Purification of Phenolic Glycolipid I from Armadillo and Human Sources

TO THE EDITOR:

Phenolic glycolipid I [1-3] has become an important tool for the specific serodiagnosis of leprosy [4-9] and is capable of inducing suppression of mitogenic responses of lepromatous leprosy patient lymphocytes [10] and thus may be implicated in the aberrant cell-mediated immune response in lepromatous leprosy. Phenolic glycolipids and the related nonantigenic and nonspecific dimycocerosylphthiocerol [11, 12] may also be implicated in the intracellular persistence of the leprosy bacillus [12]. The original protocols for the purification of phenolic glycolipid I (PGL-I) from infected armadillo tisues [2, 3, 13] resulted in preparations that were of the order of 90% pure. Because of the importance of the glycolipid and the need for large amounts of material of optimal purity, we have further modified the protocol and now present it in the form of 22 detailed steps, with three alternative strategies for final purification.

Although there is immunological evidence for the presence of PGL-I, or more precisely, its antigen determinant, 3,6-di-*O*-methyl-β-D-glycopyranoside, in human lepromatous leprosy tissue [14], direct chemical evidence is scant [15]. We now also describe in an accompanying Letter to the Editor the preparation of pure PGL-I from human lepromatous nodules and provide chemical evidence for its complete structural concordance with the product from the armadillo source.

Purification of PGL-I from infected armadillo tissue

1. *Mycobacterium leprae*-infected armadillo livers and spleens are obtainable from Dr. Eleanor Storrs, Division of Comparative Mammalogy and Biochemistry, Medical Research Institute, Florida Institute of Technology, 3325 West New Haven Avenue, Melbourne, Florida 32901, through Dr. Darrel D. Gwinn, Leprosy Program Officer, Bacteriology and Virology Branch, NIAID, Westwood Building—Room 738, 9000 Rockville Pike, Bethesda, Maryland 20205. Homogenize the tissue in 25 g lots according to the procedure of Draper [16], but without detergent [3], and in the presence of 1 mM benzamidine [14]. Centrifuge at 10,000 × *g*. The pellet is used as a source of *M. leprae*. The supernatant is lyophilized and weighed and used as a source of glycolipid.

2. Extract with 40 ml $CHCl_3$:CH_3OH (2:1) per g of material for 18 hr in a 50°C water bath in a large Erlenmeyer flask. Keep flask covered but not sealed, since $CHCl_3$ is volatile. Usually 80 g of lyophilized supernatant is processed at one time.

3. Remove flask from water bath. Filter extract through two Eaton-Dikeman, grade 515, 32 cm, fluted filter papers (VWR Scientific, P.O. Box 7900, San Francisco, California 94120) into an appropriate size vessel. Return the filter papers and residue to the original flask and re-extract with the same amount of $CHCl_3$:CH_3OH (2:1), for 18 hr at 50°C. Filter the second extract as before, combine the extracts, dry on a rotary evaporator, and weigh (typically 20 g).

4. Perform a biphasic wash using 400 ml $CHCl_3$, 200 ml CH_3OH, and 100 ml H_2O per 20 g of extract. Mix the dried extract with ½ volume of $CHCl_3$ and CH_3OH; pour into an appropriate size separatory funnel. Rinse the flask with the remaining volume of $CHCl_3$ and CH_3OH and add to the solution in the separatory funnel. Add the requisite amount of H_2O. Shake gently, and allow the mixture to separate into two distinct phases. Collect the lower organic phase, dry, and weigh (typically 8 g). Remove the interfacial residue and aqueous phase for discarding or storage.

5. Build a Florisil column using 20 g Florisil (Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennsylvania 15219) per g of organic phase crude lipid. Weigh Florisil in beaker. Use a 5 × 50 cm glass column (Chromaflex; Kontes of California, 3045 Teagarden Street, San Leandro, California 94577). The volume of Florisil will be approximately 2 cm^3/g. Fit with a stopcock, end piece, clamp, filter disk, and gasket. Set the column perpendicular with a spirit level. Pour some $CHCl_3$ into the column to check for leaks around the gasket and stopcock.

6. Add enough $CHCl_3$ (e.g., 2 ml/g) to the Florisil in a beaker to make a thin slurry. Pour the slurry into the column. Open the stopcock allowing the $CHCl_3$ to exit. Tapping the column with a cork ring helps pack Florisil as the $CHCl_3$ exits. Record the final Florisil height (e.g., 22 cm). Rinse the beaker and upper column with more $CHCl_3$. Wash the Florisil with one bed volume of $CHCl_3$ [bed volume = column radius^2 × 3.14 × Florisil height; e.g., (2.5 cm)^2 × 3.14 × 22 cm = 341 ml]. Close the stopcock when the $CHCl_3$ meniscus reaches the top of the Florisil.

7. Dissolve the dried crude lipid sample in a few ml (<10 ml) of $CHCl_3$ (warming helps to dissolve the sample). Using a Pasteur pipette, apply the sample down the sides of the column so as not to disturb the Florisil top. Rinse the flask with $CHCl_3$ (1–2 ml) to allow ap-

plication of all of the sample. Open the stopcock allowing the sample to flow into the Florisil, close the stopcock as the sample meniscus flows into the top of the Florisil.

8. Elute first with twice the bed volume of $CHCl_3$ as follows: Carefully apply $CHCl_3$ with a Pasteur pipette until approximately 2 inches of solvent are on top of the Florisil. Do not disturb the top of the Florisil bed. Carefully add the rest of the $CHCl_3$ with a funnel, pouring the solvent down the side of the column. Open the stopcock so that the effluent slowly drips into a clean, round-bottom flask. Allow the effluent to drain until the solvent meniscus reaches the Florisil top. Discard the $CHCl_3$ fraction.

9. Repeat step 8 using 2% CH_3OH in $CHCl_3$, except instead of collecting in bulk, collect the effluent in fractions (20–30 ml fractions for an initial crude lipid weight of 8 g).

10. When the 2% CH_3OH in $CHCl_3$ meniscus reaches the Florisil top, repeat with 5% CH_3OH in $CHCl_3$, collecting fractions as above.

11. Check the 2% CH_3OH fractions on silica gel TLC plates 10 cm long. Samples are spotted 0.8 cm apart with a 20 μl capillary tube. Allow one lane for standard PGL-I. Check every other tube, spotting about 20 μl per fraction. Run the plate in $CHCl_3$:CH_3OH:H_2O (45:5:0.5). Spray the plate with a fine mist of 10% H_2SO_4. Heat in an oven at 110° until spots develop (PGL-I first turns yellow, then brown).

12. If PGL-I has not been fully eluted, check the 5% CH_3OH in $CHCl_3$ fractions. Pool the PGL-I containing fractions. Evaporate and weigh the pooled impure PGL-I sample (e.g., 400 mg).

13. Build another Florisil column using 20 g Florisil/g sample using the weight of the pooled PGL-I for calculations (e.g., 80 g Florisil in a 2.5 × 50 cm column).

14. Run the column as before, collecting smaller 2% methanol fractions than before (e.g., 10 ml fractions for 400 mg of impure PGL-I).

15. Check every third fraction as before, spotting about 10 μl of fractions on silica gel thin layer plates.

16. Pool the fractions according to purity (i.e., purified PGL-I; impure PGL-I), dry, and keep separate. For easier handling, transfer the pooled PGL-I fractions, using $CHCl_3$, to screw cap test tubes, and dry under N_2.

17. Add a small amount of distilled hexane to the tubes containing the dried pooled PGL-I (e.g., for 400 mg PGL-I add about 5 ml hexane). Break up the large pieces with a glass stirring rod. An impurity will precipitate out, and this can be aided by placing the tube in the freezer for about 15 min.

18. Remove the tubes from the freezer, centrifuge at 4000 rpm for 3 min. Transfer supernatants to clean weighed tubes, keep separate. Resuspend pellets in hexane, repeat pelleting procedure.

19. Redissolve all samples in a little $CHCl_3$. Apply some of each sample to a silica gel TLC plate and run in $CHCl_3$:CH_3OH:H_2O (45:5:0.5). Spray with 10% H_2SO_4 and heat. The hexane-insoluble contaminant shows a reddish color, whereas PGL-I shows a yellowish color. R_f values are almost identical.

20. On occasion, the impure PGL-I (see step 16) looks as clean as the purified PGL-I, in which case pool them together, otherwise keep them separate. If the pellets show traces of PGL-I, repeat the hexane purification step on the pellets, otherwise they can be discarded.

21. Weigh the purified PGL-I ([17]) (e.g., 200 mg).

22. Combine the hexane supernatants and dry. Dry the pellets also.

Final Purification—Route #1. Using centrifugally accelerated preparative TLC (the Chromatotron device manufactured by Harrison Research, 840 Moana Court, Palo Alto, California 94306).

1. Using a 2 mm silica gel PF_{254} plate rotor, one can apply up to 300 mg of purified PGL-I at one time. Plates are handmade according to directions. The plate rotor is preconditioned according to directions with the first solvent, hexane. The sample is dissolved in a minimum amount (1 ml) of hexane and applied to plates according to directions).

2. For 200 mg of purified PGL-I, use 100 ml of hexane as the first solvent. Collect eluate in bulk.

3. Switch to 100 ml ether when the hexane is finished. Collect in bulk. This operation should be done under a hood.

4. Switch to 200 ml of 10% acetone-ether, when ether is finished, collect 3 ml fractions. PGL-I should emerge during this step.

5. Switch to 100 ml of 20% acetone-ether when the 10% acetone-ether is finished, collect 3 ml fractions.

6. Check fractions on analytical TLC plates.

7. Pool fractions according to purity. Typically about 80 mg of pure PGL-I is recovered.

Final Purification—Route #2. Conventional preparative TLC. (This route is especially applicable to small amounts of partially purified PGL-I, i.e., 20–30 mg.)

1. Use 20 × 20 cm, silica gel "Redi" plates from Fisher Scientific, 711 Forbes Avenue, Pittsburgh, Pennsylvania 15219. Apply approximately 10 mg of sample per plate as a thin band 1 cm from the bottom using a Kontes streaker and run the plate in ether: acetone (8:2).

2. PGL-I can be observed as the major white band, with an R_f value of 0.4–0.5, when sprayed with a fine mist of water. Areas lacking glycolipid appear translucent when sprayed.

3. Lightly score the silica gel around the bands, also scratch in a label, so bands can be identified when the plate dries.

4. After drying, scrape off the PGL-I band into a sintered glass frit filter funnel (60 ml, filter diameter = 40 mm, porosity = fine) on a bell jar vacuum apparatus (Pyrex #36060; Corning Glass Works, Corning, New York 14830).

5. Place a weighed 20 ml vial (Curtin Matheson Scientific, 4220 Jefferson Ave., Houston, Texas 77023) in the bell jar under the funnel. Elute PGL-I from silica gel with $CHCl_3$:CH_3OH (2:1). Apply suction to the bell jar.

6. Continue washing the silica gel with 10–20 ml of $CHCl_3$:CH_3OH (2:1).

7. Remove the vial. Check the purity by TLC as described above. It should be absolutely pure by this criterion ([18]).

Final Purification—Route #3. Column Chromatography. (This route is especially suited for large amounts of partially purified PGL-I, e.g., 1 g.)

1. Build a Florisil column using 20 g Florisil/g partially purified PGL-I. Weigh the Florisil in a beaker and make a thin slurry, this time using ether-hexane (1:1). Pour the slurry into an appropriate size column.

2. Dissolve the partially pure PGL-I in 2–3 ml ether-hexane 1:1. Apply the sample to the column as before. Elute first with 6 bed volumes of ether-hexane (1:1). Collect the eluate in bulk.

3. When the ether-hexane 1:1 is finished, change to ether: hexane (75:25). Elute with 12 bed volumes of 75% ether in hexane. Collect the eluate in bulk.

4. When the 75% ether in hexane is finished, change to 100% ether. Continue to elute with ether until no more PGL-I emerges from the column. Collect in 2–3 ml fractions and check purity via TLC.

5. Pool the PGL-I-containing fractions according to purity. In our experience, ether will elute only PGL-I and the product is fully pure.

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13. Details of the purification of phenolic glycolipids II ([12]) and III ([7]) are not described herein. They are minor components, apparently metabolic by-products of PGL-I, and extremely difficult to obtain. The term PGL is used in preference to Phen GL used previously.
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17. Young and Buchanan ([6]) used the deacylated PGL-I as distinct from the native material since it is more active in their ELISA protocol, although marginally less active in the protocol described by Cho, *et al.* ([8]). Pure deacylated PGL-I may be obtained by hydrolyzing a sample of fairly pure PGL-I (typically 10 mg) with 10% NaOH in benzene/methanol (1:2, 1.5 ml) in a sealed tube under N_2 at 100°C for 18 hr ([3]). The deacylated glycolipid and fatty acids are extracted from the hydrolysate with a 3 × 1 ml of $CHCl_3$ after the addition of 1 ml of 0.1 N HCl. The dried $CHCl_3$ phase is applied to a column (1.1 × 10 cm) of silicic acid/Celite (2:1) which is irrigated firstly with $CHCl_3$ (10 ml) to remove fatty acids and then with 5% CH_3OH in $CHCl_3$ to remove the deacylated PGL-I.
18. Pure PGL-I obtained by either route when examined by high performance liquid chromatography (HPLC) shows only one ultraviolet (220 nm) absorbance peak. The HPLC conditions are: Beckman Model 110 A system with a Model 332 Gradient system and a Model 160 Absorbance Detector. The column in use is an Alltech Silica Column (25 cm × 4.5 mm; 5 μm). The solvent is $CHCl_3$:CH_3OH (2:1) at flow rate of 1 ml/min.
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