Subcellular localization of *Mycobacterium leprae*-specific phenolic glycolipid (PGL-I) antigen in human leprosy lesions and in *M. leprae* isolated from armadillo liver

J. BODDINGIUS* and H. DIJKMAN

Department of Dermatology, Research Laboratory, Medical Faculty, Erasmus University, Rotterdam, The Netherlands

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Phenolic glycolipid (PGL-I), an antigen specific to *Mycobacterium leprae*, was localized subcellularly in *M. leprae* residing in human skin, in *M. leprae* isolated from armadillo liver ('isolated *M. leprae*') and outside *M. leprae* in human lepromatous skin. For a quantitative localization of PGL-I sites, specimens, including skin segments stored for 6 years in glutaraldehyde, were embedded in hydrophilic Lowicryl (K₄M) resin for ultrathin sectioning. Ultracryosections and Araldite sections of comparable specimens were used for comparison of localization results. A monoclonal antibody (F 47-21-3) directed to antigenic oligosaccharide of PGL-I was employed as primary antibody in immunogold labelling of ultrathin sections. K₄M-immunogold methods gave very satisfactory quantitative gold-labelling of PGL-I. The localization of PGL-I by this method partially corresponded with sites detectable in both ultracryosections and the qualitatively superior Araldite sections, but new sites were also localized. Cell walls in human *M. leprae* and in isolated *M. leprae* possessed many PGL-I sites, particularly in dividing organisms. PGL-I or its antigenic oligosaccharide was also found, to a lesser extent, in the bacterial cytoplasm. Capsules discernible around part of isolated *M. leprae* cells displayed heavy PGL-I labelling, sometimes clearly confined to a zone distant from the cell wall. Extrabacterial PGL-I in *M. leprae*-infected human skin was encountered (1) in phagolysosomes and cytoplasm proper of dermal macrophages containing *M. leprae*, and (2) intra- and extracellularly in epidermal areas where basal cells harboured *M. leprae* in untreated multibacillary patients.

Introduction

Phenolic glycolipids (PGLs), found in mycobacterial envelopes, are mycosides possessing unusual methylated sugars (Barksdale & Kim, 1977; Minnikin, 1982). PGL-I, the predominant PGL antigen in *Mycobacterium leprae* (Hunter & Brennan, 1981, 1983; Hunter *et al.*, 1982; Fujiwara *et al.*, 1984) may play a role in the intracellular persistence of *M. leprae* in human macrophages by scavenging toxic oxygen metabolites of antimicrobial systems of the host cell (Neill & Klebanoff, 1988). PGL-I or its antigenic oligosaccharide are used on a small scale

in the serodiagnosis of leprosy and in the monitoring of the infection (Cho *et al.*, 1983, 1986; Young & Buchanan, 1983; Agis *et al.*, 1988; Chanteau *et al.*, 1989).

Neither the surface location of PGL-I in *M. leprae*, indicated by immunofluorescence (Young *et al.*, 1984), nor possibly persisting pockets of extrabacterial PGL-I antigen in infected tissues (Boddingius & Dijkman, 1989*a*), have yet been investigated in detail in a quantitatively satisfactory way. The localization of either peribacterial (surface) or intrabacterial (non-surface) PGL-I, and of small amounts of extrabacterial PGL-I, all require immuno-electron microscopic methods. Our previous electron microscopic studies have dealt with the histopathology of human leprosy lesions (Boddingius, 1974, 1976, 1977; Boddingius *et al.*, 1989), histophysiology of lepromatous mouse nerves (Boddingius, 1984) and, recently, the locations of defined antigens – an *M. leprae*-specific lipid antigen and protein (36 kDa; 65 kDa) antigens (Boddingius & Dijkman, 1989*a, b*).

Three procedural steps are essential for immuno-

* Address to which all correspondence concerning the manuscript and requests for reprints should be sent: Dr J. Boddingius, Department of Dermatology, Erasmus University, c/o PO Box 733, 4200 AS Gorinchem, The Netherlands.

Abbreviations: GA, glutaraldehyde; GAMG₁₀, gold (10 nm) labelled goat anti-mouse IgG; mAb, monoclonal antibody; PBS, phosphate-buffered saline (0.05 g KCl, 2 g NaCl, 0.95 g NaH₂PO₄·2H₂O and 2.84 g Na₂HPO₄ in 250 ml distilled water; pH 7.3); PF, paraformaldehyde; PGL-I, phenolic glycolipid-I.

electron microscopic investigation of any *M. leprae* lipid or protein antigen not previously studied. Firstly, ultracyrosectioning and immunogold labelling of 'non-fixed' material containing the antigen(s) is done, using *M. leprae* isolated from the liver of the nine-banded armadillo (*Dasypus novemcinctus* Linn.). Secondly, very mild to 'hard' fixation techniques for the ultrastructural preservation of bacterial cells or of tissues infected by *M. leprae* are employed prior to ultracyrosectioning and immunogold labelling. In this step, the possible influence of fixation on immunogenicity of the antigen(s) should be checked by counting gold-labelled cells. Thirdly, *M. leprae* or infected tissues, after treatment with fixatives, are embedded in resin(s) in order to achieve optimal subcellular immuno-localization of antigens. The first two steps were partially carried out by us in pilot studies on 36 kDa and 65 kDa protein antigens (Boddington & Dijkman, 1989b). All three steps were partially realized in studies of PGL-I using hydrophobic Araldite resin as an embedding medium (Boddington & Dijkman, 1989a). With our new Araldite-immunogold method we obtained accurate subcellular localization of PGL-I in *M. leprae* and extrabacterially in human leprosy skin. However, the method was considered merely qualitative in character, and tissue embedding in Lowicryl was thought to be essential for any quantitative evaluation of PGL-I locations (Boddington & Dijkman, 1989a).

In the present study, Lowicryl K₄M was tested for satisfactory quantitative immunogold localization of PGL-I. Localization was carried out in *M. leprae* isolated from armadillo liver (henceforth called isolated *M. leprae*), in *M. leprae* residing in human skin, and extrabacterially in human skin and armadillo liver. Isolated *M. leprae* were dispersed in gelatin blocks for processing. The human skin segments used were part of a stock of skin and nerve biopsies which had been stored for 6 years in glutaraldehyde. Lowicryl K₄M is a hydrophilic resin which allows good access by antibodies to antigen sites. The resin can be used for tissue embedding both at low (−35 °C) and higher temperatures (up to 60 °C). Our work indicates the efficiency of K₄M embedding for the quantitatively satisfactory demonstration both of PGL-I in *M. leprae* and of extrabacterial PGL-I in human lepromatous skin. An efficient cold-room processing and embedding scheme designed by us renders Lowicryl-immunogold localization of PGL-I widely applicable and also accessible in (sub)tropical countries where leprosy is endemic.

Methods

Armadillo-derived Mycobacterium leprae ('isolated *M. leprae*'). *M. leprae* were isolated from infected armadillo liver following standard-

ized WHO/IMMLEP procedures (World Health Organization, 1980). The isolated *M. leprae* (10^{10} *M. leprae* per ml PBS) were stored at −70 °C (Royal Tropical Institute, Amsterdam, The Netherlands), and transported in liquid nitrogen to Rotterdam for use in the experiments described here.

M. leprae-gelatin blocks. Blocks containing 5×10^6 *M. leprae* per ml gelatin (5%, w/v) were prepared as described previously (Boddington & Dijkman, 1989a).

M. leprae-infected armadillo liver. After dissection from the armadillo, liver segments were stored at −70 °C (Royal Tropical Institute, Amsterdam). Segments were kept in liquid nitrogen for transfer to and storage in our laboratory in Rotterdam.

Skin biopsies. Wedge-shaped skin biopsies taken under local anaesthesia from lesions of subpolar or polar lepromatous leprosy patients presenting at the leprosy polyclinic (Department of Dermatology, Academic Hospital Dijkzigt, Erasmus University, Rotterdam), were divided into small segments immediately after excision from the patient. The segments contained epidermis, dermis and subcutaneous tissue. At the time of biopsy, the patients had been treated with dapsone (DDS; 100 mg d^{−1}) and rifampicin (600 mg d^{−1}) for 3 weeks (patient A), or with DDS, rifampicin and clofazimine (50 mg d^{−1}) for 11 months (patient B), or had not yet received anti-leprosy drug treatment (patient C).

Fixation procedures. For *M. leprae*-gelatin blocks, the following procedures were used. (a) No fixation; direct immersion in pure cryoprotectant (2.3 M-sucrose in 0.1 M-sodium phosphate buffer pH 7.3). (b) Fixation for 20 min in mild fixative added to the cryoprotectant for ultracyrotoomy [CP₂: 2.3 M-sucrose containing 2%, w/v, paraformaldehyde (PF) and 0.25%, v/v, glutaraldehyde (GA) in 0.1 M-phosphate buffer]. (c) Fixation for 2 h in mild fixative or very mild fixative consisting of a mixture of 2% PF and 0.25% or 0.1% GA in 0.1 M-phosphate buffer. This was followed by storage in 2% PF in 0.1 M-phosphate buffer; next, either processing into Lowicryl (see below) or cryoprotection in CP₂ (as above) followed by ultracyrosectioning was done. (d) Fixation for 2 h in 3% GA in 5% sucrose/phosphate buffer (for preparation, see Boddington & Dijkman, 1989a), generally followed by embedding in Lowicryl or by postfixation for 2 h in 1% (w/v) OsO₄, dehydration and embedding in Araldite (see below).

For armadillo liver, mild fixation (2% PF/0.25% GA; procedure c) with omission of storage in 2% PF was employed, followed by ultracyrotoomy; alternatively, fixation procedure d (above) and Araldite embedding were utilized.

For human skin biopsy segments, fixation procedure d was used, involving GA/OsO₄ fixation and Araldite embedding. In addition, fixation and storage (for 6 years) in 3% GA in 5% sucrose/phosphate buffer (fixation procedure e) was employed followed by Lowicryl embedding.

Lowicryl embedding. After either mild fixation (2% PF/0.25% GA) or very mild fixation (2% PF/0.1% GA; see c above), after 'hard' fixation in GA (see d above), or after fixation and long-term storage in GA (see e above), two differing dehydration and embedding schemes in Lowicryl were employed. In the *conventional low-temperature scheme*, carried out at −20 or −30 °C (Balzer type TTP010 low-temperature polymerization apparatus), a relatively long (30–60 min) immersion in graded alcohols (50, 80, 90, 100%, v/v, ethanol) and 1–2 h immersion in ethanol/Lowicryl K₄M mixtures were used. This was followed by embedding in pure K₄M (Chemische Werke Lowi, Waldkraiburg, FRG). In the *short cold-room scheme*, designed by us, dehydration and embedding were carried out at cold-room temperature (~4 °C); the steps were the same as described above for the conventional scheme but each step did not exceed 30 min. In the last step (pure K₄M) in the cold-room, a Chromato Vue Cabinet (UVP Inc., San Gabriel, USA) was

employed to photopolymerize K₄M by ultraviolet illumination. Lowicryl-embedded tissues were stored at room temperature, tightly sealed with parafilm.

Araldite embedding. Dehydration and embedding procedures of specimens fixed by procedure *d* (above) have been described previously (Boddingius & Dijkman, 1989*a*). During dehydration (step 5), overnight alc. 70% followed alc. 60%.

Ultracryosectioning and ultramicrotomy. The methods are described by Boddingius & Dijkman (1989*a*).

Monoclonal antibodies (mAbs). mAb F 47-21-3 specific for *M. leprae* phenolic glycolipid (Kolk *et al.*, 1985), was used.

Gold-labelled secondary antibody. Gold (10 nm) labelled goat anti-mouse IgG (GAMG₁₀; Janssen Life Sciences Product, Beerse, Belgium) was employed at a dilution of 1:50 in 1% BSA in PBS.

Immunogold labelling of ultrathin sections. During labelling at room temperature, grids with sections were floated upside down on liquid drops placed on wide Parafilm sheets covered with a Perspex cover. For ultracryosections and for Araldite sections, labelling schemes have been described in detail elsewhere (Boddingius & Dijkman, 1989*a*); 5% (v/v) H₂O₂ was used for the etching of Araldite sections. For Lowicryl sections, swine serum pretreatment was found unnecessary; hence after brief rinsing in PBS, grids were directly incubated for 30 min in primary antibody (mAb F 47-21-3), diluted 1:1000 in PBS. The labelling procedures were otherwise the same as for Araldite sections.

Semi-quantification of gold labelling. To assess the influence of fixation on PGL-I immunogenicity, relative frequencies were determined of gold-labelled 'non-fixed' *M. leprae* and 'fixed' *M. leprae*, using ultracryosections of *M. leprae*-gelatin blocks. Counts of gold-labelled cells were divided by total numbers (100–400) of organisms screened per grid and then multiplied by 100. Counts were carried out at a magnification of 12000× (Philips EM 300 electron microscope).

Control labelling experiments. In labelling schemes (see immunogold labelling, above), specific primary antibody (mAb F 47-21-3) either was omitted or was replaced by PBS or by control mAb (F 85-10). mAb F 85-10, directed against a distinct peptidoglycolipid of the MAIS complex (*M. avium*/*M. intracellulare*/*M. scrofulaceum*), was used at a dilution of 1:1000 in PBS.

***M. leprae*-specificity testing of mAb F 47-21-3 by immunogold labelling.** Ultracryosections of 'non-fixed' *M. tuberculosis*, *M. nonchromogenicum* and *M. leprae*, each species contained in a separate gelatin block, were subjected to identical immunogold labelling procedures (see above). *M. tuberculosis* and *M. nonchromogenicum* suspensions utilized for the preparation of gelatin blocks consisted of heat-killed (10 min at 80 °C) whole *M. tuberculosis* in PBS and of whole *M. nonchromogenicum* in PBS. Both species had been grown in Sauton's medium.

Results

PGL-I sites in isolated M. leprae and control experiments with M. tuberculosis and M. nonchromogenicum

Ultracryosections. Bacterial substructures, distinguishable in GA/OsO₄-fixed and Araldite-embedded (human) *M. leprae* (Fig. 1*a*), were not clearly imaged in 'non-fixed' ultracryosectioned isolated *M. leprae*. The location of PGL-I given below for ultracryosections is thus approximate.

Capsular material, still surrounding some of the isolated *M. leprae* in a given section, showed gold labelling throughout (see Fig. 5*a*). PGL-I gold labelling in cell wall areas was pronounced, particularly in dividing bacilli (Fig. 1*b*). Intracytoplasmic labelling of PGL-I or its antigenic oligosaccharide, although relatively sparse, was a consistent feature in ultracryosectioned *M. leprae* but, because of the electron density of the bacilli, this is not clearly visible in electron micrographs. The gelatin matrix was practically free from gold particles (Fig. 1*b*).

In control labelling experiments of ultracryosections of 'non-fixed' *M. tuberculosis* and *M. nonchromogenicum*, no gold labelling was seen on *M. nonchromogenicum* (Fig. 2*a*) and no gold labelling was present in capsular regions and cell wall areas of *M. tuberculosis* (Fig. 2*b*). When electron micrographs of 100 immunolabelled *M. tuberculosis* organisms were screened, 7% of the organisms were found to display an occasional gold particle on the cytoplasm (Fig. 2*b*). A comparable low level of gold-labelling was seen intrabacterially in *M. leprae* when control mAb F 85-10 was used as primary antibody. The great majority of *M. leprae* incubated with mAb F 85-10 were free of gold particles (Fig. 2*c*). The results indicated the absence of appreciable cross-reactivity of mAb 47-21-3 with either intrabacterial or surface glycolipids probably occurring in *M. nonchromogenicum*, or with peribacterial (surface, capsular) glycolipids in *M. tuberculosis*.

Ultracryosections of isolated *M. leprae* were also used to test semi-quantitatively the possible influence on PGL-I immunogenicity of PF/GA fixation, which is also used in the Lowicryl methods (see below). For *M. leprae*-gelatin blocks subjected to 20 min or 2 h PF/GA fixation (procedure *c* with 0.25% GA) prior to ultracryosectioning and immunolabelling, relative frequencies of gold-labelled cells were determined (Fig. 3). After 2 h PF/GA fixation, satisfactory numbers of gold-labelled *M. leprae* organisms were still present (Fig. 3). Comparable 2 h PF/GA fixation of infected armadillo liver segments, followed by ultracryosectioning, resulted in distinct PGL-I gold-labelling both on *M. leprae* and outside the bacilli. Such extrabacterial PGL-I was found in the electron-transparent substance in which bacilli were 'floating' in vacuoles of armadillo liver parenchymal cells (illustrated in Boddingius & Dijkman, 1989*b*). This showed that PF/GA fixation of *M. leprae*-infected tissues allows satisfactory demonstration of PGL-I both intra- and extrabacterially.

Araldite sections of isolated *M. leprae* and of infected armadillo liver. Immunogold labelling experiments showed that after 'hard' fixation in GA/OsO₄ and Araldite embedding, PGL-I immunogenicity in infected

Fig. 1. (a, c) Araldite sections of 'hard'-fixed human *M. leprae* either non-labelled (a) or after immunogold labelling of PGL-I (c). (b) Ultracyrosection of 'non-fixed' *M. leprae* isolated from armadillo liver; immunogold labelling of PGL-I. Bars, 100 nm. (a) In the intact, transversely sectioned *M. leprae* cell are visible: nucleoplasm (N), cytoplasm (C), double cell membrane (▶), and peripherally to the membrane the electron-dense part of the cell wall and the cell wall boundary (B). Skin biopsy, lepromatous patient (patient A). (b) Isolated *M. leprae* (M) caught in the process of division and displaying gold labelling of PGL-I in the cell wall (▶), in capsular areas (→) and, occasionally, in the bacterial cytoplasm. (c) Gold labelling of PGL-I (▶) at several sites in the wall of the longitudinally sectioned *M. leprae* cell. Dermal macrophage; same skin biopsy as in (a).

tissues is retained to an acceptable degree (see Boddington & Dijkman, 1989b). This is less so for cell wall PGL-I in isolated *M. leprae*, while *M. leprae* capsules are not detectable (see Boddington & Dijkman, 1989a).

Lowicryl sections. After very mild fixation (procedure c with 0.1% GA) and PF storage of isolated *M. leprae* in gelatin, followed by embedding in Lowicryl (using the long low-temperature scheme or the shorter cold-room

Fig. 2. Control gold labelling of *M. nonchromogenicum* and *M. tuberculosis* with mAb F 47-21-3 (*a, b*) and of *M. leprae* with mAb F 85-10 (*c, d*) and with GAMG₁₀. Gold labelling is absent on the mycobacterial organisms (*a, c, d*) and in the macrophage cytoplasm (*d*). Bars, 100 nm. (*a*) Ultracryosection of 'non-fixed' *M. nonchromogenicum* organisms (Mn) dispersed in gelatin. (*b*) Ultracryosection of 'non-fixed' *M. tuberculosis* organisms (Mt) dispersed in gelatin and occurring in clusters. Gold labelling is absent on cell walls and capsular areas, but a sporadic gold particle is seen on the bacterial cytoplasm of a few organisms (→). (*c*) Ultracryosection of 'non-fixed' isolated *M. leprae* (Ml) in gelatin. (*d*) Lowicryl section with transversely cut (1, 2) and obliquely sectioned (3) *M. leprae* in a dermal host macrophage in a human lepromatous skin lesion (patient C). Lowicryl cold room embedding.

Fig. 3. Semi-quantitative evaluation of the influence of fixation on the immunogenicity of PGL-I in isolated *M. leprae*. , No fixation; , mild fixation for 20 min in CP₂ during cryoprotection; , mild PF/GA fixation (2% PF/0.25% GA) followed by 20 min in CP₂ during cryoprotection.

scheme) the following was observed. Peri- and intrabacterial PGL-I gold-labelling intensities were higher than seen in ultracyosections and Araldite sections, while labelled bacterial structures largely appeared to be identical. After Lowicryl-immunogold labelling procedures, dividing *M. leprae* organisms displayed many more PGL-I sites in the cell wall (Fig. 4a) than were discernible in ultracyosectioned dividing *M. leprae* (Fig. 1b). Also the transverse cell walls of the incompletely separated daughter cells showed gold labelling in Lowicryl preparations and the cytoplasm was labelled in several places (Fig. 4a). In addition, peri-bacterial (capsular) PGL-I was more clearly localized in Lowicryl

sections (Fig. 5b) than in ultracyosections (Fig. 5a). In some Lowicryl-embedded *M. leprae*, capsular PGL-I gold labelling was confined to a narrow zone distant from the cell wall (Fig. 5b). Another finding of interest in Lowicryl sections was that numerous gold particles, not clearly associated with bacterial structures, were found quite evenly dispersed in all of the 'vacuolar spaces' (Fig. 4c) in which individual *M. leprae* or small groups of *M. leprae* resided in the gelatin matrix. Comparable vacuolar spaces surrounding *M. leprae* in ultracyosections (Fig. 1b) or in Araldite sections contained very few or no gold particles. No appreciable differences were seen between gold labelling of PGL-I after low-temperature Lowicryl embedding (Fig. 4a) and after cold-room Lowicryl embedding (Fig. 5b) of *M. leprae* taken from the same suspension.

M. leprae-gelatin blocks which had been 'hard'-fixed in GA (procedure d) before Lowicryl embedding showed labelling results comparable to those obtained after very mild fixation.

Thus none of the fixation procedures employed, including storage for 1–7 d in 2% PF, impaired PGL-I immunogenicity to an unacceptable degree.

Intra- and extrabacterial PGL-I in human leprosy skin biopsies embedded in Lowicryl or Araldite

Araldite sections. In sections of 'hard' (GA/OsO₄)-fixed and Araldite-embedded human skin (patient A; fixation procedure d) PGL-I gold labelling was seen, in dermal macrophages containing *M. leprae*, in the following sites: (1) on cell walls of electron-dense bacilli representing viable *M. leprae* (Figs 1c, 5c) and on cell walls of degenerating *M. leprae* (Fig. 5c, d), (2) on debris, possibly of bacterial origin, occurring in phagolysosomal embedded isolated *M. leprae*, however, showed a more precise immuno-localization of PGL-I (Fig. 5b), suggesting that capsular PGL-I mainly occurs in a confined zone about 85 nm from the cell wall. This 'PGL-I zone' seems to agree with the position suggested for phenolphthiocerol-based mycosides in mycobacterial envelopes

Fig. 4. Immunogold labelling of Lowicryl sections with mAb F 47-21-3 and GAMG₁₀. (a) Isolated *M. leprae* caught in the process of division. Note many gold labelled PGL-I sites in the cell wall (→), including the new transverse cell walls (⇒) which separate the daughter cells. In several places the bacterial cytoplasm contains gold-labelled sites (▶) which represent either PGL-I sites or locations of its antigenic oligosaccharide. Fixation procedure c (2% PF/0.1% GA); low-temperature Lowicryl embedding. Bar, 50 nm. (b) Electron-dense *M. leprae* residing in human dermal macrophage in skin lesion (patient C). Note PGL-I labelling in cell wall areas of *M. leprae* (→) and, outside the bacillus, in the electron-transparent substance (ETS) of the phagolysosomal vacuole. Fixation (6 years) in 3% GA. Lowicryl cold-room embedding. Bar, 100 nm. (c) Heavy gold labelling (→) of (?)soluble PGL-I in vacuoles which contain isolated *M. leprae* (M). Cell walls (▶) and cytoplasm of *M. leprae* are also labelled. The gelatin matrix (G) is practically free from gold particles. Fixation procedure c (2% PF/0.1% GA). Lowicryl cold-room embedding. Bar, 100 nm. (d) Extrabacterial PGL-I (→) occurring in the electron-transparent substance (ETS) in a phagolysosomal vacuole also containing *M. leprae* (M) with gold-labelled PGL-I sites. Note that PGL-I is not expressed on the exterior of the macrophage's cell membrane (▶). The extracellular space (ES) does not show PGL-I labelling. Fixation (6 years) in 3% GA. Lowicryl cold-room embedding. Bar, 100 nm.

large vacuoles (Fig. 5*d*), (3) on the boundary between phagolysosomal vacuoles and the macrophage cytoplasm proper (Fig. 5*c*), and (4) in small vacuoles of *M. leprae*-containing lysosomes, which have a finely foamy appearance (Fig. 5*d*). The cytoplasm proper of dermal macrophages only occasionally showed gold labelling. In the skin biopsy of patient B (treated for nearly 1 year with anti-leprosy drugs), PGL-I labelling was seen in a few places only, notably in large phagolysosomal vacuoles containing bacterial debris in 'foamy' dermal macrophages.

Lowicryl sections. Prior to Lowicryl embedding, skin biopsy segments had been 'hard'-fixed in GA followed by storage for 6 years in this fixative (fixation procedure *e*). The first contact with GA-fixative had been within seconds of excision of the biopsy from the patient.

In dermal macrophages, intrabacterial PGL-I gold labelling was seen in the cytoplasm and cell wall of *M. leprae* and just outside the bacillus, possibly in the former capsular area (Fig. 4*b*). Approximately 20% of the *M. leprae* organisms in a given skin section showed gold labelling. Extrabacterial PGL-I gold labelling was seen in two major positions in the dermal host macrophage: (1) in the electron-translucent substance surrounding the 'PGL-I positive' bacilli in phagolysosomal vacuoles (Fig. 4*b, d*), and (2) in the cytoplasmic stroma of the macrophage. PGL-I gold labelling was not a consistent feature in the electron-translucent substance of all the *M. leprae*-containing vacuoles: in the same 'foamy' macrophage, some *M. leprae*-containing vacuoles were abundantly labelled, other vacuoles not at all. The outer surface of the cell membrane of dermal macrophages containing *M. leprae* and extrabacterial PGL-I showed no PGL-I gold-labelled sites (Fig. 4*d*). Certain other cells, containing many *M. leprae* bacilli, and surrounded by a basal lamina, showed no PGL-I gold labelling. These cells were possibly Schwann cells of tiny dermal nerves, as such cells were readily observed in non-labelled Araldite sections of the same biopsy.

In the epidermis, extrabacterial PGL-I gold labelling was found in the cytoplasm of *M. leprae*-containing epidermal basal cells and in extracellular spaces between

such basal cells. The skin segments used were too narrow to contain adequate numbers of Langerhans' cells for the possible involvement of these cells in PGL-I expression to be evaluated.

No significant differences in amounts of gold labelling of PGL-I were seen when we compared results obtained after conventional low-temperature Lowicryl embedding and after cold-room embedding of segments from the same biopsy. Some reduction in the intensity of the electron beam was necessary during examination of Lowicryl sections of cold-room embedded specimens in order to avoid disruption of the electron-translucent substance in *M. leprae*-containing macrophages.

Ultrastructural details of bacilli and of host cells, in both types of Lowicryl sections, were not preserved to such a satisfactory degree as in skin segments which had been post-fixed with OsO₄ and embedded in Araldite.

Control labelling of Lowicryl and Araldite sections

When mAb F 47-21-3 was omitted or was replaced by PBS or by control mAb F 85-10 (Fig. 2*d*), which is not directed against PGL (see Methods), no gold labelling was observed in any of the sections.

Discussion

In previous studies on PGLs, the localization of PGL-I in *M. leprae* and in *M. leprae*-infected tissue has been rather superficial. As PGL-I was easily separable mechanically from *M. leprae*, the antigen was thought to occur in the bacterial capsule, and this was supported by immunofluorescent staining of whole *M. leprae* (Young *et al.*, 1984).

Our Lowicryl-immunogold procedures presented here, and immunolabelling results obtained on ultracryosections, give semi-quantitative results and show convincingly that PGL-I occurs in the bacterial capsular area. In ultracryosections, ragged material thought to represent remnants of capsular material showed randomly distributed gold labelling (Fig. 5*a*). Some of the Lowicryl-

Fig. 5. Comparison between immunogold labelling of PGL-I in 'non-fixed' ultracryosectioned isolated *M. leprae* (*a*), in very mildly fixed and PF-stored Lowicryl- (cold-room) embedded isolated *M. leprae* (*b*), and in *M. leprae* residing in 'hard'-fixed and Araldite-embedded human skin, also showing extrabacterial PGL-I (*c, d*). (*a*) PGL-I labelling (→) is seen throughout the bacterial capsular material of isolated *M. leprae* (M). Note that the gelatin matrix (G) is free from labelling. Bar, 140 nm. (*b*) Longitudinally sectioned isolated *M. leprae* (L) and transversely cut organism (T) showing gold labelling on cell wall areas (→) and on capsular areas (→). Note that most of the gold particles in capsular areas of L are confined to a zone located approximately 85 nm from the cell wall. Bar, 100 nm. (*c*) Human dermal host macrophage displaying gold particles on the cell wall (→) of electron-dense *M. leprae* and on the cell wall of a degenerating *M. leprae* (O→), at the boundary between lysosome and macrophage cytoplasm and in the macrophage cytoplasm (MP) (⇔). Bar, 100 nm. (*d*) Extrabacterial PGL-I (→) is present on (?) bacterial debris in vacuolar space (VS) also containing *M. leprae*. Cell walls show PGL-I labelling (→) in degenerating *M. leprae*, and PGL-I is also labelled (●→) in the finely vacuolar lysosome (L). Sporadic labelling is seen on the macrophage cytoplasm (MP; O→). Bar, 100 nm.

on the basis of the likely location of envelope components (Minnikin, 1982; Brennan, 1988). In these models of the mycobacterial envelope, phenol-phthiocerol-based mycosides are located in a hydrophobic interaction region, in the periphery of the envelope, with their hydrophilic oligosaccharide moieties projecting outside this region.

In addition, we have localized PGL-I (or its antigenic oligosaccharide) in other substructures of *M. leprae*, predominantly in the cell wall and, to a lesser extent, in the bacterial cytoplasm. The gold-labelled sites seen in the *M. leprae* cytoplasm could in part represent sites of synthesis of specific oligosaccharide; they deserve further study. The very sporadic labelling in the cytoplasm that was observed in about 7% of the *M. tuberculosis* organisms screened may represent specific labelling, as it was not observed in *M. nonchromogenicum*. If so, this would seem to suggest a slight cross-reactivity of mAb F 47-21-3 with intracytoplasmic compounds in *M. tuberculosis*. The significance of this observation remains to be elucidated. There was no cross-reactivity of mAb F 47-21-3 with capsular (envelope) compounds of *M. tuberculosis* and *M. nonchromogenicum*, thus supporting the finding of *M. leprae* specificity of mAb F 47-21-3 in immunofluorescence tests on whole mycobacteria (Kolk *et al.*, 1985).

Hydrophilic Lowicryl (K₄M) resin, used here as an embedding medium for immunodetection of PGL-I, offers reasonably good resolution for transmission electron microscopy of, amongst others, bacteria and mitochondria (Armbruster *et al.*, 1982). K₄M resin has not previously been used for the immunocytochemical study of glycolipids; it is generally used for immunodemonstration of proteins (e.g. enzymes) in tissues (Roth *et al.*, 1981; Roth & Berger, 1982; Kellenberger *et al.*, 1987). One important finding we made is that Lowicryl sections give more satisfactory quantitative immunogold labelling of PGL-I than do ultracryosections or Araldite sections. Secondly, it was shown that neither (very) mild nor 'hard' fixation, even 'hard' fixation of very long (6 years) duration, influenced PGL-I immunogenicity to an unacceptable degree. This discovery offers numerous possibilities for the future use (for the immunocytological or histological demonstration of PGL-I) of a wide range of leprosy tissues stored in various fixatives. Izumi *et al.* (1985) previously showed PGL-I seropositivity in infected human lepromatous liver kept for a long time in formalin.

The PGL-I sites shown here in Lowicryl sections compared well with, and were present in larger amounts than, those observed in ultracryosections and Araldite sections of isolated *M. leprae*. An abundance of PGL-I sites was seen in the cell wall of many isolated *M. leprae*, particularly in dividing organisms, in which new cell wall

and capsular material must be synthesized. Dividing *M. leprae* organisms were encountered very infrequently: not more than one pair of longitudinally sectioned dividing *M. leprae* was seen in all sections examined of a given *M. leprae*-gelatin block. This prevented a more quantitative evaluation of PGL-I in dividing organisms. Precise quantification of PGL-I immunogold labelling was not attempted in our study. However, the heavy labelling we obtained in individual isolated *M. leprae* by the Lowicryl-immunogold method is compatible with the biochemical report by Cho *et al.* (1986) that 2% of the mass of *M. leprae* is composed of PGL-I.

The subcellular location of PGL-I in *M. leprae* located *in situ* in human skin lesions agreed well with that seen on isolated *M. leprae*. However, while most of the isolated *M. leprae* examined in a given Lowicryl section showed gold labelling, only some (at most 20%) of the *M. leprae* residing in skin segments of an untreated multibacillary patient showed gold-labelled sites in Lowicryl sections. In view of the large amounts of solid (viable) *M. leprae* occurring in these skin segments this was an unexpected finding. Possible explanations are that mAb F 47-21-3, directed against PGL-I from isolated *M. leprae*, reacts best with PGL-I in isolated *M. leprae*, and less well with human *M. leprae*, or that the long (6 years) duration of 'hard' GA fixation to which the human *M. leprae* had been subjected reduced the immunogenicity of PGL-I. However, the presence of heavily labelled *M. leprae* (Fig. 4b) next to non-labelled *M. leprae* organisms in Lowicryl sections of human skin, seems to disallow the second possibility and to weaken the first. A third possibility, which we consider the most likely explanation, is that the *M. leprae* organisms isolated from armadillo liver were all in a biochemically active state in which ample PGL-I is present in the cell wall and capsule, while many of the *M. leprae* residing in macrophages in human skin were in a biochemically less active state. The possible effect of the nature of the host tissue on PGL-I quantities should be investigated further. This could be done by comparing labelling results obtained with freshly excised or liquid-nitrogen-stored segments from infected armadillo liver with those obtained with freshly excised segments of skin lesions (and, where possible, with autopsied or formaldehyde-stored liver) from untreated multibacillary patients.

The present study does not differentiate between the possible occurrence of soluble PGL-I antigen and membrane- (or otherwise structurally-) bound PGL-I. Our observations, in Lowicryl sections, of abundant extrabacterial PGL-I in all the vacuoles containing isolated *M. leprae* within gelatin blocks suggest that our Lowicryl embedding procedures may have prevented soluble PGL-I from diffusing out of *M. leprae*-gelatin blocks into lipid-solvents employed in dehydration.

Whether the PGL-I sites demonstrated in Lowicryl sections in the human dermal macrophage cytoplasm proper likewise present soluble PGL-I remains to be elucidated.

Our observations of extrabacterial PGL-I are in agreement with earlier conclusions that PGL-I must be present extrabacterially in *M. leprae*-infected armadillo liver (Hunter & Brennan, 1983) and in skin from humans infected with leprosy (Young, 1981), since the biochemically shown quantities of PGL-I or of bacterial lipid were far in excess of that expected from the bacillary load.

Sites of extrabacterial PGL-I occurrence in human skin were identified in Lowicryl-embedded specimens, both in a very satisfactory quantitative manner and in a wider distribution than seen in the qualitatively superior Araldite sections. Extrabacterial PGL-I was found in the basal epidermal cells containing *M. leprae* and just outside these cells in an untreated multibacillary patient. The many *M. leprae*-containing dermal macrophages encountered failed to show gold labelling at the outside of the macrophage cell membrane (Fig. 4*d*). This suggests that in the skin areas of multibacillary patients studied here, PGL-I was not expressed by macrophages. In this context, the low amount of HLA-DR antigen expressed by inflammatory cells in hyperbacillary forms of leprosy (Collings *et al.*, 1985) is interesting. The possible association between these phenomena should be investigated by double immunolabelling to identify both antigens simultaneously. The varying degree of extrabacterial PGL-I gold labelling seen, in Lowicryl sections, in *M. leprae*-containing vacuoles of the same dermal macrophage may reflect differences, in either metabolism or growth, between individual clusters of *M. leprae*.

The drawback of the Lowicryl method in immunolabelling studies of tissues is the poorer preservation of ultrastructural details. To complement each other in PGL-I localization studies, Lowicryl and Araldite embedding should preferably be carried out concomitantly for segments of the same skin biopsy or leproma.

In a preliminary test, we were unable to demonstrate satisfactorily the *M. leprae*-specific 36 kDa and the 65 kDa protein antigens in very mildly fixed and low-temperature Lowicryl-embedded isolated *M. leprae*. A combination of storage of isolated *M. leprae* in the deep-freeze, subsequent thawing, and ensuing fixation may be responsible for these negative findings. The only technique thus far found suitable for both these protein antigens has been ultracryosectioning of 'non-fixed' specimens (Boddington & Dijkman, 1989*b*). We recommend that for future simultaneous demonstration of PGL-I and protein antigens in any study on possible differences in the expression of lipid and protein antigens by macrophages or Langerhans' cells, freshly excised and 'non-fixed' *M. leprae* or *M. leprae*-infected

tissue segments are sectioned by advanced ultracryotomy. Next, double-immunogold labelling should be done as described by Geuze *et al.* (1981).

In conclusion, the results presented here show the suitability of Lowicryl embedding for the quantitative demonstration of intra- peri- and extrabacterial PGL-I antigen sites in skin from human leprosy cases and in *M. leprae* isolated from armadillo liver. Araldite embedding gave qualitatively the best immunogold labelling results. The cold-room Lowicryl embedding variant, employing a cost-effective apparatus, proved to be highly efficient for demonstration of PGL-I. Similar methods to those established here for one important lipid, PGL-I, deserve to be tested for another immunodominant glycolipid of *M. leprae*, namely lipo-arabinomannan (LAM-B; Hunter *et al.*, 1986), which has to date been the subject of only a single light-microscopic investigation (Khanolkar *et al.*, 1989).

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