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ISOLATION AND PARTIAL CHARACTERIZATION OF GLYCOLIPIDS AND A CARBOHYDRATE FROM THE SECONDARY GRANULES AND PLASMA MEMBRANE OF POLYMORPHONUCLEAR LEUKOCYTES

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Summary

Chloroform/methanol extracts of the secondary granule and plasma membrane fractions of polymorphonuclear leukocytes have been shown to contain both non-polar and polar carbohydrate-containing materials. The ratio of the polar to the non-polar material was much higher in the plasma membrane than the secondary granule fraction. The non-polar material contains at least two ceramide-like glycolipids and accounts for most of the broad band of periodic acid/Schiff-positive material which migrates at the dye front in sodium dodecyl sulfate electrophoretic gels of granule and plasma membrane extracts. The polar material appears to be a single substance containing no fatty acids or sialic acid and is composed of glucose, hexosamine and a carboxylic acid derivative of pentose. Expressed on a per mg of protein basis, the amount of carbohydrate associated with the polar material in the plasma membrane fraction was about five times that of the secondary granule fraction.

Introduction

Polymorphonuclear leukocytes contain two types of cytoplasmic granules, namely primary and secondary, each of which contain a variety of antibacterial agents [1]. During phagocytosis these granules fuse with the internalized plasma membrane surrounding phagocytic vacuoles. After fusion takes place the contents of the cytoplasmic granules are secreted into the lumen of the vacuole where bacterial killing and digestion take place [2]. The molecular

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mechanisms involved in the secretory function of cytoplasmic granules during phagocytosis are poorly understood. A knowledge of the biochemical composition of the cytoplasmic granules and plasma membrane would be useful in elucidating such mechanisms.

Previous studies in our laboratory, using dodecyl sulfate polyacrylamide gel electrophoresis showed that most of the major glycoprotein components of the post-nuclear particulate fraction of polymorphonuclear leukocyte homogenates are associated with the secondary granules and plasma membrane fractions. A rapidly migrating, broad band of carbohydrate-containing material was also observed in the gel patterns of dodecyl sulfate-soluble secondary granule and plasma membrane fractions. On the basis of these properties this material was tentatively identified as glycolipid [3]. We report here studies aimed at isolating and characterizing the carbohydrate-containing substances found in chloroform/methanol extracts of the secondary granule and plasma membrane fractions of polymorphonuclear leukocytes.

Materials and Methods

Cell preparation

Leukocytes were obtained from male, strain 13 guinea pigs (400—500 g) as described previously [3].

Subcellular fractions

The secondary granule and plasma membrane fractions were prepared by differential centrifugation of leukocyte homogenates [3]. These fractions were washed three times with 0.34 M glycerol to remove all detectable amounts of sucrose as measured by the anthrone method (see below).

Extraction and partition

Secondary granule and plasma membrane fractions were extracted with chloroform/methanol and the extracts were separated by partition with 0.1 M KCl into aqueous and non-aqueous phases [4]. The two phases were washed, evaporated under reduced pressure in a rotary evaporator at 40° C and reconstituted in a suitable volume of chloroform/methanol (2 : 1).

Polyacrylamide gel electrophoresis

Subcellular components and chloroform/methanol extracts of such components were analyzed for glycoproteins and glycolipids on sodium dodecyl sulfate-polyacrylamide gels as reported previously [3].

Thin-layer and column chromatography

Thin layer chromatography (TLC) was carried out according to Stahl [5] using glass plates coated with silica gel G (Camag, Berlin, Germany). The solvents used were all of analytical grade. Various reagents were used to identify the glycolipids and carbohydrates separated by TLC [4,6].

The polar fraction was separated on Florisil 6-100/PR (Sigma Chem. Co., magnesium silicate, activated at 670° C) column (0.9 × 12 cm) using stepwise elution with chloroform/methanol/water. The flow rate of the column was

1 ml/min; 5-ml fractions were collected and the carbohydrate content was measured in each fraction as described below.

Gas-liquid chromatography and mass spectrum analysis

Carbohydrate-containing fractions were hydrolyzed and prepared for gas-liquid chromatography (GLC) essentially as described by Yang and Hakomori [7]. The analyses were carried out in a Finnigan 9500 gas-liquid chromatograph equipped with a flame ionization detector. The borosilicate glass column (180 cm long, 4 mm inner diameter) was packed with 3% ECNSS-M on 100/120 mesh gas-chrome QB (Applied Science Laboratories, Inc., State College, PA). The separation was run at the following temperatures: injection, 200°C; detector, 250°C; column initial, 170°C, maximum, 210°C with 2°C/min programming.

Peak fractions from the GLC analyses were further analyzed by mass spectrometry using a Finnigan 10150 gas chromatograph-mass spectrometer equipped with a chemical detector and using methane as the carrier gas.

Analytical and chromatographic methods

Total carbohydrate. The total carbohydrate was determined by the anthrone method using mannose as the standard [8].

Sialic acid. The carbohydrate material was hydrolyzed in 0.1 M H_2SO_4 for 1 h and the sialic acid content was determined by using Warren's thiobarbaturic acid method [9].

Uronic acid. The uronic acid was determined colorimetrically by the modified method of Dische [10].

Hexosamine. Hexosamine was liberated by hydrolysis with 6 N hydrochloric acid for 18 h at 80°C and the HCl was removed under reduced pressure in a rotary evaporator. The hexosamine content in the hydrolysate was determined by the Elson-Morgan method [10].

Protein determination. The protein content of the secondary granule and of the membrane fraction was determined by the method of Lowry et al. [11] before and after glycolipid extraction.

Results

Gel electrophoresis of the sodium dodecyl sulfate extracts of the secondary granule and plasma membrane fractions

Fig. 1A and B show that sodium dodecyl sulfate extracts of the secondary granule and plasma membrane fractions, respectively, contain periodic acid/Schiff-positive material which migrates in a broad band near the dye front in the electrophoretic gels. (See gel 2 in Fig. 1A and B.) This material was completely extracted from the subcellular fractions by chloroform/methanol (gels 4 and 6 in Fig. 1A and B). The chloroform/methanol extracts contained both polar (gel 7) and non-polar (gel 8) periodic acid/Schiff-positive components in both the secondary granule and plasma membrane fractions. However, on a cell equivalent basis the non-polar component accounted for most of the periodic acid/Schiff-positive component extracted by chloroform/methanol from both of these subcellular fractions.

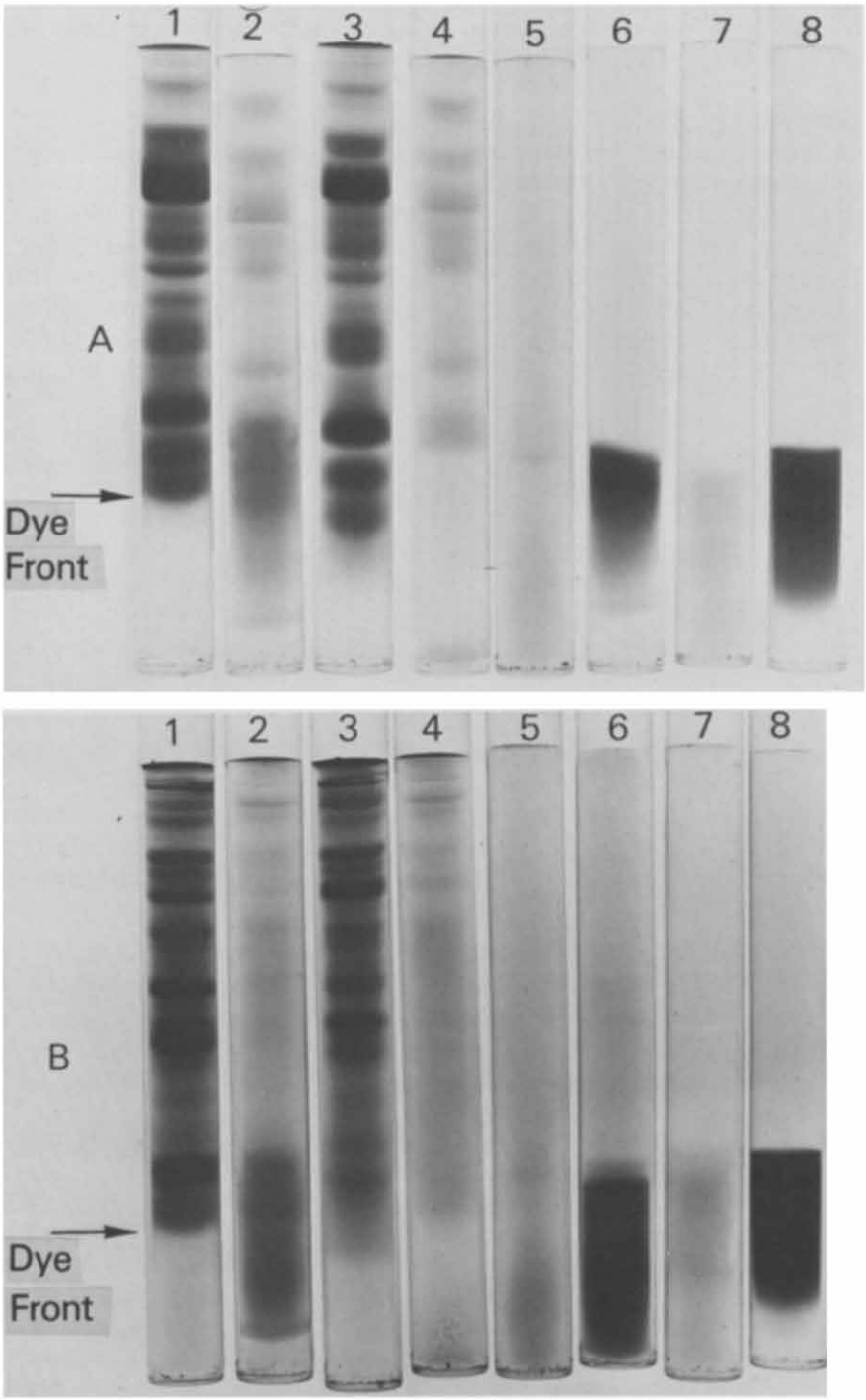

Fig. 1. SDS-polyacrylamide gel electrophoresis of components of the secondary granule (A) and plasma membrane (B) fraction of polymorphonuclear leukocytes. In (A) and (B) the electropherograms are as follows: subcellular fraction before (1,2) and after (3,4) removal of glycolipids by chloroform/methanol extraction, chloroform/methanol-extracted components (5,6); chloroform/methanol-extracted polar (7) and non-polar (8) components. Gels 1, 3, and 5 are stained for protein with Coomassie blue and gels 2, 4, 6—8 for carbohydrate by the periodic acid/Schiff method. Gels 1—4 contain sample equivalent to $2 \cdot 10^8$ cells. Gels 5—8 contain sample equivalent to $4 \cdot 10^8$ cells.

Total and chloroform/methanol extractable carbohydrate contents of the secondary granule and plasma membrane fractions

Results of analyses of the secondary granule and plasma membrane fractions for total carbohydrate shown in Table I indicate that the plasma membrane fraction contains about 50 times as much carbohydrate/mg protein as the secondary granule fraction. Table I also shows that the secondary granule fraction differs markedly from the plasma membrane fraction with respect to the ratio of the carbohydrate-containing polar and non-polar components in the chloroform/methanol extracts of these subcellular fractions. The data also indicate that the plasma membrane contains approximately four times more chloroform/methanol-extractable carbohydrate than secondary granules and this difference is accounted for by elevated levels of both the polar and non-polar components.

Characterization of the polar and non-polar components of chloroform/methanol extracts of the secondary granule and plasma membrane fractions

Table II shows that the polar substance isolated from both the secondary granule and the membrane fractions contains hexosamine. The polar material from secondary granules and plasma membranes also gave a strong, positive reaction in the colorimetric test for uronic acid. Sialic acid could not be detected in the polar component from either subcellular fraction and, if present, is less than 0.3 μg and 0.1 μg/100 μg carbohydrate in the secondary granule and plasma membrane fractions, respectively. Analysis of the polar substance by TLC, using four different solvent systems (Table III), revealed only one carbohydrate-positive spot (Fig. 2). This spot was negative for iodine-staining lipids, ceramides (benzidine/hypochlorite stain) and free amino groups (ninhydrin reagent). When the polar material was eluted from a Florisil column, using different mixtures of chloroform/methanol/water, only one peak of carbohydrate-positive material was obtained (Fig. 3). GLC analysis of the acid hydrolyzates of the polar material revealed the presence of two carbohydrates; glucose and an unidentified sugar (see Fig. 4). The latter had a retention time less than the hexoses and greater than the pentoses. No fatty acids could be detected in the GLC pattern suggesting the absence of fatty acid-containing lipids in the polar material. Mass spectrum analyses confirmed the presence of

TABLE I

TOTAL CARBOHYDRATE CONTENT IN DIFFERENT EXTRACTED FRACTIONS OBTAINED FROM SECONDARY GRANULES AND PLASMA MEMBRANE

The mean ± S.E. of three experiments is shown.

Subcellular fraction	Carbohydrates (μg/100 μg protein)			
	Total	Chloroform/methanol extract		
		Polar (P)	Non-polar (NP)	P/NP
Secondary granules	29 ± 4.6	3.9 ± 0.8	4.2 ± 0.7	0.92
Plasma membranes	1518 ± 352	20.6 ± 6.5	8.6 ± 1.3	2.4

TABLE II

ANALYSIS FOR VARIOUS COMPONENTS OF THE POLAR MATERIAL OBTAINED FROM SECONDARY GRANULES AND PLASMA MEMBRANE

The mean ± S.E. of three experiments is shown.

Subcellular fraction	Component (μg/100 μg total carbohydrate)		Colorimetric test for uronic acid
	Hexosamine	Sialic acid	
Secondary granules	13.0 ± 2.4	undetectable	positive
Plasma membrane	8.5 ± 2.2	undetectable	positive

TABLE III

SOLVENTS USED IN THE TLC ANALYSIS OF THE POLAR MATERIAL OBTAINED FROM SECONDARY GRANULES AND PLASMA MEMBRANE

Solvent	Composition ratio (v/v)	Polar, carbohydrate-containing substance (R_F X 100)
Chloroform/methanol/water	50 : 21 : 3	15
Chloroform/methanol/conc. NH_4OH	6 : 4 : 1	27
n-Propanol/water/conc. NH_4OH	80 : 13.7 : 6.7	22
1,2-Dichloroethane/methanol/water	97 : 3 : 0.1	0

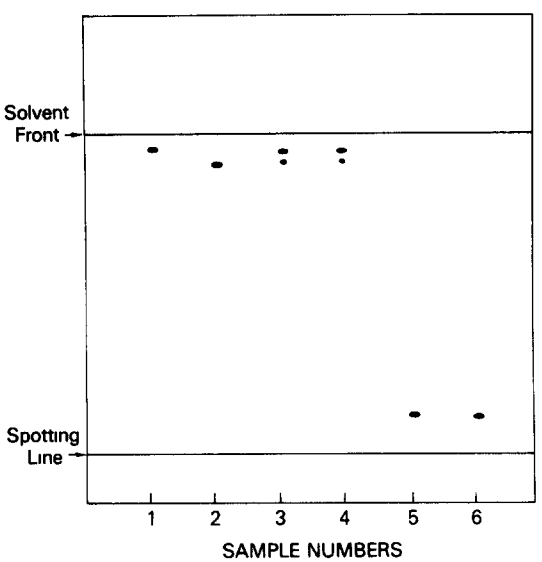

Fig. 2. Thin-layer pattern of the polar and non-polar components in chloroform/methanol extracts of secondary granules and plasma membrane. Samples 1 and 2 are standard ceramides I and II, respectively. Samples 3 and 4 are the non-polar fractions of chloroform/methanol extracts of secondary granule and plasma membrane, respectively. Samples 5 and 6 are the polar fractions of chloroform/methanol extracts of secondary granule and plasma membrane, respectively. The solvent is chloroform/methanol/water (50 : 21 : 3). Anthrone/phosphoric acid reagent was used to visualize carbohydrate-containing compounds.

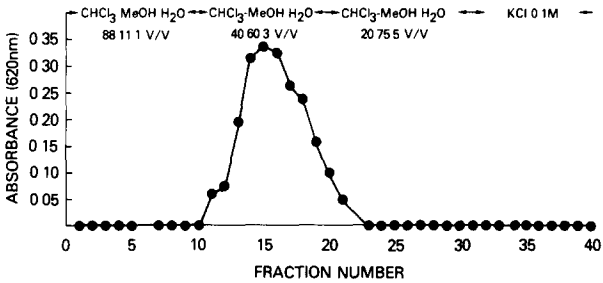

Fig. 3. The elution of the membrane polar, carbohydrate-containing compound with chloroform/methanol/water from a Florisil column.

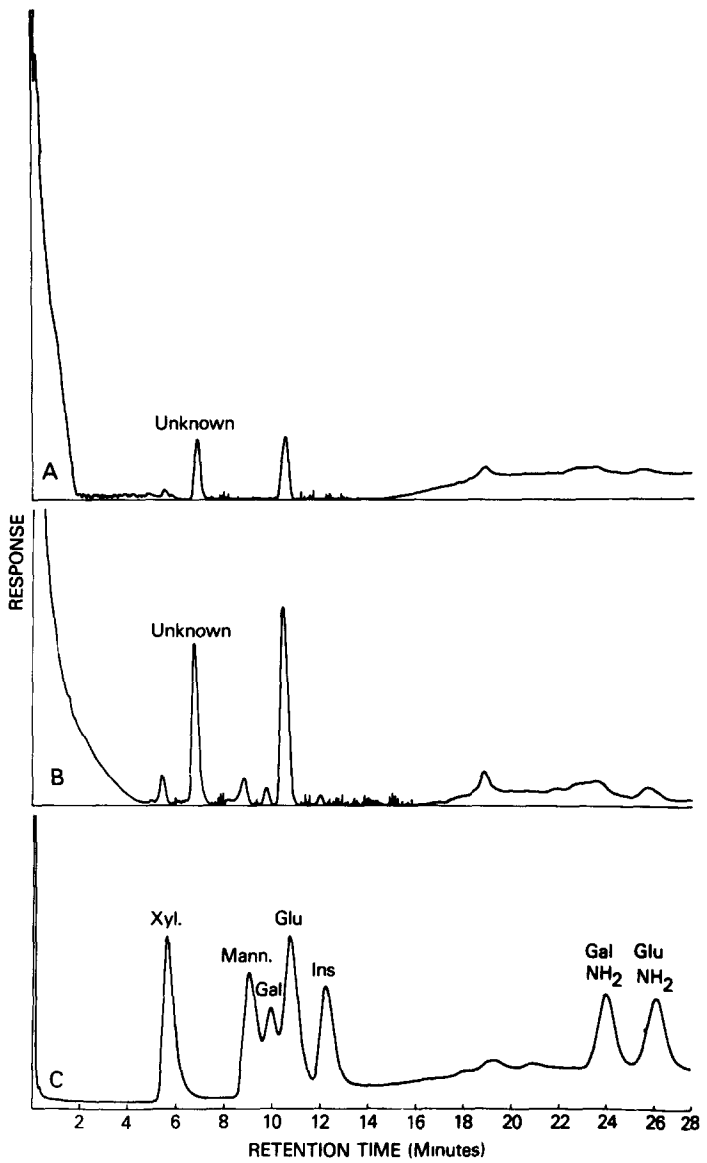

Fig. 4. Gas-liquid chromatographic pattern of the alditol acetate derivatives of the hydrolyzate of the membrane polar material (A). (B) Same as (A) except the sample contains a trace amount of standard alditol acetate monosaccharides. (C) The pattern of the standard alditol acetate monosaccharides.

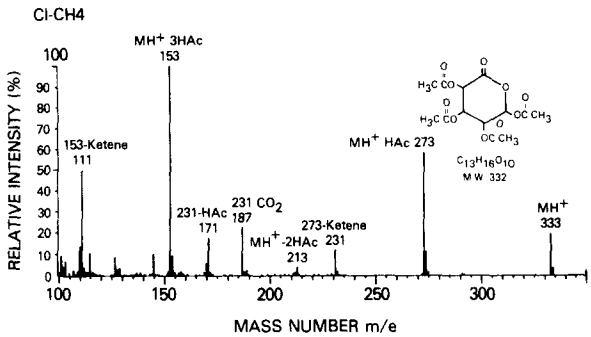

Fig. 5. The *m/e*, methane chemical ionization mass spectrum of the unknown compound detected by GLC in the acetylated hydrolysate of the membrane polar component (see Fig. 4). The mass spectrum pattern shows that the molecular weight of the acetate derivative of the unknown compound is 332 and the suggested formula is shown at the right top of the figure. In the mass spectrum the protonated form of the unknown molecule (333 mass number) is referred to as MH^+. The different mass fragments were obtained by loss of either acetic acid (HAc, $M_r = 60$), ketene ($M_r = 42$), CO_2 ($M_r = 44$), or combinations of these molecules from the protonated unknown compound.

glucose in the polar material and showed that the unidentified sugar might be a carboxylic acid derivative of a pentose (Fig. 5). The results of all the above analyses carried out on the polar substance were the same for both the secondary granule and plasma membrane fractions.

Separation and analysis of the non-polar components in the chloroform/methanol extracts of the secondary granule and plasma membrane fractions by TLC revealed the presence of two carbohydrate-positive spots which had R_f values corresponding to those of ceramides I and II (Fig. 2) and which were also iodine positive (data not shown). The non-polar fractions also contained other iodine-positive substances which were negative for carbohydrate and had R_f values corresponding to standards containing phospholipid, cholesterol, and cholesterol ester (data not shown). The results of these TLC studies were the same, qualitatively, for extracts of the secondary granule and the plasma membrane fractions.

Discussion

The results of the present study show that both the secondary granule and plasma membrane fractions of polymorphonuclear leukocytes contain non-polar and polar, carbohydrate-containing substances extractable with chloroform/methanol. The plasma membrane fraction is characterized by a much higher ratio of the polar to non-polar, carbohydrate-containing material than that of the secondary granule fraction. The non-polar fraction contains at least two glycolipids of the ceramide type which can be separated by TLC. These non-polar glycolipids account for most of the broad band of periodic acid/Schiff-positive material which, as reported previously [3], migrates at a high relative mobility in sodium dodecyl sulfate electrophoretic gels of extracts of the secondary granule and plasma membrane fractions. Ceramides have also been reported by others to be present in lipid extracts of whole leukocytes [12,13].

The polar, carbohydrate-containing component in both subcellular fractions appears to be single substance since it could not be resolved into more than one component by TLC in four different solvent systems or by chromatography on Florisil columns. This substance which, to our knowledge has not been reported previously in leukocytes, consists of two sugars detectable by GLC and mass spectrometry, namely, glucose and an unidentified compound which may be a carboxylic acid derivative of a pentose. Although no fatty acids or iodine-staining lipids could be detected in this substance, the fact that it is soluble in chloroform/methanol suggests the presence of some hydrophobic moiety in the molecule. Colorimetric analyses also suggest the presence of hexosamine and the absence of sialic acid in this polar compound. On a per mg of protein basis, the carbohydrate of the polar material appeared to be much richer in the plasma membrane fraction than in the secondary granule fraction.

At present, it remains to be determined whether the glycolipids and polar, carbohydrate-containing compound in secondary granules and the plasma membrane of leukocytes play any role in the fusion of these organelles during phagocytosis. In this connection it will be of interest to determine whether the glycolipids and/or carbohydrates are localized on the outer surface of the limiting membrane of secondary granules where they might come in contact with the plasma membrane which surrounds phagocytic vacuoles.

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