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Lysosomal Membranes of Rabbit Polymorphonuclear Leukocytes as a Model to Study Intracellular Membrane Proteins

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Lysosome membranes from rabbit polymorphonuclear leukocytes free from other cellular contaminants and with minimal enzymatic adsorption have been obtained Chemical constituents of these membranes were in the same proportion as in the plasma cell membranes except for the smaller number of polypeptides. Molecular weights of the 12 lysosome membrane polypeptides, ranged from 9,200 to 480,000 as estimated by acrylamide gel electrophoresis, seven of which gave a positive periodic acid-Schiff (PAS) staining. Lysosome membranes were solubilized by using formic acid as a disrupting agent and 8 M urea for maintaining proteins in solution during the purification work. Proteins behaved nicely when applied to gel filtration or isoelectric focusing and three of them (mol. wts. 9,200, 37,800, 145,000) were isolated and chemically characterized. Carbohydrate content of the isolated proteins, was higher than in the whole membrane, at expenses of neutral sugars and methyl pentoses but with smaller amount of sialic acid. Amino acid composition of 9,200 MW protein was rich in arginine and non-polar aminoacids and that of the two others was rich in glutamic acid and glycine. This procedure represents a good approach to the study lysosome membrane proteins, from which a better understanding of the phagocytosis process could be obtained.

Chemical understanding of the cell membrane proteins has at least two technical disadvantages, which makes that our knoledge about this field is poorer than other areas of biochemistry. They are related with the difficulties in isolating membranes free of other cellular components and in the development of membrane-proteins solubilizing procedures, which allow the isolation of both peripheral and integral membrane proteins.

In this regard the rabbit polymorphonuclear leukocytes offer a favorable sys-

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tem to obtain lysosomal membrane free of other cell structures hence representing a good model to study the chemical characteristics of intracellular membranes.

Detergents have been used extensively (10, 18, 21, 28) in the solubilization procedures, but they pose two problems: *a*) limitation of the fractionation methods that can be applied, and *b*) the difficulty of removing the last traces of detergents out of the protein. Other technical approaches have been assayed with limited success, using as solubilizing agents guanidine hydrochloride (3), formic acid (20) and organic solvents (23). A remarkable exception was the isolation and characterization by MARCHESI *et al.* (24, 25) of the major glycoprotein of the human red blood cells.

In this paper we describe a procedure for membrane protein solubilization which allowed us the isolation of three proteins from lysosomal membranes of rabbit polymorphonuclear leukocytes.

Materials and Methods

Collection of cells. Polymorphonuclear leukocytes were obtained from peritoneal exudates of New Zealand white rabbits, which had received 4 h before a 200 ml peritoneal infusion of 0.1 % glycogen (Sigma) in 0.9 % sodium chloride solution. Cells were washed twice with salt balanced 119 medium (Difco, Long Island) and 0.25 M sucrose respectively, disrupted by osmotic pressure (0.34 M sucrose), and the lysosomes released from these cells were isolated by differential ultracentrifugation (6).

Preparation of the lysosome membranes. Membranes were obtained by suspending lysosomes in 0.07 M Tris HCl buffer, pH 8.6 and sonified at 0° C for 15 seconds at 75 watts in a Model W-140C sonifier (Heat System Co., Melville, New York). Partial delipidation of membranes was obtained by repeated ultrasonic treatment during 25 seconds each time. Membranes were sedimented in a Model 50 Spinco rotor at 15,000 rpm for 10 minutes in a preparative model L Spinco Ultracentrifuge. Hydrolytic enzymes present in the supernatant were discarded and the procedure was repeated until enzymatic activities were detected in negligible amounts.

Solubilization and Purification of the lysosomal membrane proteins. Lysosome membrane suspensions were made in 0.06 M mercaptoethanol and then concentrated formic acid was added slowly with constant stirring to a final concentration of 35 % by volume. This solution of membrane proteins was immediately dialyzed against a 8 M urea solution buffered to pH 8.6 with 70 mM Tris HCl; the dialysis was continued until the protein solution reached a pH of 8.6. The solubilized membrane proteins, were purified using ascending chromatographic systems with sephadex G-200 or agarose 2 B (Pharmacia, Sweden) eluted with 70 mM Tris-HCl buffer, pH 8.6, urea 8 M and 1 M guanidine hydrochloride.

In some cases, electrophoresis was carried out in a watercooled isoelectric focusing column, Type 8101 (LKB, Sweden).

Disc electrophoresis was performed according to Davis method (7), modified by FAIRBANKS *et al.* (10), using 11.6 % acrylamide gels at pH 7.4 in 1 % SDS, 0.15 % ammonium persulfate and 0.05 % Temed. Gels were stained for proteins with Coomassie brilliant blue and for carbohydrates by the periodic acid Shiff (PAS) procedure. Prior gel electrophoresis, membrane protein preparation were incubated with 0.1 M mercaptoethanol at 37° C during 2 h and then 0.1 M iodoacetamide and 1 % SDS, at 37° C during 2 h were added. Overnight dialyses was made against the same buffer used for electrophoreses (40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, 1 % SDS, pH 7.4). In some experiments, last pro-

cedure was omitted, and protein samples were pretreated before gel electrophoresis with 1 % SDS, 1 % mercapto-ethanol, 4 M urea and boiled during 5 minutes, to avoid some protein aggregations. Similar results were obtained with both experimental approaches. Molecular weight markers for SDS gel calibration with range molecular weight between 1,450 to 480,000 were obtained from Schwarz-Mann.

Chemical analysis. Proteins were determined by the method of LOWRY *et al.* (22). Lipids were extracted in 2: 1 chloroform-methanol. Lipid phosphorus was determined by the method of FISKE and SUBAROW (12), and total cholesterol according to ABELL *et al.* (1). Neutral sugars were determined by the method of ROE (27), hexosamines according to BELCHER *et al.* (4), N-acetylhexosamines by SCHIFFMAN *et al.* method (29), sialic acid determinations according the procedure of AMINOFF (2) and methyl-pentoses by the DISCHE and SHETTLES method (8).

Enzyme assays. Acid phosphatase was assayed by the method of BESSEY *et al.* (5), β-glucorunidase according to FISHMAN *et al.* (11) and catepsin by the method of GIANNETO and DE DUVE (13).

Amino acid analysis. Proteins were hydrolyzed at 110° C for 20 h in high vacuum sealed tubes with 5.7 M HCl and analyzed in an automatic Jeol JLC 5AH and 6AH amino acid analyzers.

Results

Purity of cell and lysosomal membrane preparations. The percentage of leukocytes in the cells of the peritoneal exudates was of 95 % or greater. Lysosomes prepared from these cells were almost free of other cytoplasmic organelles as it has been demonstrated before by negative assay for cytochrome oxidase or by electron microscope studies (14).

The purity of the final membrane preparation was assessed by testing for several enzymes. It was confirmed that the final membrane contained from 0-5 % of the total lysosomal activity in the case of catepsin, the same for β-glucorunidase and 5-10 % in the case of acid phosphatase.

Disc electrophoresis pattern and chemical analysis of the lysosomal membrane proteins before and after the solubilization procedure. Before and after the solubilization procedure, lysosomal membrane proteins gave an identical SDS-gel electrophoretic pattern. In both cases, the same electrophoretic mobility of the 12 polypeptides was observed; seven of which had PAS positive staining. Molecular weight stimations in 11.6 % acrylamide SDS gels were: 9.2 K, 13 K, 18 K, 25 K, 37 K, 54 K, 73 K, 82 K, 105 K, 145 K, 250 K and 480 K. Polypeptides of 480 K, 145 K, 37 K, 25 K, 18 K, 13 K and 9.2 K molecular weight, gave a positive carbohydrate staining. Since glycoproteins bind proportionally less SDS than marker proteins and as a consequence have slower mobility than polypeptides of similar size, glycoprotein molecular weight determinations are only approximate. However, this different electrophoretic mobility was improved running electrophoresis with low porosity gels.

As shown in table I, the percentage of phospholipids and cholesterol in lysosomal membranes were of 33 % and 8.6 % respectively. Partial delipidation of these membranes achieved by ultrasonic treatment decreased significantly the lipid content to 9.5 % phospholipids and 2.4 % of cholesterol. Total carbohydrate content in lysosome membranes was 9.4 % distributed as 4.6 for neutral sugars, 2.3 % for hexosamines, 1 % of N-acetylhexosamines and sialic acid and 0.5 % of methyl-pentoses. Slight differences in the chemical

Table I. *Chemical composition (%) of the lysosomal membrane of rabbit polymorphonuclear leukocytes.*

Constituent	Before delipidation	After delipidation	After delipidation and solubilization
Proteins	48.9	73.8	77.1
Phospholipids	33.0	9.5	8.0
Cholesterol	8,6	2.4	2.9
Neutral sugars	4.6	7.0	5.2
Hexosamines	2.3	3.6	3.4
N-acetyl hexosamines	1.0	1.5	1.7
Sialic acid	1.0	1.4	1.1
Methyl pentoses	0.5	0.8	0.6

composition of lysosome membrane were found after the solubilization procedure. Similar content in phospholipids, cholesterol, hexosamines and N-acetylhexosamines, sialic acid and methyl-pentoses were determined before and after the solubilization procedure.

Purification of lysosomal membrane proteins. Initial solubilized membrane protein preparation were applied to an ascending column of Sephadex G-200 fine, eluted with 70 mM Tris-HCl buffer pH 8.6, 8 M urea and 1 M guanidine hydrocloride. Two main peaks and a final trail (fig. 1, A), were obtained. As verified by gel electrophoresis polypeptides eluting in peak II were of intermediate molecular weight and when they were rechromatografied on Sephadex G-200 superfine (figure 1, B) three new and better defined peaks were obtained. After rechromatography of the top peak 2 (B) on Sephadex G-200 superfine (fig. 1, C), a single peak was obtained, in which only a polypeptide of 37,800 molecular weight was detected. This polypeptide had an identical electrophoretic mobility that the major protein of the lysosome membranes with a positive periodic acid- Schiff (PAS) reaction.

Polypeptides present in peak I obtained

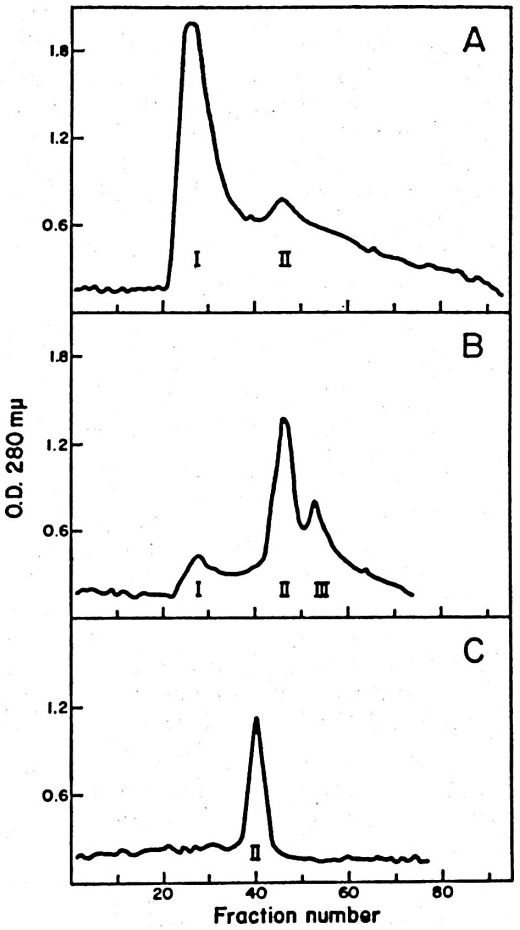

Fig. 1. *Gel filtration of lysosomal membrane proteins from rabbit polimorphonuclear leukocytes.*

The material was applied to columns (2.6 cm × 100 cm) of Sephadex G-200 equilibrated with 70 mM Tris-HCl buffer, pH 8.6, urea 8 M and 1 M guanidine hydrochloride. 4 ml fractions were collected.

after chromatography on Sephadex G-200 fine (fig. 1 A), were applied to ascending agarose 2B column and after that they were distributed in three new peaks (figure 2). Proteins eluting in the second peak of the last separation procedure were applied to an isoelectric focusing system, and

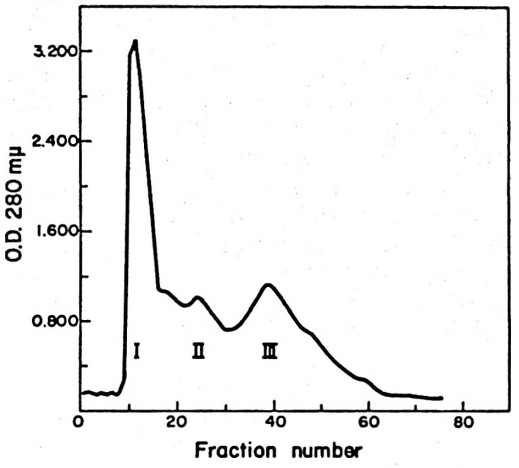

Fig. 2. *Chromatographic pattern of the higher molecular weight lysosome membrane proteins.*
The material was applied to columns (2.6 cm × 100 cm) of Agarose 2B, equilibrated with 70 mM Tris-HCl buffer, pH 8.6, urea 8 M and 1 M guanidine hydrochloride. 4 ml fractions were collected.

Table II. *Amino acid composition (moles/mole of protein) of three lysosomal membrane proteins, from polymorphonuclear leukocytes.* N.D. = Not determined; Methionine, Cysteine and Triptophan were not determined. Molecular weights from gel electrophoresis.

Amino acid	Proteins (molecular weights)		
	A (9,200)	B (37,800)	C (145,000)
Lysine	5.6	5.9	3.4
Histidine	2.9	1.9	0.9
Arginine	28.8	2.5	2.6
Aspargine	14.7	4.9	5.8
Threonine	5.6	2.7	2.3
Serine	7.0	10.8	6.6
Glutamine	19.9	6.6	7.8
Proline	9.4	8.7	2.3
Glycine	17.4	4.8	6.8
Alanine	17.3	3.8	3.3
Valine	10.1	1.9	3.0
Isoleucine	11.4	1.8	2.9
Leucine	21.5	3.2	5.0
Tyrosine	1.2	1.4	0.3
Phenylalanine	8.7	N.D.	1.4

in the eluates of pH 5 (fig. 3) a single polypeptide of 145,000 molecular weight was detected as verified by gel electrophoresis. Also, when the final trail obtained from Sephadex G-200 fine column (fig. 1, A) was applied to Sephadex G-200 superfine, 4 small peaks were detected.

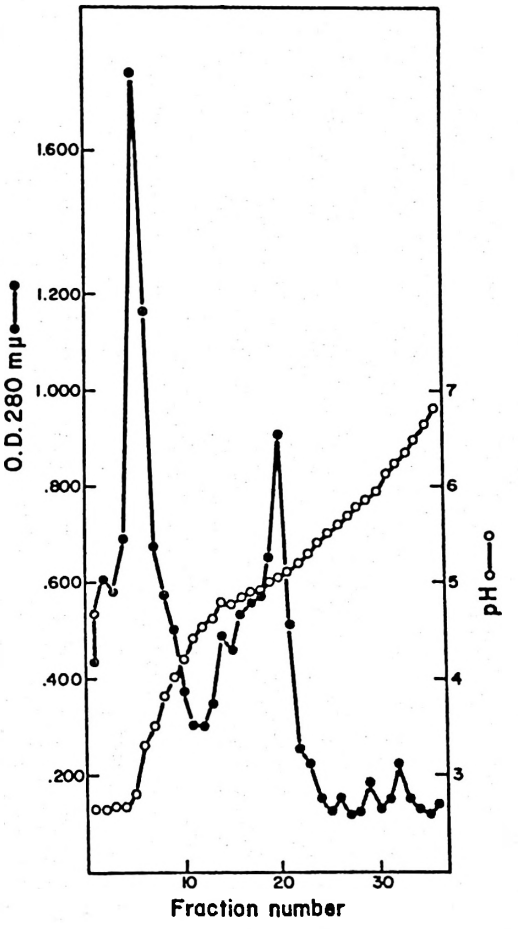

Fig. 3. *Preparative isoelectric focusing of the lysosome membrane proteins collected from the eluates of peak II of Agarose 2B column.* Isoelectric focusing was performed in a sucrose gradient containing 1 % ampholines with a 110 ml LKB column. Focusing was started at 800 V for two hours and continued at 1,200 V for an additional 46 hours.

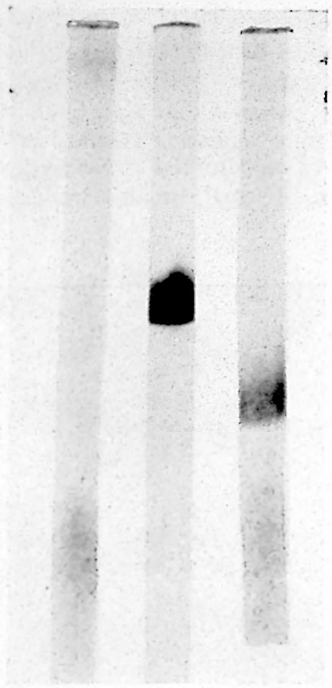

Fig. 4. *SDS polyacrylamide gel electrophoresis of the three isolated lysosome membrane proteins.*

Polypeptides were distributed according their molecular weight and in the fourth one eluted only the small polypeptide (9,200 M.W.) of the initial preparation.

Purified polypeptides were identified as single bands by gel electrophoresis with identical mobility that those observed in the initial lysosome membrane preparation (fig. 4). The three purified polypeptides gave a positive PAS reaction. Amino acid composition of these 3 polypeptides have been presented in table II. Protein A (9,200 M.W.) has a high content of arginine and also of leucine, glutamine, glycine and alanine. Protein B (37,800 M.W.) had a higher content of serine and proline, and protein C (145,000 M.W.) in glutamic acid and glycine.

Percentage of carbohydrates in protein A was of 19.6 % which was distributed as 12.4 % of neutral sugars, 3.6 % and 1.1 % of hexosamines and N-acetyl hexosamines respectively, 0.2 % of sialic acid and 2.3 % of methyl pentoses. Carbohydrate content of protein B was of 18.2 %, with a 10.1 % of neutral sugars, 1.5 % of hexosamines and 3.6 % of N-acetyl-hexosamines, 0.6 % of sialic acid and 2.4 % of methyl-pentoses. Protein C contained 13.6 % of carbohydrates, with 10.1 % of neutral sugars, 3.44 % of hexosamines and 0.12 % of sialic acid.

Discussion

Glycogen has a powerful stimulating effect on rabbit polymorphonuclear leukocytes migration into the peritoneum. In fact, when peritoneal exudates were drained 4 h after glycogen administration 95 % of the cells were made of polymorphonuclear leukocytes. These cells can be broken easily under mild conditions and since they are rich in lysosomes and poor in mitochondria a nearly pure preparation of lysosomes was obtained. In this connection, lysosomal preparations gave no reaction for cytochrome oxidase and when examined in the electron microscope only a few mitochondria were observed. Therefore, lysosomes membranes from rabbit polimorphonuclear leukocytes appear to be a good model for the chemical study of intracellular membranes. This is particularly so, because they can be obtained free of other cellular contaminants, and also possess a single unit membrane as seen in the electron microscope (26). Furthemore, only minimal enzymatic adsorption to these membranes was found. Chemical constituents of these membranes are very similar to that of plasma cell membranes. However, only 12 polypeptides were identified by acrylamide gel electrophoresis, which contrast with the approximately 40 polypeptides in the red blood cell ghosts (15) and in the plasma hepatocytes membrane (19), and in gen-

eral with a greater number in almost all plasma cell membranes. As a general biological phenomenon, it has been suggested (16) a close correlation between the number of proteins and the enzymatic activities in the cell membranes. Therefore, the smaller number of polypeptides in the lysosomal membranes could mean that their proteins fullfill only structural and recognition purposes. This would be further supported by the lack of enzymatic activities in the membrane of the lysosome (14). As it is well known, phagocytosis involve a first step of bacterial attachement to leukocyte plasma membrane followed by ingestión within a phagocytic vacuole. In a second stage, killing and digestion occur after fusion of the phagocytotic vacuole with lysosomes. Obviously a better understanding of the whole process may arise from the study of the chemical structure of both plasma and lysosomal membranes. Since recognition may be a key step in phagocytosis it seems cogent to suggest that specific binding sites are present in the lysosome membrane in order to recognize the phagocytic vacuole. Thus, in an attempt to correlate structure and function of lysosomes, we have developed a procedure for solubilization of the membrane proteins of these organelles. By chemical analysis and gel electrophoresis studies, before and after the solubilization procedure, we proved that membrane constituents remain in a similar proportion, thus validating this method for further purification steps. As shown in this paper, proteins in solution behaved nicely when applied to gel filtration or isoelectric focusing and three of them were isolated and chemically characterized. Also, GRANDA *et al.* (15) have been successful in applying this procedure to the purification of proteins from human red blood cells ghosts.

In protein A (9,200 M.W.) non-polar amino acids were found in greater percentage than the others, except for arginine which was the amino acid more broadly distributed. This unusual amount of arginine is similar to those observed in human plasma very low density lipoproteins (30, 31). After the treatment of the main protein B (37,800 M.W.) of lysosome membranes with cyanogen bromide, 3 polypeptides of 17,800, 13,000 and 7,000 molecular weights were obtained; two of them containing carbohydrates. More information about the physicochemical properties of this protein and its relation with the structure of lysosome membrane, is in progress.

The carbohydrate content of the three isolated lysosome membrane proteins was higher than in the whole membrane at expenses of neutral sugars and methylpentoses in proteins A and B. However, in the initial membrane preparation sialic acid was determined in greater amount than in the purified proteins. Sialic acid residues of glycoproteins are responsible of the negative charge at the cell surface (9). As for lysosome membranes, the N-acetylneuraminic acid is responsible of its strong negative charge and it is of great importance in maintaining a lower pH inside rather than in the surrounding medium. In fact, the acidity of lysosomes is the consequence of the selective permeability of lysosomal membrane to cations and protons and also by a Donnan equilibrium resulting from the intralysosomal accumulation of non-difusible anions as sialic acid bound to glycoproteins of the membrane (17).

Finally we could conclude that we have validated a method, which allows to isolate and chemically characterize the constituents of lysosomal cell membranes. This methodological approach may be of potential usefulness to a better understanding of the structure-function relationships at the membrane level.

Resumen

Se obtienen membranas lisosomales a partir de leucocitos polimorfonucleares de conejo, li-

bres de contaminantes subcelulares y con una minima adsorción enzimática. A excepción de un menor número de polipéptidos, los componentes químicos de estas membranas se encuentran en la misma proporción que en las membranas plasmáticas. Tras electroforesis en gel de acrilamida se determinaron los pesos moleculares (P.M.) de los 12 polipéptidos de las membranas lisosomales, encontrándose que oscilaban entre 9.200 y 480.000 daltons. Siete de estos polipéptidos dieron una tinción positiva con el reactivo de Schiff (PAS). Las proteínas de estas membranas fueron solubilizadas con ácido fórmico y mantenidas en solución con urea 8 M durante el proceso de purificación. Con la aplicación de métodos de filtración con gel y electroenfoque, tres de estas proteínas (P.M. de 9.200, 37.800 y 145.000) fueron aisladas y caracterizadas químicamente. El contenido en hidratos de carbono de estos polipéptidos fue mayor que en las membranas, a expensas de azúcares neutros y metil pentosas, pero con una menor concentración en ácidos siálicos. La composición de aminoácidos fue rica en arginina y aminoácidos no polares en el polipéptido con P.M. de 9.200 y con un gran contenido en ácido glutámico y glicocola en los otros dos. Este procedimiento puede significar un modelo útil para el estudio químico de sus proteínas, y como consecuencia de ello, para una mejor comprensión del proceso de fagocitosis.

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