

Most *Mycobacterium leprae* Carbohydrate-Reactive Monoclonal Antibodies Are Directed to Lipoarabinomannan

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Each of more than 30 monoclonal antibodies that had been raised against *Mycobacterium leprae* and previously classified as reactive with carbohydrate was shown to be directed against lipoarabinomannan, a prominent, highly pervasive, myo-inositol-phosphate-containing, cross-reactive antigen within the leprosy bacillus. Some of the antibodies preferentially bound to the lipopolysaccharide of *M. leprae* rather than to that of *Mycobacterium tuberculosis*, suggesting the presence of distinguishing structural features. The presence of alkali-labile inositol 1-phosphate in the lipopolysaccharide from *M. tuberculosis* and its apparent absence from the *M. leprae* product may account for the difference.

In a recent report (7), we presented the chemical characteristics of a lipopolysaccharide from *Mycobacterium leprae* and *Mycobacterium tuberculosis* which we called lipoarabinomannan B (LAM-B), a molecule previously referred to in discussions of the tubercle bacillus as arabinomannan since as a product of alkaline extraction, it was usually ester-free; arabinomannan of *M. tuberculosis* has long been recognized as an antigen (4, 12-16). There are two other major carbohydrate-containing molecules in *M. leprae* and *M. tuberculosis*, resolvable by gel electrophoresis of ethanol extracts (7). One of these was originally called LAM-A (7); however, it is virtually devoid of arabinose (S. W. Hunter, unpublished observations) and hence is now called lipomannan (LM). The other is composed of a mixture of the common mycobacterial phosphoglycolipids, the phosphatidylinositol mannosides (PIM) (6) (S. W. Hunter and G. A. Hirschfield, unpublished observations). Western blot (immunoblot) analysis using a murine monoclonal antibody and human lepromatous leprosy sera identified LAM as the only antigen in the extracts; LM and PIM were not reactive (7). Similar polyacrylamide gel-immunoblot results have been reported by others (1-3, 8, 9, 11); however, in only one instance (3) did human sera bind to bands which resemble our LM and PIM. In the absence of parallel chemical characterization by these earlier workers, it now seems obvious that LAM is the predominant, highly pervasive antigen of *M. leprae*.

After the World Health Organization-sponsored workshop on monoclonal antibodies to *M. leprae* (H. D. Engers and Workshop Participants, Letter, Infect. Immun. 48:603-605, 1985), we screened the antibodies provided by participants at the workshop against the ethanol-soluble carbohydrates of *M. leprae* and *M. tuberculosis*; several reacted much more readily in an enzyme-linked immunosorbent assay (ELISA) with the products from *M. leprae* than with those from *M. tuberculosis*. In response to this unexpected finding, we obtained 13 monoclonal antibodies which had been generally classified as reactive with carbohydrates (Engers et al., Letter) from several laboratories and tested them in parallel with 7 of our antibodies which were known to be reactive with LAM (7) to attempt to answer two questions: do all of

these antibodies recognize only LAM among the soluble carbohydrates of *M. leprae*, and do all show a preference for the *M. leprae*-derived LAM over that from *M. tuberculosis*? We used a combination of immunoblotting and plate ELISA to answer these questions.

The antibodies of the 900 series (901, 903, 904, 906, 908, 920, and 922) were all independent isolates from a fusion described previously (7). A female BALB/c mouse was primed with whole *M. leprae* in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) intraperitoneally and given several boosts of crude LAM extract. Spleen cells were fused with SP20 myelomas by a protocol described elsewhere (7) by using polyethylene glycol (E. Merck AG, Darmstadt, Federal Republic of Germany). Hybrid cells were selected (10) and screened by ELISA for secretion of antibodies reactive with a 70% ethanol extract of delipidated *M. leprae* (7). Six antibodies were isolated and cloned by limiting dilution. Characterization of the antibodies revealed that all six were of the IgG3 subclass and that they could not be distinguished from one another by any comparative ELISA. The heavy-chain types were established in a plate ELISA by using commercially available antibodies (Zymed Laboratories, South San Francisco, Calif.). Line 906 was subcloned two times to produce 906.43, from which ascitic fluid was produced by injection of 10⁷ cells into a pristane (2,4,6,10-tetramethylpentadecane; Aldrich Chemical Co., Inc., Milwaukee, Wis.)-primed BALB/c mouse (7).

The SA1 series of monoclonal antibodies (A6C, A10G, A11C, A12B, A12C, A12D, B10E, C94, D1F, D11E, D11G, and C11H) was produced from a single fusion described by Young et al. (17). The cloned cell lines were chosen initially for binding to a sonic extract of *M. leprae* (5). A group of antibodies which recognized protease-resistant antigens were then rescreened on a crude LAM preparation derived from *M. leprae* by ethanol reflux and phenol extraction (7). Twelve different antibodies capable of binding to this preparation were selected for further testing as ascitic fluids. All were typed as IgG1. Antibody L9 was supplied to us as an ascitic fluid by W. Britton after a similar test on crude LAM prepared as described previously (7). This IgG3 antibody has been described as part of a larger collection (1).

Ethanol extracts of *M. leprae* and *M. tuberculosis*, each containing the full array of soluble bacterial carbohydrates, were electrophoresed in polyacrylamide gels, and the pat-

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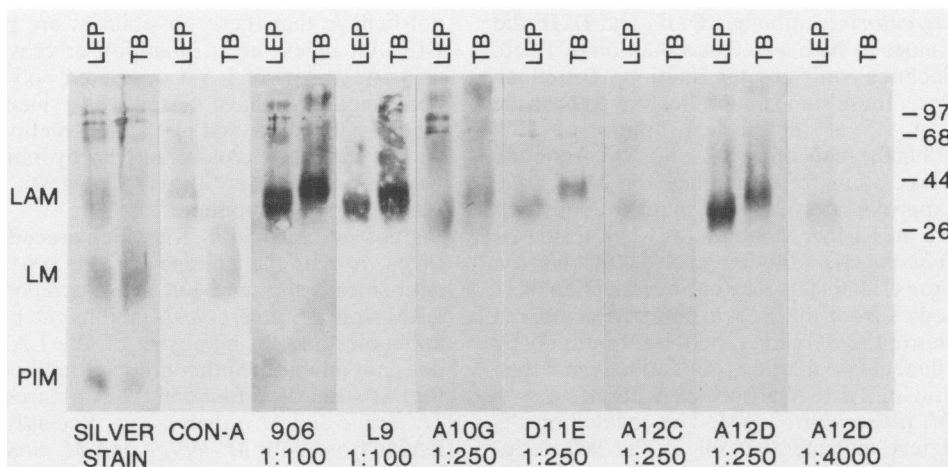


FIG. 1. Results of immunoblotting of polyacrylamide gels of LAM-containing preparations with various monoclonal antibodies. Polyacrylamide slab gels (5 by 8 cm) were polymerized by using 15% acrylamide with 0.016% bisacrylamide (both from U.S. Biochemical, Cleveland, Ohio) as described previously (7). LAM-containing crude preparations were dissolved in loading buffer at a concentration of 2 mg/ml, and 3 μ l was loaded into each well of a Mini Gel device (Bio-Rad Laboratories, Richmond, Calif.) and electrophoresed at a 20-mA constant current. Silver staining was a composite of two methods previously described (7). The polyacrylamide gel patterns were transferred to nitrocellulose (Bio-Rad) in Tris-glycine-methanol buffer by using a constant 4 V/cm field. The nitrocellulose sheets were blocked in 2% (wt/vol) polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) and immersed in antibody or lectin overnight. Antibodies were diluted from ascitic fluid in PBS containing 0.005% (vol/vol) Tween 20 (Sigma) and 1% (vol/vol) normal goat serum (GIBCO Laboratories, Grand Island, N.Y.); biotinylated concanavalin A (Vector Laboratories, Burlingame, Calif.) was diluted 1:500 in PBS containing 0.05% Tween (PBS-Tween). After being rinsed in PBS-Tween, bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse antiserum (immunoglobulin G [IgG], IgM, and IgA) (Cooper Biomedical, Inc., West Chester, Pa.). Bound concanavalin A was detected with horseradish peroxidase-conjugated streptavidin (Zymed). Color development was done with the Bio-Rad 4-chloro-1-naphthol reagent in the presence of H₂O₂ as recommended by the manufacturer. LEP, *M. leprae*; TB, *M. tuberculosis*. Molecular weight markers (in thousands) are indicated on the right.

terns were transferred to nitrocellulose sheets for immunostaining (Fig. 1). These initial extracts were deliberately not further processed (7) and were overloaded onto gels to test the antigenicity of all soluble, electrophoresable carbohydrates. Antibody 906.43, all 12 antibodies of the SA1 series, and the L9 antibody were used to probe the gel patterns; all were used as diluted ascitic fluids at an arbitrary 1:250 dilution. A representative sample of the results, along with a silver-stained gel and a gel probed with concanavalin A, is shown in Fig. 1. In all instances, the antibodies were reactive only with LAM, although some binding to more slowly migrating material could sometimes be seen. Binding to the LM and PIM bands was not observed. Antibodies 906.43 and L9 (both IgG3) reacted equally well with LAM from *M. leprae* or *M. tuberculosis*, whereas the SA1 series antibodies generally reacted better with *M. leprae*-derived LAM. These antibodies were also deliberately overloaded so that binding to the mannosyl units of LM or PIM could be detected, if such existed. When antibody A12D was used at a final dilution of 1:4,000, the binding was seen to be directed exclusively to LAM of *M. leprae*. This was true for five of the SA1 series antibodies tested in this manner (results not shown) and is expected for all of them based on plate ELISA data (see below). Note also that whereas concanavalin A reacted with LAM and LM from *M. leprae*, binding to LAM from *M. tuberculosis* was weak, again pointing to structural differences between the LAMs from the two species.

Five of the monoclonal antibodies (906.43, L9, A10G, D11E, and D11G) were titrated on pure LAM plated on polystyrene plates at 50 ng per well (Fig. 2). Whereas antibodies 906.43 and L9 showed nearly equivalent binding to LAMs from *M. leprae* and *M. tuberculosis*, all antibodies from the SA1 fusion were clearly much more reactive with *M. leprae*-derived LAM. For instance, when titrated on

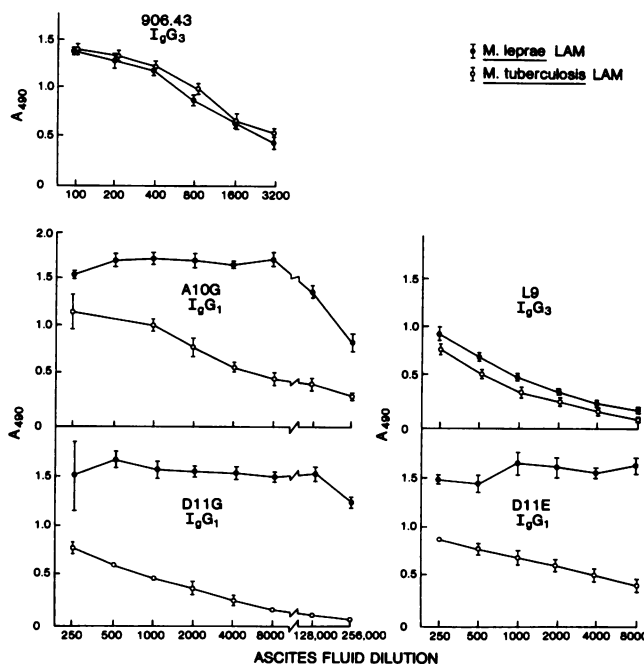


FIG. 2. Titration of pure LAM with five monoclonal antibodies. LAM extracted from lyophilized *M. leprae* or *M. tuberculosis* with refluxing 70% ethanol was purified by the use of a phenol biphasic wash and anion-exchange chromatography in detergent-containing buffer (7). LAM quantitation is in micrograms per milliliter, assayed as mannose equivalent units by the phenol-H₂SO₄ method as described previously (7). Pure LAM was plated onto U-shaped wells of polystyrene plates (Dynatech Laboratories, Inc., Alexandria, Va.) by incubating 50 ng of LAM in 50 μ l of phosphate-buffered saline in each well. The datum points are means for four wells (standard deviations are shown by bars).

LAM from *M. tuberculosis*, antibodies D11G and D11E did not achieve A_{490} values as high as 1.0 at a dilution of 1:250, and the values declined with greater dilution. Tested on LAM from *M. leprae*, these same antibodies stayed near the saturation value of 1.5 at the highest dilutions tested (1:256,000 and 1:8,000, respectively) (Fig. 2). Antibody A10G showed a less dramatic preference for *M. leprae*-derived material: approximately 250-fold more antibody was required to achieve an ELISA value of 1.0 when tested on LAM from *M. tuberculosis* (dilution of 1:1,000) than on LAM from *M. leprae* (1:256,000). The entire collection of 12 monoclonal antibodies from the SA1 fusion was compared with the 7 antibodies of the 900 series and the L9 antibody by testing the ascitic fluid at two dilutions per antibody and then comparing the ratio of ELISA absorbance for *M. leprae* LAM to that for *M. tuberculosis* LAM. The collated results of this simplified test indicate that all of the 900 series antibodies and L9 can be grouped together, reacting equally well with the LAM from either source. However, all of the SA1 antibodies form a second group, being up to fivefold more reactive with the LAM from *M. leprae* than with that from *M. tuberculosis*. Thus, the 30 anti-LAM antibodies fall into two distinct groups. One group, comprising the 900 series monoclonal antibodies and antibody L9 (1), are all of the IgG3 subclass and reacted equally well with the LAMs of *M. leprae* and *M. tuberculosis*. The 12 antibodies in the SA1 series are of the IgG1 subclass and were much more readily reactive with the LAM of *M. leprae* than the LAM of *M. tuberculosis*. This distinct grouping of antibodies forces us to conclude that the LAM molecules extracted from the two species are different. The difference in concanavalin A binding and the slight but reproducible difference in electrophoretic mobility also indicate some qualitative distinction between the two LAM molecules.

An ELISA reading is an empirical value which is dependent on the amount of antigen on the plate, the affinity of the antibody-antigen interaction, and the kinetics of binding. Many antibodies display diffusion-controlled binding kinetics in solution, with affinities strong enough for binding to be considered irreversible for practical purposes. For these antibodies, the ELISA reading is a measure of antigen when antibody is saturating and a measure of antibody when antigen is in excess. Antibodies of high affinity having slow on-rate kinetics will give ELISA readings which are a more complex function of both antibody and antigen concentrations unless care is taken to be certain that equilibrium is reached (at long times). Low-affinity antibodies are characterized by a low on-rate/off-rate ratio; the individual rates may be fast or slow. If both rates are slow, then the ELISA reading will be a measure of affinity if equilibrium is reached (at long times), but if both rates are fast, dissociation of bound antibody may change from the equilibrium value during the washing steps and again be a complex function of antibody and antigen concentrations.

The straightforward interpretation of the data presented here is that two distinct epitopes exist, one defined by the 900 series antibodies and L9 and the other defined by the SA1 series. The first is present in LAMs of both *M. leprae* and *M. tuberculosis*. The second is found on the LAM from *M. leprae*, but not necessarily exclusively so. Since the SA1 series of antibodies showed significant binding to the LAM from *M. tuberculosis* at high concentration (a 200-fold increase in concentration for antibody A10G; Fig. 2), an epitope similar to that on the *M. leprae* product exists in *M. tuberculosis*. The SA1 antibodies would be interpreted as having lower affinity for this *M. leprae*-like structure. We do

not believe that these possibilities are resolvable by using ELISAs, unless clear inhibition or cross-reactivity studies using fragments of LAM or related polysaccharides can be performed. We have tested yeast mannan, mannose, α -methylmannoside and α - and β -methylarabinofuranoside without detecting cross-reactivity by inhibition ELISA. The work of Misaki et al. (12) suggests an $\alpha(1\rightarrow5)$ -linked arabinofuranosyl epitope.

Another explanation, for which precedents are not known to us, is that the epitope for the 900 and SA1 series of antibodies is the same but that a component present on the LAM from *M. tuberculosis* inhibits the binding of one set of antibodies and, being absent on the LAM from *M. leprae*, does not inhibit the other group of antibodies. Some support for this scenario is found in the structures of the LAMs from *M. leprae* and *M. tuberculosis*. Recently, we reported that LAMs from both *M. leprae* and *M. tuberculosis* are unexpectedly complex, containing arabinose, mannose, glycerol, and *myo*-inositol 1-phosphate, in addition to lactate, succinate, palmitate, and 10-methyloctadecanoate (7). Since then, we have found that the long-chain fatty acids, glycerol and inositol phosphate, in the LAMs from both *M. leprae* and *M. tuberculosis* occur as covalently bound phosphatidylinositol (S. W. Hunter and P. J. Brennan, unpublished observations). However, in addition, LAM from *M. tuberculosis* also contains alkali-labile inositol phosphate (7), presumably in phosphodiester linkage to some of the sugar units; inositol phosphate in this form appears to be absent from the *M. leprae* product. Accordingly, it can be suggested, pending further evidence, that the presence of such inositol 1-phosphates on the LAM from *M. tuberculosis* partially inhibits binding of the SA1 series antibodies to $\alpha(1\rightarrow5)$ -linked arabinofuranosyl, the primary epitope, but does not affect recognition of the same epitope by the 900 series of antibodies. In view of the dominant immunogenicity of LAM, its copiousness, and its peculiar pervasiveness in all subcellular fractions of *M. leprae*, these are important questions that perhaps only detailed chemical analysis will answer.

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LITERATURE CITED

1. Britton, W. J., L. Hellqvist, A. Basten, and R. L. Raison. 1985. *Mycobacterium leprae* antigens involved in human immune responses. *J. Immunol.* **135**:4171-4177.
2. Chakrabarty, A. K., M. A. Maine, and P. H. Lambert. 1982. SDS PAGE analysis of *M. leprae* protein antigens reacting with antibodies from sera from lepromatous patients and infected armadillos. *Clin. Exp. Immunol.* **49**:523-531.
3. Coates, S. R., D. Hansen, G. Schecter, G. Slutkin, P. Hopewell, L. Affronti, and D. F. Echenberg. 1986. Identification of *Mycobacterium tuberculosis* antigens in Seibert fractions by immunoblotting. *J. Clin. Microbiol.* **24**:126-130.
4. Daniel, T. M., and B. W. Janicki. 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. *Microbiol. Rev.* **42**:84-113.
5. Gillis, T. P., and T. M. Buchanan. 1982. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. *Infect. Immun.* **37**:172-178.
6. Goren, M. B., and P. J. Brennan. 1979. Mycobacterial lipids: chemistry and biological activities, p. 69-193. *In* G. P. Youmans (ed.), *Tuberculosis*. The W. B. Saunders Co., Philadelphia.

7. Hunter, S. W., H. Gaylord, and P. J. Brennan. 1986. Structure and antigenicity of the phosphorylated lipopolysaccharides of the tubercle and leprosy bacilli. *J. Biol. Chem.* **261**:12345–12351.
8. Ivanyi, J., S. Sinha, R. Aston, D. Cussell, M. Keen, and U. Sengupta. 1983. Definition of species-specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. *Clin. Exp. Immunol.* **52**:528–536.
9. Klatser, P. R., M. M. VanRens, and T. A. Eggelte. 1984. Immunochemical characterization of *Mycobacterium leprae* antigens by the SDS-polyacrylamide gel electrophoresis immunoperoxidase technique (SGIP) using patients' sera. *Clin. Exp. Immunol.* **56**:537–544.
10. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **6**:511–519.
11. Miller, R. A., and T. M. Buchanan. 1984. Production and characterization of a murine monoclonal antibody recognizing a shared mycobacterial polysaccharide. *Int. J. Lepr.* **52**:461–467.
12. Misaki, A., I. Azuma, and Y. Yamamura. 1977. Structural and immunochemical studies on D-arabino-D-mannans and D-mannans of *Mycobacterium tuberculosis* and other *Mycobacterium* species. *J. Biochem.* **82**:1759–1770.
13. Ohashi, M. 1970. Studies on the chemical structure of serologically active arabinomannan from mycobacteria. *Jpn. J. Exp. Med.* **40**:1–14.
14. Seibert, F. B., M. Stacey, and P. W. Kent. 1949. An antigenic polysaccharide, "polysaccharide II," isolated from tuberculin. *Biochim. Biophys. Acta* **3**:632–640.
15. Tsumita, T., R. Matsumoto, and D. Mizuno. 1960. Chemical and biological properties of the hemagglutination antigen, a lipopolysaccharide, of *Mycobacterium tuberculosis, var. hominis*. *Jpn. J. Med. Sci. Biol.* **13**:131–138.
16. Weber, P. L., and G. R. Gray. 1979. Structural and immunochemical characterization of the acidic arabinomannan of *Mycobacterium smegmatis*. *Carbohydr. Res.* **74**:259–278.
17. Young, D. B., M. J. Fohn, S. R. Khanolkar, and T. M. Buchanan. 1985. Monoclonal antibodies to a 28,000 mol. wt. protein antigen of *Mycobacterium leprae*. *Clin. Exp. Immunol.* **60**:546–551.