Surface Membrane Electrokinetic Properties of Polymorphonuclear Leucocytes: Subpopulation Heterogeneity and Phagocytic Competence

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In cells which display plasma membrane-mediated motile activities (locomotion, pseudopodia formation, chemotaxis, phagocytosis, etc.) a variety of intrinsic plasma membrane constituents, some of which contribute to the cell's electrokinetic properties, have been indicted as participants in the motile phenomena. In particular, some attention has been focused upon certain membrane sialoglycoproteins as possible candidates for transmembrane control of the disposition and level of organisation of cytoskeletal elements such as actin, actin binding protein, and α-actinin. In some way, not yet understood, these structural and contractile proteins interact to produce force generation for the motile event.

A relationship between surface membrane electrical charge and motile phenomena has been speculated upon by a number of researchers. It appears that in many motile cells there is an inverse relationship between surface membrane electronegativity (zeta potential) and functional competence. For example, as early as 1966, Weiss and his colleagues showed that removal of sialic acid groups from the surfaces of monocytes and macrophages by neuraminidase resulted in enhanced phagocytic activity. In a similar study, Lichtman and Weed [6] showed that mature granulocytes from marrow, characterised by a high neuraminidase-labile negative surface charge density,

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were more rigid, less adhesive to glass and showed a slow rate of spreading and poor phagocytic capacity when compared with circulating blood polymorphonuclear leucocytes (PMNL), which were less electrophoretically mobile and showed a higher phagocytic rate. More recently Henricks et al [13] confirmed that neuraminidase-treated PMNL phagocytosed more opsonised staphylococci and showed a higher production of superoxide than control PMNL not exposed to the enzyme.

In the present paper, a study has been made of the phagocytic capacity of electrophoretically separated PMNL isolated from porcine blood. Four surface charge-dependent subpopulations were taken from the free flow electrophoretic profile and their phagocytic capacities quantified using opsonised paraffin oil droplets into which [3H]glycerol had been incorporated. An inverse relationship between this functional property and surface membrane electronegativity has been confirmed. This characterisation will form the basis for further studies directed towards understanding membrane glycoproteins and other constituents in transmembrane control of cytoskeletal organisation during phagocytic events.

MATERIALS AND METHODS
Preparation of Cells [1]

Fresh pig blood from a local abbatoir was anticoagulated with one-tenth volume of acid-citrate-dextrose and mixed with one-half volume of 6% dextran (MW-500,000). After allowing the agglutinated erythrocytes to settle (about 45 min), the supernatant plasma was removed and centrifuged at 130 g for 20 min. The pellet was gently resuspended in a Krebs-Ringer-buffer containing 13 mM glucose, DNase (100 μg/ml) and a protease inhibitor cocktail added consisting of 0.021 mM leupeptin, 0.0029 mM pepstatin, 0.2 mM PMSF, and 0.014 mM TPCK (final concentrations). The resuspended cells were loaded onto Ficoll-Hypaque in 100-ml centrifuge tubes (15 ml Ficoll-Hypaque cushion and about 60 ml cell suspension) and centrifuged at 400 g for 30 min. The sedimented cells (PMNL and some residual erythrocytes) were rapidly but gently resuspended in 20 ml of distilled water to lyse the erythrocytes. After about 30 sec, an equal volume of Krebs-Ringer-buffer containing 0.3 M NaCl was added to restore isotonicity. The suspension was then centrifuged at 130g for 10 min to collect PMNL. The lysis/wash-step was repeated several times to get a final PMNL suspension free of red cells.

Separation of PMNL by High-Voltage Free-Flow Electrophoresis

PMNL were washed and resuspended in chamber buffer (10 mM triethanolamine, 280 mM glycine, and 30 mM glucose, pH 7.35) containing DNase (100 μg/ml) and the protease inhibitor cocktail to give a final cell count of 25.10^7 PMNL/ml. The material was then applied to an Elphor VAP 5 free-flow electrophoresis apparatus (Bender and Hobein, Munich, FRG). A 100 mM triethanolamine buffer (pH 7.35) was used for the electrode buffer. The voltage was maintained at 1,150–1,160 V which generally gave a current of 135–155 mA at a chamber temperature of 6.0–7.0°C. The sample was injected at a flow rate of 1.2-1.4 ml/h, and the chamber buffer flow rate was maintained at 3.8 ml/h per fraction throughout the run.

Measurement of Phagocytosis

Phagocytosis by PMNL was determined by measuring the uptake of opsonised [3H]glycerol-labelled paraffin oil droplets; an aliquot containing 10 μCi (370 KBq)

[^{3}H]glycerol, [1(3)-^{3}H]-glycerol 92.5 GBq/mmol (Amersham International, UK) was mixed with 1 ml of paraffin oil. The oil was stirred into 3 ml of Krebs-Ringer-buffer containing 20 mg/ml bovine serum albumin. After sonicating the mixture for 3 min, a microscopically homogeneous preparation resulted. For quantifying phagocytosis, PMNL at a concentration between 2 and 7.3.10^7 cells/ml in RPMI-medium were incubated at 37°C under gentle agitation with the [^{3}H]glycerol oil droplets (volume ratio of oil to cells was 1:4.3). After 30 min incubation, 1 ml was taken, and 1.4 ml of icecold 0.15 M NaCl containing 1 mM NEM was added. The PMNL were washed several times by centrifugation at 400g for 10 min and finally resuspended in 1 ml of 2% Triton X-100. The suspension was transferred into vials, 10 ml of Beckman HP/b scintillation cocktail were added, and the radioactivity measured in a Phillips scintillation counter.

Electron Microscopy

The PMNL were fixed in glutaraldehyde, dehydrated, embedded in araldite, sectioned and poststained in uranyl acetate, and lead citrate and examined in a Corinth 275 transmission electron microscope. For scanning electron microscopy, the PMNL were fixed in glutaraldehyde (pH 7.3, cacodylate buffer), dehydrated, mounted, and coated with gold.

Measurement of G- and F-Actin

Actin in PMNL was measured by a DNase I inhibition assay in a modification [2] of the method described by Blikstad et al [3]. Measurement of the cytosol and cytoskeletal distribution of total cell actin was carried out using high-speed supernatants (at least 9×10^6 g min) and pellets prepared from the Triton X-100 homogenates after treatment of both fractions with 1.5 M guanidinium chloride to depolymerise the F-actin.

RESULTS

Using dextran/Ficoll-Hypaque, it was found that 394.5 $\pm$ 109.10^7 PMNL (n = 20) could be isolated from one litre of pig blood. The preparations were free of erythrocytes and platelets, and the cells had a typical morphology with a lobed nucleus and a cytoplasm rich in granules (Fig. 1).

For the separation of subpopulations, the cells were washed and resuspended in chamber buffer containing DNase and protease inhibitors. The addition of DNase and protease inhibitors was essential; otherwise the PMNL start to aggregate in the sample syringe or, during the injection, into the electrophoresis chamber. The separation profile consistently gave a broad peak extending over about 25 fractions as measured by extinction (E^{500}) readings (Fig. 2), and the whole of the separation profile was located on the anodal side of the entry port to the chamber. The PMNL were collected into four pools by subtending a perpendicular from the top of the peak to the base line and a horizontal at half peak height (Fig. 2). Each pool was centrifuged at 130g and resuspended in buffer to give a cell concentration sufficient for the measurement of phagocytic activity and cellular actin.

Scanning electron microscopy revealed no significant differences in the appearance of the cells in the different PMNL pools (Fig. 3), except perhaps the cells in the middle fractions in the profile (Fig. 3, P2 and P3) appeared to be slightly more
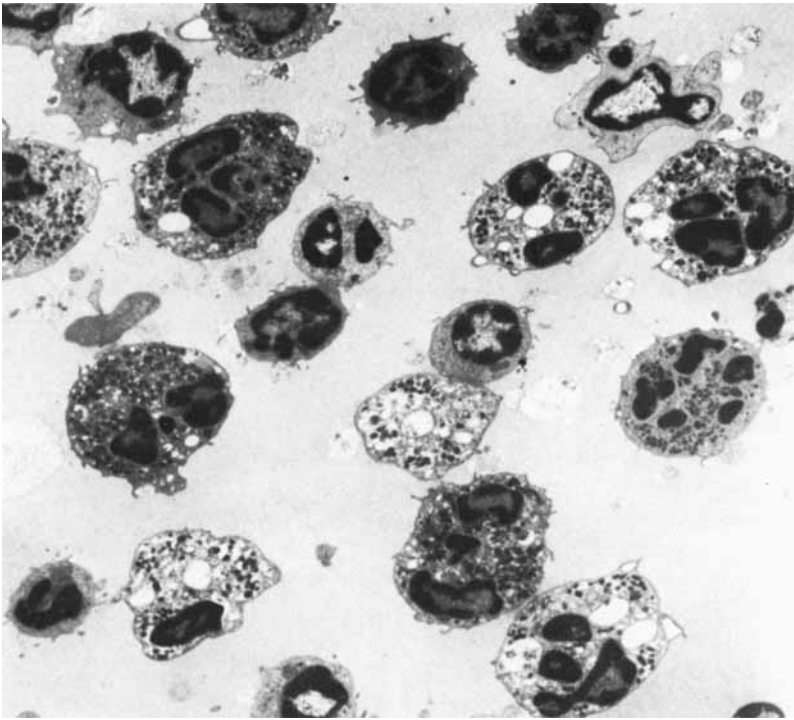
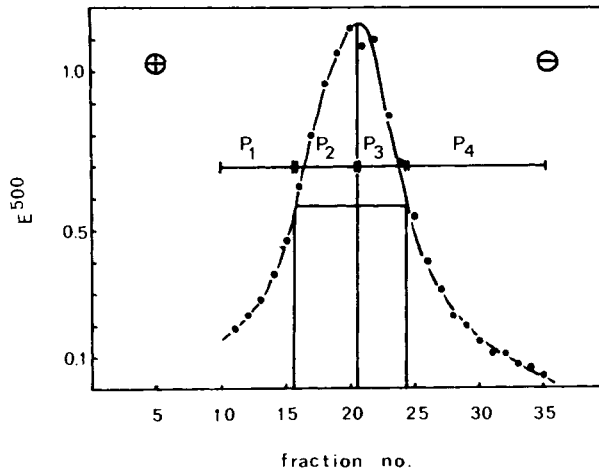Fig. 1.   Transmission electron micrograph of pig PMNL, ×3,000.

Fig. 2.   Free-flow electrophoresis profile showing distribution of PMNL as assessed by measuring the turbidity (E^{500}) of the chamber fractions.

spherical and less ruffled than those in the leading and trailing fractions of the electrophoretic separation (P1 and P4). The four pools located at exactly their original peak positions when they were re-electrophoresed (Fig. 4). Addition of the E^{500} - values from the electrophoretic profiles of the pools P1 and P3 (Fig. 4B) and P2 and P4 (Fig. 4C) gave essentially the same profile (Fig. 4D) as that from the original PMNL suspension (Fig. 4A).

After electrophoresis, the cells were viable as assessed by measuring their phagocytic capacity. In the present studies we have used a modification [4] of the quantitative phagocytic method developed by Stossel et al [5]. Paraffin oil labelled with [^{3}H]glycerol was sonicated in Krebs-Ringer-buffer in which bovine serum albumin was included for opsonisation. Phagocytosis of these particles by the PMNL was rapid and reached a plateau within 15 min of incubation at 37°C (Fig. 5). Measurements of the phagocytic uptake of the labelled oil made at 30 min of the four different pools revealed significant differences (Fig. 6).The least electronegative cells (fraction P4) had approximately twice the phagocytic capacity of the most electronegative cells (fraction Pl) and there was a clear inverse relationship between electrophoretic mobility and phagocytic competence across the profile.

Quantitative determinations of the amount of total cellular actin and cytoskeletal actin were made on the four different PMNL pools not subjected to a phagocytic stimulus. As shown in Table I, there was no significant difference in the amount of actin associated with the cytoskeleton in the different cell pools when expressed as a percentage of the total cell actin. The unseparated control PMNL contained 12.6 ± 5 μg total actin/10^6 PMNL (n = 6), of which 9.0 ± 3.9% was located in the Triton X-100 insoluble core as determined after depolymerization of F-actin with 1.5 M guanidinium chloride. There was no consistent trend in cytoskeletal actin content (expressed as percentage of total cell actin) across the electrophoretic profile in four different samples of pig PMNL separated into subpopulations.

DISCUSSION

The circulating PMNL are heterogeneous in their surface charge which is reflected in different levels of functional competence. As reported by Lichtman and Weed [6], immature PMNL possess a high negative charge density and are characterised by a low adhesiveness, a low propensity to aggregate, and a very low rate of phagocytosis. Mature PMNL are characterised by a lower surface electronegativity, a higher adhesiveness, a higher aggregatability, and a higher rate of phagocytosis.

Under normal conditions only a few immature PMNL are present in circulating blood, but in certain pathological conditions they can increase in number and could contribute to a variety of abnormal cellular reactions. A proportion of PMNL, for example in patients suffering from chronic granulocytic leukaemia, do not adhere well to glass or to nylon and are poorly phagocytic [7–9]. The surface charge of PMNL in vivo can also be modified secondarily by drugs, antibodies, and biologically active substances and, for example, Harthus and Hannig [10] demonstrated changes in surface charge density of PMNL induced by several lymphokines. Both receptor-induced changes in the topography of membrane constituents contributing to the cell's zeta potential and adventitious binding of components in the plasmatic environment (ions and proteins) may well be important features in the expression of surface-charge-dependent subpopulation heterogeneity.
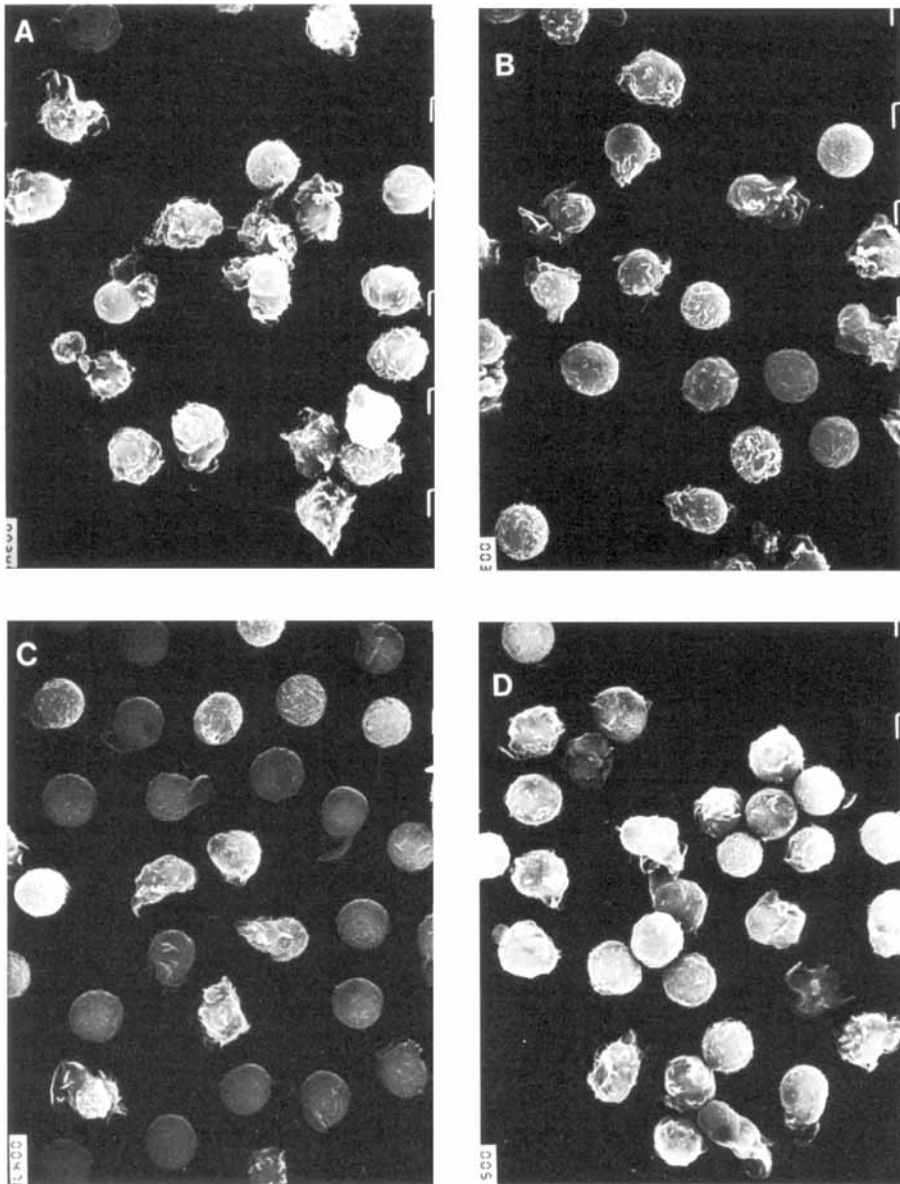
Fig. 3. Scanning electron micrographs of the four pooled subpopulations of pig PMNL **A** = pool P1; **B** = pool P2; **C** = pool P3; **D** = pool P4, ×2,500.

In order to study the biochemical and the functional heterogeneity of PMNL differing in their surface charge, separation methods on the preparative scale are required to obtain subpopulations in sufficient yield for functional and analytical testing. In this study, continuous free-flow electrophoresis has been used to collect cell subfractions from the circulating pool of PMNL according to their differing electrophoretic mobilities. Free-flow electrophoresis has been previously employed
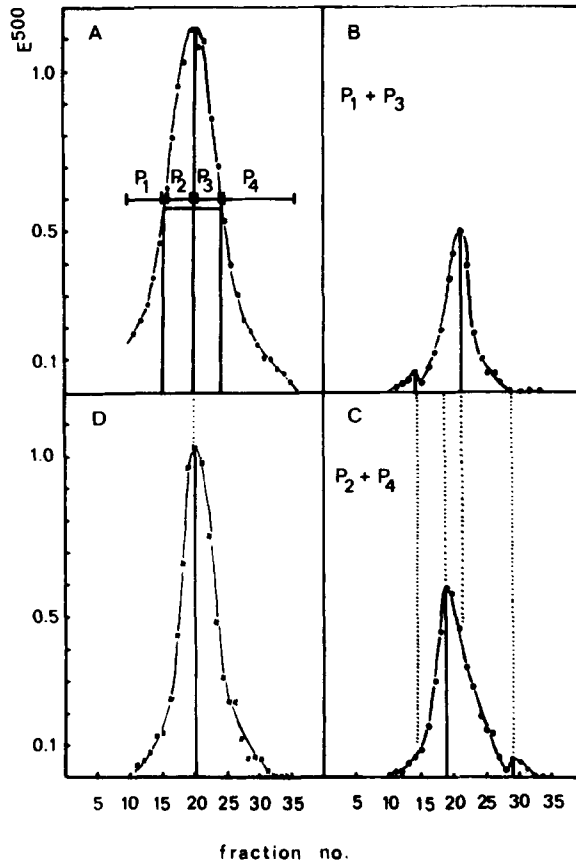
Fig. 4. Free-flow electrophoresis profile of pig PMNL (A), as well as the profiles of the re-electrophoresed mixed pools P1 and P3 (B) and P2 and P4 (C). D = the summation of the E^{500} values of the electrophoresis re-runs B and C.

by Heidrich and Hannig [11] to isolate different cell organelles and membranes, and at the whole cell level, lymphocyte subpopulations have been successfully separated into T and B cell subclasses by several authors [12]. To our knowledge there have been no previous reports of the separation of viable PMNL by free-flow electrophoresis so as to allow a quantitative appraisal of the phagocytic competence of subpopulations.

The pig PMNL separated by free-flow electrophoresis in this study produced a single broad peak, and the cells in the subfractions appeared to be morphologically normal. In some experiments the subfractions were re-electrophoresed. They located at exactly their original positions in the electrophoretic profile. For routine purposes, however, re-electrophoresis was not found to be necessary, although such a procedure might well reveal more significant analytical differences.

The functional viability of the PMNL was assessed by measuring their phagocytic capacity by the uptake of [³H]glycerol-labelled oil droplets. This assay is a more sensitive modification of the Oil Red "O" method first introduced by Stossel et al [5]; and a full characterisation of the procedure has been reported elsewhere [4]. As
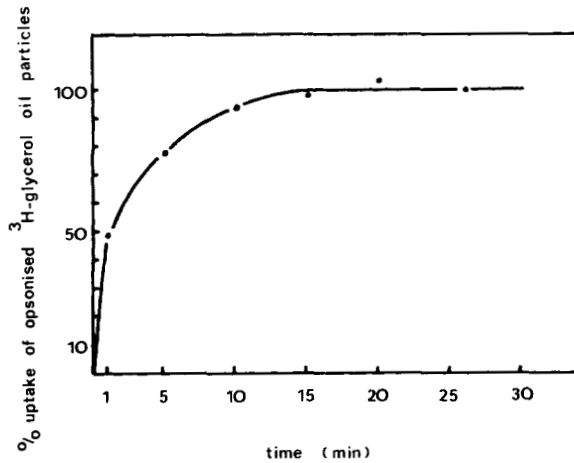
Fig. 5. Time curve for the uptake of opsonised [^{3}H]-glycerol-labelled oil droplets by phagocytosing neutrophils.

PMNL-pools after high-voltage
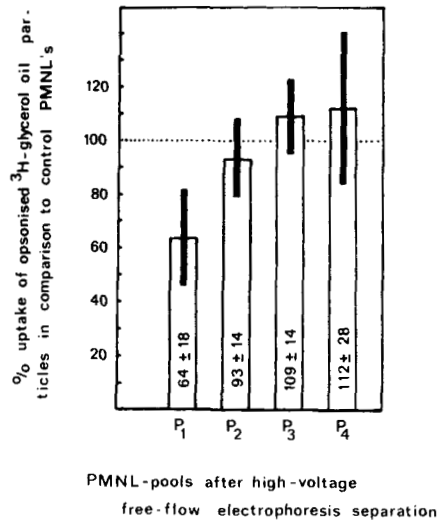
free-flow electrophoresis separation

Fig. 6. Phagocytic capacity of the PMNL pools (P1–P4) collected after free-flow electrophoresis. The horizontal line (100%) represents the phagocytic capacity of the whole PMNL population before electrophoresis.

seen in Figure 5, PMNL phagocytose the labelled particles quickly, reaching a plateau after 15 min of incubation.

In the assays of the subpopulations, the least electronegative PMNL had twice the phagocytic capacity of the most electronegative cells. These results are in general agreement with those of Lichtman and Weed [6]. It is important to recognise, however, that oil ingestion procedures represent a "frustrated phagocytic process" since, following internalisation, phagolysosomes are not formed [15]. It must be assumed that the plateau in the oil uptake curve represents the limit in plasma membrane surface area the cell can internalise without membrane recycling. It is, however, the recognition and endocytic events which are believed to be under

TABLE I. Distribution of Actin (F-actin) in the Triton-Insoluble Cytoskeletal Cores of the Polymorph Subpopulations[a]

	Cytoskeletal actin expressed as percentage of total actin content (n)
Total PMNL	9.0 ± 3.9^a (6)
P1 subpopulation	8.7 ± 0.9 (4)
P2 subpopulation	8.6 ± 3.6 (4)
P3 subpopulation	9.6 ± 2.6 (4)
P4 subpopulation	9.4 ± 5.0 (4)

[a]Values are mean $\pm$ SD.

cytoskeletal control [16]. Recently, Hendricks et al [13] reported an enhanced phagocytic capacity of PMNL treated with neuraminidase. The surface negative charge of PMNL which is at least in part due to the ionised carboxyl group of N-acetylneuraminic acid, can be substantially diminished by the use of this enzyme, and desialyation may be a contributory factor in juxtaposition of the particle and the membrane preparatory to internalisation.

A correlation between phagocytic capacity and the cytoskeletal associated actin content of resting PMNL could not be established in the present studies, although several workers have shown that actin polymerization occurs and some redistribution of cell actin takes place during phagocytosis with PMNL [14-16]. Whether changes in the disposition of cytosolic G- and F-actin, or the level of organisation of the polymer (ie, degree of cross-linking), occurs during the phagocytic event in the subpopulations remains to be investigated.

In conclusion, the present studies have shown that PMNL are separable into functionally viable charge-dependent subpopulations by continuous flow electrophoresis. These subpopulations differ markedly in their phagocytic behaviour with an established inverse relationship between phagocytic capacity and surface membrane electronegativity. This first study of PMNL subpopulation separation on the basis of surface charge heterogeneity will permit more detailed studies of locomotion, phagocytosis, chemotaxis, receptor status, and other aspects of membrane biochemistry in relation to the cell's electrokinetic properties and transmembrane links of surface constituents with intracellular elements involved in motile phenomena.

ACKNOWLEDGMENTS

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