Transmembrane Linkage between Surface Glycoproteins and Components of the Cytoplasm in Neutrophil Leukocytes

PETER SHETERLINE and COLIN R. HOPKINS
Department of Histology and Cell Biology (Medical), The University of Liverpool, Liverpool L69 3BX, England

ABSTRACT An experimental approach is described that enables the analysis of interactions between exogenous surface ligands and components of the cytoplasm in neutrophil leukocytes. Neutrophils treated with the nonionic detergent Lubrol PX, under controlled conditions, yield intact detergent-insoluble ghosts. Morphological analysis of neutrophil ghosts shows that they retain the original dimensions of the cell and consist almost entirely of a peripheral filamentous network, representing the submembranous cortical web, concentric to nuclear remnants. All intracellular membrane-bounded organelles, plasma membrane, and background cytoplasmic electron density are absent.

Biochemical analysis of the ghosts shows that <10% of enzyme markers for the soluble and granule fractions remain, and that >90% of total cell phospholipid is removed during detergent extraction. The major proteins remaining in the ghosts comigrate, on polyacrylamide gels in the presence of SDS, with chicken gizzard actin, myosin, filamin, and a 110-kdalton protein.

Patches and caps induced on neutrophils with either fluorescein isothiocyanate-concanavalin A or ferritin-concanavalin A retain their original location and morphology on ghosts after lysis, as determined by both fluorescence and electron microscopy. In similar experiments, but using 125I-labeled lectins, 37% of total cell bound concanavalin A (Con A) and 25% succinylated Con A remain attached to the ghosts.

A major 125I-labeled membrane glycoprotein (80 kdaltons) is associated with ghosts prepared from intact neutrophils iodinated in the presence of exogenous lactoperoxidase. Further 125I-labeled membrane glycoproteins (217, 170, and 147 kdaltons) become associated with ghosts prepared from iodinated cells treated before lysis with Con A, but not with succinylated Con A.

These data taken together suggest that linkages exist in neutrophils between proteins exposed on the outer surface of the plasma membrane and the peripheral filamentous network independent of the presence of lipid bilayer. The implications of these findings for surface motile phenomena will be discussed.

Since the wide acceptance of a membrane structure based on the fluid mosaic model proposed by Singer and Nicolson (35), it has become apparent that the lateral mobility of some membrane proteins may be constrained or influenced by their interaction, through the lipid bilayer, with components of the cytoplasm (14). This property of certain membrane proteins can be inferred from their behavior in the presence of multivalent ligands (34) and from lateral diffusion data obtained in photobleaching experiments (30, 31, 36). In some cases these phenomena can be enhanced or inhibited by treatment of cells with agents that interfere with microtubule or microfilament integrity (13, 14), thus leading to the predominant view that these structures are somehow involved either directly or indirectly in the anchorage or movement of membrane proteins. Data supporting this view have emerged from several laboratories, showing redistribution of actin and myosin, correlated with redistribution of membrane proteins by ligand. In particular, Singer and his colleagues have shown that actin, myosin, and \( \alpha \)-actinin are preferentially located or retained directly subadjacent to membrane protein clusters induced by ligand on the cell surface (9, 16). In fibroblasts, addition of multivalent lectins or antibodies directed against specific transmembrane
proteins causes clustering of their respective target proteins and their relocation over stress fibers (2, 3), further consolidating the view that actomyosin-containing structures influence the localization of certain membrane proteins. Recently, Flanagan and Koch (15) showed that microfilaments, isolated from detergent-lysed cells pretreated with anti-Ig antisera, were physically linked, either directly or indirectly to antibody, presumably via its antigen receptor Ig.

The evidence presented above suggests that interaction can occur between cytoplasmic components and several membrane proteins, including defined transmembrane proteins and unidentified lectin-binding glycoproteins. Thus this interaction may be regarded as a common feature of membrane structure. However, although it has inspired much speculation (1, 12, 23, 24), the nature of this interaction is little understood. However, although it has inspired much speculation (1, 12, 23, 24), the nature of this interaction is little understood. Despite the evidence presented above, interactions between membrane proteins and components of the cytoplasm cannot be retained on detergent-insoluble ghosts prepared from neutrophils. Preliminary identification of components involved in the interaction and some properties of the interaction are described.

MATERIALS AND METHODS

Isolation and Incubation of Neutrophils

Neutrophils were routinely prepared from blood collected directly from the jugular vein of pigs and prevented from coagulating by the addition of citrate and glucose. Erythrocytes were removed by gelatin sedimentation from blood, previously freed by centrifugation at 550 g for 20 min at 23°C from platelets, lymphocytes, and monocytes. The procedure is essentially that described by Henson (18) except that the optimal concentration of gelatin for agglutination of erythrocytes from pig's blood is 0.75% wt/vol. The purity of the cellsuspension in the jugular vein of pigs and prevented from coagulating by the addition of citrate described in the interaction and some properties of the interaction are.

Preparation of Detergent-insoluble Ghosts

The standard lysis procedure was performed on ice in a medium containing 4% wt/vol polyethylene glycol (1,000 mol wt), 1.5% wt/vol Lubrol PX, 60 mM KCl, 50 mM PIPES, 1 mM MgCl₂, 2 mM PMSF, and 10 μg/ml chymotrypsin adjusted to pH 6.8 at 23°C by addition of NaOH. Cells were sedimented in a Beckman microfuge (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.), followed by removal of supernate and resuspension of cells by gentle aspiration into a plastic automatic pipette tip.

Lectins

Concanavalin A (Con A) was prepared from jack bean meal (Sigma [London] Chemical Company Limited, Poole, Dorset, U. K.) by affinity chromatography on Sephadex G-100 essentially according to the method of Olson and Leiner (29). Fluorescein isothiocyanate (FITC)-derivatized Con A was obtained from Miles Research Laboratories, St. Louis, Missouri, U. S. A., and succinylated Con A, and its rhodamine isothiocyanate (RITC) derivative, RITC-succinylated Con A, were obtained from Vector Laboratories, Burlington, Calif.

Various iodination procedures were evaluated to produce lectins with both a high specific ¹²⁵I radioactivity and maximum biological activity. Both chloramine-T- and -lactoperoxidase-catalyzed (see references 21 and 27, respectively) iodination yielded products with high specific radioactivities, but the latter procedure yielded lectin with a higher biological activity as judged by binding to and specific elution from immobilized ligand. Lectins Con A and succinylated Con A (100 μg) were incubated in 50 μl of 50 mM sodium phosphate pH 7.0 at room temperature with 1.25 μM lactoperoxidase and 0.18 mM H₂O₂ for 15 min. ¹²⁵I-labeled lectin was separated from free iodine by molecular exclusion chromatography on Bio-Gel P-60 (Bio-Rad Laboratories Inc., Richmond, Calif.). The iodinated lectin was then mixed with native lectin to achieve a convenient specific radioactivity for binding studies, usually ~5 x 10⁶ cpm/μg. Because one of the uses for the lectins was to evaluate the binding parameters of cell surface glycoproteins, it was necessary to obtain an accurate value for the specific radioactivity of biologically active lectin. To this end the mixed native and iodinated lectins were subjected to affinity chromatography on an appropriate matrix, and bound lectin was eluted with 0.1 M glucose. The lectin peak was then dialyzed against Dulbecco's solution A containing 0.1 mM CaCl₂ and MgCl₂, and the specific radioactivity of the nondiffusible material estimated by using the protein assay of Lowry et al. (26) with lectin as standard.

Antisera were raised to Con A in rabbits by injection of 1 mg of Con A emulsified in either complete (first injection) or incomplete (subsequent injections) Freund's adjuvant at multiple sites on the dorsal surface of rabbits. Antisera were evaluated by immuno/diffusion in agarose against both Con A and succinyl Con A in the presence of 0.1 M ouabain.

Characterization of Lectin-binding Sites

Preliminary experiments showed that binding of lectins to washed, paraformaldehyde-fixed cells was identical to binding to live cells at 0°C. For convenience, and to avoid use of lectin by pinocytosis, binding experiments were performed on cells fixed and stored at 4°C in 2% (wt/vol) paraformaldehyde in 0.876% wt/vol NaCl, pH 7.4. Fixed cells were washed twice, then incubated at 0°C in Dulbecco's solution A, containing for 0.1 mM MnCl₂ and 0.1 mM CaCl₂, with no glucose or PMSF, at a cell density of 5 x 10⁵/m³. ¹²⁵I-Lectin was added at initial concentrations in the range 1-300 μg/ml. Cells required 30 min to equilibrate with Con A; after incubation, 50-μl aliquots of cells were centrifuged for 1 min in a Beckman microfuge through 100 μl of a 60:40 mixture of silicone oil MS550 and dinonylphthalate (SG 1.03) in 400-μl tubes. The cells pelleted, leaving the supernate on the silicone oil cushion. The free lectin concentration was calculated by removing a sample of supernate for determination of ¹²⁵I radioactivity. Bound lectin was determined by cutting off the tip of the tube just above the pellet and assaying for radioactivity in a gamma counter. Data were then subjected to Scatchard analysis using a computer program adapted from one originally devised by Dr. P. J. England, Department of Biochemistry, University of Bristol, U. K., which fits a linear regression line by the reduced common axis method of York (41).

Iodination of Cells

Cells were suspended into Dulbecco's solution A containing 16.5 mM glucose and 0.1 mM PMSF at 5 x 10⁵ cells/ml. Proteins exposed on the outer surface of the plasma membrane were iodinated in the presence of 100 μCi/ml ¹²⁵I (Radiochemical Centre, Amersham, Buckinghamshire, U. K.) 25 μg/ml lactoperoxidase and 140 μM glucose oxidase for 20 min at 0°C (20). Cells were washed twice in Dulbecco's solution A containing 0.1 mM PMSF, 5.5 mM glucose and 0.1 mM KI. Omission of glucose oxidase or glucose reduced incorporation of ¹²⁵I into TCA-insoluble material by >90%. Omission of lactoperoxidase caused an ~60% reduction in labeling, presumably because of the presence of myeloperoxidase released from cells in the medium.

Electrophoresis and Autoradiography

Whole cells freeze-thawed three times and detergent-insoluble ghosts were either added to Laemmli sample buffer (24) containing 2 mM PMSF or treated with DNase before solubilisation. Samples were triturated for a few minutes, then warmed to 100°C for 2 min, and allowed to cool, then 2 μl of 400 mg/ml iodoacetamide per 100-μl sample was added to alkylate sulfhydryl groups, and left for 15 min at room temperature. Gels were stained in Coomasie Blue and destained with acetic acid. The gels were scanned for autoradiography using a 2% vol/vol glycerol gel after destaining and before drying. Dried gels were exposed to Kodak NS-ZT film for between 3 and 10 d.

Assay Procedures

Protein was assayed by the method of Lowry et al. (26), using bovine serum albumin as the standard. DNA was estimated colorimetrically by the method of Burton (11). Lactate dehydrogenase was assayed by following the oxidation of NADH at 340 nm in the presence of pyruvate (7) by cell lysates (freeze-thawed three times) and detergent-insoluble ghosts. Myeloperoxidase was assayed by following the change in optical density at 440 nm by oxidation of o-tolidine (4). The reaction was carried out in 1.4 ml of 0.1 M sodium citrate, pH 5.0, containing

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glycerol in phosphate-buffered saline (PBS), and were fixed for 10 min at 23°C by addition of paraformaldehyde (2% wt/vol). Cells were usually centrifuged onto glass slides, mounted in 90% vol/vol glycerol in phosphate-buffered saline (PBS), and observed in a Zeiss photomicroscope as described in Materials and Methods. Bar, 20 μm.

Preparation of Cells for Microscopy

Cells incubated with fluorescent lectins or destined for phase-contrast microscopy were fixed for 10 min at 23°C by addition of paraformaldehyde (2% wt/vol). Cells were usually centrifuged onto glass slides, mounted in 90% vol/vol glycerol in phosphate-buffered saline (PBS), and observed in a Zeiss photomicroscope III equipped with epifluorescence optics. Images were recorded onto Ilford FP4 film.

FIGURE 1 Plastic 1-μm section of typical neutrophil preparation. Neutrophils were prepared from pig's blood and treated for microscopy as described in Materials and Methods. Bar, 20 μm.

Results

Neutrophils isolated from pig's blood by gelatin sedimentation of erythrocytes constitute >95% of the total cells and have a viability of >95%, as judged by exclusion of trypan blue. Eosinophils generally constituted the greatest single contaminating species but rarely exceeded 2% of the total cell population. The balance was made up from small numbers of erythrocytes, lymphocytes, and monocytes; a typical preparation is shown in Fig. 1. Neutrophils retained a high viability at room temperature for ~6 h, although experiments were generally initiated within 1 h of isolation.

Evaluation of the Lysis Procedure

It has been established elsewhere (see reference 19), and confirmed in this laboratory (38), that treatment of cells attached to culture dishes with nonionic detergents under controlled conditions removes the majority of membrane-bounded organelles and soluble proteins. The detergent-insoluble material remaining on the dish consists mainly of microfilaments, microtubules, and 10-nm filaments, which retain a distribution similar to that seen in fixed and permeabilized cells. A similar approach for the preparation of detergent-insoluble ghosts from neutrophil leukocytes in suspension is described here.

A variety of lysis media and conditions were evaluated on the basis of the following criteria: (a) the balance between retention of known contractile and cytoplasmic structural proteins and the efficiency of removal of presumed soluble proteins and granule contents, (b) the removal of total (chloroform/methanol soluble) lipid, (c) the disappearance of phase-contrast cytoplasmic granularity and unmasking of the nucleus, and (d) the retention of lectin-induced patches.

From the use of these criteria in preliminary experiments, the standard lysis conditions described in Materials and Methods were adopted. The data in Table I show that >50% of the DNA is retained in the detergent-insoluble ghosts and that removal of soluble components (represented by lactate dehydrogenase) and of granule contents (represented by myeloperoxidase and acid phosphatase activity) was extremely efficient. Only 10% of total phospholipid is retained by the ghosts. Thin-layer chromatography on silica gel plates indicated that the lipid remaining associated with the ghosts was entirely methanol soluble, (b) the disappearance of phase-contrast cytoplasmic granularity and unmasking of the nucleus, and (d) the retention of lectin-induced patches.

From the use of these criteria in preliminary experiments, the standard lysis conditions described in Materials and Methods were adopted. The data in Table I show that >50% of the DNA is retained in the detergent-insoluble ghosts and that removal of soluble components (represented by lactate dehydrogenase) and of granule contents (represented by myeloperoxidase and acid phosphatase activity) was extremely efficient. Only 10% of total phospholipid is retained by the ghosts. Thin-layer chromatography on silica gel plates indicated that the lipid remaining associated with the ghosts was entirely representative of total cell lipid (data not shown). Proteins remaining in the detergent-insoluble ghosts were separated by electrophoresis on polyacrylamide gels in the presence of SDS, and proteins were tentatively identified by comparison with those separated from chicken gizzard myofibrils (Fig. 2). The major proteins in the ghosts comigrated with gizzard actin, myosin, filamin, and a 110-kdalton protein. During preliminary lysis experiments it was noticed that recovery of proteins with the electrophoretic mobilities of actin-binding protein and myosin was rather variable, suggesting proteolysis during lysis. Whole-cell lysates were found to exhibit considerable proteolytic activity at neutral pH. Samples of freeze-thawed cells were assayed for protease activity, using azocasein as substrate, in the presence of a variety of inhibitors. The data in Table II suggest that there is major chymotrypsin-like protease activity present in whole-cell lysates, which can be substantially inhibited by using either PMSF or disopropylfluorophosphate (DFP) (irreversible but relatively slow acting serine protease inhibitors), together with chymostatin (a noncovalent but fast-acting peptide inhibitor of chymotrypsin-like proteases). These inhibitors were, therefore, included at 2 mM and 10 μg/ml, respectively, in the lysis medium.

Bands comigrating with α- and β-tubulin were noticeably weak or absent in SDS gels of ghost proteins. Attempts were made to enhance the stability of microtubules during lysis by the addition of EGTA, GTP, dimethylsulfoxide, and glycerol to the lysis medium and/or by extracting cells at 37°C instead of 0°C. These additional precautions, however, only marginally
improved the retention of tubulin in the detergent-insoluble ghosts and were not routinely adopted. It seems likely either that microtubules are particularly sensitive to the low levels of proteolytic activity remaining in the presence of inhibitors or, because neutrophil microtubules, which extend centripetally from the centrioles to near the cell periphery, are only 3–4 μm long, that even small losses by depolymerization from the distal end are sufficient to deplete the ghosts of these structures.

It is also notable that retention of proteins with the electrophoretic mobilities of actin-binding protein, myosin, and the 110-kdalton protein in ghost preparations, as judged by staining density of bands on polyacrylamide gels (Fig. 2), is relatively efficient, whereas large amounts of protein with the electrophoretic mobility of actin are lost. This is consistent with previous suggestions that much of the actin in nonmuscle cells is maintained in a soluble pool (10). The only other major polypeptide, 86,000 mol wt, which is also the major periodic acid/Schiff-positive band, is of unknown origin.

![Image of SDS polyacrylamide gels showing bands of proteins](https://example.com/image.png)

**FIGURE 2** SDS polyacrylamide gels of proteins from detergent-insoluble ghosts and from whole cells. Proteins from (a) 5 × 10⁸ neutrophils and (b) 10⁷ ghosts were separated on 6% (wt/vol) polyacrylamide gels and stained with Coomassie Blue. The scale indicates approximate molecular weight × 10⁻⁵. Mean values of molecular weight for ghost proteins given in Table VIII.

Three-times freeze-thawed neutrophils at 2.5 × 10⁷ cells/ml were incubated with azocasein at 10 mg/ml for 2 h at 37°C in the absence and presence of inhibitors and at the concentrations detailed above. Proteolysis was assessed by measuring the absorbance at 340 nm of dye released into a 5% wt/vol TCA-soluble fraction. DTT, dithiothreitol; TLCK, tosyllysylchloromethylketone; TPCK, tosylphenylalanylchloromethylketone; TPCK, tosylphenylalaninylchloromethylketone.

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>DNA</th>
<th>Phospholipid</th>
<th>LDH</th>
<th>Myeloperoxidase</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>µg</td>
<td>nmol P</td>
<td>U/min</td>
<td>ΔOD₄₅₀/min</td>
<td>ΔOD₄₅₀/min</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----</td>
<td>---------</td>
<td>--------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Cells</td>
<td>47</td>
<td>9.6</td>
<td>11.50</td>
<td>2.56×10⁻³</td>
<td>3.4×10⁻³</td>
</tr>
<tr>
<td>Ghosts</td>
<td>10</td>
<td>5.5</td>
<td>1.25</td>
<td>1.12×10⁻³</td>
<td>5.0×10⁻⁴</td>
</tr>
<tr>
<td>% Removed by extraction</td>
<td>79</td>
<td>43</td>
<td>89</td>
<td>99.6</td>
<td>85</td>
</tr>
</tbody>
</table>

Cells and detergent-insoluble ghosts were prepared, and appropriate numbers of cells or ghosts were assayed as indicated above using procedures described in Materials and Methods. These data are given as the mean of triplicate analyses on a representative ghost preparation.

**Table II**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc.</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>0.1 mM</td>
<td>41</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.0 mM</td>
<td>62</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>0.1 mM</td>
<td>7</td>
</tr>
<tr>
<td>DFP + Chymostatin</td>
<td>1 mM + 20 µg/ml</td>
<td>85</td>
</tr>
<tr>
<td>PMSF + Chymostatin</td>
<td>1 mM + 40 µg/ml</td>
<td>80</td>
</tr>
<tr>
<td>PMSF + Chymostatin + pepstatin 10 µg/ml + 20 µg/ml</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>PMSF + Chymostatin + DTT</td>
<td>1 mM + 10 µg/ml + 5 mM</td>
<td>74</td>
</tr>
<tr>
<td>PMSF + Chymostatin + EDTA</td>
<td>1 mM + 20 µg/ml + 2 mM</td>
<td>72</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>40 µg/ml</td>
<td>20</td>
</tr>
<tr>
<td>TPCK</td>
<td>1 mM</td>
<td>21</td>
</tr>
<tr>
<td>TLCK</td>
<td>1 mM</td>
<td>19</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>50 µg/ml</td>
<td>9</td>
</tr>
<tr>
<td>DTT</td>
<td>5 mM</td>
<td>11</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>5 mM</td>
<td>16</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
<td>19</td>
</tr>
<tr>
<td>p-Amino benzamidine</td>
<td>2 mM</td>
<td>3</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100 µg/ml</td>
<td>25</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>1 mg/ml</td>
<td>12</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>100 U/ml</td>
<td>0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>1,000 U/ml</td>
<td>39</td>
</tr>
</tbody>
</table>

Cells and detergent-insoluble ghosts were centrifuged onto glass slides and examined by phase-contrast microscopy (Fig. 3). The ghosts show a dramatic loss of the phase-dense margin corresponding to the cell periphery and of the cytoplasmic granularity. The characteristic multilobed nuclei that are masked by large numbers of cytoplasmic granules in whole cells are clearly visible in the detergent-insoluble ghosts and appear to retain a normal morphology.

Thin sections of epoxy resin-embedded material were also examined by transmission electron microscopy. These images show clearly that the general dimensions of the cell are retained by the detergent-insoluble ghosts (Fig. 3). The ghosts appear to consist almost entirely of a filamentous network, which corresponds to the cortical microfilament network, concentric to the...
nucleus. Membrane-bounded organelles, organized lipid bilayers and cytoplasmic background density are absent. In some cases, as shown in Fig. 3, the centrioles and some poorly preserved microtubules remain, but in general microtubules were rarely seen, in agreement with the electrophoretic data. The nuclear morphology remains relatively unchanged; it re-
tains not only its shape and position relative to the cell periphery but also appears to contain heterochromatin with a distribution similar to that seen in sections of intact cells.

**Effects of Lectin on Neutrophil Cell Surface Motility**

Application of Con A to neutrophils results in a defined sequence of events. The lectin rapidly forms sizable clusters on the cell surface, followed in a proportion of the cells by relocation of the patches into caps (Fig. 4). Endocytosis takes place slowly (t½, ~10 min) at the sites of both patching and capping. The effects of Con A can be compared with those induced by succinylated Con A, a divalent derivative of Con A (17). Succinylated Con A does not induce either patching or capping on neutrophils, but if cells treated with succinylated Con A are incubated with anti-Con A IgG, then the degree of patching and capping is restored to that observed at a similar concentration of Con A. These data suggest that the induction of patches and caps by lectin on neutrophils is highly dependent on valency. Studies on the binding parameters of lectin-binding sites confirm that both Con A and succinylated Con A bind to a similar number of sites on the cell surface (Table III).

The nature of the patching and capping phenomena on neutrophils was investigated by using a variety of inhibitors. Capping could be completely inhibited by reducing the total cellular ATP content below 20% (data not shown) of control levels in the presence of iodoacetate or 2-deoxyglucose. Capping was also prevented by incubation of neutrophils in the presence of cytochalasin D, which prevents growth onto prenucleated F-actin polymer (25), trifluoperazine, which prevents Ca²⁺ activation of target enzymes in the presence of calmodulin, notably in this context of myosin light-chain kinase (32), and A23187, a Ca²⁺ ionophore (Table IV). These data are consistent with the hypothesis that the force driving redistribution of patches into caps derives from an interaction between actin and myosin. None of these treatments influenced the formation of patches on neutrophils.

**Lectin Attachment to Detergent-insoluble Ghosts**

The work of Flanagan and Koch (15) had previously shown that linkage between microfilaments and clustered cell surface Ig on lymphocytes was stable in the presence of nonionic detergent. To determine whether lectin-induced patches or caps remained associated with detergent-insoluble ghosts, neutrophils were treated with FITC-Con A at 37°C, to allow patching and capping, and then lysed. In both cases the appearance and distributions of both patches and caps on whole cells and on ghosts prepared from the same batch of cells were identical (Fig. 5). If cells were treated with FITC–succinylated Con A, the lectin remained evenly distributed over the surface of neutrophils. Detergent-insoluble ghosts prepared from these cells also showed reduced but even fluorescence around the periphery.

The relationship between lectin-induced patches and components of the detergent-insoluble ghosts was examined in more detail by electron microscopy (Fig. 6). Cells were incubated as before but with ferritin–Con A before lysis. The

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**Table III**

Characterization of Lectin-binding Sites on Neutrophils

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Dissociation constant (Ka + SEM x 10⁶ M⁻¹)</th>
<th>No. of sites/cell (±SEM x 10⁻⁴)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>6.56 ± 0.49</td>
<td>8.99 ± 0.42</td>
<td>0.974</td>
</tr>
<tr>
<td>Succinylated Con A</td>
<td>99.9 ± 5.56</td>
<td>7.33 ± 0.23</td>
<td>0.904</td>
</tr>
</tbody>
</table>

Iodinated lectins were prepared, and equilibrium binding assays performed as described in detail in Materials and Methods. Data was analyzed by using a computer program for calculating the dissociation constant and number of binding sites/cell on the basis of a reduced axis linear regression on data in the form of a Scatchard analysis. The correlation coefficient reflects closeness of fit of the data to the calculated regression.

**Table IV**

Effects of Various Agents on Con A–induced Capping by Neutrophils

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentration</th>
<th>No. of caps as percentage of total cells, with and without colchicine (10⁻⁶ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A23187</td>
<td>10⁻⁵</td>
<td>13 → 76</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>2 × 10⁻⁶</td>
<td>12</td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>2.5 × 10⁻²</td>
<td>7</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>2 × 10⁻⁵</td>
<td>8</td>
</tr>
</tbody>
</table>

Cells were incubated at 10⁻⁶/ml in the absence or presence of colchicine for 30 min at 37°C. 2-Deoxyglucose was added at t = 0 min and other inhibitors at t = 20 min. At t = 30 min, 20 µg/ml FITC–Con A was added and cells incubated for a further 5 min at 37°C. Samples of cells (10⁶) were fixed in paraformaldehyde. The percentage of total cells displaying caps was estimated from counts of at least 200 cells or ghosts. No qualitative differences in the appearance of caps in control and treated cells were observed.
FIGURE 5  Retention of patched and capped FITC-Con A on detergent-insoluble ghosts. Cells were treated with FITC-Con A at 20 μg/ml for 5 min at 37°C. Samples of cells were fixed in 2% (wt/vol) paraformaldehyde, the remainder lysed according to the schedule in Materials and Methods, then fixed. Cells and ghosts were centrifuged onto slides and examined by fluorescence microscopy. (a) Capped and patched neutrophils. (b) Ghosts prepared from a similar preparation of cells. Bars, 5 μm.

majority of the ferritin-Con A maintains a location in the ghosts clearly reminiscent of that observed in sections of whole cells either on the cell surface as patches or within endocytic vesicles or invaginations of the plasma membrane. As there was no evidence for the presence of the intervening plasma membrane in any of the sections examined, in contrast to previous studies (12), retention of lectin on the ghosts must occur by means independent of the presence of lipid bilayer.

Two approaches were adopted for further analysis of the nature of lectin attachment to detergent-insoluble ghosts. First, neutrophils were incubated with 125I-labeled lectins and allowed to patch and cap. Cells were then lysed, and the proportion of the original cell bound lectin remaining on the ghosts was assessed. The data are presented in Table V. It can be seen that although 40% of the total cell-bound Con A survives lysis, only 25% of the total succinyl Con A remains associated with the ghosts. The distribution of retained lectin in the ghosts has been described above (Figs. 5 and 6). Patches also remained attached to ghosts prepared from cells treated with agents that block the redistribution of patches into caps, i.e., as in Table IV (data not shown).

In the second approach, receptive groups exposed on the outer surface of the plasma membrane were labeled with 125I in the presence of lactoperoxidase and glucose oxidase. Preliminary iodination experiments confirmed, in common with the experience of others (28, 40), that large amounts (~30%) of 125I were taken up by the cells during iodination. However, covalent modification occurred almost exclusively to protein exposed on the cell surface. No labeling of intracellular proteins with electrophoretic mobilities corresponding to actin, myosin, or actin-binding protein was observed, and in the absence of extracellular glucose oxidase 125I incorporation was reduced below 10%.

The degree of labeling of lipid molecules exposed at the outer surface of the cells was determined by separating chloroform-soluble material from whole cells and comparing the total radioactivity in this extract with total TCA-insoluble material. Less than 15% of the 125I was found associated with the chloroform/methanol-extracted material. Lipids were further separated by thin-layer chromatography; Table VI shows that the proportion of 125I associated with (unidentified) glycolipids in ghost preparations from lectin-treated cells was similar to that in control preparations, suggesting that a preferential association of particular 125I glycolipids with these ghosts is unlikely.

If ghosts were prepared from iodinated cells incubated in either Dulbecco's solution A or medium containing lectins, significantly more TCA-insoluble 125I was associated with ghosts prepared from cells incubated with lectin before lysis (Table VII). However, because 26% of the total 125I-labeled TCA-insoluble material remains attached to ghosts prepared from cells incubated in the absence of lectin, the nature of this associated label was examined further.

Proteins from ghosts prepared from iodinated cells were separated on polyacrylamide gels in the presence of SDS, then subjected to autoradiography. It can be seen from Fig. 7 and Table VIII that eight consistently labeled proteins could be identified on autoradiograms of total cell proteins separated on gels. However, only one major labeled species remains associated with ghosts isolated from control cells. This protein, which has a molecular weight of 80,000, thus appears to be the predominant labeled component that is both exposed on the outer surface of the plasma membrane and attached, in the absence of ligand, to the detergent-insoluble ghosts. The possibility that the 80-kdalton protein is 125I-labeled lactoperoxidase was excluded by showing that (a) lactoperoxidase does...
Figure 6. Location of ferritin-Con A on capped neutrophils and in detergent-insoluble ghosts prepared from a similar preparation. Cells were incubated with ferritin-Con A (50 μg/ml Con A equivalent) for 5 min at 37°C. Detergent-insoluble ghosts were prepared from samples of treated cells, and both cells and ghosts were fixed and prepared for electron microscopy as detailed in Materials and Methods. (a) Capped neutrophil. Bar, 1 μm. (b) Ghost prepared from capped neutrophil. Note the retention of the surface features of the capped neutrophil in the ghosts. Bar, 1 μm. (c) Unstained thick (interference color green) section of capped region of ghosts showing retention of the vesicular distribution of ferritin-Con A. Bar, 200 nm. (d) Thin section (silver interference color) stained with lead citrate of similar region showing the filamentous nature of the peripheral web. Bar, 200 nm.
not comigrate with the 80-kdalton protein (Table VIII, (b)). The labeling efficiency of the 80-kdalton protein is not reduced in the presence of competing sugar, 0.1 M α-methyl-D-mannoside for 5 min at 37°C. Total cell and ghost proteins were associated with electrophoresis in the presence of SDS on 7% (wt/vol) polyacrylamide gels; then the dried gels were subjected to autoradiography. The scale to the right of the gels shows approximate molecular weights × 10^{-3}, derived from standard curves.

TABLE V

<table>
<thead>
<tr>
<th>Lectin</th>
<th>% Cell-bound 125I-lectin</th>
<th>No. binding sites/ghost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>3.70 ± 4.8 (4)*</td>
<td>3.3 x 10^5</td>
</tr>
<tr>
<td>Succinyl Con A</td>
<td>2.5 ± 1.7 (4)*</td>
<td>1.8 x 10^5</td>
</tr>
</tbody>
</table>

Cells at 5 x 10^7/ml were incubated with 125I-lectin in the absence or presence of competing sugar, 0.1 M α-methyl-D-mannoside for 5 min at 37°C. Cells were washed once at 0°C. 50-μl aliquots of cells were then centrifuged through a silicone oil cushion. Detergent-insoluble ghosts were prepared from remaining cells and similar aliquots centrifuged through silicone oil. The bottoms of the tubes were cut off and assayed for 125I in a gamma spectrometer. Specific lectin binding was taken to be the difference between that observed in the absence and presence of competing sugar.

The values given above are the percentage of lectin bound to cells remaining after preparation of ghosts. The significance of differences between two groups of data was determined by applying Student's t test.

* P < 0.05 Student's t test. Numbers in parentheses = number of trials.

TABLE VI

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Cells</th>
<th>Ghosts</th>
<th>Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>7.5</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>15.6</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>27.4</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>10.4</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>38.5</td>
<td>4.5</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Chloroform/methanol-soluble lipid was extracted from 125I-labeled cells, ghosts prepared from the same, and the extract subjected to thin layer chromatography. Spots were located and their 125I content assayed as described in Materials and Methods. The glycolipid values are given as if for a single lipid; this entry, however, consists of three discreet but unidentified spots. The distribution of 125I within the group was similar in all cases. Other lipids were tentatively identified by comigration with standard lipid samples. The amounts of TCA-insoluble 125I and chloroform-soluble 125I associated with cells and ghosts are given as follows for 10^6 cells (or ghost equivalents): (a) cells, TCA-insoluble 125I, 152,568 cpm, chloroform-soluble, 21,996 cpm; (b) ghosts, TCA-insoluble 29,844 cpm, chloroform-soluble, 3,175 cpm; ghosts prepared from Con A-treated cells, TCA-insoluble 35,244 cpm, chloroform-soluble 3,430 cpm.

TABLE VII

<table>
<thead>
<tr>
<th>Lipid</th>
<th>TCA-insoluble 125I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As % of total cell</td>
</tr>
<tr>
<td></td>
<td>125I</td>
</tr>
<tr>
<td>Control cells</td>
<td>100</td>
</tr>
<tr>
<td>Control ghosts</td>
<td>26.8 ± 5.7 (14)</td>
</tr>
<tr>
<td>Con A ghosts</td>
<td>36.3 ± 6.2 (14)</td>
</tr>
<tr>
<td>Succinyl Con A ghosts</td>
<td>27.2 ± 5.3 (14)</td>
</tr>
</tbody>
</table>

Cells were iodinated at 5 x 10^7/ml in ice in the presence of lactoperoxidase and glucose oxidase, then washed twice in ice-cold Dulbecco's solution A containing 0.1 mM KI. Washed cells were then incubated with lactin for 5 min at 37°C, washed twice in ice-cold medium, and 200-μl aliquots (10^6 cells) were added to ice-cold 5% wt/vol TCA containing 1 mM KI. Lactoperoxidase was then added to equivalent aliquots and these too were added to 5% wt/vol TCA. After standing on ice for 10 min TCA-insoluble material was collected by centrifugation and washed twice in further volumes of 5% wt/vol TCA. Pellets were then assayed for 125I radioactivity. The significance of differences between two groups of data was determined by applying Student's t test.

* P < 0.001 Student's t test.

Ghost and cell proteins were separated in the presence of SDS on polyacrylamide gels and stained with Coomassie Blue; dried, then subjected to autoradiography. Molecular weights given are the mean ± SEM for four separate preparations and are derived by comparison with the mobility of myosin, 200,000 daltons; phosphorylase b, 95,000 daltons; bovine serum albumin, 68,000 daltons; tubulin, 54,000 and 56,000 daltons; actin, 42,800 daltons.

* Nos. 1–8, indicate labeled bands on autoradiograms shown in Fig. 7, lanes b–e.

† Letters A–F correspond to Coomassie-Blue-stained bands in the gels shown in Figs. 2, lane b and Fig. 7, lane a. Band A comigrates with chicken gizzard filamin. Band B comigrates with chicken gizzard myosin heavy chain. Band F comigrates with chicken gizzard actin. Band D stains positively for carbohydrate with periodic acid-Schiff's reagent. The lactoperoxidase used in these experiments had a molecular weight of 87,283 ± 5,067 as measured by its relative mobility in polyacrylamide gels.

(c) small amounts (<10% control) of 125I are incorporated into the 80-kdalton protein if lactoperoxidase is omitted from the iodination medium. The iodinated band consistently ran in
The experiments described above were initiated to find a means of studying the molecular events occurring at the cell surface after binding and redistribution of lectin. The approach evaluated the possibility that interactions between membrane proteins and components of the cytoplasm occur in neutrophils, and, further, that these interactions can be retained and studied on detergent-insoluble ghosts.

The biochemical and morphological data show that extraction of neutrophils with Lubrol PX removes the majority of the cytoplasmic matrix components, intracellular organelles, and lipid bilayers of membrane boundaries. Only 20% of the total cell protein remains in the ghosts. For the most part this residual protein can be accounted for by the proteins, actin, myosin, actin-binding protein, a 111-kdalton protein that comigrates with a prominent band from gizzard muscle, and an unidentified 86-kdalton glycoprotein. Because the only structures observed in the ghosts are the peripheral filamentous network and a nuclear remnant, it is probable that the majority of the contractile related proteins are located in the filamentous network.

Treatment of neutrophils with Con A causes rapid clustering of lectin-binding sites on the cell surface followed, in a proportion of the cells, by relocation of these clusters into caps at one pole of the cell. Capping can be blocked by a range of inhibitors that would be expected to interfere with actomyosin-independent motile systems. Because we can show by vectorial labeling of the cell surface that the majority of the contractile proteins are located on the cytoplasmic side of the plasma membrane, it is likely that an interaction of clusters with the peripheral network occurs through the lipid bilayer of the plasma membrane.

Comparison of cells and ghosts from cells treated with FITC- or ferritin-labeled Con A, by light and electron microscopy, show that lectin is exclusively located in clusters on the ghosts and that the size and spatial distribution of these clusters is essentially unchanged by the lysis procedure. Because electron microscopy shows that the majority of the lectin at the time of lysis is either on the surface of neutrophils or in invaginations of the plasma membrane open to the surface, it is unlikely that the patches are merely trapped within the filamentous network.

The lectin-binding species within clusters have been only partially characterized, but ghosts prepared from iodinated neutrophils treated with lectin did not contain more 125I-glycoproteins than control ghosts; thus it is likely that the majority of these binding sites are glycoproteins. Identification of surface glycoproteins associated with these clusters was attempted by vectorial labeling of intact cells with 125I. Autoradiograms show eight or more consistently iodinated proteins exposed on the intact cell surface only one of which, the 80-kdalton protein, is efficiently retained by the ghosts. An additional three or four iodinated proteins are retained by ghosts prepared from iodinated cells incubated in the presence of Con A. These additional proteins are presumably associated with the lectin-induced clusters. The selectivity of association between surface iodinated proteins and the ghosts argues against the possibility that small amounts of intact plasma membrane survive detergent extraction. These results differ from those of Ben-Ze'ev et al. (6) who found, using mild lysis conditions, that many surface labeled proteins were retained by the extracted cells.

Quantitative estimates obtained by sectioning tube gels (data not shown) suggests that ~30% of the total cell complement of the 125I-labeled 80-kdalton protein remains associated with the ghosts from untreated cells. We interpret this data to mean that this 80-kdalton protein is a component of the plasma membrane normally attached to the peripheral filamentous network in the cytoplasm of neutrophils.

In ghosts prepared from iodinated neutrophils treated with succinylated Con A, the autoradiograms show an autoradiographic pattern identical to that of control ghosts, in that the 80-kdalton protein remains attached to the ghosts but additional lectin associated proteins are absent. Because succinylated Con A does not cause clustering, we conclude that although cross-linking of surface glycoproteins is not required for attachment of the 80-kdalton protein to the ghosts, it is obligatory for retention of the additional lectin-binding proteins. The data in Table V further show that 24% of cell-bound succinylated Con A is retained by the ghosts. It is thus possible that the 80-kdalton protein is itself a binding site for Con A and that the attachment of the three or four other iodinated glycoproteins, observed in ghosts prepared from cells treated with Con A, could occur by direct cross-linking of these glycoproteins to the 80-kdalton protein. The increased efficiency of retention of the 80-kdalton protein on patched or capped ghosts through the lysis procedure, could reflect the binding advantage derived from cooperativity.

In the lateral plane, the 80-kdalton protein may be important in establishing connections with mobile binding sites exposed to the external surface before their relocation or internalization. The 80-kdalton protein fulfills the major requirements of the universal cross-linker "protein X" postulated by Bourguignon and Singer (8). However, because the 80-kdalton protein may be itself a Con A-binding protein, we are unable to use lectins to explore the possibility that the protein becomes associated with ligand induced clusters in the manner suggested by these authors. With other ligands this aspect is open to direct experimentation.

The 80-kdalton protein is both exposed on the outer surface of the plasma membrane and attached to the peripheral filamentous network, this protein must therefore itself, or in conjunction with other proteins, span the lipid bilayer. Because contraction generated by components within the peripheral filamentous network will tend to pull the network away from the plasma membrane, direct attachment of the network to a transmembrane protein (complex) may provide an important form of anchorage. Thus the 80-kdalton protein molecules may act in the manner of floats on a seine net, and, while prevented from being plucked out of the bilayer by hydrophobic/hydro-
philic interactions, would be free to move laterally within the constraints imposed by their attachment to components within the cytoplasm. Contraction of the peripheral filamentous network would then act against the fluid volume within the cell via the lipid bilayer.

This model can provide a simple conceptual description of certain phenomena of surface motility and gives rise to predictions relating to the location of both the float protein and the contraction (relaxation) stimulus, aspects of which can be tested experimentally.

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