as strongly with *M. leprae* resonicate as with *M. leprae* sonicate in immunoblotting experiments ([2]). Therefore, the relative concentrations of the antigens in the two samples were examined in an inhibition ELISA. Both the *M. leprae* sonicate and resonicate inhibited the bind-

TABLE 1. *Mycobacterial antigen inhibition of Mab binding to* M. leprae *sonicate in ELISA.*

Mab	Protein concentration (μg/ml) required for 50% inhibition of maximum binding with				
	M. leprae sonicate	*M. leprae* resonicate	*M. bovis* (BCG)	*M. tuberculosis*	*M. smegmatis*
L4	28	40	4	15	54
L9	4	4	32	115	32

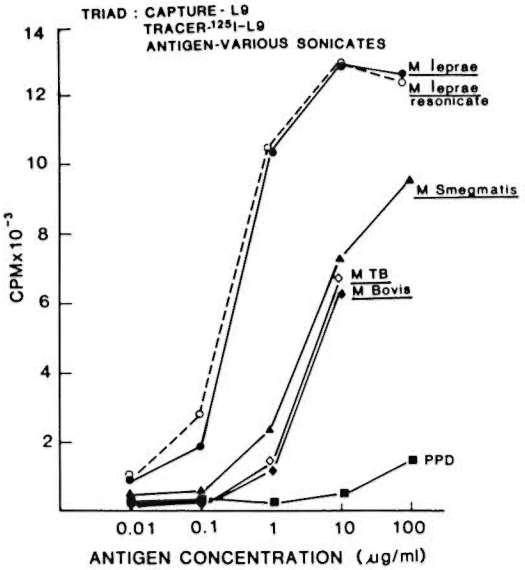

FIG. 4. Antigen capture assay in which L9 immobilized on a PVC tray bound various mycobacterial sonicates which, in turn, reacted with ^{125}I-L9. Antigens were *M. leprae* sonicate (●), *M. leprae* resonicate (○), *M. bovis* sonicate (◆), *M. tuberculosis* sonicate (◇), *M. smegmatis* sonicate (▲) and PPD (■). Standard deviation from each point was less than 10%.

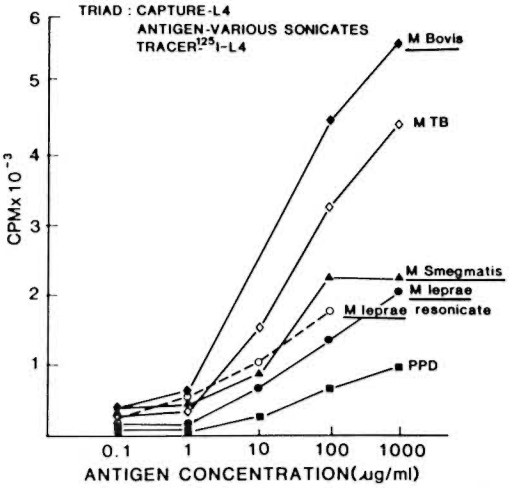

FIG. 5. Antigen capture assay in which L4 was the capture and tracer antibody and the mycobacterial sonicates varied. Antigens same as in Figure 4 legend. Standard deviation from each point was less than 10%.

ing of L9 to *M. leprae* sonicate to an equivalent degree (Fig. 3). Thus, the protein concentration associated with 50% inhibition of maximum binding was 4 µg/ml for each (Table 1). In the case of L4, similar concentrations of *M. leprae* sonicate and resonicate were required to inhibit the binding of L4 to *M. leprae* (Table 1).

Previously, differences in the intensity of binding of the Mabs with various mycobacterial sonicates in immunoblots had been observed. L9 reacted weakly with *M. bovis* and *M. tuberculosis* sonicates and the antigen identified had a slightly higher M_r of 35–45 kD ([2]). By contrast, L4 reacted strongly with a LMW band of similar mobility in the three mycobacterial species. Therefore, we examined the inhibitory effect of other mycobacterial sonicates on the binding of L9 and L4 to *M. leprae*. *M. bovis*, *M. tuberculosis* and *M. smegmatis* inhibited the binding of L9 to *M. leprae*, but at a higher concentration of sonicate than with either *M. leprae* sonicate or resonicate (Table 1). However, less *M. bovis* sonicate was required for a 50% inhibition of L4 binding to *M. leprae*, indicating that the LMW antigen was more abundant in the *M. bovis*

TABLE 2. *Antigen capture assay using L9 or L4 as capture and tracer antibodies and* M. leprae *sonicate as antigen.*

Antigen concentration	Tracer Mab bound (cpm ± S.E.M.)[a]			
	^{125}I-L9		^{125}I-L4	
	10 µg/ml	1 µg/ml	10 µg/ml	1 µg/ml
Capture Mab				
L9	11,192 ± 348	6,582 ± 117	1, 828 ± 80	672 ± 47
L4	14,990 ± 459	6,888 ± 224	220 ± 12	113 ± 12
Control				
K-1-21	120 ± 60	0	0	0

[a] 50 µl samples tested as described in text in Materials and Methods. Mean of test triplicate minus the cpm bound to BSA control well incubated without antigen.

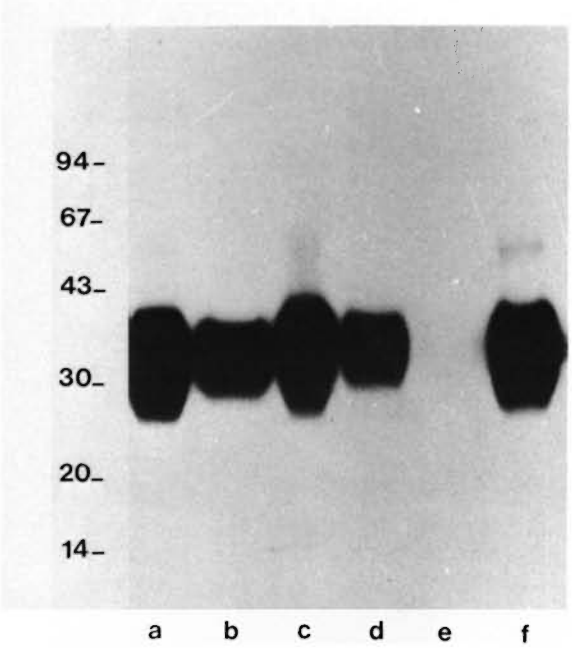

FIG. 6. Immunoblot analysis with L9 of fractions from L9 and L4 Mabs-affinity columns which were reacted with *M. leprae* sonicate. Tracks a = original *M. leprae* sonicate; b = L9 column, pH 7.4 fraction containing unbound antigen; c = L9 column, pH 11.5 fraction; d = L9 column, pH 2.0 fraction; e = L4 column, pH 7.4 fraction containing unbound antigen; f = L4 column, pH 11.5 fraction. Position of protein molecular weight markers (in kD) are shown on left hand side.

preparation (Table 1). A similar pattern was seen when the binding of L4 to *M. bovis* sonicate was examined in the inhibition ELISA. *M. bovis* sonicate was more efficient than *M. leprae* in inhibiting the binding of L4 to *M. bovis*.

Antigen capture assay. The structural relationship between the 30–40 kD and LMW carbohydrate determinants was examined in an antigen capture assay with the corresponding two Mabs L9 and L4. L9 could be used as both capture and tracer Mab to detect the 30–40 kD antigen in *M. leprae* sonicate down to a concentration of 10 ng protein/ml (Fig. 4). L4 was as efficient as L9 in capturing the 30–40 kD molecule which could, in turn, bind ^{125}I-L9 (Table 2). This suggested that the two determinants were located on molecules associated in the aqueous phase. In order to confirm this, the reverse experiment was conducted which showed that L9 could capture the LMW antigen as revealed by ^{125}I-L4 tracer (Table 2). However, *M. leprae* antigen captured by L4 did not bind further ^{125}I-L4 except at high concentrations, indicating that there were a limited number of L4-defined determinants on the LMW molecule of the *M. leprae* sonicate (Fig. 5). Therefore, although the 30–40 kD and LMW antigens appeared to be separate antigens in SDS-PAGE, they were clearly associated and able to be immobilized by either L9 or L4.

The same assay was used to examine the binding of L9 or L4 to different sonicates (Fig. 4). Equivalent amounts of L9 and L4 reactive antigen were present in *M. leprae* sonicate and resonicate, confirming that these Mab-defined determinants were located in the cell wall of the mycobacterium. Less L9 reactive antigen was captured from *M. bovis* or *M. tuberculosis* sonicates compared to *M. leprae*, and there was a small amount of the 30–40 kD antigen in the PPD (Fig. 4). By

TABLE 3. *Antigen capture assay with intact bacilli.*

Bacilli concentration	Tracer Mab bound (cpm ± S.E.M.)[a]					
	^{125}I-L9			^{125}I-L4		
	10^9/ml	10^8/ml	10^7/ml	10^9/ml	10^8/ml	10^7/ml
Capture Mab						
L9	2290 ± 144[b]	508 ± 18[c]	83 ± 48	285 ± 60	150 ± 24	86 ± 55
L4	573 ± 17	172 ± 22	102 ± 43	409 ± 47	234 ± 92	40 ± 57
BSA control	335 ± 25	34 ± 27	0	477 ± 44	151 ± 21	15 ± 47

[a] 50 μl samples tested as described in text in Materials and Methods. Mean of test triplicates minus cpm bound to BSA control well incubated without antigen.

[b] Differences to BSA control incubated with same concentration of bacilli significant at $p < 0.005$ (Mann-Whitney rank sum test).

[c] Differences to BSA control incubated with same concentration of bacilli significant at $p < 0.01$ (Mann-Whitney rank sum test).

contrast, more ^{125}I-L4 bound to *M. bovis* or *M. tuberculosis* than to *M. leprae* sonicate (Fig. 5).

Whole bacilli were also substituted for sonicate in order to test whether the determinants recognized by either Mab were on the surface of intact organisms. When the bacilli were incubated in wells coated with L9, significantly more ^{125}I-L9 was subsequently bound to the immobilized bacilli than if the bacilli had been incubated in control wells (Table 3). This only occurred if the higher concentrations of whole bacilli, 10^9/ml and 10^8/ml, were used. By contrast,^{125}I-L4 did not bind to the bacilli in wells coated with either L4 or L9 (Table 3). These results indicated that only the 30–40 kD antigen was exposed on the surface of the bacilli. The difference in appearance of the L9 reactive bands shown in Tracks b and d (Fig. 6) of the blots is presumably due to the fact that the 30–40 kD band is composed of several different antigens ([2,12]).

Immunofluorescence. The location of the two antigens was examined directly by immunofluorescence with the same Mabs and a fluorescinated second antibody. L1, L9, and L10, which recognized the 30–40 kD antigen, stained the majority of organisms with a granular pattern. This occurred with either supernatant or ascites fluid diluted to 10^{-4}, but the intensity was less than that seen with a pool of human lepromatous leprosy sera. On the other hand, no fluorescence was detected with any of the three antibodies (L3, L4, B6) directed against the LMW antigen, confirming the conclusion from the antigen capture assay with whole bacilli.

Affinity chromatography. The relationship between the two antigens was further analyzed by Mab-affinity chromatography. *M. leprae* sonicate (5 mg) was applied sequentially to the L9 and L4 columns. The eluates and unbound residual fractions were concentrated and examined by SDS-PAGE and immunoblotting with L9 and L4 (Fig. 6). The 30–40 kD antigen was eluted from the L9 column by both alkaline and acidic buffers (Tracks c, d). However, the unbound fraction still contained immunoreactive antigen (Track b). When this unbound fraction was passed over the L4 column, it was depleted of the 30–40 kD antigen (Track e) which was subsequently eluted from the column with 0.05 M diethylamine. Immunoblotting of the fractions with L4 indicated that the LMW antigen had bound to both L9 and L4 columns. A similar result was obtained when *M. bovis* sonicate was used with the 30–40 kD and LMW antigen being eluted from either L9 or L4. Thus, the determinants recognized by the two antibodies on the 30–40 kD and LMW antigens were present in a common complex.

DISCUSSION

These six antibodies were shown to define two different determinants on related, cell-wall carbohydrate antigens, both of which were present in *M. bovis* and *M. tuberculosis* as well as *M. leprae*. The fact that the determinants recognized by the Mabs were carbohydrate in nature was demonstrated by the resistance to protease and by the sensitivity to oxidation with periodate at low concentrations (Figs. 1 and 2). Despite being similar in composition the two determinants could be distinguished on the basis of their location on antigens of differing molecular weights when examined by polyacrylamide electrophoresis in the presence of SDS. There was no difference in the M_r of the antigens in reducing and nonreducing conditions, indicating there were no covalent disulfide bonds between the 30–40 kD and 4.5–6 kD antigens. Furthermore, there was no crossinhibition between the three Mabs (L1, L9, and L10) which reacted with the 30–40 kD antigen and the three Mabs (L3, L4, and B6) recognizing the 4.5–6 kD LMW band ([2]). In addition, even though both antigens were apparently associated with the cell wall, being present in equivalent concentrations in sonicate and resonicate (Figs. 3–5), only the 30–40 kD antigen could be detected by the immunofluorescence and capture assay on the intact bacilli. Nevertheless, the two antigens were clearly related in a structural sense since they could be eluted from either L9 or L4 Mab-affinity columns (Fig. 6), which suggested they were associated in aqueous solution presumably by non-covalent bonds. Consistent with this conclusion were the findings from the capture assay in which L9-immobilized antigen could bind ^{125}I-L4 and vice versa (Table 2). Thus, the LMW and 30–40 kD antigens appeared to be derived from a common cell-wall molecule. Covalent bonds linking the

two antigens could have been split by the sonication process, leaving them associated non-covalently in a molecular complex which could be immobilized by either L9 or L4. However, when the complex was examined under the denaturing conditions of SDS-PAGE, the two antigens had different mobilities. Thus, they probably both originate from the polysaccharide side chains which are attached to the peptidoglycan backbone of mycobacterial cell walls ([7, 9]).

Both arabinogalactan (AG) and arabinomannan (AM) have been isolated from mycobacteria ([1, 5]). AG was demonstrated to be a structurally important constituent of the cell wall in the seven mycobacterial species studied by Misaki and co-workers and its general structure and major antigenic determinant have now been defined ([25]). The molecular weight of AG derived from *M. bovis* was found to be 31 kD ([26]). In the case of *M. leprae,* Draper showed that the cell walls contained arabinose and galactose in the molar ratio 3:1, and considered that AG with attached mycolic acid was a major part of the wall ([8]). AM, on the other hand, was thought to be a cytoplasmic rather than a cell-wall polysaccharide and that it may have contaminated previous cell-wall preparations ([9,10]). Recent data, however, indicate that AM may be a significant cell-wall polysaccharide as well. Miller and Buchanan isolated Mab AM8-2c from a fusion using mice primed with AM purified from *M. smegmatis* ([22]). The molecular weights of the two bands identified by AM8-2c were 80–90 kD and 30–40 kD, and the latter had a similar appearance to the L9-defined band in immunoblots of *M. leprae* sonicates. The binding site of AM8-2c encompassed more than one sugar residue. AM isolated from *M. smegmatis* reacted strongly with human leprosy sera, indicating there was crossreactivity between antisera to the cell-wall polysaccharide of *M. smegmatis* and *M. leprae* ([23]). This indirect evidence for the presence of AM in the cell wall of *M. leprae* has now been confirmed following the isolation of phosphorylated lipoarabinomannan-B (pLAM-B) from *M. leprae* ([16]). Thus, the purified pLAM-B proved to have a similar molecular weight (35 kD) and appearance in immunoblots to the L9-defined antigen, and it reacted with human antisera.

On the basis of these findings, it was possible that the L9- and L4-defined determinants could, in fact, be located on pLAM-B. To test this, pLAM-B purified from *M. tuberculosis* was reacted with L9, L4, and a third Mab, 906.4, directed against LAM from *M. leprae* in ELISA, inhibition RIA and antigen capture assays. Both the LAM and 906.4 were kindly provided by Dr. P. J. Brennan (Colorado State University). The results indicated that L9 did indeed bind to LAM, on which it reacted with a separate epitope to that recognized by 906.4. Furthermore, according to the antigen inhibition assay, there was an increased concentration of the L9-defined determinant in LAM from *M. tuberculosis* compared to *M. leprae* sonicate. By contrast, no binding of L4 to pLAM-B was demonstrable (data not shown). This could have been due to the location of the L9- and L4-reactive epitopes on different polysaccharides. However, in view of the fact that the two determinants co-eluted from the Mab affinity columns, the more likely explanation is for the epitopes to be on the same molecule and for the L4-defined determinant to have been destroyed during preparation of pLAM-B. When taken in conjunction with the data showing the binding of L9 to intact bacilli by immunofluorescence (see results), these findings provide additional support in favor of the presence of AM in the cell wall.

The results described here and those of other investigators point to differences in the species distribution of the epitopes defined by L4, L9, and AM8-2c. AM8-2c was strongly reactive with all species except *M. tuberculosis* ([22]). Both the inhibition ELISA and capture assay data indicated that the L4-defined epitope on the LMW antigen was more abundant in *M. bovis* and *M. tuberculosis* (Table 1). Possibly this fragment is more readily solubilized by sonication in these two species. Conversely, the L9 defined epitope on the 30–40 kD band was abundant in *M. leprae*; whereas both *M. bovis* and *M. tuberculosis* sonicates displayed a lower level of reactivity with L9. Misaki and co-workers ([24]) have demonstrated that species variation between AM purified from different mycobacterial species resided in the secondary side chains which consisted mainly of mannose. The LMW

fragment would appear to be derived from the part of the AM molecule common to the mycobacterial species studied.

The antigen capture assay was most effective when ^{125}I-L9 was the tracer antibody and either L9 or L4 was the capture antibody (Table 2). This indicated that the L9-defined epitope was a repeating determinant on the 30–40 kD molecule. By contrast, the LMW fragment in *M. leprae* sonicate was more efficiently bound by ^{125}I-L4 if first captured by L9, suggesting there are a limited number of L4 binding sites on this fragment. A similar radiometric assay with a single Mab directed against the circumsporozoite proteins of malaria parasites was equally effective because there were multiple common epitopes within that molecule as well ([32]). The sensitivity of the capture assay with L9 was greater than for any combination of Mabs directed against the protein antigen of *M. leprae,* including the 18 kD, 60–65 kD, and 70 kD antigens (unpublished observations). The value of this assay in measuring mycobacterial antigens in body fluids is currently being assessed.

The cell-wall-associated polysaccharide antigens are strong B-cell immunogens in man ([7]). Thus, the titer of antibody against *M. smegmatis* AM in patients with lepromatous leprosy was found to be proportional to the bacterial load of the patients ([23]). We have shown that the mean inhibitory titers of leprosy sera in Mab inhibition assays with L4 were greater than those obtained with Mabs directed against protein antigens ([2]). Even though the L4- and L9-defined determinants were present in *M. tuberculosis,* the inhibitory titers of the tuberculosis sera tested were not significantly raised above that of the controls. In other words, the serological response in lepromatous leprosy to these common polysaccharide antigens appeared to be an effective marker of disease status.

The role of cell-wall polysaccharide in stimulating T cells is less clear. Arabinogalactans and arabinomannans have elicited immediate-type skin reactions in sensitized guinea pigs ([14]), but with two exceptions they have failed to elicit delayed-type hypersensitivity in the same species ([4, 5]). In human studies, the mycobacterial polysaccharides have failed to stimulate proliferation of lymphocytes or to elicit skin reactions from tuberculin-positive subjects ([6, 27]). On the contrary, AM purified from *M. tuberculosis* was shown to suppress antigen-specific proliferative responses by a macrophage-dependent method ([11]). The capacity of antigenic determinants on protein-free, cell-wall polysaccharide of *M. leprae* to either stimulate or to suppress the cell-mediated immune response to *M. leprae* in man awaits elucidation.

SUMMARY

Monoclonal antibodies (Mabs) raised against *Mycobacterium leprae* sonicate defined two different determinants on related, cell-wall-associated, carbohydrate antigens common to *M. leprae, M. bovis* (BCG), and *M. tuberculosis.* Antigen inhibition ELISA and antigen capture assays demonstrated that the two antigens were present in a cell-wall fraction, *M. leprae* resonicate. There was species variation in the distribution of the antigens; the 4.5–6 kD antigen was more abundant in *M. tuberculosis* and *M. bovis,* while the 30–40 kD antigen was more concentrated in *M. leprae* preparations. Although both were present in the cell wall, only determinants on the 30–40 kD antigen were accessible on intact bacilli. The results from capture assays and Mab affinity chromatography with both L9 and L4 indicated that the 4.5–6 kD antigen was probably a fragment of the larger molecule. Both antigens are significant immunogens in the human B-cell response to *M. leprae.*

RESUMEN

Se prepararon anticuerpos monoclonales contra un extracto soluble del *Mycobacterium leprae,* los cuales identificaron a 2 determinantes antigénicos diferentes presentes en carbohidratos asociados a la pared celular del *M. leprae, M. bovis,* BCG, y *M. tuberculosis.* Los ensayos de ELISA para inhibición por antígeno y para captura de antígeno, demostraron que los 2 antígenos estuvieron presentes en la fracción correspondiente a la pared celular del *M. leprae.* Se encontró variación en la distribución de los antígenos de acuerdo a las especies; el antígeno 4.5–6 kD estuvo más concentrado en las preparaciones de *M. tuberculosis* y de *M. bovis,* mientras que el antígeno 30–40 kD fue más abundante en el *M. leprae.* Aunque ambos estuvieron presentes en la pared celular, sólo los determinantes del antígeno 30–40 kD fueron accesibles en los bacilos intactos. Los resultados de los ensayos de captura y la cromatografía

de afinidad tanto con L9 como con L4, indicaron que el antígeno 4.5–6 kD fue probablemente un fragmento del de mayor peso molecular. Ambos antígenos son inmunogénicos en los humanos.

RÉSUMÉ

Des anticorps monoclonaux (Mabs) dirigés contre des mycobactéries de la lèpre (*Mycobacterium leprae*) traitées aux ultrasons ont permis de mettre en évidence deux déterminants distincts sur des antigènes hydrocarbonés associés à la paroi cellulaire, communs à *M. leprae*, à *M. bovis* (BCG) et à *M. tuberculosis*. Un test de détection d'inhibition par ELISA et un test de capture d'antigène ont démontré que les deux antigènes étaient présents dans une fraction de paroi cellulaire obtenue après une répétition du traitement de *M. leprae* aux ultrasons. On a constaté une variation dans la distribution des antigènes, selon l'espèce de mycobactérie. L'antigène 4.5–6 kD était plus abondant chez *M. tuberculosis* et *M. bovis*; l'antigène 30–40 kD était plus concentré dans les préparations de *M. leprae*. Alors que les deux antigènes étaient présents dans la paroi cellulaire, seuls les déterminants mis en évidence sur l'antigène de 30–40 kD étaient accessibles chez des bacilles intacts. Les résultats des essais de capture, de même que l'affinité chromatographique des anticorps monoclonaux pour L9 et L4, indiquaient que l'antigène 4.5–6 kD était probablement un fragment d'une molécule volumineuse. Les deux antigènes se sont révélés être des immunogènes significatifs pour induire une réponse des cellules B humaines à *M. leprae*.

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REFERENCES

1. Azuma, I., Kimura, H., Niinaka, T., Aoki, T. and Yamamura, Y. Chemical and immunological studies on mycobacterial polysaccharides. 1. Purification and properties of polysaccharides from human tubercule bacilli. J. Bacteriol. **95** (1968) 263–271.
2. Britton, W. J., Hellqvist, L., Basten, A. and Raison, R. L. *Mycobacterium leprae* antigens involved in human immune responses. 1. Identification of four antigens by monoclonal antibodies. J. Immunol. **135** (1985) 4171–4177.
3. Chakrabarty, A. K., Maire, M. A. and Lambert, P. H. SDS-PAGE analysis of *M. leprae* protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos. Clin. Exp. Immunol. **49** (1982) 523–531.
4. Chaparas, S. D., Thor, D. E., Godfrey, H. P., Baer, H. and Hedrick, S. R. Tuberculin-active carbohydrate that induces inhibition of macrophage migration but not lymphocyte transformation. Science **170** (1970) 637–639.
5. Daniel, T. M. The antigenicity in guinea pigs and monkeys of three mycobacterial polysaccharides purified by affinity chromatography with concanavalin A. Am. Rev. Respir. Dis. **110** (1975) 634–641.
6. Daniel, T. M. and Hinz, C. F. Reactivity of purified proteins and polysaccharides from *Mycobacterium tuberculosis* in delayed skin test and cultured lymphocyte mitogenesis assays. Infect. Immun. **9** (1974) 44–47.
7. Daniel, T. M. and Janicki, B. W. Mycobacterial antigens: a review of their isolation, chemistry and immunological properties. Microbiol. Rev. **42** (1978) 84–113.
8. Draper, P. Cell walls of *Mycobacterium leprae*. Int. J. Lepr. **44** (1976) 95–98.
9. Draper, P. The anatomy of mycobacteria. In: *The Biology of the Mycobacteria*. Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, vol. 1, pp. 9–52.
10. Draper, P. Wall biosynthesis: a possible site of action for new antimycobacterial drugs. Int. J. Lepr. **52** (1984) 527–532.
11. Ellner, J. J. and Daniel, T. M. Immunosuppression by mycobacterial arabinomannan. Clin. Exp. Immunol. **35** (1979) 250–257.
12. Engers, H., Abe, M., Bloom, B. R., Mehra, V., Britton, W., Buchanan, T., Khanolkar, S. K., Young, D. B., Closs, O., Gillis, T., Harboe, M., Ivanyi, J., Kolk, A. H. J. and Shepard, C. C. Results of a World Health Organization (WHO) sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **48** (1985) 603–605.
13. Gillis, T. P. and Buchanan, T. M. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **37** (1982) 172–178.
14. Goren, M. B. Immunoreactive substances of mycobacteria. Am. Rev. Respir. Dis. **125** Part 2 (1982) 50–69.
15. Greenwood, F. C., Hunter, W. M. and Glover, J. S. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89** (1963) 114–123.
16. Hunter, S. W., Gaylord, H., Stewart, C. and Brennan, P. J. Isolation and preliminary characterization of the major intracellular carbohydrate-containing antigens of *Mycobacterium leprae*. Int. J. Lepr. **53** (1985) 712–713.
17. Imai, K., Moriya, Y., Fujita, H., Tsujisaki, M., Kawaharada, M. and Yachi, A. Immunologic characterization and molecular profile of carcinoembryonic antigen detected by monoclonal antibodies. J. Immunol. **132** (1984) 2992–2997.
18. Ivanyi, J., Sinha, S., Aston, R., Cussell, D., Keen, M. and Sengupta, U. Definition of species specific

and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. Clin. Exp. Immunol. **52** (1983) 528–536.

19. KLASTER, P. R., VAN RENS, M. M. and EGGELTE, T. A. Immunochemical characterisation of *Mycobacterium leprae* antigens by the SDS-PAGE immunoperoxidase technique (SGIP) using patients' sera. Clin. Exp. Immunol. **55** (1984) 537–544.
20. KOLK, A. H. J., MINH, L. H., KLATSER, P. R., EGGELTE, T. A., SJOUKJE, K., DE JONG, S. and VAN LEEUWEN, J. Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis, M. bovis* (BCG) and *M. leprae*. Clin. Exp. Immunol. **58** (1984) 511–521.
21. LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227** (1970) 680–685.
22. MILLER, R. A. and BUCHANAN, T. M. Production and characterization of a murine monoclonal antibody recognizing a shared mycobacterial polysaccharide. Int. J. Lepr. **52** (1984) 461–467.
23. MILLER, R. A., HARNISCH, J. P. and BUCHANAN, T. M. Antibodies to mycobacterial arabinomannan in leprosy: correlation with reactional states and variation during treatment. Int. J. Lepr. **52** (1984) 133–139.
24. MISAKI, A., AZUMA, I. and YAMAMURA T. Structural and immunochemical studies of D-arabino-D-mannans and D-mannans of *Mycobacterium tuberculosis* and other *Mycobacterium* species. J. Biochem. (Tokyo) **82** (1977) 1759–1770.
25. MISAKI, A., SETO, N. and AZUMA, I. Structure and immunological properties of D-arabino-D-galactan isolated from cell walls of *Mycobacterium* species. J. Biochem. (Tokyo) **76** (1974) 15–27.
26. MISAKI, A. and YUKAWA, S. Studies on cell walls of *Mycobacteria*. II. Constitution of polysaccharides from BCG cell walls. J. Biochem. (Tokyo) **59** (1966) 511–520.
27. ROOT, R. K., WOLFF, S. M. and BAER, H. Skin reactivity of human subjects to a polysaccharide component of BCG culture filtrate. Proc. Soc. Exp. Biol. Med. **136** (1971) 403–407.
28. SHAPIRO, M. and ERICKSON, R. P. Evidence that the serological determinant of H-Y antigen is carbohydrate. Nature **290** (1981) 503–505.
29. TOWBIN, H., STAEHELIN, T. and GORDON, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76** (1979) 4350–4354.
30. YOUNG, D. B., FOHN, M. J., KHANOLKAR, S. R. and BUCHANAN, T. M. Monoclonal antibodies to a 28,000 molecular weight protein antigen of *Mycobacterium leprae*. Clin. Exp. Immunol. **60** (1985) 546–552.
31. YOUNG, R. A., MEHRA, V., SWEESTER, D., BUCHANAN, T., CLARK-CURTISS, J., DAVIS, R. W. and BLOOM, B. R. Genes for the major protein antigen of the leprosy parasite *Mycobacterium leprae*. Nature **316** (1985) 450–452.
32. ZAVALA, F., COCHRANE, A. H., NARDIN, E. H., NUSSENZWEIG, R. S. and NUSSENZWEIG, V. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J. Exp. Med. **157** (1983) 1947–1957.