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THE ISOLATION FROM RAT PERITONEAL LEUKOCYTES OF PLASMA MEMBRANE ENRICHED IN ALKALINE PHOSPHATASE AND A B-TYPE CYTOCHROME

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SUMMARY: A plasma membrane fraction more than 10-fold enriched in 5'-nucleotidase and alkaline phosphatase was prepared from peritoneal polymorphonuclear leukocytes. This fraction was highly enriched in MgATPase and a b-type cytochrome. High NADPH-duroquinone reductase activity was observed in the leukocytes, in addition to a lipid with quinone-like properties, neither of which cofractionated with plasma membrane. Therefore, we propose the possibility that an electron transport chain which functions to produce microbicidal oxygen metabolites is subdivided between the plasma membrane and one of the cytoplasmic membranes in non-phagocytizing cells.

The vital function of leukocytes in preventing microbial infection of the body is well known and dramatically demonstrated by chronic granulomatous disease of children in which leukocytes are abnormal and infections result in early death (1). Since the pioneering studies of Karnovsky and Cohn (2-5), it has been clear that phagocytosis and oxygen consumption are important aspects of the microbicidal process. NADH dehydrogenase, NADPH dehydrogenase, glutathione peroxidase, myeloperoxidase, superoxide and hydroxyl radical have been implicated (6-8). Although histochemical evidence suggests that dehydrogenase activity is associated with the plasma membrane (9), subcellular fractionation of the electron transport chain that functions to produce the lethal oxygen metabolites has not been possible. As an initial step towards *in vitro* reconstitution of this electron transport chain from individual components, we have isolated the plasma membrane fraction and report here that it is enriched in a b-type cytochrome which has recently been implicated by others to be necessary for production of oxygen metabolites (10-11).

METHODS

Leukocytes. Polymorphonuclear leukocytes were elicited in rat peritoneum by injection of sterile sodium caseinate (Difco Laboratories, Detroit) as

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described (12). About 15 h after treatment, cells were collected by peritoneal lavage with 0.9% sodium chloride and centrifugation at 1000 xg for 3 min. Red cells were removed by resuspending in cold water for 30 sec, restoring iso-osmolarity with saline and recentrifuging. Microscopic examination showed that polymorphonuclear leukocytes were the only major cell type; macrophages were prominent only after 3-4 days of treatment.

Plasma Membrane (Preparation I). The leukocytes were washed once each with saline and with 1 mM sodium carbonate, 50 mM potassium chloride and 250 mM sucrose and then resuspended in the latter using a volume of 1 ml per rat (about 1 ml per 20 mg of protein). The cells were diluted 5-fold by addition of cold water and, after 5 min on ice, were homogenized by 5 strokes of a type A pestle in a Dounce tissue grinder. Isoosmolarity was restored by addition of concentrated sucrose solution and the homogenate was centrifuged at 2000 xg for 3 min. The extract was decanted and saved; the unbroken cells and nuclei were rehomogenized. The two extracts were combined; after removing an aliquot, the combined extract was centrifuged at 100,000 xg for 35 min in a Beckman ultracentrifuge using a 50 Ti rotor. The pellets were resuspended in 5 ml of 1 mM sodium bicarbonate, 50 mM potassium chloride and 250 mM sucrose, and 4 ml was layered on top of a 20-50% (w/w) linear sucrose gradient and centrifuged at 100,000 xg for 20 min in a Beckman ultracentrifuge using an SW 27 rotor. In this gradient, the granules go deep; therefore, plasma membrane was collected from the upper region of the gradient by pumping 60% sucrose into the bottom.

Digitonin Treatment (Preparation II). The above preparation was not suitable for demonstrating a digitonin shift because the gradient was not centrifuged to equilibrium. For this, leukocytes were homogenized in a hypo-osmotic solution of 1 mM sodium bicarbonate, 0.5 mM calcium chloride as above but without restoring isoosmolarity. The extract was centrifuged at 20,000 xg for 20 min in a Sorvall centrifuge with an SS-34 rotor, and the pellets were resuspended in 1.0 mM sodium bicarbonate, 0.5 mM calcium chloride and recentrifuged. The washed pellets were resuspended in 5 ml of the same solution; 4 ml of which were layered on a 20-50% (w/w) linear sucrose gradient, with 100 mM potassium chloride throughout, and centrifuged at 100,000 xg for 2 h. Fractions of density about 1.15 g per ml contained plasma membrane markers, were collected by pumping 60% sucrose into the bottom and were treated with low concentrations of digitonin as described (13). Recentrifugation in the same way yielded the markers at densities of about 1.17 g per ml. Thus, a 0.02 g per ml digitonin shift, characteristic of plasma membrane (13), was observed.

Assays. Alkaline phosphatase (14) and 5'-nucleotidase (15) were assayed as described. ATPase was measured in 1.5 mM ATP, 3.0 mM $MgCl_2$, 50 mM potassium chloride, 1 mg of bovine serum albumin per ml buffered with 20 mM potassium morpholinopropane sulfonate, pH 7, incubated at 37°C for 20 min. The enzyme reaction was stopped by addition of trichloroacetic acid to 3% (w/v), and released phosphate was determined with ascorbate-ammonium molybdate (16). NAD(P)H-duroquinone reductase activity was measured with 50 mM morpholino-ethanesulfonic acid, pH 6, and 0.2 mM each NAD(P)H and duroquinone by following the absorbance at 340 nm using a Zeiss recording spectrophotometer. Protein was measured after precipitation with 5% (w/v) trichloroacetic acid by the Lowry method (17). Ubiquinone-like compounds were determined by oxidized vs. borohydride reduced difference spectra using a Cary 118 scanning spectrophotometer and the extinction coefficient of ubiquinone (18).

TABLE I

Isolation of Plasma Membrane Fractions from Rat Peritoneal Leukocytes

Preparation	Fraction	Protein	Marker Enzyme Activities		
			5'-AMPase	ATPase	Alk. P'ase
		(mg)	(μmol/min)		
I	Homogenate	62.1	2.37	10.1	3.62
	Plasma membrane	0.80	0.33	1.06	0.52
	(Enrichment		10.8	8.2	11.2)
II	Homogenate	45.8	1.30	5.34	2.00
	Plasma membrane	0.47	0.24	0.98	0.44
	(Enrichment		18.2	17.8	21.4)

Polymorphonuclear leukocytes were collected and fractions were obtained and assayed as described in Methods. Abbreviations: 5'-AMPase, 5'-nucleotidase; Alk. P'ase, alkaline phosphatase.

RESULTS

Polymorphonuclear leukocytes collected from the peritoneal cavities of rats treated with sodium caseinate were homogenized and subfractionated by differential and sucrose density gradient centrifugation to obtain a fraction enriched 10-fold in 5'-nucleotidase activity (Table I, preparation I). This fraction was similarly enriched in alkaline phosphatase activity; thus, in rat polymorphonuclear leukocytes, alkaline phosphatase is located in the plasma membrane as it is in rabbit (19) and in contrast with human neutrophils (20). Furthermore, the plasma membrane fraction had high MgATPase activity; the enrichment was nearly as great as the other markers and suggests that as much as 80% of the total leukocyte ATPase activity may be located in the plasma membrane. This surprising result can be explained in part by the complete absence of mitochondria in peritoneal polymorphonuclear leukocytes and is similar to that reported previously for human neutrophils (21).

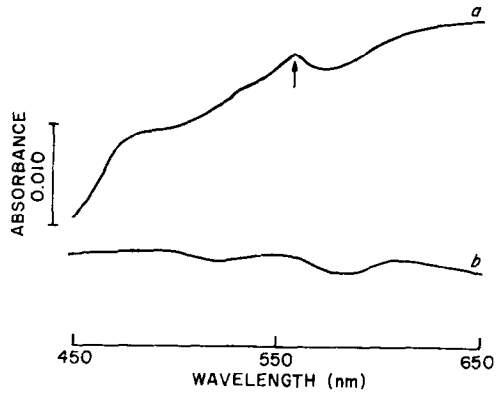

Fig. 1. Difference Spectrum of Plasma Membrane. Plasma membrane (preparation I) was in 0.5% Tween 80, 50 mM potassium phosphate, pH 7, 0.5 mM EDTA at 0.09 mg protein per ml and placed in matched cuvettes. A few grains of sodium dithionite were added to one cuvette, and then the reduced vs. oxidized spectrum was recorded. Reduction was complete within 5 min -- Trace a. Reduced vs. oxidized buffer blanks -- Trace b.

Digitonin treatment of fractions enriched in 5'-nucleotidase activity gave a 'digitonin shift' to heavier densities by 0.02 g per ml which is in good agreement with other plasma membrane studies (13, 20, 22). However, the digitonin preparation (Table I) was significantly more enriched in all the plasma membrane marker enzyme activities. It is not clear whether the greater enrichment is due to greater purity per se or due to a loss of extrinsic proteins which are associated with the plasma membrane in the intact cell. Cytoplasmic granules are prominent in leukocytes and are probably the major contaminant in both preparations; however, N-acetyl-β-D-glucosaminidase measurements, used to mark the azurophilic granules, indicated less than 10% contamination in both.

The plasma membrane preparations had a pink color and gave a b-type cytochrome visible spectrum (Fig. 1). Spectra of all subcellular fractions were analyzed by the Williams method (23). The enrichment of cytochrome in plasma membrane fractions (Table II) indicates that this is its predominant subcellular location. It is important to emphasize that the precision of the cytochrome determination is limited by uncertainty about the actual extinction

TABLE II

B-type Cytochrome Content of Leukocyte Subcellular Fractions

Preparation	Fraction	B-type Cytochrome (nmol/mg)
I	Homogenate	0.3
	Plasma membrane	3.1 (10X)
II	Homogenate	0.08
	Plasma membrane	1.2 (15X)

The fractions were prepared as in Table I. Cytochrome contents were calculated by the Williams method (23) and are approximate because maximum absorbance was at 558-560 nm rather than 562 nm.

coefficient and that the Williams method was developed for mitochondria. The gradual decrease in absorbance at lower wavelengths (Fig. 1) may be due to another chromophore, with maximum absorbance in the oxidized state at about 400 nm. We are investigating whether this is the flavin of a D-amino acid oxidase such as that reported to occur in the human polymorphonuclear leukocytes (24). It is clear, however, that a b-type cytochrome is located in the plasma membrane; this is an important observation in view of previous suggestions, based on spectral analysis of whole cells (10, 11), that this cytochrome is necessary for killing of microbes.

The presence of a cytochrome in the plasma membrane has added significance if an electron transport chain, that functions to produce the oxygen metabolites associated with phagocytosis, is divided between plasma membrane and an internal membrane in the non-phagocytizing or resting leukocyte. It is interesting that the resting leukocytes of this study are very active in NADPH-duroquinone reductase activity (Table III). In view of the association of cytochrome b and ubiquinone in mitochondria (25), it is surprising that,

TABLE III

NAD(P)H-Duroquinone Reductase Activities of Peritoneal Leukocytes and Liver Mitochondria

Sample	Duroquinone Reductase	
	NADH	NADPH
	(μmol/min-mg)	
Leukocytes	0.15	0.52
Liver mitochondria	0.16	0.002

Samples were prepared as in Table IV, and reductase activities were measured as described in Methods.

although leukocytes contain quinone (Table IV), enrichment does not occur in the plasma membrane fraction.

DISCUSSION

The plasma membrane of rat peritoneal polymorphonuclear leukocytes was isolated and found to contain 5'-nucleotidase, alkaline phosphatase, MgATPase

TABLE IV

Quinone Content of Peritoneal Leukocytes and Rat Liver Mitochondira

Sample	Quinone
	(nmol/mg)
Mitochondria	1.7
Leukocytes	0.15

Polymorphonuclear leukocytes were prepared as described in Methods. Rat liver mitochondria were isolated by differential centrifugation (15). Quinone content was determined by extraction with petroleum ether and by absorbance (oxidized vs. borohydride reduced) measurements using an millimolar extinction coefficient of 12.25 (18).

and a b-type cytochrome. The location of cytochrome in this membrane has implications in terms of the importance of membrane fusion in triggering the production of microbicidal metabolites. Others have reported that a b-type cytochrome is functional in the production of these metabolites (10, 11); furthermore, trifluorothienylbutanedione, which is competitive with ubiquinone (26), is an inhibitor of the respiratory burst associated with production of microbicidal metabolites (27). Since an enrichment of quinone was not found in the plasma membrane, we propose the existence of an electron transport chain which is subdivided between plasma and internal membranes in quiescent, non-phagocytizing cells. We predict that the functional electron transport chain is formed by membrane fusion during phagocytosis. This prediction can be tested by isolation of fused, or phagolysosomal, membranes from phagocytizing cells and by in vitro fusion of isolated plasma and granule membranes.

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