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Note

One-step column chromatographic procedure for purification of mycobacterial glycopeptidolipid antigens

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A number of mycobacterial lipid antigens have been isolated and reported in the last several years [1—3]. Of particular importance have been a group of glycopeptidolipid (GPL) antigens which are associated with the *Mycobacterium avium—M. intracellulare—M. scrofulaceum* (MAIS) serocomplex [1, 4]. The MAIS group of mycobacteria have lately been associated with life-threatening infections in individuals suffering from a new disease, acquired immune deficiency syndrome (AIDS) [5—7]. Although the pathogenesis of the MAIS group is not well understood, it is thought that the superficial L_1 layer may play an important role as a protective barrier following phagocytic uptake by host macrophages [8]. It has been demonstrated that the GPL antigens are a major component of the L_1 layer [9] both preceding and immediately following phagocytosis by macrophages [10, 11]. Because of their association with the superficial L_1 layer, the GPL antigens may play some role in the pathogenesis of these non-tuberculosis mycobacteria.

In an attempt to study the pathogenic aspects of the GPL antigens by monitoring their postphagocytic degradation and distribution within host macrophages, our laboratory has been engaged in isolation and purification of large quantities of these antigens. One of the major obstacles in our program has been the time and cost involved in purifying sufficient quantities of these antigens for appropriate studies. Current procedures involve the use of silicic acid-Celite column chromatography followed by DEAE-cellulose column chromatography [1, 9]. In some cases, repurification on these supports and/or preparative thin-layer chromatography (TLC) is necessary to complete the isolation process [1, 9]. These procedures take several weeks to complete, and in the case of DEAE-cellulose, the column support has to be converted to the acetate form prior to use [1, 9].

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In order to improve the availability of large amounts of the GPL antigens, we investigated the applicability of a short-column chromatographic procedure to our problem [12]. Although in this publication purification of the GPL antigens from serovar 20 is described, we hope to illustrate its general applicability by expanding it to include the isolation of GPL antigens from other MAIS serovars.

EXPERIMENTAL

Isolation of glycopeptidolipids

The GPL antigens were isolated from serovar 20 of the MAIS serocomplex by procedures described in previous publications [9—11]. Briefly, serovar 20 was cultivated in Middlebrook 7H9 (Difco, Detroit, MI, U.S.A.) supplemented with OADC (Difco). After cultivation, the mycobacteria were autoclaved, harvested by centrifugation and lyophilized. Lipids were extracted from the lyophilized cells using the extraction procedure of Folch et al. [13] and stored in chloroform at −20°C until further use. Presence of the antigens in each batch of lipid was confirmed by development on analytical precoated silica gel 60 TLC plates (E. Merck, Darmstadt, F.R.G.) using chloroform—methanol—water (60:12:1) (solvent A) and detection with orcinol-sulfuric acid reagent as described previously [4, 9]. When detected by this procedure, the GPL antigens produce a characteristic yellow-gold color and can therefore be differentiated from other lipid components [1].

Preparation of short column

A Chromaflex column (Kontes, Vineland, NJ, U.S.A.) (250 mm × 25 mm I.D.) equipped with a 500-ml reservoir on the top and a Chromaflex adapter with Luer joint bearing a Luer Kel-F Hub (Kontes) on the bottom was used for fractionation of lipids.

Silica gel H, 10—40 μm (Sigma, St. Louis, MO, U.S.A.) was activated at 120°C for 2 h and chloroform added until a slurry was formed. The slurry was added to the column and packed by applying pressure with nitrogen and vibration with a vibro-graver. Final column dimensions were 20 cm × 2.5 cm and ratio of lipid to support was 1:100.

Purification of GPL antigens

Crude lipid extracts were dissolved in chloroform (100 mg/ml) and applied directly to the column. Maximum amount of lipid applied to any one column was 500 mg, although it was found that smaller amounts could be used and purification of the GPL antigens achieved. The columns were developed with chloroform until all of the pigments were eluted. The eluting solvent was then changed to chloroform—methanol (90:10) (solvent B) and 3-ml fractions were collected on an LKB Ultrorac fraction collector (LKB, Rockville, MD, U.S.A.). Individual fractions were dried under a stream of nitrogen, reconstituted in chloroform and monitored by TLC (solvent A) to locate the fractions containing the GPL antigens. Presence of individual GPL antigens was confirmed by spraying TLC plates with orcinol-sulfuric acid reagent and observing the characteristic yellow-gold color produced [1, 9]. Previously purified serovar 20 GPL antigens were used as standards [9—11].

RESULTS

To achieve good separation of the GPL antigens on a TLC plate, crude lipid is routinely chromatographed by development in solvent A (Fig. 1, lanes A and Q) [9]. As suggested by Hunt and Rigby [12], the proportion of the more polar component in the TLC solvent system was decreased to improve separation with the short-column method. Thus, the solvent used for elution of the GPL antigens in this study was solvent B. In preliminary work it was found that separation of the GPL antigens using solvent B was improved if crude lipid was first eluted with chloroform.

Prior elution with chloroform offered two advantages. First, pigments and other apolar components were removed from the crude lipid fraction and secondly, elution of the pigments served as a visual check of column uniformity without the use of dyes or markers. The pigments could be visualized as narrow moving bands which began to form after approx. 50—100 ml of chloroform had eluted from the column.

Following elution of pigments, which required 300—350 ml of chloroform (Fig. 1, lane B) 500 ml of solvent B were added and fractions were collected at 15-min intervals with the column flow-rate set at 12 ml/h. Fractions were

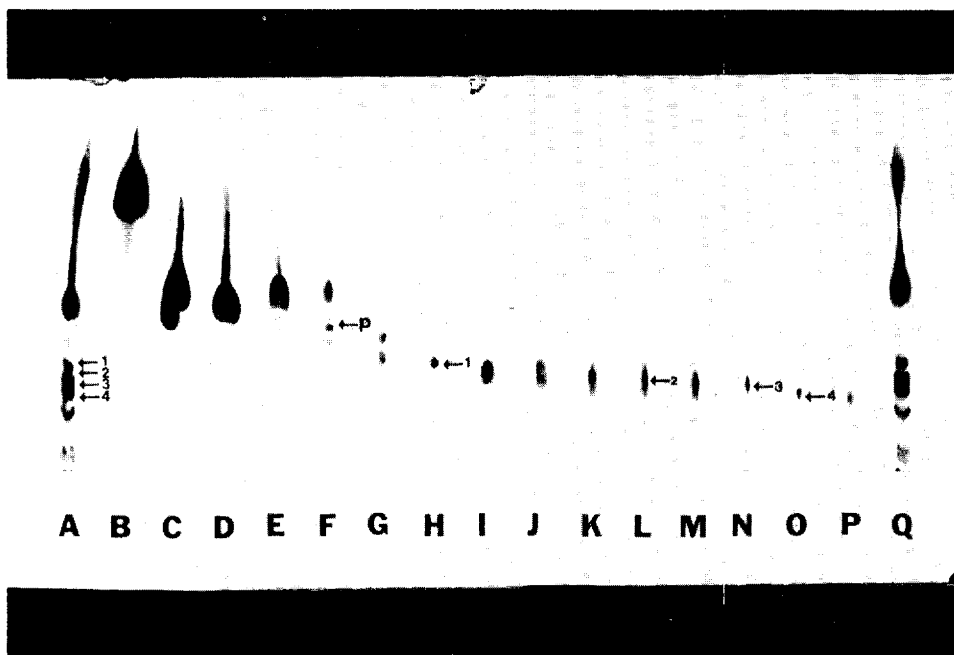

Fig. 1. Thin-layer chromatographic plate representing fractions collected from short column. Lanes A and Q represent crude lipid before elution on short column. Lane B represents the chloroform fraction collected prior to addition of solvent B and lanes C—P represent fractions collected after addition of solvent B. The numbers 1, 2, 3 and 4 indicate GPL antigens. Lanes H—O represent those fractions containing only GPL antigens. Phospholipids are indicated in lane F (p). Plate was developed in solvent A and components detected by spraying with orcinol-sulfuric acid reagent. GPL antigens turn a yellow-gold color when treated in this manner.

monitored by TLC in solvent A and those fractions containing the four GPL antigens (GPLs 1, 2, 3 and 4) [9] were pooled and stored in chloroform at −20°C until further use. The GPL antigens routinely came off in fractions 57 (S.D., ± 7) through 96 (S.D., ± 13) as shown in Fig. 1 (lanes G—O), and yields of pure GPL antigens amounted to 10.4% (S.D., ± 1.1) of total lipid as determined from four successive analyses. Fractions containing pure antigens are represented in lanes H—O. It was possible to achieve partial separation of individual GPL antigens (lanes H, L, N and O) and to remove phospholipids which migrate just in front of GPL 1 (lane F). The amounts of purified antigens are comparable to the 10—13% yields achieved with the conventional methods. However, it is not possible to assess that accurately because with the conventional methods it is a common practice to pool fractions from successive analyses and rechromatograph them.

There are several advantages that the short-column procedure has over the conventional methods. First, the need for three column supports is reduced to only one. In addition to saving money, this also eliminates the need to prepare the acetate form of DEAE-cellulose, a procedure which requires additional time and supplies. Secondly, GPL antigens can be purified in sufficient quantities by one chromatographic development taking only three days. One-step purification of the GPL antigens is not possible with the conventional methods. Thirdly, use of the short-column procedure reduces the amount of solvents necessary for purification of the antigens and reduces the potential loss of antigens which is associated with multiple-column analyses.

We believe that 10.4% recovery of the GPL antigens may be further improved to eliminate the interference of two persistent contaminants one of which has the mobility slightly higher than GPL 1 (Fig. 1, lane G) and the other slightly lower than GPL 4 (Fig. 1, lane P). The parameters which are being considered are increase in flow-rate (i.e. pressures in slight excess of atmospheric) and solvent gradient elution.

DISCUSSION

A problem which has hindered our studies has been the time necessary to purify sufficient quantities of the GPL antigens to investigate their immunological and pathological properties. Previous methods have required that the lipids first be fractionated on silicic acid-Celite columns and then further purified on columns of DEAE-cellulose (acetate form) [9—11]. In most cases, separations have to be repeated in order to achieve purification, thereby causing these procedures to take four to eight weeks for completion.

The results of this investigation demonstrate that the short-column chromatographic procedure [12] can be used to purify sufficient amounts of the GPL antigens in one tenth the time that it would take using current procedures. By using the short-column technique not only is total purification time reduced but the necessity for multiple column supports is eliminated. The procedure allows individual chromatographic analyses to be completed in three days. This makes it possible to maintain a constant supply of purified GPL antigens in sufficient quantities for further investigations regarding their immunological and pathological attributes. Because the GPL components represent similar

serovar-specific antigens in the MAIS complex [1], this laboratory is currently applying the short-column chromatographic procedure to the purification of antigens from additional serovars.

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