THE ISOLATION OF A PURIFIED PLASMA MEMBRANE FRACTION FROM RABBIT PERITONEAL LEUCOCYTES BY REVERSIBLE ADHESION TO NYLON FIBRES

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1. Introduction

The polymorphonuclear leucocyte (PMN-leucocyte) is a highly motile, phagocytic and secretory cell with a surface membrane responsive to a wide variety of foreign and naturally occurring mediators capable of triggering these processes. Although neutrophil chemotaxis and phagocytosis are fairly well understood phenomena at the descriptive level, the molecular events which take place within the plasma membrane for the receipt and translation of surface signals and the transport processes responsible for the maintenance of a satisfactory intracellular milieu are far from clear. Moreover there is a paucity of information about the extent of the association of cytoskeletal elements with membrane constituents and how these may be involved in the cells' motile phenomena.

To explore the structural and enzymatic basis for the contribution of the PMN-leucocyte plasma membrane to the cells' functional behaviour, the isolation of a surface membrane free from contamination by intracellular features is a necessary prerequisite. Most current procedures involve some form of mechanical rupture applied to the whole cells, followed by a subcellular fractionation by differential centrifugation or gradient centrifugation in a variety of media [1–4]. In a number of reports using different cell breakage techniques the low enrichment values for surface labels and/or marker enzymes reflect the technical problems in avoiding contamination by intracellular elements in the final plasma membrane preparation and the development in our understanding of the PMN-leucocyte plasma membrane has been frustrated by the need for cautious interpretation of such data.

In 1975 a procedure was briefly reported [5] for the isolation of phagocytic cell plasma membrane using the adhesion of the cells to nylon wool fibres. The procedure was based upon a filtration leucophoresis technique which is widely used for obtaining human neutrophils for clinical transfusion [6]. We have adapted this procedure for the isolation of a plasma membrane fraction from rabbit peritoneal PMN-leucocytes. It is a simple, rapid and low cost procedure which results in a plasma membrane vesicle population in high yield and which is considerably better enriched in surface labels and marker enzymes than some of the more time consuming techniques at present recommended.

Abbreviations: EDTA, ethylenediaminetetra acetic acid; EGTA, ethyleneglycol-bis(β-amino-ethyl ether)*N,N'*-tetra-acetic acid; 5'-nucleotidase, EC 3.1.3.5. (5'-ribonucleotide phosphohydrolase)

2. Materials and methods

Peritoneal polymorphonuclear leucocytes were elicited in rabbits by intraperitoneal injection of 500 ml 0.1% oyster glycogen and the cells were harvested after 16 h by washing out the peritoneal cavity with 250 ml sterile saline [7]. The cells were washed twice in isotonic Tris buffer (5 mM Tris, 0.154 M NaCl, 1 mM $CaCl_2$, pH 7.4, 290 mOsmol) and resuspended in this buffer at a cell/volume ratio of ~1:5. The yield by this procedure was $1.5–2.5 \times 10^9$ cells for 3.5–5.0 kg rabbits. In some experiments the cells were labelled with ^{125}I-labelled *Lens culinaris* lectin as in [8]. A cell suspension (5 ml at $1–2 \times 10^8$ cells/ml) in isotonic Tris buffer was applied to 0.88 g nylon wool (3 denier scrubbed nylon-wool fibre; Travenol Labs., Norfolk) loosely packed in the barrel of a 10 ml

plastic syringe held vertically. This wool fibre had been pre-washed with 7 bed vol. isotonic Tris buffer. The syringe nozzle was sealed and the syringe incubated in a water bath at 37°C for 30 min. After removal from the water bath any unattached cells were eluted from the packed fibre by 5 bed vol. isotonic Tris buffer (37°C). The cells were then lysed by the passage of 10 bed vol. hypotonic Tris buffer (25 mM Tris, 1 mM $CaCl_2$, pH 7.4, 30 mOsmol, 4°C) containing a protease inhibitor cocktail consisting of 2 mM EGTA, 0.2 mM PMSF, 2 μg leupeptin/ml and 2 μg pepstatin/ml. The lysis procedure removed most nuclear and cytoplasmic elements. The surface membrane attached to the fibre was removed by passage of 15 bed vol. 54 mM EDTA (4°C) with agitation provided by 3 short strokes of the syringe plunger during each passage. Residual nuclear material was removed from the membrane suspension by centrifugation (800 × *g* for 10 min at 4°C) and membrane collected as a pellet by centrifugation (100 000 × *g* for 90 min at 4°C).

The following enzymes were determined: 5′-nucleotidase [9]; thiocholine esterase [10]; and acid and alkaline phosphatase measured fluoremetrically using 4-methyl umbelligeryl-phosphate [11]. Lactate dehydrogenase was used as a cytosol marker [12]. Protein was measured as in [13] and DNA content monitored according to [14].

Scanning electron microscopy (SEM) was performed on the whole cells attached to the nylon fibres, after fixation with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) and critical point drying in acetone [15]. Samples for transmission electron-microscopy were fixed in a similar manner and stained [16].

3. Results and discussion

Fig.1 shows a scanning electron micrograph of nylon wool fibre strands teased out from the column immediately following the post-attachment isotonic wash. The leucocytes appear to remain reasonably spherical without significant spreading and higher magnifications showed negligible fragmentation. Using a Coulter model D counter the proportion of cells held in the fibre column was measured by counting the number of cells eluted during the isotonic washing and expressing these as a % of those applied. The % retained was 95 ± 6%, mean and standard deviation from 4 expt. This high level of retention was con-

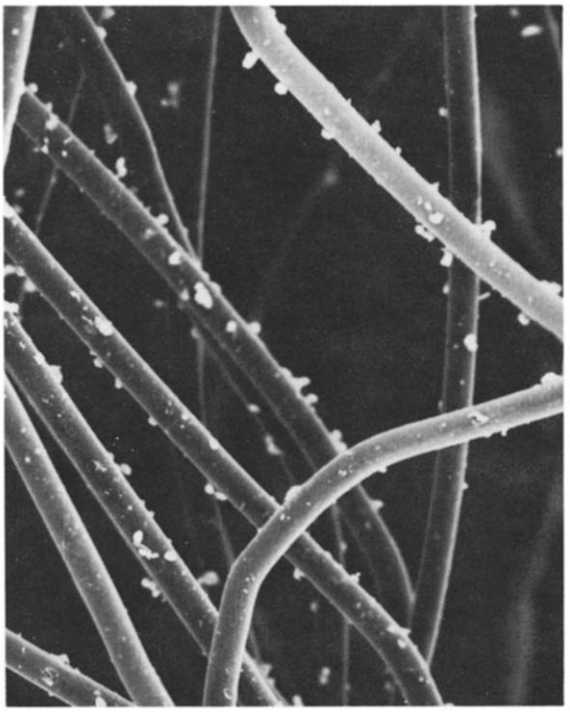

Fig.1. Scanning electron micrograph of nylon fibres teased from the column after isotonic Tris buffer washing. (Magnification × 400.)

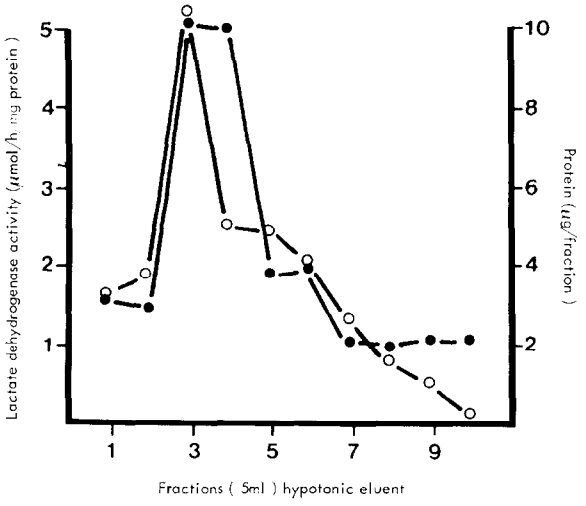

Fig.2. Lactate dehydrogenase activity (○) and released protein (●) in hypotonic eluent fractions.

firmed in a further 4 expt. using cells prelabelled with ^{125}I-labelled *Lens culinaris* lectin. Again the % of cells retained was >92%. This procedure, however, tends to underestimate the retention since there is some dissociation of the lectin label from the cells during the isotonic washing. In one experiment in which the PMN leucocytes were slightly contaminated with erythrocytes a comparison of the volume distribution curves of the cells applied to the column and those which were not retained showed that erythrocytes have little or no affinity for the nylon wool fibres.

The next stage in the isolation of the plasma membrane involves a disruptive procedure in which the attached cells are lysed in situ with a hypotonic buffer. The efficiency of this lysis was monitored by the progressive release of lactate dehydrogenase, and by the protein content measured in the eluates from each passage through the column. In a typical preparation (fig.2) maximal lysis occurred between the 2nd and 3rd passage of hypotonic buffer and the eluted lactate dehydrogenase activity had fallen to a negligible level after 10 passages.

Various concentrations and combinations of EGTA and EDTA were used to determine the optimal conditions for removal of the attached membrane fragments from the nylon wool (table 1). EDTA at a concentration of 1 in 50 (54 mM) was found to be the most suitable giving the greatest yield of protein from the column whilst at the same time the specific activity of the surface membrane markers, 5′-nucleotidase and lectin, expressed with respect to a whole cell homogenate reflected high enrichment. Under these conditions the yield of membrane is 1.0–1.5 mg protein/10^9 cells applied to the column.

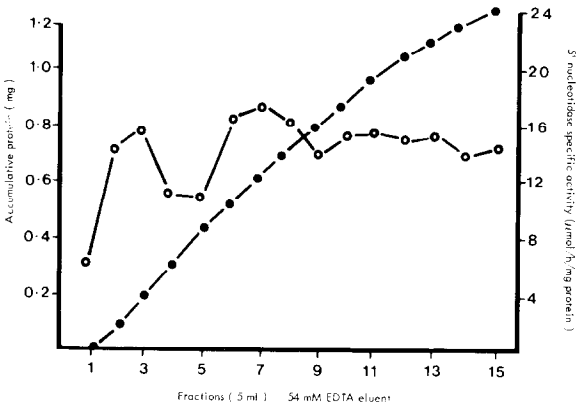

Fig.3. 5′-Nucleotidase (○), specific activity and accumulative protein (●) from successive elutions with 54 mM EDTA.

In the experimental work to establish the optimum conditions the membrane collected by each EDTA elution was analysed for total protein and for the specific activity of surface markers. Fig.3 shows a typical plot of accumulated protein and 5′-nucleotidase specific activity throughout the elution procedure. It is clear that as progressively more membrane protein is removed by each elution the specific activity of the nucleotidase remains reasonably constant suggesting that the high purification values for the membrane preparation are not due to the selective removal of 5′-nucleotidase-rich membrane domains. Similar results were obtained with elution of ^{125}I-labelled lectin.

In addition to nucleotidase activities and lectin label various other marker enzymes for intracellular membranes and organelles were also assayed and table 2 shows a list of the findings with some of these.

Table 1
Effect of different concentrations and combinations of EDTA and EGTA on the elution of the membranes from the nylon wool fibre (no. expts in parenthesis)

Conditions	Protein yield (mg/10^9 cells)	-Fold enrichment in spec. act. of:	
		^{125}I-Labelled lectin	5′-Nucleotidase
EDTA 0.54 mM	0.41 ± 0.1 [3]	17 [2]	31 ± 2.5 [3]
EDTA 5.4 mM	0.51 ± 0.05 [3]	16.5 [2]	28 ± 2 [3]
EDTA 54 mM	1.48 ± 0.2 [3]	18 [2]	28 ± 2.5 [3]
EGTA 5.4 mM	0.05 [2]	13 [2]	13 [1]
EGTA 54 mM	0.19 [2]	20.5 [2]	20 [2]
EDTA 5.4 mM + EGTA 5.4 mM	0.39 [1]	14 [2]	16 [1]

Table 2
Enrichment of surface membrane markers and other enzymes in rabbit PMN-leucocyte membranes

Enzyme	No. determinations	-Fold enrichment with respect to homogenate[a] activities (mean ± SD)
^{125}I-Labelled *Lens culinaris*	9	15 ± 4
5′-Nucleotidase	15	25 ± 5
Thiocholinesterase	3	9 ± 4
Phosphodiesterase	3	4 ± 1
Alkaline phosphatase	3	<0.1
Acid phosphatase	3	<0.1
Lactate dehydrogenase	4	<0.01
Deoxyribonucleic acid	1	<0.01

[a] For these studies a homogenate of the PMN-leucocyte was prepared by pestle homogenisation. Enrichment values were calculated with respect to the specific activities of the various constituents measured in these homogenates

The enrichment in the activity of 5′-nucleotidase in the membrane fraction generally fell in the range 20–32-fold enriched (25 ± 5 for 9 determinations) and the lectin 15 ± 4-fold enriched (9 determinations), indicating a level of purification which is difficult to achieve by more conventional density gradient procedures. The surface membrane preparation also shows negligible contamination with lactate dehydrogenase, the soluble phase marker enzyme or with alkaline and acid *p*-nitrophenyl phosphatase and DNA. A phosphodiesterase measured fluorometrically with *bis*-umbelliferyl phosphate [11] and a thiocholine esterase activity showed, respectively, 4- and 9-fold enrichment (means of 3 determinations) in the membrane fractions probably indicating a multisite location of these enzymes in the polymorphonuclear leucocyte.

Transmission electron microscopy (fig.4) of the membrane fractions showed sealed vesicles of varying diameters with some amorphous and stranded material interspersed between. Some of this material may represent associated cytoskeletal components.

In conclusion this simple and rapid procedure gives a highly purified plasma membrane fraction in good yield with high enrichment of surface markers. It may have considerable value in the study of surface membrane constituents and their interrelationships in experimental studies with the PMN-leucocyte. It is not known at present however if the attachment phase to the nylon wool fibre represents a simulated incomplete phagocytic process which may be accompanied by some perturbation of the membrane topography. This aspect is currently being investigated.

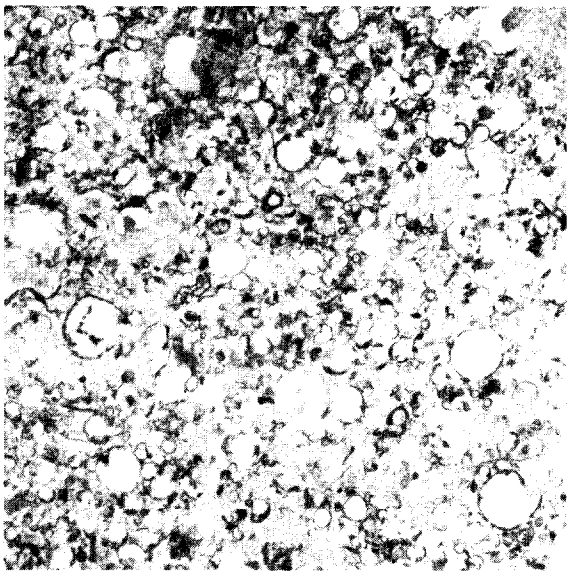

Fig.4. Transmission electron micrograph of 100 000 × *g* pellet from EDTA elution of the nylon fibre column. (Magnification × 13 333.)

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