COMPARISON BETWEEN ENDOCYTOSIS AND INTRACANALICULAR SEQUESTRATION OF CELL-SURFACE ANTIGENS IN HUMAN PLATELETS

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BRENT JENNINGS

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Supervisors: Dr Errol Holland

Prof. Lutz Thilo

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I, Brent Jennings, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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SUMMARY

Human platelets respond to various macromolecules in the plasma. Uptake of specific ligands, and antibodies to various epitopes on the platelet plasma membrane, has been observed. The platelet canalicular system has been shown to be involved with this uptake. Recently, investigators have speculated on the role of endocytosis in platelets to account for the presence of plasma proteins such as fibrinogen and immunoglobulin within platelet organelles. Antibodies binding to cell-surface antigens on platelets can lead to a redistribution of these antigens. When antibodies, specific for platelet cell-surface receptors, bind to platelets they may either undergo endocytosis into intracellular vacuoles, or may merely become sequestered within the canalicular system of platelets.

The present study investigated whether endocytosis occurs in platelets. Such a process would lead to the endocytic uptake of a fluid-phase marker and would involve internalization and recycling of cell surface membrane.

A fluid-phase marker (FITC-dextran) was used to measure any constitutive endocytic activity. In addition, a suitable membrane marker was used to determine whether membrane internalization occurred. This involved a technique whereby radioactive galactose was covalently attached to cell-surface glycoconjugates.

A monoclonal antibody to the platelet receptor, GPIIbIIIa, was used in conjunction with the membrane marker in order to determine if membrane internalization was involved during the subsequent redistribution of the receptor-antibody complex. Immunocytochemical techniques using electron-dense probes were employed to localise the sites to which this receptor-antibody complex became redistributed.

In comparison with reported rates of endocytic uptake of fluid-phase marker in other cell types, no significant endocytic activity could be detected with platelets, after taking their relatively small volume into account. Similarly, membrane internalization was not detected with resting platelets. Following challenge of the platelets with anti-GPIIbIIIa antibody, no membrane internalization could be measured during redistribution of the receptor-antibody complex.

The compartment to which the receptor-antibody complex was redistributed could be identified morphologically as the canalicular system.

The present data provide evidence for a process of sequestration of receptor-antibody in the canalicular system of resting platelets. It remains possible that other mechanisms exist within the platelet system for uptake of extracellular material as this study dealt exclusively with the platelet response to a specific antibody. These results may have implications with respect to the interaction of platelets with anti-platelet antibodies in the normal state, as well as with

clinical disorders involving elevated levels of platelet-associated IgG.

As far as can be deduced from the available literature, these data represent the first use of a covalent membrane marker in conjunction with uptake of macromolecules to study endocytic events in human platelets.

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CONTENTS

CHAPTER 1

INTRODUCTION

The aim of this thesis was to gain quantitative and qualitative information about the membrane events that lead to uptake of extracellular material in human platelets. Also, to describe any constitutive endocytic activity occurring with normal platelets in the resting state, using assays for measuring fluid-phase uptake and membrane labelling techniques. These techniques will be used in conjunction with a monoclonal antibody to a platelet membrane receptor, to determine whether or not endocytosis occurred during redistribution of the receptor antibody complex. Before discussing evidence relating to this question it is necessary to describe platelet morphology and other relevant aspects.

1.1 PLATELET MORPHOLOGY

Blood platelets are derived from megakaryocytes. The mechanism by which they are formed and released into the circulation is still subject to controversy, but probably involves the network of tubules and fissures that communicate with the surface of the megakaryocyte and ultimately contribute towards the membranes of mature platelets (1,2). A summary of megakaryocyte development is presented by Hovig (1).

The human platelet, an apparently simple cell lacking a nucleus, has a discoid shape in the circulation and is relatively small, with dimensions of about 3,6 x 0,9 μm. The volume of platelets varies, with a mean of 5,2 to 7,7 μ^3 (3).

The cytoplasmic content of platelets has great similarities to that of the parent cell. A schematic diagram summarizing some of the more prominent structures observed in human platelets is depicted in Figure 1. Unlike cells such as leucocytes and macrophages, which have a surface characterized by villous projections and ridges, the platelet surface is smooth. The plasma membrane is enriched in membrane proteins, a higher proportion of which is located on the exoplasmic face of the membrane when compared to other cell types (1). This relates to the platelets' role in the recognition of surfaces and agonists involved with platelet activation (cf. 1.2.1).

The platelet has a cytoskeleton of filaments and microtubules. Actin filaments constitute a large percentage of the total protein. Filaments are rearranged and polymerized for pseudopod extension and for the contractile process that squeezes the granules towards the cell centre during surface activation. A peripheral band of microtubules is important for the maintenance of the discoid shape in the resting state (1).

The platelet has α-granules, containing various proteins and growth factors, dense granules, and glycogen particles. Lipid droplets may be seen, as well as the occasional mitochondrion or remnants of Golgi apparatus (1,4,5).

There is a network of tubules that ramify throughout the cytoplasm, the membranes of which are in continuity with the plasma membrane. This forms the open canalicular system (CS) which is in communication with the surrounding medium (2), and

can be seen as a network of small vacuoles or tubules in ultrathin sections of platelets (cf. Figure 1). The three-dimensional reconstruction of the CS by Kawakami and Hirano (6) allows a perspective of the CS as a whole, including the opening, or pore, on the plasma membrane. The CS membranes cannot be referred to as true, internal membranes, because they are in continuity with the plasma membrane, but they retain a degree of individuality with respect to their glycoprotein composition (7).

It has been suggested that the CS represents a reservoir of sequestered membranes that can be evaginated following platelet activation (8). On the other hand, others believe that the CS cannot contribute to increased plasma membrane surface area during spreading. They argue that the CS would have to be torn apart for the membranes to undergo evagination (9).

The role of the CS in platelet secretion is controversial. It has long been upheld that the CS can serve as a route for uptake and secretion (7-12). Discharge of granular contents into the CS during secretion has been questioned as bovine platelets, while showing normal secretion, are almost devoid of CS (13). The potential importance of the CS in human platelets as sequestering units is great. Circulating platelets in an adult represent a membrane surface area of about 25 m^2, to which should be added the area of the extensive CS (10).

1.2 PLATELET MEMBRANE PROTEINS

1.2.1 Functions

Platelets are responsible for the initiation of and the structural basis for the haemostatic plug which prevents bleeding. The cell undergoes transformation to a form which adheres to other platelets and to surfaces, secreting a variety of mediators. In doing this, the platelet expresses binding sites for adhesive macromolecules on the plasma membrane (14).

A variety of platelet interactions are required for haemostasis and thrombosis to occur, many of which involve membrane proteins. Most of these proteins are glycoprotein receptors, as platelet function depends primarily on adhesion events. The interactions of platelets are specialised adaptations of adhesive mechanisms used by a wide variety of cells (15).

1.2.2 The platelet GPIIbIIIa

The glycoprotein IIbIIIa complex comprises proteins of the integrin receptor family (15). The complex is composed of one molecule of GPIIb (140 kD) which consists of disulfide-linked light and heavy chains, and one molecule of GPIIIa (150 kD). The complex is present as a Ca^{2+}-dependent heterodimer consisting of a globular head domain and two tail domains, the tips of which constitute the hydrophobic sites associated with the membrane. There are two short cytoplasmic domains (15).

On activated platelets, the GPIIbIIIa complex serves as a receptor for fibrinogen, fibronectin, von Willebrand factor, vitronectin, and thrombospondin, and mediates platelet aggregation, adhesion and spreading (15). Normal platelets contain about 50 000 molecules of the complex, randomly distributed on the surface of resting platelets (14,15). An α-granule pool of GPIIbIIIa has also been identified and is associated with the granule membrane (16). The complex is present on megakaryocyte membranes with a similar, random distribution as on resting platelets (17).

1.2.3 Interactions between membrane glycoproteins and the platelet cytoskeleton

Many cell-surface glycoproteins are known to interact in a transmembrane manner with the cell cytoskeleton, especially after aggregation of the proteins by multivalent ligands. As well as actin, the cytoskeleton has myosin and actin binding protein (18). In addition to activating agents such as thrombin, ligands (e.g. concanavalin A) can induce interaction between surface glycoproteins and the internal cytoskeleton (19). In surface-activated platelets, bound fibrinogen undergoes redistribution on the cell surface accompanied by cytoskeletal reorganisation, suggesting a link between surface receptors and the cytoskeleton (20,21).

Subsequent studies confirm the receptor-cytoskeleton link (22,23), demonstrating that the use of actin depolymerization agents (e.g. cytochalasin B) causes previously redistributed,

clustered receptors to form a patch-like pattern, a process thought not to be passive (22). Interactions of platelet surface glycoproteins with the cell cytoskeleton thus play an important role during receptor-ligand redistribution on resting and activated platelets.

1.2.4 Interactions between monoclonal antibodies and membrane glycoproteins

In addition to ligand-induced receptor reorganization, binding of specific antibodies to one of various platelet surface antigens results in receptor mobility and organized redistribution (24). Monoclonal antibody induced activation of platelets involves Fc receptor-dependent cell-cell interactions (25). Another reported finding is that activation of platelets by a monoclonal antibody to GPIIbIIIa involves cross-linking of an Fc receptor with GPIIbIIIa (26-28).

Even when platelets are in the resting state, antibodies directed against membrane receptors are redistributed to pronase-resistant, intracellular sites (29). A study involving monoclonal antibodies to platelet GPIb and GPIIbIIIa (chosen because of their importance in platelet immune interactions) showed that antibody binding results in redistribution of GPIIbIIIa, whereas GPIb does not redistribute. The redistribution of GPIIbIIIa occurred in a patch-cap manner followed by apparent internalization (24). This was postulated to have a role in the clearing of anti-platelet antibodies (in a membrane clearing process) by the process of endocytosis.

1.3 EVIDENCE RELATING TO ENDOCYTIC ACTIVITY IN PLATELETS

Receptor-mediated and fluid-phase endocytosis via coated pits and coated vesicles provides a general mechanism for selective uptake of specific macromolecules into cells from the surrounding fluid (30,31). Platelets have a great deal of membrane surface area in contact with the surrounding plasma, especially if the membranes of the canalicular system are taken into account. The importance of platelets as internalizing elements of the blood is potentially great, considering the high density of receptors contained in the platelet plasma membrane and the significance of platelet-macromolecule interactions. This includes interactions with anti-platelet IgG (24,32).

1.3.1 Phagocytosis, receptor-ligand redistribution and the canalicular system

There have been many studies claiming to demonstrate phagocytic activity with human platelets using markers such as large and small latex particles, colloidal carbon, thorium and cationised ferritin (9, 33-36). An early study demonstrates that latex particles are not phagocytosed in the usual sense, but are instead sequestered in the channels of the canalicular system (CS) (12). Another study subsequently demonstrates acid phosphatase activity in the latex-containing "organelles", pointing towards the platelet as a true phagocyte (36). Small latex particles, taken up in the CS without causing significant changes in morphology are observed to localize within dilated, vacuolar structures which appear to be altered CS, and not

separate "phagosomes". The membranes of these vacuoles retain continuity with the cell surface (9,35). Information on the mechanism by which platelets sequester latex particles thus remains controversial. Various mechanisms have been suggested including two separate pathways for endocytosis of large and small particles involving the CS and small membrane invaginations (11).

Tracers such as antibodies and cationised ferritin are sequestered from the cell surface into the CS, resulting in a cleared surface membrane (10). This clearing phenomenon was the subject of studies using platelets spreading on a substratum (glass). It involves a continuous centripetal movement of membrane (22,37), with attached markers (such as fibrinogen-gold particles) being centralized and sequestered in the CS (10,23,38). The clearing of receptor-ligand complexes from the platelet surface membrane to the CS, as measured by fibrinogen-gold labelling, occurs independently of platelet activation (8).

1.3.2 Endocytic activity in relation to the origin of granule proteins

Certain plasma proteins (e.g. fibrinogen) found in platelets and megakaryocytes have their origin exclusively in the blood plasma and no mRNA coding for fibrinogen can be detected in platelets or megakaryocytes (39). This has led to the assumption that the fibrinogen content of secretory granules is derived from endocytosis of plasma fibrinogen (39,40).

Megakaryocytes have been suggested to acquire some of their α-granule proteins (such as IgG, albumin and fibrinogen) from the plasma. Distinct mechanisms have been suggested to exist within the megakaryocyte for this purpose (32). Proteins may be transferred via a coated vesicle route from the cell surface to endosomes and then to either lysosomes, Golgi, or secretory granules. Other evidence suggests that in addition, α-granule proteins in megakaryocytes may be derived from endogenous synthesis via the normal biosynthetic pathway and trafficked to the early secretory granule (32). This complex form of processing by the maturing megakaryocyte appears to have several pathways for processing granule proteins. Platelets lack a functional protein synthesis apparatus and are thus thought to possess a limited form of the processing pathways mentioned above (32).

Using fluid-phase markers (protein-stabilised colloidal gold particles), Behnke (41) has shown structures resembling coated pits and coated vesicles within mammalian platelets. These data propose the possible existence of an endocytic pathway responsible for transferring small particulate matter to platelet granules. Monoclonal antibodies have also been used to characterise endocytosis in resting platelets. Monoclonal antibodies against GPIIbIIIa are taken up in an energy dependent manner to apparent intracellular vacuoles as demonstrated by morphologic analysis. Two models are proposed by the author to accommodate the data obtained in this study (42; cf. Figure 14).

1.4 EXPERIMENTAL APPROACH

As has been discussed, controversy exists as to whether uptake occurs by means of endocytosis, or merely involves sequestration within the CS. The aim of this study was to detect fluid-phase uptake and membrane internalization in relation to clearance of antibodies bound to GPIIbIIIa on human platelets.

As a marker for the platelet membrane the exogalactosylation technique was used. This technique has previously provided a stable membrane marker by way of binding radioactive galactose covalently with surface glycoconjugates of eukaryotic cells. The label can be removed enzymatically with ß-galactosidase (43,44). A fluid-phase marker (FITC-dextran) was used to study fluid-phase uptake. A monoclonal anti-GPIIbIIIa antibody was used to study receptor-ligand internalization with platelets. The antibody, designated 3B3, (45), was used in conjunction with the exogalactosylation technique and along with a secondary, fluorochrome-labelled antibody.

Immunocytochemistry using electron-dense probes was employed in an attempt to visualise the precise intracellular compartment to which the internalized receptor-ligand complex was redistributed (46,47).

CHAPTER 2

MATERIALS AND METHODS

2.1 THE ISOLATION OF HUMAN PLATELETS

Choice of anticoagulant, as well as the presence of a protective protein such as albumin, is important for the prevention of activation of platelets due to cell-cell and cell-substratum interaction. Citrate-citric acid-dextrose is commonly used as an anticoagulant due to its calcium chelating ability, as well as the effect it has of lowering the pH of the blood to 6,5; platelets do not aggregate as readily at this pH (50). Contact with glass surfaces is to be avoided as platelets are activated by glass. A Tris-containing buffer is inhibitory to some platelet functions and should not be used. Media similar to those used in tissue culture are preferred (5,50). Prostaglandin E_1 (PGE_1) in the medium helps to prevent platelet aggregation during manipulation, especially when fluctuations in temperature are introduced (24,51).

Platelets were collected from normal, healthy donors who had not taken any drugs over the previous 10 days that may affect platelet function (e.g. aspirin). Collection was done from a forearm vein with a 19-gauge winged infusion set using mild venous occlusion. Blood was allowed to run into a 50 ml polycarbonate tube coated with and containing 8 ml of anticoagulant citrate-citric acid-dextrose (CCD) (93 mM Trisodium citrate,7 mM citric acid, 140 mM dextrose, pH 6,5, with 0,35% BSA, fraction 5) at 37°C. Care was taken to prevent frothing of the mixture.

The whole blood was spun at low speed (200 x g) in a Beckman model TJ-6 benchtop centrifuge at room temperature for 20 minutes to separate the erythrocytes. Platelet-rich plasma (PRP) was removed, taking care to avoid disturbing the white blood cell layer, and combined with an equal volume of warm CCD. The pH of the PRP was adjusted to 6,5 by adding 0,2 M citric acid. The PRP was centrifuged at 900 x g for 15 minutes at room temperature to pellet the platelets. Resuspension of the platelets was done using a small-bore pipette with gentle action. Platelets were washed twice with prepared Hanks Balanced Salt Solution supplemented with 0,1% BSA and 1 µg/ml prostaglandin E_1 (Sigma) (HBSS P/B), and were counted with a Technicon H1 cell counter (Bayer Diagnostics).

Care was taken with the cells to avoid contact with glass surfaces and rapid fluctuations in temperature and pH. Platelets prepared in this manner were viable (also after the labelling procedures on ice cf below) as shown by their susceptibility to activation and spreading if plated onto glass coverslips, and observed with the microscope at 1000 x magnification (Wild Herbrugg, Switzerland).

2.2 THE EXOGALACTOSYLATION TECHNIQUE

2.2.1 Cell-surface labelling

Platelets were washed twice with 5 ml of HBSS P/B at room temperature and cooled on ice. Radioactive galactose was covalently linked to terminal N-acetyl glucosamine moieties on the platelet surface with galactosyl transferase (43,44) as

follows: For a typical volume of 70 µl, the labelling solution was made up in HBSS P/B and contained 20 µCi/ml UDP-[6-^{3}H] galactose (ammonium salt, code: TRK 513, Amersham, England), $MnCl_2$ (5 mM) and galactosyl transferase (0,5 U/ml), (from bovine milk, code: G-5507, Sigma, USA).

Typically, 130 µl of cells cooled to 4°C were added to the reaction mix, also at 4°C, and mixed. The concentration of cells in the reaction mixture was in the order of 1 x 10^9 cells/ml (from 50 ml of blood). The incubation period was 20 minutes at 4°C. At the end of this period, the mixture was diluted 10-fold and washed four times with cold HBSS P/B.

2.2.2 Detection of radioactivity

Cells were solubilised by treatment with 0,5% Triton X-100 (Sigma, USA) and the tritium was counted using liquid scintillation cocktail (Beckman, Palo Alto, California) in a Packard 1900 CA scintillation counter (Packard Instruments Inc, Canberra). Counting efficiency was about 50%.

2.2.3 Glycosidase treatment of cell surface

The glycosidases were in the form of a crude bacterial extract and contained ß-galactosidase and neuraminidase activity (Thilo, personal communication). The ß-galactosidase activity of the enzyme mixture was determined by monitoring the hydrolysis of O-Nitrophenyl-ß-D-galactopyranoside (ONPG) (1 mg/ml) via absorbance at 420 nm (Molar Extinction Coefficient = 2,5).

2.2.3 (i) Pretreatment conditions: In order to improve the level of incorporation of galactose into glycoconjugates, the cell surface was treated for 10 minutes with glycosidases (0,1 U/ml ß-gal activity) at 4°C (43), to remove existing terminal N-acetyl glucosamine residues. The reaction was stopped by 10-fold dilution in cold buffer.

2.2.3 (ii) Removal of cell-surface label: The same glycosidase solution (0,1 U/ml ß-gal activity) used for pretreatment was used to remove label from the cell surface. This was done for 15 minutes at 4°C. To quantify release of label, the cell suspension with glycosidases was diluted as above, and an aliquot removed to measure the total radioactivity per volume. The remainder of the suspension was centrifuged at 4°C to pellet the cells, after which an aliquot of equal volume was sampled from the supernatant to measure the concentration of radioactivity released from the cell surface. The quotient of the concentrations of radioactivity in the supernatant and the complete cell suspension gave a measure of the fraction of label that was released by glycosidase treatment.

2.3 ANALYSIS OF LABELLED MEMBRANE PROTEINS BY SDS-PAGE

This was performed according to Maizel (50), using 1,5 mm thick, 25 cm long 5-13% polyacrylamide gradient slab gels. Rainbow protein molecular weight markers (Amersham International PLC, England) were used. The proteins were: myosin (200 000), phosphorylase b (92 500), Bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase

(30 000), trypsin inhibitor (21 500) and lysozyme (14 300). Alternatively, a mixture of high (MW-SDS-200 Kit, Sigma) and low (MW-SDS-70L Kit, Sigma) molecular weight markers were used. ^{3}H-labelled membrane samples to be analysed on the gel, as well as molecular weight markers, were added to equal volumes of two-fold concentration sample buffer (10% glycerol, 2,3% SDS, 5% ß-mercaptoethanol, 0,002% bromophenol blue, 0,042 M Tris-HCl pH 6,3) and boiled for one minute.

Gels were run overnight at a constant 12 mA, and were fixed and stained for 30 minutes with a 46% methanol, 8% glacial acetic acid, 0,1% Coomassie brilliant blue R mix. Destaining overnight in 20% methanol, 8% acetic acid followed. Rehydration was with 8% acetic acid, 3% glycerol for 2 hours followed by washing in distilled water for 30 minutes before treatment with the fluorographic reagent, Amplify (Amersham International PLC, UK), for 20 minutes. Gels were then dried in a slab gel drier (Model SE 1150, Hoefer Scientific Instruments, San Francisco).

Alternatively, gels were soaked in 1 M sodium salicylate pH 7,4, for 30 minutes, after a 30 minute incubation in distilled water to remove excess acetic acid, before drying. The dried gel was exposed to Kodac XAR-5 film (Sigma Chemicals Company, St. Louis, USA) at -70°C to obtain a fluorograph.

2.4 MEMBRANE INTERNALIZATION ASSAY USING THE EXOGALACTOSYLATION TECHNIQUE

Labelled cells were incubated in HBSS P/B at 37°C and 75 rpm in an orbital shaker bath (TS-611A, Yih Der Instruments, Taiwan). Aliquots of about 1 x 10^8 cells were removed as a function of time and diluted in 10-fold volume cold buffer, then placed on ice to stop endocytic activity. The cells were then treated with glycosidases (0,075 U of ß-Gal/ml) at 4°C for 15 minutes in order to remove cell surface label. This fraction of label susceptible to release was measured in order to assess the amount that had been internalized and was no longer on the surface.

The release of bound radioactivity in the absence of ß-Gal was determined from a parallel set of aliquots in order to distinguish between the processes of enzymatic label removal by ß-gal, and shedding to the medium.

In a separate experiment, labelled cells were incubated for 3 hours in HBSS P/B at 37°C and at 4°C. Aliquots of each were then treated with or without glycosidases (0,075 U of ß-gal/ml) for 15 minutes at 4°C as above. The percentage radioactivity released into the medium by ß-gal could then be determined and the value obtained with the sample incubated at 4°C could be compared with that at 37°C. This allows the discovery and quantification of membrane internalization that may have occurred at 37°C.

2.5 UPTAKE OF FLUID-PHASE MARKER

Fluorescein isothiocyanate-dextran (FITC-dextran 40 kD, Sigma) was used as a fluid-phase marker. FITC-dextran was made up in HBSS P/B. A suspension of platelets in HBSS P/B was added to give a final concentration of 10^9 cells/ml with FITC-dextran at 10 mg/ml. The suspension was incubated at 37°C for various times (controls incubated at 4°C) and endocytosis stopped by dilution in ice-cold buffer.

After the incubation period, the cells were washed four times in ice-cold buffer, counted, and treated with 0,5% Triton X-100 for 20 minutes to disrupt all cell membranes. If the mixture was cloudy, loose membranes and debris were sedimented in a benchtop microfuge at 10 000 rpm for 10 minutes until clear. The fluorescence intensity was measured by excitation at 470 nm and emission at 520 nm in a Perkin-Elmer LS-5 luminescence spectrophotometer.

An aliquot of the original incubation medium containing the fluid-phase marker was diluted a thousandfold, from which aliquots of 1 to 5 ml were removed and the fluorescence measured. This was used to set up a standard curve indicating volume FITC-dextran taken up compared with fluorescence intensity.

2.6 PURIFICATION OF MONOCLONAL ANTIBODY 3B3

The monoclonal antibody, 3B3, binds exclusively to the human fibrinogen receptor, the IIbIIIa glycoprotein complex found on

the membranes of human platelets and megakaryocytes. The antibody belongs to the murine IgG 1 subclass and was produced by fusing spleen cells from Balb/c mice with NS-1 myeloma cells using polyethylene glycol (45). 3B3 was a generous gift of the Department of Clinical Science and Immunology, UCT. The antibody was supplied either as a precipitate from mouse ascites fluid stabilized with ammonium sulphate or in a frozen PBS-glycerol solution taken directly from hybridoma medium.

2.6.1 Affinity chromatography

After desalting, the solution was filtered through a 0,22 µm Millex-GS filter (Millipore) before being loaded on a Protein G column (Pharmacia LKB), which was first stabilised with 5-10 column volumes of 20 mM phosphate buffer pH 7. Chromatography was performed in a cold room at 4°C. The column was washed with phosphate buffer (20 mM, pH 7) and the bound IgG eluted with Glycine-HCl buffer (0,1 M, pH 2,7) into 1 M Tris-HCl, pH 9, to neutralize the final antibody solution in 1 ml fractions. Protein was measured via absorbance at 280 nm (Unicam SP 1800 spectrophotometer), the relevant fractions pooled and the final concentration of protein determined (Molar Extinction Coefficient of 1mg/ml = 0,69). The pooled solution of IgG was supplemented with 0,1% BSA, aliquotted and stored at -20°C.

2.6.2 Purity analysis by SDS-PAGE

An ammonium sulphate precipitate of protein from mouse ascites fluid containing 3B3, as well as affinity purified 3B3 were prepared for SDS-PAGE as described in section 2.3. The samples

prepared for SDS-PAGE as described in section 2.3. The samples were run on a 5-20% polyacrylamide gel.

2.7 INDUCTION OF MEMBRANE INTERNALIZATION USING 3B3

2.7.1 Measurement of internalization using the exogalactosylation technique

Platelets were labelled as described in section 2.2.1 and incubated at 37°C in HBSS P/B at 5 x 10^8 cells/ml (control samples were incubated at 4°C). The antibody 3B3 was then added to a final concentration of 2 µg/ml. Parallel samples were incubated without 3B3. Cells were incubated for 3 hours in the orbital shaker.

The amount of internalized membrane was determined from the amount of label released into the medium by glycosidases (0,08 U/ml ß-gal activity) as described in section 2.4.

2.7.2 Immunofluorescence microscopy

Immunofluorescent staining of platelets was performed as described by Wencel-Drake (42,51) with minor modifications: Platelets, suspended in HBSS P/B, and preincubated for 40 minutes on ice or at 37°C with 3B3 (2 µg/ml) (control samples had no 3B3), were washed and fixed with 3% paraformaldehyde (Merck) for 45 minutes on ice. Unreacted aldehyde was blocked with 1,5% L-lysine (BDH Chemicals, Poole, England) and the cells were washed.

To visualise membrane glycoproteins tagged with 3B3 that have been interiorized, the cells were then treated with 0,1% Triton X-100 for five minutes to render them permeable to the secondary antibody. The platelets were then incubated with this secondary antibody, a fluorescein-labelled goat $F(ab')_2$ anti-mouse IgG (a gift of the Department of Clinical Science and Immunology) for 20 minutes at room temperature. The cells were then allowed to adhere to poly-L-lysine coated (12 kD, Sigma) glass coverslips and washed. The coverslips were mounted on mounting medium containing 1 mg/ml paraphenylenediamine (Sigma) in a 1:1 mixture of glycerol:PBS. Platelets were observed under a fluorescent microscope (Zeiss Axioskop: Exciter filter BP 450-490, Barrier filter LP 520) at 630 x magnification.

2.8 DETECTION OF LABEL IN THE CANALICULAR SYSTEM BY SELECTIVE REMOVAL OF PLASMA MEMBRANE LABEL

Platelets labelled by exogalactosylation were warmed to 37°C for 45 minutes to allow membrane internalization to take place. They were then cooled on ice and an aliquot was fixed with 3% paraformaldehyde for 45 minutes and washed. Platelets that were either at 4°C or fixed were then treated with glycosidases (0,1 U/ml ß-gal activity) at 4°C for 20 minutes. Label released thus, was determined as described in section 2.2.3.

Platelets were treated as above but in the presence and absence of 3B3 in order to determine whether or not incubation with the

antibody caused a difference in the amount of label protected from glycosidases.

2.9 PREPARATION OF PLATELETS FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

Platelets, suspended in HBSS P/B, were fixed with 3% paraformaldehyde in White's saline (cf. Table 1) for 30 minutes then washed in White's saline before being fixed with 1,25% glutaraldehyde in White's saline for 20 minutes (5). Cells were washed with sodium cacodylate buffer (0,1 M, pH 7,4; with 5 mM $CaCl_2$, 5 mM $MgCl_2$) and post-fixed with 1% osmium tetroxide in cacodylate buffer, for 20 minutes. After washing with cacodylate buffer, cells were stained with 0,5% tannic acid in cacodylate for 5 minutes (52).

Table I. Stock solutions for White's saline

A	NaCl	14,0 g
	KCl	0,75 g
	$MgSO_4$	0,55 g
	$Ca(NO_3)_2.4H_2O$	1,5 g
	Add distilled H_2O to 100 ml	
B	$NaHCO_3$	1,1 g
	$Na_2HPO_4.7H_2O$	0,22 g
	KH_2PO_4	0,052g
	Phenol red	0,01 g
	Add distilled H_2O to 100 ml	
	Mix and pH to 7,4	

Before use, mix 5% A, 5% B in distilled H_2O.

After White JG (5).

Platelets were washed with cacodylate buffer and resuspended in 20 µl distilled water. 2% NOBLE agar (cooled to 50°C in a water bath, after boiling) was then added to the cells which were centrifuged to a pellet in a 50°C water jacket. The tubes

were placed on ice for one hour with 70% ethanol covering the agar. The agar could then carefully be removed and the embedded cell pellet chopped into cubes 1 to 2 mm in size (53).

The samples were dehydrated in 70%, 90% and 100% ethanol and transferred to propylene oxide (Merck). The samples were then embedded in EPON-ARALDITE (20% Araldite; TAAB, 27% Agar 100; Agar Scientific, 53% DDSA; Bio Rad) resin and polymerized at 65°C for 48 hours.

Thin (70 nm) sections were cut onto Cu grids with an ultramicrotome (Ultracut E, Reichert-Jung) and contrasted using 8% uranyl acetate (Merck) and lead citrate. Grids were then washed on drops of distilled water and air dried. Sections were viewed under an electron microscope at an accelerating voltage of 75 kV (Hitachi H-600 TEM).

2.10 PRE-EMBEDDING LABELLING WITH GOLD-CONJUGATED 3B3

2.10.1 Preparation of colloidal gold

Colloidal gold of 10 nm diameter was prepared according to the method of Slot and Geuze (54,55). All glasswear was thoroughly cleaned with chromic acid and rinsed with hot water. Briefly, a solution of gold chloride (Merck) and a reducing mixture of 1% citrate, 1% tannic acid and 25 mM K_2CO_3 were heated to 60°C. The reducing mixture was added to the gold solution while stirring and boiled for 5 minutes, then allowed to cool. The colloidal gold so formed was checked for clumping and uniformity of size by mounting on formvar-coated EM grids and

viewed under the Hitachi electron microscope at 100 000 x magnification with an accelerating voltage of 100 kV.

2.10.2 Iodination of antibody

The monoclonal antibody, 3B3, was iodinated by the iodogen tube method (56). In an Eppendorf 600 µg 3B3, 2 µl of sodium iodide (^{125}I, 100 mCi/ml Amersham) and 400 µl of Hepes-saline (10 mM HEPES, 140 mM NaCl pH 7.3) were mixed and then transferred to an iodogen tube for 10 minutes on ice. The labelling mix was then transferred to Spectrapor dialysis tubing (12-14000 mw cut off, SA Scientific products) which was washed three times, each for 45 minutes, in saline-EDTA (150 mM NaCl, 2 mM EDTA pH 7,4). The dialysis bag was then placed in 1,5 l of saline-EDTA and left to dialyse for 3 days with daily changes of the wash solution. When the free iodine had dialysed out, the dialysate was removed from the tubing. Cold 3B3 was added to increase the protein concentration which was determined by the method of Lowry (57). Iodinated protein was counted in a Crystal II multidetector RIA-counter (Packard, Canberra) and the specific activity determined.

2.10.3 Conjugation of antibody with colloidal gold

This was performed according to the method of Geoghegan and Ackerman (58). Adsorption isotherms were determined for the immunoglobulin in order to determine the minimum amount of protein and optimal pH conditions required for stabilization of the colloidal gold. Briefly, protein solutions of different concentrations were made up in different pH buffers and mixed

with gold colloid solutions at different pH's in a 48-well test plate (Falcon, Beckman Dickinson, New Jersey). A solution of NaCl was added to a final NaCl concentration of 1,3%, mixed and left to stand for 5 minutes and the colour change from red to blue assessed, the blue colour indicating that the gold has flocculated. The minimum amount of protein required for stabilization of the gold is taken from the red coloured well with the lowest amount of protein.

The pH of the colloid was adjusted to 7,5 (from the isotherms) with 0,05 M K_2CO_3 and the colloid was centrifuged at 1400 x g for 5 minutes in a polycarbonate tube to remove any debris. 80 µg of antibody was dialysed on a floating polycarbonate membrane against distilled water for 1 hour before being added, dropwise to the gold colloid while stirring. BSA was added to 1% and the mixture was centrifuged in polycarbonate tubes at 25000 rpm at 15°C for 1 hour in an SW 65 Ti rotor (Beckman Ultracentrifuge, Palo Alto, California). Supernatant was discarded leaving about 10% of initial volume which was decanted, leaving particles stuck to the bottom of the tube. This was layered on top of a 0,02 M Borate buffer (pH 7,5 with 1% BSA) with a 90% sucrose solution as a "cushion" at the bottom of a polyallomer tube. The tubes were centrifuged at 25 000 rpm at 15°C for 30 minutes in a SW 40 rotor (Beckman). The probe was recovered as a small, mobile, red pellet which was washed with Borate buffer (0,02 M, with 1% BSA) pH 7,5 (58-60).

2.10.4 Binding of gold labelled versus unlabelled antibody

2.10.4 (i) Radioactivity analysis: The radioactive antibody-gold probe prepared in section 2.10.3 was assessed for its binding efficiency to platelets. Platelets were incubated in HBSS P/B at room temperature for 90 minutes with either the iodinated probe, or iodinated 3B3 that was not conjugated with colloidal gold. The cells were then washed with cold HBSS P/B. The amount of radioactivity associated with the cells was measured after each wash in the gamma counter.

2.10.4 (ii) Immunomarking of platelets: Platelets that were treated as described in section 2.10.4 (i) were processed for transmission electron microscopy as described in section 2.9.

2.11 POST-EMBEDDING IMMUNOCYTOCHEMISTRY

3B3 that had bound to platelets during incubation in suspension could be localised in thin sections after fixation and processing of the cells for TEM. This technique involves the use of secondary or tertiary antibodies to target the antigen (in this case 3B3) embedded in the resin of the ultrathin sections (47,61).

2.11.1 Dot Blot analysis

The immunoreactivity of the antibody probes to be used was tested using a nitrocellulose dot blot technique (59,62).

2.11.1 (i) Rabbit anti-mouse (RAM) with goat anti-rabbit (GAR-5):

3B3 at two concentrations (0,9 mg/ml and 0,1 mg/ml) were applied to two different wells on a nitrocellulose dot blot apparatus and allowed to dry. The nitrocellulose membrane was removed and incubated in a blocking solution of 5% BSA in Tris-saline (20 mM Tris, 150 mM NaCl, pH 7,4) on a rocking table (Hoefer Scientific Instruments, San Francisco) for 30 minutes. The membrane was then incubated with a 1:125 dilution of RAM IgG (Janssens Life Sciences Products, Belgium) supplemented with 1% normal goat serum (a gift from the Department of Clinical Science and Immunology, UCT) for 1 hour and washed in Tris buffer (20 mM Tris with 0,1% BSA), pH 7,4, for 2 x 10 minutes. The membrane was then stained with a 1:200 dilution of GAR-5 (Janssens) overnight, after which it was washed with Tris buffer for 2 x 10 minutes.

The bound gold was visualised by silver staining according to the method of Moermans et al (59). Nitrocellulose strips were washed in distilled water and then in citrate buffer (0,2 M, pH 3,82) after which they were protected from light with foil. A developer was prepared (10 ml 2 M citrate pH 4,85; 15 ml 16% hydroquinone; 0,7% silver lactate, the components being mixed just prior to use) and added to the tube with the nitrocellulose for 15 minutes. The product was fixed with 1:10 diluted photographic fixer (Ilford) for 2 minutes and washed with distilled water before being air dried.

2.11.1 (ii) Goat anti-mouse (GAM-10): Serial, double dilutions of 3B3 and serial, double dilutions of iodinated 3B3 were added to the nitrocellulose and allowed to dry. The nitrocellulose membrane was incubated in a blocking soluton of 5% BSA in Tris-saline (20 mM Tris, 150 mM NaCl, pH 7,4) for 30 minutes. The membrane was then incubated with a 1:100 dilution of GAM-10 in Tris buffer (20 mM Tris with 0,1% BSA) pH 8,2 for 3 hours. After washing, the membrane was treated with the silver enhancement stain as described in section 2.11.1 (i).

The dried nitrocellulose was exposed to Kodac XAR-5 film (Sigma) for 3 hours to obtain an autoradiograph.

2.11.1 (iii) Goat anti-mouse (GAM-10) with whole cells: Platelets were incubated for 45 minutes at 37°C with 3B3 at two concentrations (0,9 mg/ml and 0,1 mg/ml) in HBSS P/B and washed with ice cold buffer. Platelets were then fixed with 2,5% glutaraldehyde for 20 minutes and washed. Unreacted aldehyde was blocked with 0,6% lysine overnight. Cells were then applied to two separate wells on a nitrocellulose dot blot apparatus and allowed to dry. The nitrocellulose membrane was then treated as described in section 2.11.1 (ii).

2.11.2 On-grid immunomarking

Resin blocks containing cells incubated with 3B3 were sectioned onto Nickel grids for post-embedding immunocytochemistry. These sections were etched with either a 10% solution of sodium metaperiodate (Merck) or a 5% solution of hydrogen peroxide for

5-7 minutes and washed on drops of distilled water, or jet-washed with a gentle stream of water from a wash-bottle.

2.11.2 (i) RAM with GAR-5: Etched sections were treated with RAM IgG (with 1% normal goat serum) for 1 hour and jet-washed with Tris buffer (20 mM Tris with 0,1% BSA, pH 7,4). The sections were then incubated on drops of GAR-5 in Tris buffer (20 mM Tris with 0,1% BSA, pH 8,2) for 4 hours and jet washed in Tris buffer, pH 7,4. This was followed by fixation with 2% glutaraldehyde in sodium cacodylate buffer for 10 minutes and washing with distilled water. The sections were contrasted using uranyl acetate and lead citrate, washed with distilled water, air-dried and viewed under the electron microscope.

2.11.2 (ii) GAM-10: Etched sections were first blocked with 1% normal goat serum in Tris buffer, pH 7,4, and then incubated with GAM-10 for 4 hours. Sections were then jet-washed with Tris buffer supplemented with 0,1% BSA and fixed with 2% glutaraldehyde. After washing with distilled water, sections were contrasted with uranyl acetate and lead citrate, washed again and viewed under the electron microscope, after being air-dried.

2.12 BIOTINYLATION OF SURFACE MEMBRANE PROTEINS

Platelets were incubated in HBSS P/B contaning 0,5 mg/ml NHS-SS-Biotin (Pierce) for 1 hour on ice, after which they were washed with ice cold HBSS P/B. The cells were then incubated with iodinated streptavidin (iodinated by the same method used for the immunoglobulin, described in section 2.10.2) on ice for

30 minutes. Control samples were incubated with non-biotinylated cells. Platelets were then washed with ice-cold HBSS P/B and the cell-associated radioactivity determined at each wash.

At the fourth wash, dithioerythritol (Merck) was added to a concentration of 30 µM, 10-fold the amount needed to fully reduce the disulphide bond in the NHS-SS-Biotin (63). After 15 minutes, the cells were washed and the amount of radioactivity removed was counted.

The biotinylation reaction was also performed with mouse macrophages of the $P388D_1$ cell line in a separate experiment identical to that described above. DTE as well as ß-mercaptoethanol was used to remove radioactivity bound to the cells.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 THE EXOGALACTOSYLATION TECHNIQUE

Radioactive galactose (^{3}H-galactose) was introduced onto terminal N-acetylglucosamine moieties of surface membrane glycoproteins.

Characterization of membrane interactions and endocytosis with platelets in terms of this membrane marker required a series of control experiments, namely:

(i) To apply the membrane marker to cell-surface glycoproteins with and without glycosidase pretreatment.

(ii) To remove the membrane marker efficiently with glycosidases.

(iii) To characterize the molecular composition of the labelled glycoprotein species with and without glycosidase pretreatment.

3.1.1 Application of the membrane marker to the cell surface

In order for this labelling technique to be of any use experimentally, the labelling intensity had to be sufficiently high to enable quantification of any endocytic activity. The amount of label incorporated could be increased by pretreating the cell surface with a glycosidase mixture containing neuraminidase and ß-galactosidase (ß-gal) which would remove existing, terminal sialic acid and galactose molecules, creating more sites for incorporation of radioactivity.

The optimal labelling conditions had to be established. Figure 2 shows the kinetics of cell-surface labelling. Washing efficiency was tested by measuring the amount of radioactivity found in the supernatant after each wash (Figure 3). This showed that a small amount (3-5%) of label was repeatedly removed by each wash. This probably relates to label associated with the membranes of cells disrupted during resuspension of the platelets with the pipette, as well as label trapped in crevices or channels of the cells.

The effect of different concentrations of Mn^{2+} in the labelling mixture can be seen in Figure 4. Labelling intensity proved to be highest at a Mn^{2+} concentration of 5 mM, the concentration at which subsequent labelling was conducted. At concentrations below 5 mM $MnCl_2$, manganese ions were the rate-limiting factor to labelling. At concentrations above 5 mM, no increase in labelling intensity was detected up to 15 mM, a concentration above which Mn^{2+} may be toxic to the platelets.

Two buffers were assessed for their suitability as labelling media: Hepes-saline (10 mM Hepes, 150 mM NaCl, pH 7,4) supplemented with 0,1% BSA and 1 µg/ml PGE_1, and HBSS P/B. This was also done to assess whether or not the phosphate ions in the HBSS were complexing with the manganese ions in the labelling mix, leading to the formation of insoluble manganese phosphate. This would effectively remove the Mn^{2+} from solution with a consequent lowering of labelling intensity. From Figure 4 it would appear that this is not the case for

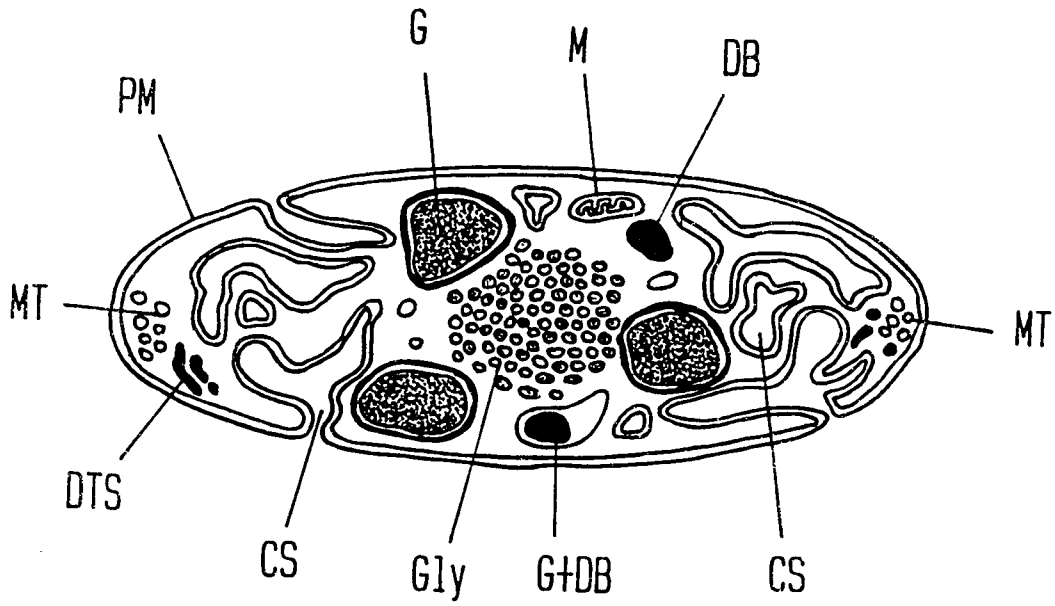


Fig. 1. Schematic diagram summarizing structures observed in discoid platelets.

A number of ultrastructural features may be observed in cross-sections of platelets. The cells have a trilaminar plasma membrane (PM) and may exhibit the circumferential band of microtubules (MT) that are responsible for maintaining the discoid shape of circulating blood platelets. Glycogen particles (Gly) are usually centralized. Organelles such as mitochondria (M) and remnants of Golgi vesicles may also be seen, as well as α-granules (G) and granules containing dense bodies (G + DB). Membrane systems include the dense tubular system (DTS) which serves as the platelet sarcoplasmic reticulum, and the canalicular system (CS). The canalicular system may either appear as membrane-bound vesicles or as channels connected to the plasma membrane.

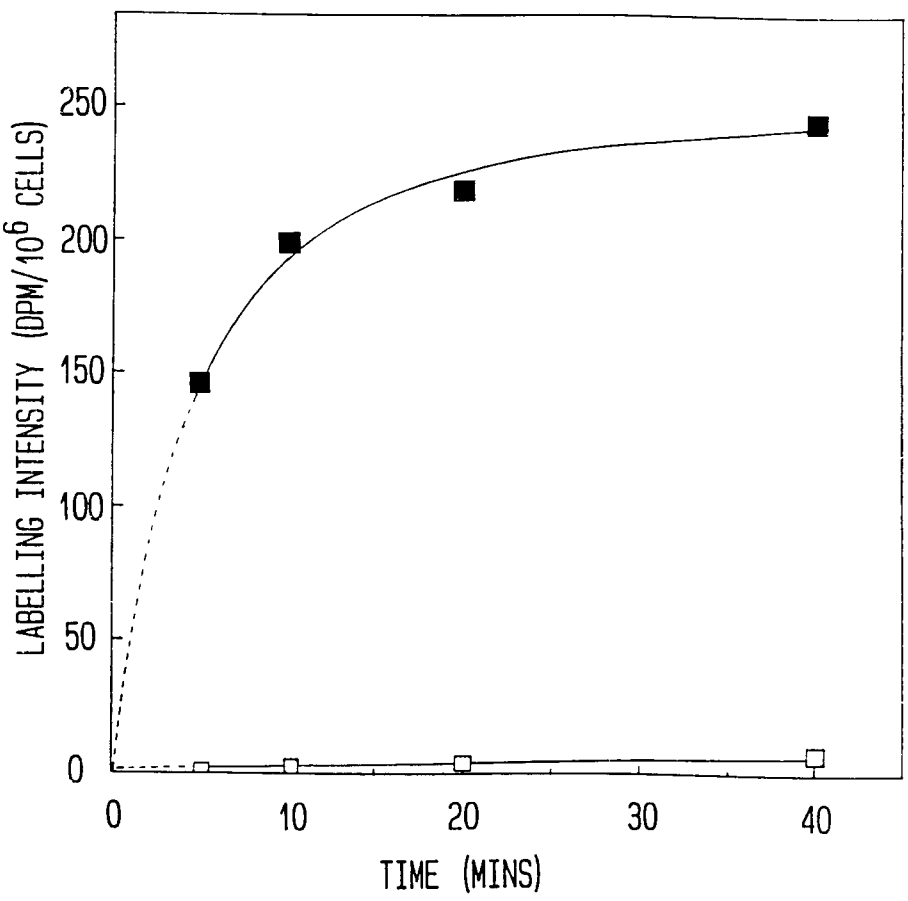


Fig 2. Kinetics of cell-surface labelling with radioactive galactose.

Platelets were incubated with galactosyltransferase and radioactive UDP-galactose for 20 minutes on ice (■). A parallel sample was incubated for 20 minutes without galactosyltransferase (□). Aliquots (of about 25 x 10^6 cells) were removed at various times and diluted in cold buffer to stop the reaction. Platelets were then washed and cell-associated radioactivity determined.

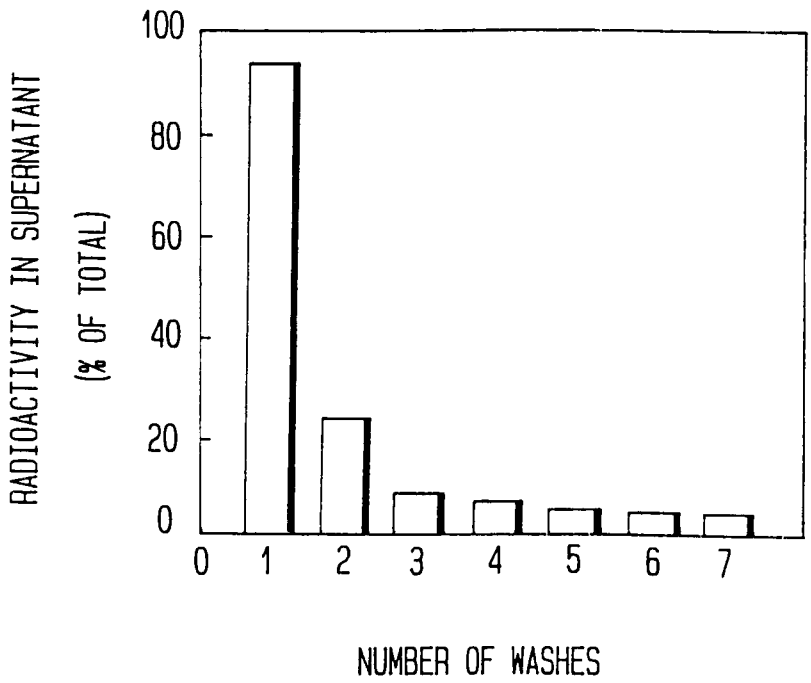


Fig. 3. Post-labelling washing efficiency.

Platelets were labelled as described in Figure 2. At each wash step, the amount of radioactivity in an aliquot of supernatant after centrifugation was determined, and expressed as a percentage of an equal volume aliquot of cell suspension (about 1×10^8 cells).

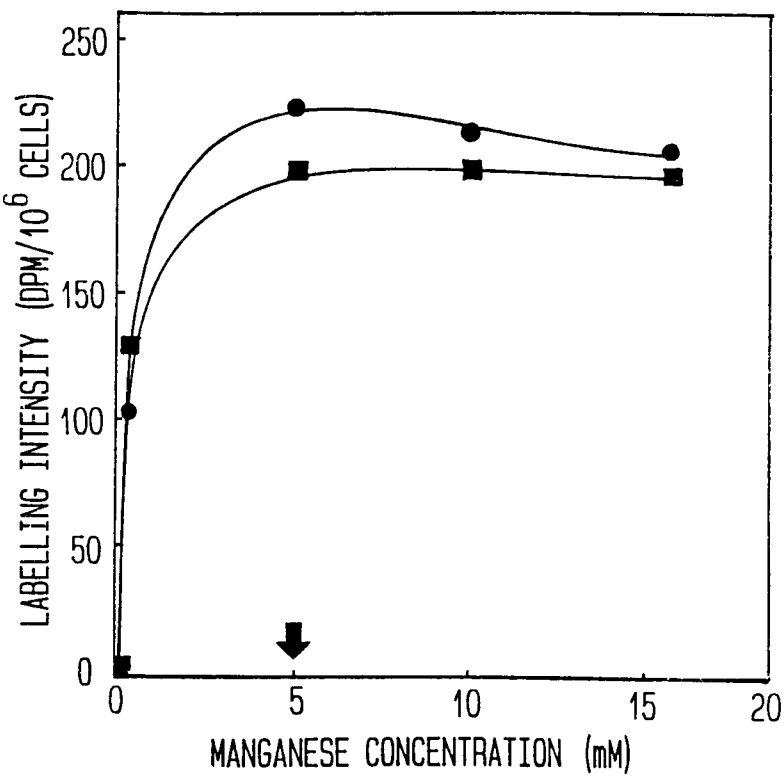


Fig. 4. Effect of Mn^{2+} concentration on labelling intensity.

Platelets were resuspended in Hepes-saline (■) or Hanks Balanced Salt Solution (●) and cooled on ice. Platelets were then labelled, as described in Figure 2, in aliquots (of about 1×10^8 cells) containing increasing concentrations of Mn^{2+}. After washing with cold buffer, cell-associated radioactivity was quantified. Arrow represents concentration of Mn^{2+} used in subsequent experiments, unless otherwise stated.

5 mM $MnCl_2$. HBSS P/B was used as the buffer of choice due to its suitability as a medium for platelets (5).

Following pretreatment of the cells with glycosidases for 10 minutes, the labeling intensity was increased by as much as 10-fold (Figure 5).

Discussion

The pretreatment of platelets was not employed for subsequent experiments, even though the significant increase in cell surface label would be preferable from a quantification point of view. It was considered preferable to subject the cells to as few manipulations as possible. Also, after pretreatment of the cells and subsequent labelling, platelets became difficult to resuspend and tended to form small aggregates, making accurate counting of cell numbers in the H1 counter difficult.

Although the labelling intensity was greater when platelets were preincubated with glycosidases (approximately 10-fold), the amount of label associated with the cells without pretreatment was still sufficient (200-400 DPM/10^6 cells). Also, the increase in labelling intensity had to be weighed against the extra manipulations to which the cells were subjected. The labelling intensity also tended to vary with platelets from different donors. The molecular basis for this difference is not known but care was taken to perform any single experiment or set of similar experiments using the same donor.

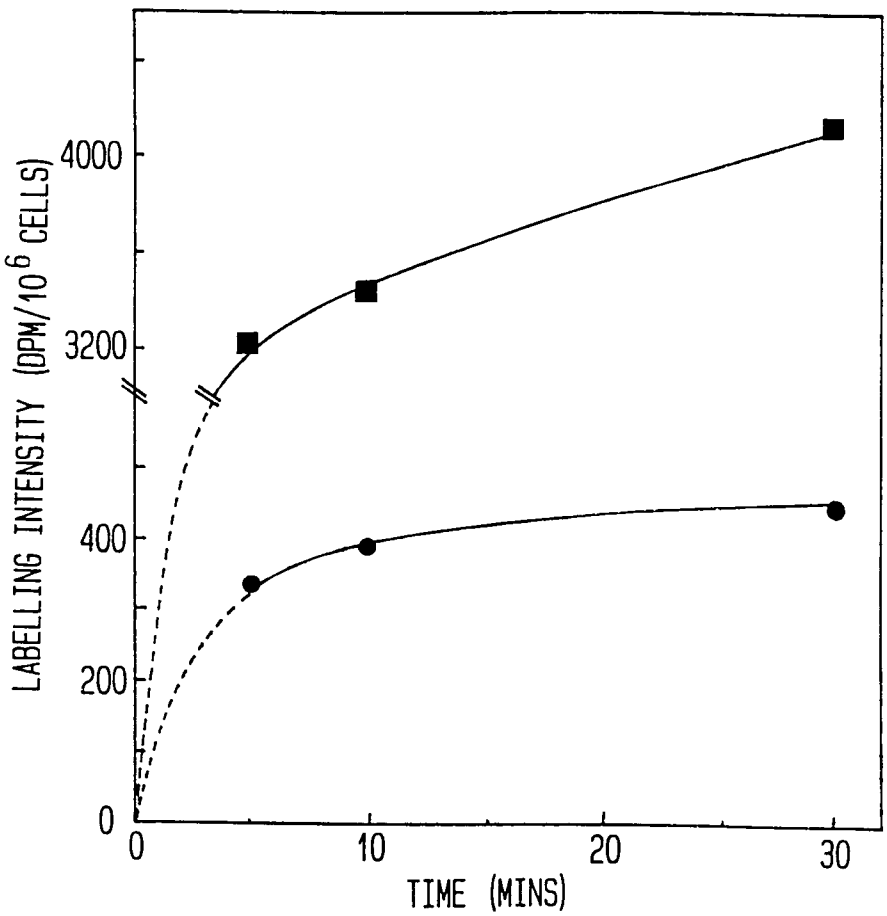


Fig. 5. Effect of pre-treatment with neuraminidase and ß-galactosidase on the platelet surface labelling intensity.

Platelets were either labelled as described in Figure 2 (●) or pre-treated with ß-Gal (0.1 unit/ml) for 10 minutes in the cold before labelling (■). Aliquots of about 1 x 10^8 cells were removed as a function of time and the cell-associated radioactivity determined.

3.1.2 Removal of label with glycosidases

Figure 6 shows the kinetics of label removal from the cell surface by glycosidases. After 20 minutes treatment with glycosidases on ice, 95% of label could be removed. After 120 minutes, 4-5% of the label remained resistant to glycosidase removal.

Discussion

Efficient removal of surface label was essential to subsequent experiments involving endocytosis studies. A concentration of ß-gal of between 0,075 and 0,1 U/ml was employed to remove surface label. An amount of label appeared to be released from the cells in the absence of glycosidase treatment. Whether this related to label from disrupted cells, label remaining in cell crevices and channels or label actively "shed" from the platelets, was uncertain. In subsequent experiments, the amount of label released in the absence of ß-gal was taken into account when determining the label actually removed by direct enzymatic action of the glycosidases. This label was commonly in the order of 5-15%, more being released at 37°C than at 4°C.

A time of 20 minutes was employed for pretreatment of the cells with glycosidases, as well as for the use of glycosidases to remove bound label. From Figure 6 it can be seen that a significantly larger amount of label cannot be released with longer treatment of glycosidases. Also, prolonged incubation of platelets with these enzymes was deleterious to the cells;

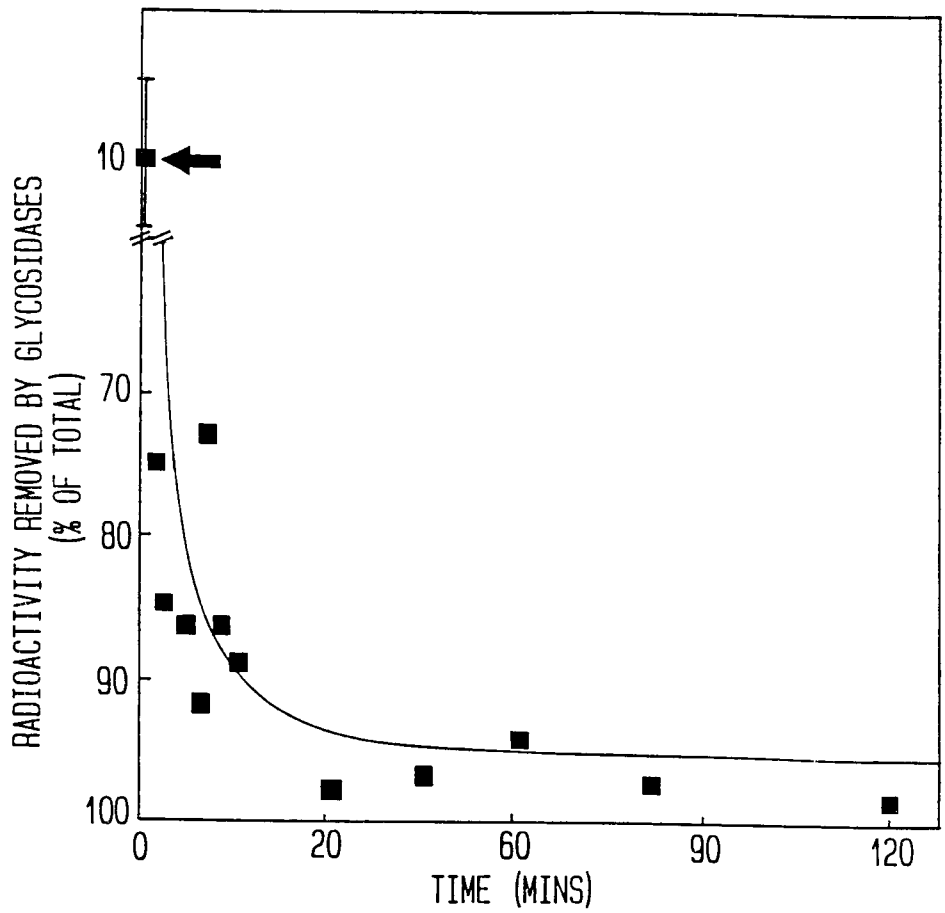


Fig. 6. Removal of cell-surface label by ß-galactosidase.

Platelets were labelled as described in Figure 2. Platelets were then treated with ß-galactosidase at 0.075 units/ml on ice. Aliquots were then removed into cold buffer and the radioactivity determined. After centrifugation, radioactivity of supernatant was measured in order to determine the fraction of label released. Symbols (■) represent the results of two separate experiments. Arrow represents the amount of label released in the absence of ß-gal as averaged from results in separate experiments (Figures 7,8,9), s = 4,0.

platelets tended to form small aggregates that were difficult to resuspend and caused a decrease in the counting efficiency with respect to cell number.

There appeared to be a fairly high variation in the amount of label shed from the platelets in the absence of glycosidase treatment. At 4°C, label shed was about 10%. Cells at 37°C tended to shed label at a level about 10-11% higher. The cause of this apparent "shedding" of label is not known but a similar phenomenon is known to occur with other cell types (64, and N. Godenir, personal communication) and is thought, in part, to result from an active process. The shedding could include unbound label escaping from the canalicular system channels where the effects of washing of the platelets could be slowed down by diffusion.

This shed label was either determined in separate samples of each subsequent experiment and subtracted from the amount of label removed by glycosidases, or the platelets were washed prior to their treatment with glycosidases, to eliminate the effect of shedding.

3.1.3 Analysis of the molecular weight composition of platelet membranes as labelled by the exogalactosylation technique

The platelet cell surface was labelled with and without pretreatment with glycosidases as described in Figure 5. The composition of labelled molecular species was analysed by SDS-PAGE. Figure 7 shows that the degree of labelling of the cells

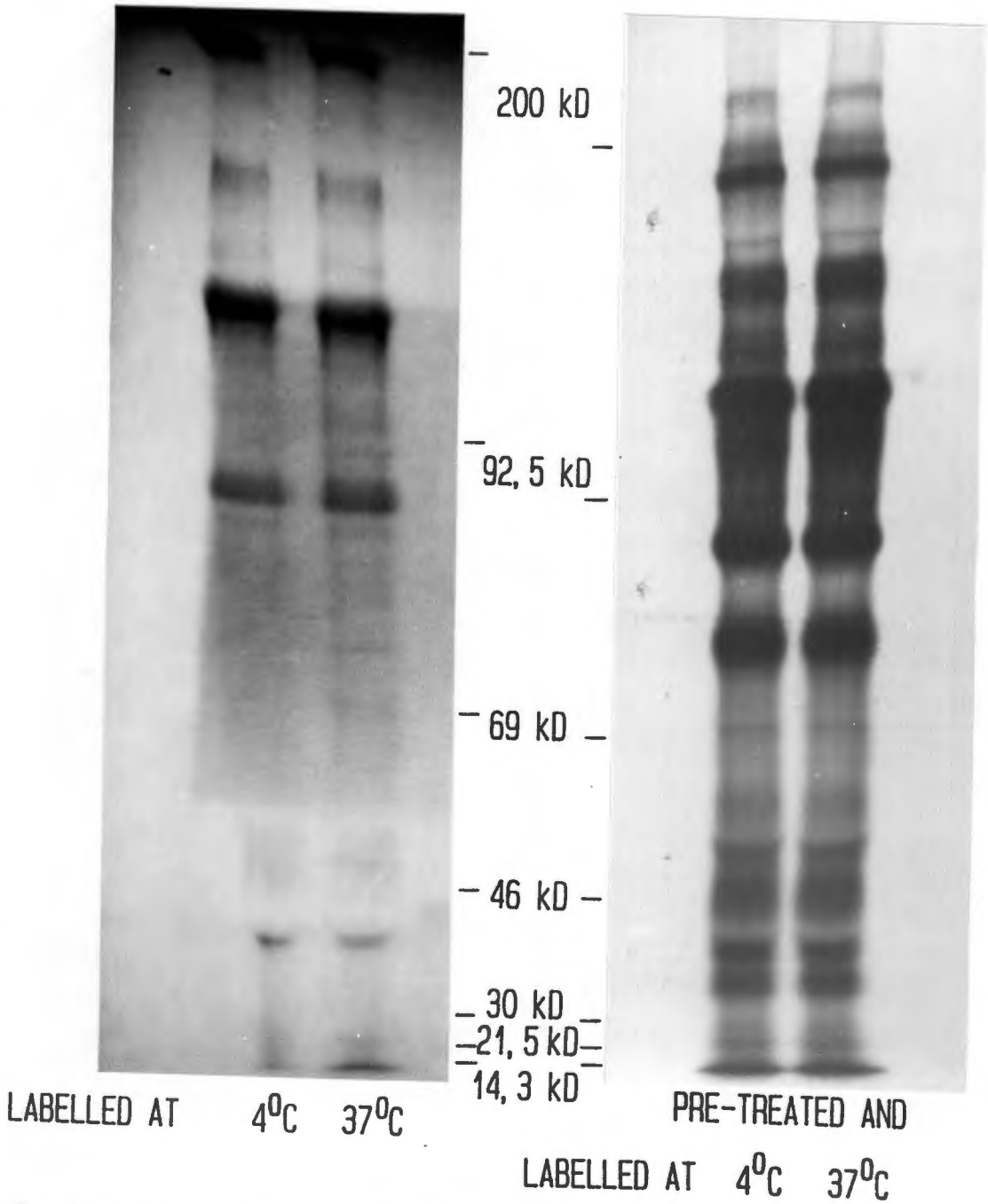


Fig. 7. Fluorography of radiolabelled platelet membranes.

Platelets were treated as described in Figure 5. In addition, aliquots of pre-treated and non pre-treated cells were labelled at 37°C. Platelets were prepared for electrophoresis under reducing conditions in a 5-13% polyacrylamide gel. The dried gel was processed for fluorography and exposed at -70°C for 8 days (pre-treated cells) and for 34 days (non pre-treated cells).

pretreated with glycosidases was much greater. The number of molecular species radiolabelled by the pretreatment method was greater than that labelled without pretreatment. Cells labelled at 4°C show no difference in the degree of labelling, or in the number of labelled species relative to cells labelled at 37°C.

Discussion

Of interest was the observation that labelling the cells at 37°C had no effect on the labelling intensity or on the number of M_r species labelled. After 20 minutes, little increase in labelling intensity occurred. That there was little or no difference in the number of labelled species after labelling at 37°C has some interesting implications. At 37°C, the cells would be expected to endocytose some of the labelling mixture, possibly causing intracellular membranes of the endocytic pathway to become labelled. This would, in turn, conceivably label molecular species not present on the cell surface and alter the composition of the labelling profile as seen in Figure 7. This did not occur. Whether this would happen with other cell types known to have an active endocytic mechanism at 37°C, can only be speculated upon.

3.2 CHARACTERIZATION OF CONSTITUTIVE ENDOCYTIC ACTIVITY

The exogalactosylation technique (43), as described in section 3.1, was used to determine the existence of constitutive endocytic activity of resting human platelets. Endocytosis implies that internalization of membranes takes place during

coated pit and endosome formation. This would imply that labelled components of the plasma membrane are redistributed to internal sites of the cell. These sites would be inaccessible to the glycosidases employed to remove this label as the enzymes are presumably unable to traverse the cell membrane bilayer. There is thus reduction in the amount of label removed after glycosidase treatment. This should follow a time-dependent course as more membrane is internalized.

Complementing membrane internalization analysis, uptake of fluid-phase components was also characterized with platelets in the resting state. When endosomes are formed at the plasma membrane, part of the surrounding medium of the cells is taken up and traverses the lumen of the endocytic pathway. This can be analysed with the use of fluid-phase markers.

3.2.1 The use of the exogalactosylation technique to measure labelled membrane uptake with resting cells

After labelling the platelets at 4°C as described previously, they were warmed to 37°C and incubated in buffer. Figure 8 (bars) was expected to indicate a time-dependent decrease in amount of label accessible to removal by glycosidases from the cell surface. No such decrease was observed. This indicates that over 20 minutes, there was no perceived endocytosis of membrane. An average of 75% of label was removed by ß-gal.

In the event that endocytic activity was a very slow process with platelets, the cells were allowed to endocytose for 3

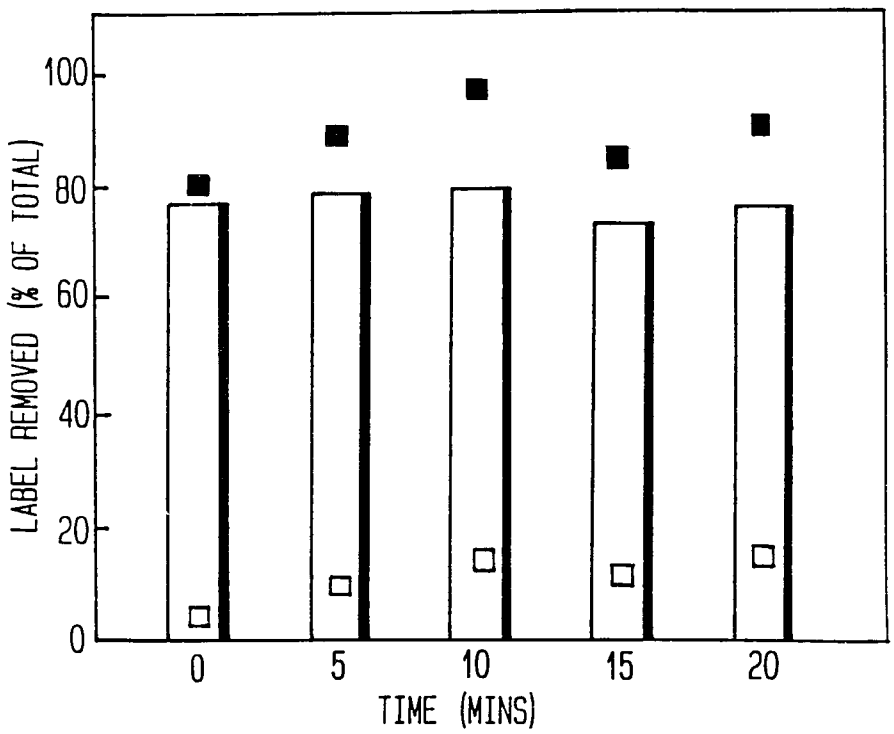


Fig. 8. Label accessible to removal by ß-galactosidase, after incubation of platelets at 37°C.

Platelets were labelled as described in Figure 2. After warming to 37°C, aliquots were removed as a function of time and cooled on ice to stop endocytosis. In order to remove surface bound (accessible) label, cells were incubated with ß-gal in the cold, and the amount of label released was measured (■). In addition, platelets were incubated without ß-gal, in the cold, and the amount of label released was measured (□). The values represented by open symbols were subtracted from those with closed symbols to give the amount of label released by ß-gal (represented by bars).

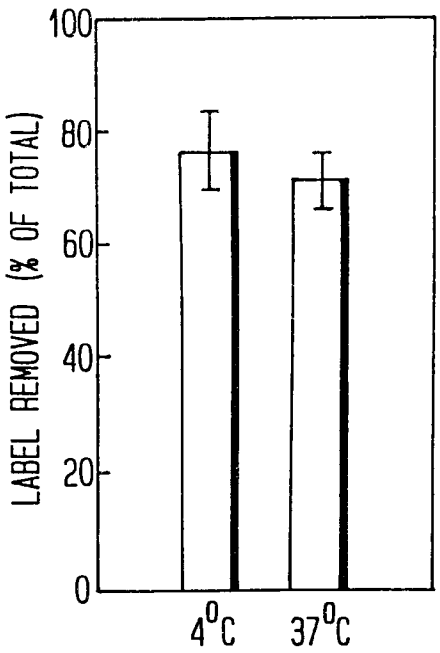


Fig. 9. Determination of membrane internalization: removal of surface-bound label by ß-galactosidase, after incubation at 4°C and 37°C for 3 hours.

Platelets were labelled as described in Figure 2 and incubated for 3 hours at 4°C or at 37°C. Aliquots from each were then treated with ß-galactosidase in the cold to remove accessible label. The percentage label released was determined by centrifugation as described in Figure 4. Percentage label "shed" from cells without ß-gal treatment was subtracted from percentage label released by ß-gal. The final value obtained is expressed with error bars representing the standard deviation of 12 samples (three separate experiments).

hours at 37°C. As shown in Figure 9, after 3 hours at 4°C, about 76% of label (s = 7%) was removed. With cells at 37°C, 71% of label (s = 5%) was removed. Although the mean was less with the cells at 37°, there was no statistical significance between the values as seen by the standard deviation of the mean.

Discussion

No significant difference between label accessible to glycosidase release was detected between cells incubated at 4°C, and cells incubated at 37°C. Even a low endocytic rate should have been detected after 3 hours using this technique (44).

By looking at the number and intensity of molecular species labelled using the exogalactosylation technique at 4°C, as seen in Figure 7, it is possible that endocytosis may involve the internalization of membrane species that were either labelled poorly, or not at all. This leaves the possibility that endocytosis could be observed after first pretreating the cells with glycosidases, to increase the number of labelled species (Such an experiment could not be performed because the pretreated cells were in a state of partial activation and could not be demonstrated to be fully viable after pretreatment).

3.2.2 Uptake of fluid-phase marker as a measurement of endocytic activity with resting cells

Because no membrane internalization could be measured by the exogalactosylation technique, a fluid-phase uptake assay was employed. FITC-dextran was used as the fluid-phase marker (65).

Uptake of FITC-dextran (10 mg/ml) at 4°C versus 37°C is shown in Figure 10. Curves indicated a very low apparent rate of uptake (1,8 fl/10^6 cells/min). The rate of uptake of FITC-dextran at 4°C was only marginally lower than that at 37°C (0,47 fl/10^6 cells/min lower). In Figure 10, the 4°C curve is shown by the broken line.

Discussion

Even in relation to the small volume of the platelet (mean cell volume = 5,2 - 7,7 fl) the rate of endocytic volume uptake was negligibly small when compared to the rates of endocytosis of other cell types (Table II). These results confirmed the findings that membrane internalization could not be detected by the exogalactosylation experiments.

It would thus appear that if endocytosis with resting platelets was occurring, in a basal or constitutive form, the rate of the process is extremely low. Such a slow process would appear to have little physiological function. Many workers have discussed circulating platelets as endocytic cells (9-14) but no such activity could be demonstrated in this study.

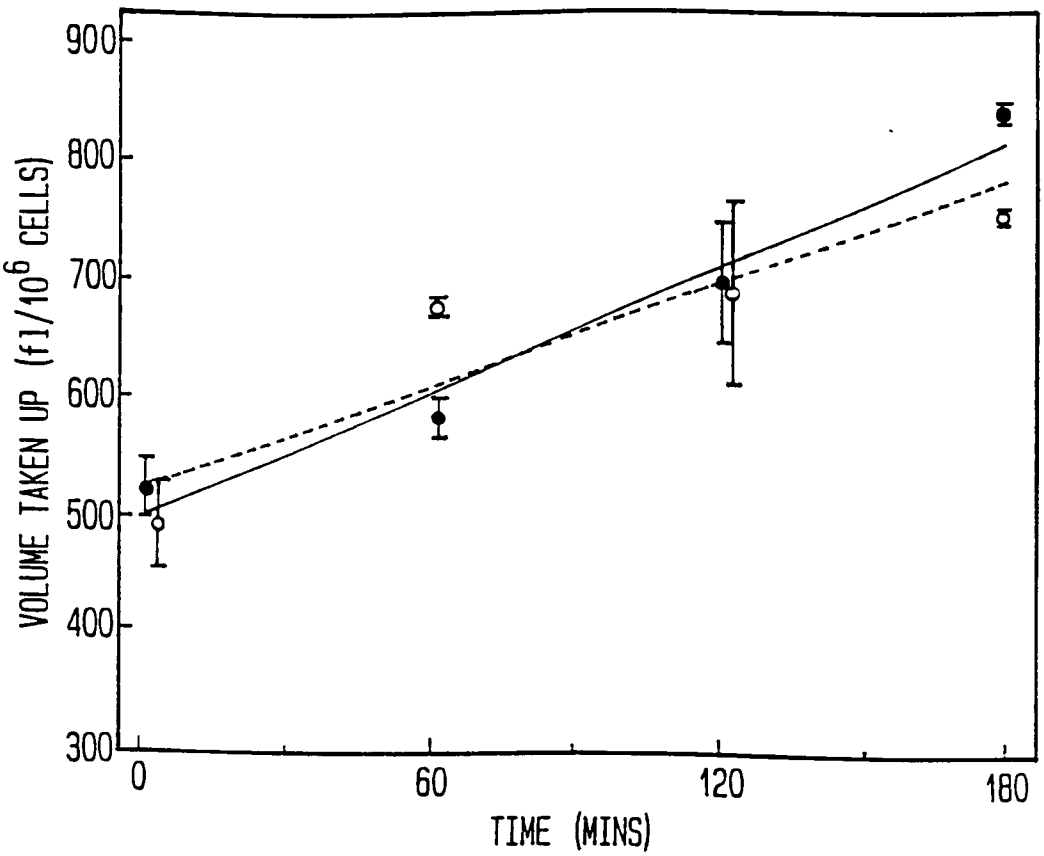


Fig. 10. Fluid-phase uptake assay by platelets.

Platelets were incubated with the fluid-phase marker, FITC-dextran at 4°C (o), and at 37°C (•). Aliquots were removed, as a function of time, into cold buffer to stop endocytosis. Platelets were then washed, counted, and treated with Triton X-100. Membrane debris was sedimented and fluorescence intensity measured on a fluorimeter. Error bars show an average of two samples. Concentration of FITC-dextran used in the incubation was 10 mg/ml. (•): Slope = 1.85 fl/10^6 cells/min. (o): Slope = 1.38 fl/10^6 cells/min.

Table II. Kinetic data on pinocytic rates

V, volume; S, surface area; d, diameter; v, volume uptake rate.

Cell type	Cell dimensions			Internalization
	$V(\mu m^3)$	$S(\mu m^2)$	$d(\mu m)$	$v(\mu m^3/min)$
Mouse peritoneal macrophages	395	825	9,1	1,2
Mouse macrophage cell line, J774	1662	3925	14,7	2,8
Guinea-pig alveolar macrophages	1750	2250	15	8,6
Mouse macrophage cell line, $P388D_1$	900	1300	12	2,5
Mouse fibroblasts, L cells	1765	2100	15	0,9
Human fetal lung fibroblasts IMR-90	2500	2670	16,8	5,3
*Human platelets	6,5	22,2	3,6x0,9	$<10x10^{-7}$

From Thilo L. (44)
* This study.

3.3. THE USE OF A MONOCLONAL ANTIBODY TO INDUCE MEMBRANE INTERNALIZATION

It had been reported previously that antibodies to the fibrinogen receptor of human platelets, the so-called GPIIbIIIa complex, are internalized in a time-dependent fashion. Also, this internalization required active metabolic function and did not occur at 4°C (42). Antibodies against other membrane glycoproteins (i.e. GPIb) were not internalized (24). Several different monoclonal and polyclonal antibodies to GPIIbIIIa were used with apparently identical results (42).

The monoclonal antibody against GPIIbIIIa, designated 3B3 (45), was used in this study to observe if specific internalization of this receptor complex occurred using the exogalactosylation technique. Assuming the 3B3 binding elicited the same effect as antibodies used in previous studies, the internalization process should become detectable. It was thus necessary to:

(i) purify the monoclonal antibody

(ii) determine whether 3B3 was indeed binding to the platelets under the experimental conditions used and whether redistribution occurred in the same way as for other antibodies reported in previous studies.

(iii) measure whether the presence of 3B3 caused stimulated internalization of labelled membrane.

3.3.1 Purification of the antibody and analysis of purity

The monoclonal antibody, 3B3, was purified by affinity chromatography on a protein G column. The purity was assessed by analysis on an SDS polyacrylamide gel (Figure 11).

3B3-rich mouse ascites fluid was harvested and pooled and the proteins precipitated with ammonium sulphate. The molecular weight composition of reduced proteins from the IgG-rich fraction is shown in lane 2 of the gel in Figure 11. The IgG heavy chains of 50 000 D as well as light chains of about 24 000 D appear. Several bands appear in addition to the IgG chains including a large band in the region of 66 000 D. This is the albumin present in the mixture. Lane 1 shows the composition of proteins from the pooled fractions eluted off

the column and shows only the IgG chains present. A very faint band in the 66 000 region reflects a small amount of albumin.

3.3.2 Stimulated internalization of labelled membrane by platelets incubated with monoclonal antibody

The exogalactosylation technique was used in an attempt to measure endocytosis of labelled membrane after the antibody 3B3 was added to a suspension of cells and warmed to 37°C. Figure 12 indicates the amount of membrane accessible to removal by glycosidases. 76,5% (s = 5,5) and 75,5% (s = 4,5) of label was removed after the 4°C and 37°C incubation, respectively. In the presence of 3B3, 74% (s = 4,0) and 72% (s = 5,0) of label was removed after the 4°C and 37°C incubation, respectively. The differences between these values were not statistically significant.

Discussion

It would appear that even in the presence of antibody to the receptor complex GPIIbIIIa, no detectable endocytic activity took place. This is contrary to expectations and differs from previous studies that claim to show internalization of the receptor-antibody complex (24,42).

3.3.3 An immunofluorescence technique to localise internalized receptor-antibody complex

An immunofluorescence study was employed to test the binding of the antibody to the cells (i.e. whether or not the 3B3 in fact

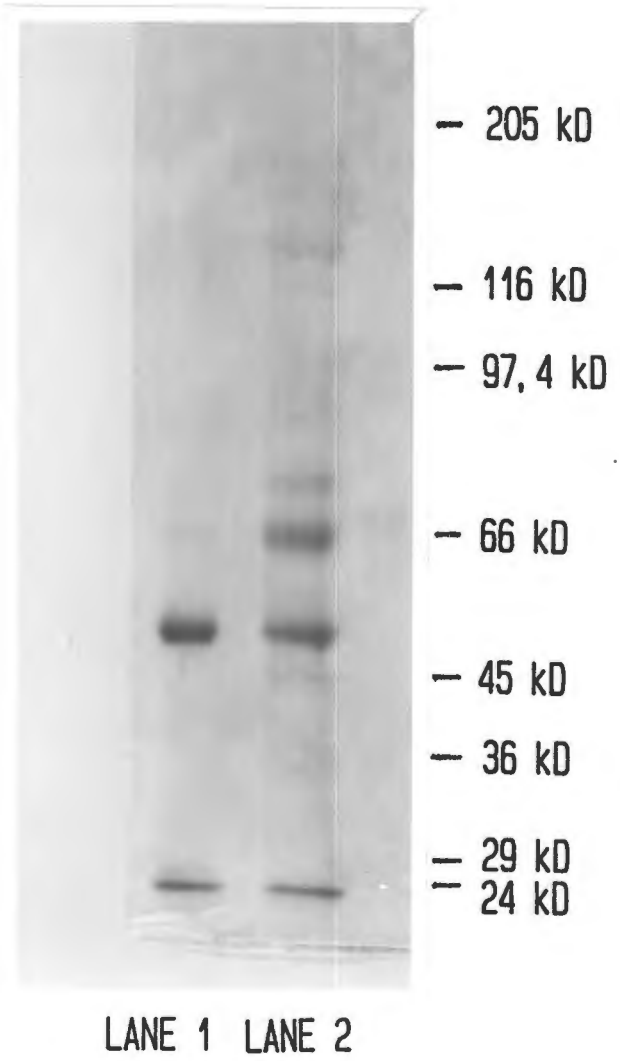


Fig. 11. Purity analysis by SDS-PAGE, of affinity purified 3B3.

The monoclonal anti-human GPIIbIIIa IgG, 3B3, was raised in mice and affinity purified on a protein G column as described in the Methods. The purity of the antibody was analysed on a 5-20% polyacrylamide gel under reducing conditions and stained with coomassie blue. The purified antibody was loaded onto lane 1 of the gel. An ammonium sulphate precipitate of the antibody from ascites fluid (prior to affinity chromatography) was loaded onto lane 2 of the gel as a crude preparation.

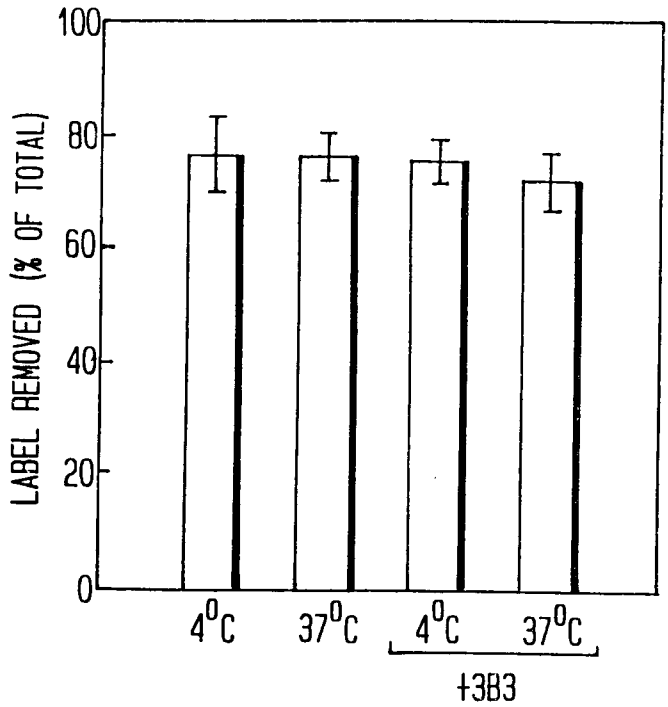


Fig. 12. Membrane internalization in the presence and absence of monoclonal antibody, 3B3.

Platelets were labelled as described in Figure 2 and cooled on ice, or warmed to 37°C. Platelets were then incubated in the presence and absence of anti-GPIIbIIIa antibody, 3B3 for 3 hours. Subsequently, ß-galactosidase was used to remove surface-bound label. The fraction of removed radioactivity was calculated as described in Figure 4. Values shown were corrected for label shed into the medium during the 3 hour incubation. Error bars represent the standard deviation of four samples from two separate experiments.

recognised the GPIIbIIIa). It could also be used to determine if internalization of the antibody took place and if this process was temperature dependent. The experimental approach was essentially the same as that described by Wencel-Drake et al. (42,51). The results are summarized in Figure 13.

Platelets incubated with 3B3 and then stained with goat anti-mouse IgG-F(ab')$_2$-FITC showed a bright green fluorescent pattern. Controls (not shown) treated in the same way but in the absence of 3B3 showed only a background fluorescence attributable to autofluorescence of the paraformaldehyde (this was of a more yellow-green colour) and very low levels of non-specific binding of the fluorochrome-IgG. The controls are not shown as the automatic camera could not be used to take micrographs of such low-light images at the same exposure as the micrographs of fluorescing platelets. These results indicated that the 3B3 was indeed binding to the platelets and could be visualised with the fluorescent secondary antibody.

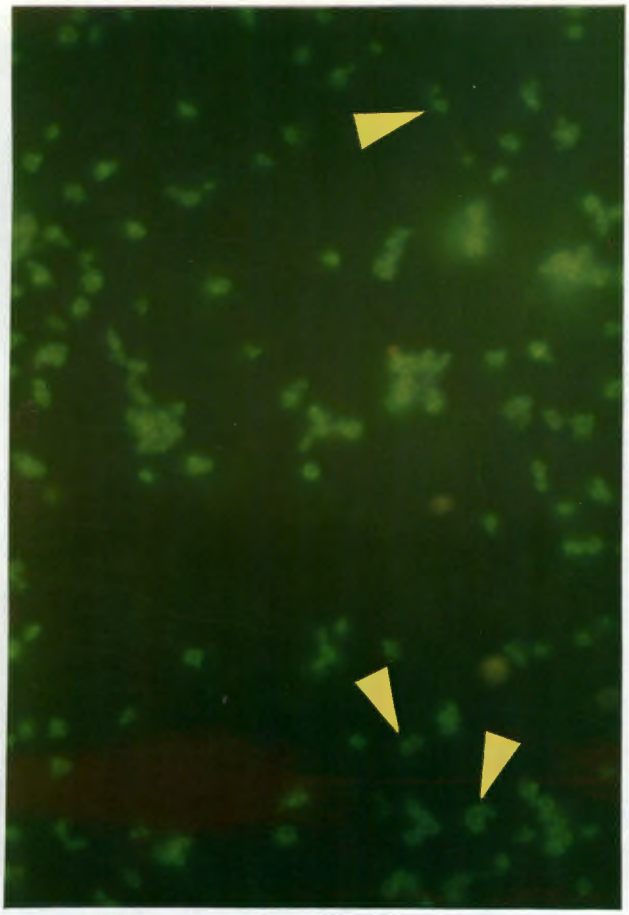
Platelets not permeabilised with Triton X-100 prior to staining with secondary antibody showed a rim-pattern fluorescence (Figure 13B, - TX 100) indicating antibody bound to the surface of the cell. The permeabilised cells (Figure 13B, + TX 100) showed, in addition, fluorescent areas within the cell indicating that the 3B3 had been internalized. Platelets labelled with 3B3 in the cold and permeabilised before staining showed a rim-pattern fluorescence. Uptake of 3B3 thus appeared to be an active metabolic process.

Fig. 13. Immunofluorescence microscopy of platelets incubated with 3B3.

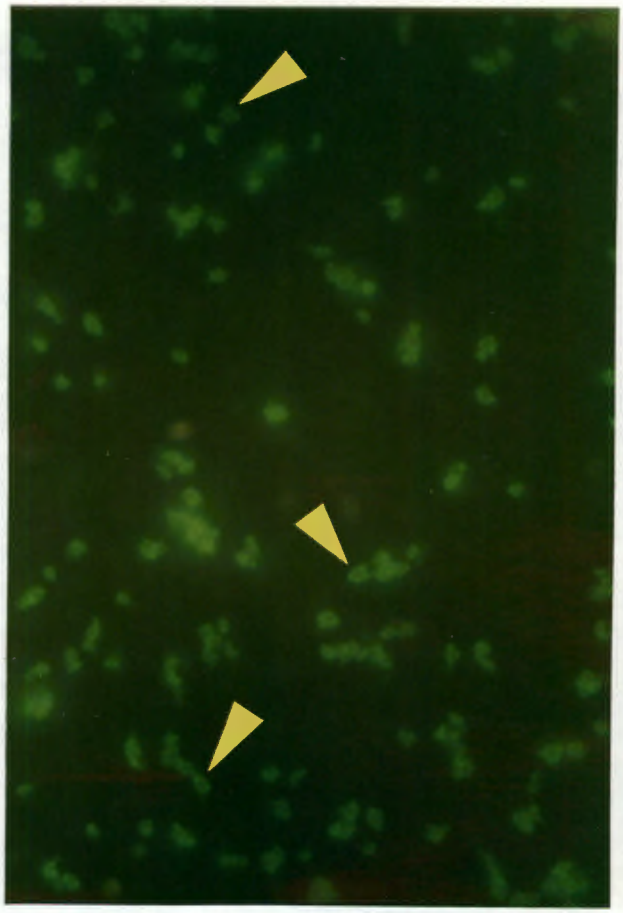
Platelets were incubated for 40 minutes in the presence (A;B) and absence (not shown) of 3B3, on ice (A) and at 37°C (B). Platelets were then fixed with 3% paraformaldehyde on ice for 45 minutes. Aliquots of A and B were rendered permeable by brief (5 minutes) treatment with Triton X-100 (indicated in the figure as +TX-100). Permeabilised and non-permeabilised platelets were then incubated for 20 minutes with goat anti-mouse IgG-F(ab')$_2$-FITC, at room temperature. Platelets were then allowed to adhere to poly-L-lysine coated coverslips and observed under the fluorescence microscope at 630 x magnification. Platelets incubated in the absence of 3B3 exhibited low levels of non-specific, background fluorescence (micrographs not shown - see Results).

Both permeabilised and non-permeabilised platelets incubated with 3B3 at 37°C are shown (B: ± TX-100). Only permeabilised cells incubated with 3B3 at 4°C are shown (A). Two panels of each treatment are shown to provide a larger field of view. As in any one field only some cells will be in the correct plane of focus, arrows indicate particularly clear examples.

A rim-pattern fluorescence can clearly be seen with the platelets incubated on ice (A), as well as with those incubated at 37°C without subsequent permeabilisation (B: -TX-100). Interior staining can be seen in the platelets incubated at 37°C and followed by permeabilisation, before staining.



A



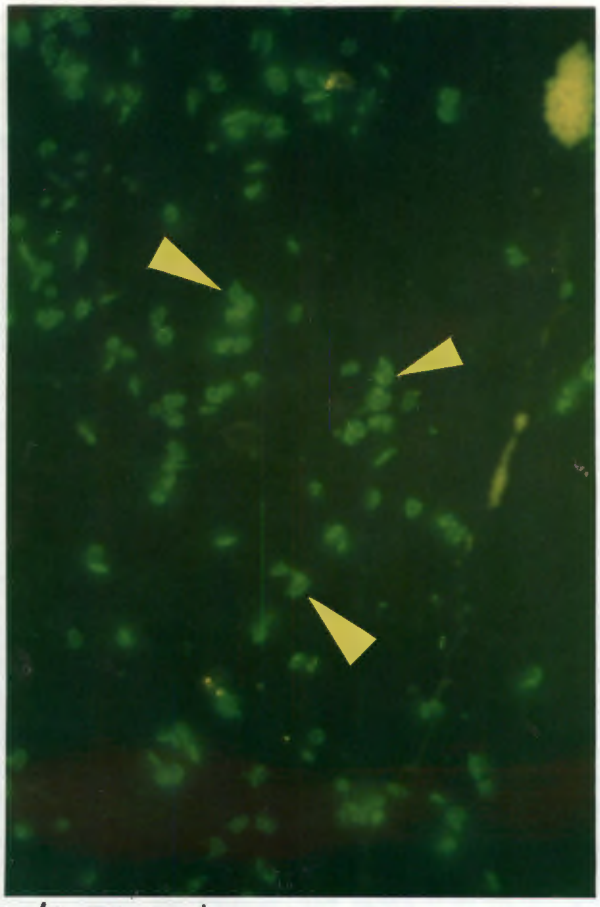
A

FIG. 13. FOR LEGEND SEE p55

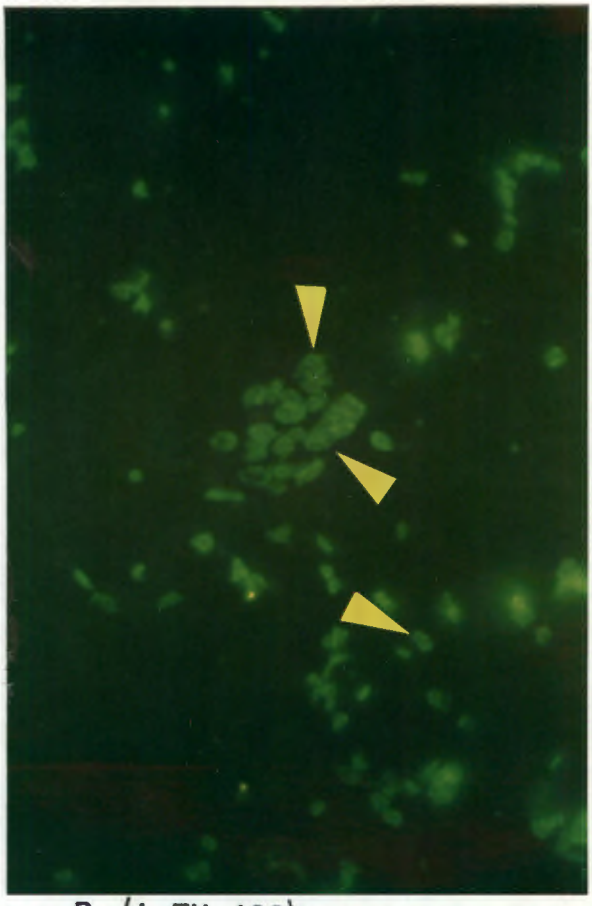
Discussion

The results are essentially the same as those reported for other antibodies by Wencel-Drake (42). The 3B3 was recognising GPIIbIIIa on the platelet membrane, binding to it and was being redistributed to sites within the cell in a process requiring a functional cell metabolism.

Only after treatment with Triton, was internalized 3B3 accessible to staining with secondary antibody. This may lead to the deduction that the 3B3 had been transferred to an intracellular, membrane-bound site such as an endosome or lysosome, i.e. endocytosis has occurred. However, the exogalactosylation technique results shown in Figure 12 indicated that no endocytosis was occurring. This apparent

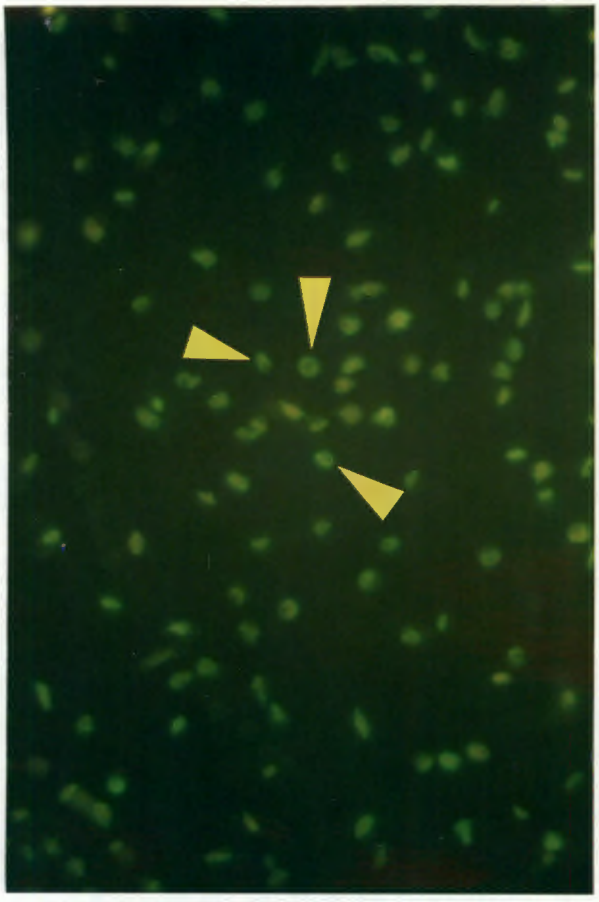


B (+ TX-100)



B (+ TX-100)

B (- TX-100)



B (- TX-100)

FIG. 13. FOR LEGEND SEE p55

anomaly can be explained if the possibility is taken into account that the 3B3 has not been endocytosed but sequestered in the canalicular system. This possible mechanism would provide for the clearing of particulate matter from the platelet surface, as reported in other studies (10,29,34,37). For the sake of discussion, the term interiorized (rather than internalized, which implies true endocytosis via coated vesicles etc) is used to describe the fate of the 3B3 bound to the platelet surface receptors.

Two models were proposed to accommodate the data discussed by earlier workers. These models are summarized in Figure 14 (42). Previous authors were apparently unable to use either model A or model B to fully accommodate data. The present study gave evidence for model B (Figures 12 and 13). An anomaly that still persisted was that if sequestration into the canalicular system had occurred, why was anti-GPIIbIIIa antibody in both the Wencel-Drake study and the present study only accessible to staining with secondary antibody after Triton treatment, as the canalicular system is in continuity with the platelet's surrounding medium? The finding that the antibody was transferred to an intracellular compartment accessible only after permeabilisation, has been used as the evidence for model A in Figure 14 (42). It was thus necessary to discover whether fixation with paraformaldehyde prevented entry of molecules such as enzymes and antibodies (e.g. ß-gal and secondary antibody - FITC conjugates) to the canalicular system. This would provide evidence for model B and account for the findings in the present study.

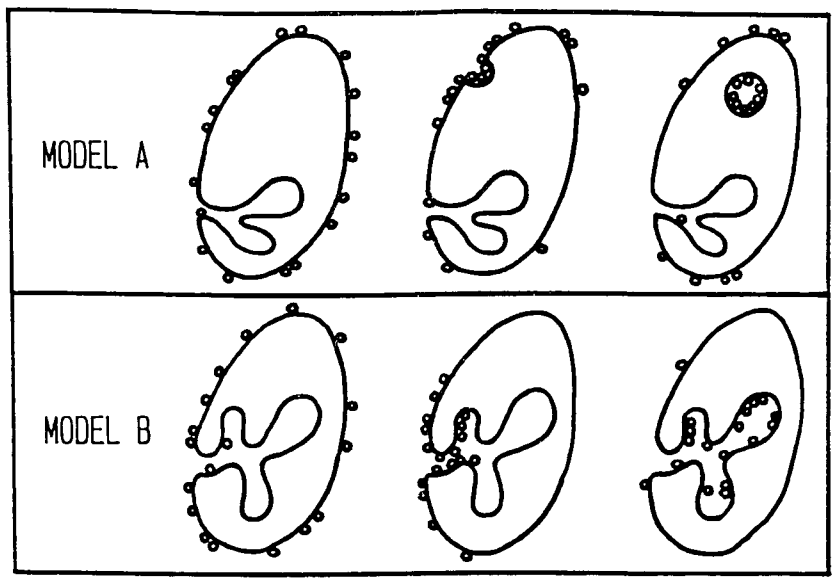


Fig. 14. Models for internalization of receptor-ligand complex GPIIbIIIa-3B3.

Two model systems are illustrated. In Model A, labelled GPIIbIIIa (open circles) is actively endocytosed into vesicles which may be transferred to the canalicular system, granules or back to the plasma membrane. Alternatively, in Model B, labelled GPIIbIIIa (open circles) is cleared from the surface by an active mechanism and sequestered in the canalicular system, which in thin section, at the electron microscope level, or at the level of resolution of the light microscope would appear vesicular in nature. (Wencel-Drake 1990).

3.3.4 Measuring canalicular system label protected from glycosidases by fixation

Platelets labelled using the exogalactosylation technique as before were treated with 3B3. Cells were cooled and either fixed with paraformaldehyde or maintained at 4°C on ice. Results are shown in Figure 15. After treatment with glycosidases, 77% of label was removed with the cells on ice (a value in agreement with data in Figure 12). Only 55% of label was removed after the cells were fixed. Thus, after fixation, 22% of label was glycosidase resistant. It is unlikely that this was due to the fixation preventing removal of cell-surface material as such. It has been shown, for macrophages, not to be the case (45). The exogalactosylation labels only the terminal ends of glycoprotein moieties, a molecular region not normally affected by paraformaldehyde.

An attempt was made to detect any differences in the amount of label retained in the canalicular system after treatment with and without 3B3. If the 3B3 was being sequestered in the canalicular system, the possibility existed that this would alter the fraction of the glycosidase-resistant label. Figure 16 indicates little difference (26% protected in the absence of 3B3, 25% protected after incubation with 3B3).

Discussion

From Figure 15 and Figure 16 it can be deduced that paraformaldehyde fixation renders the label bound to the

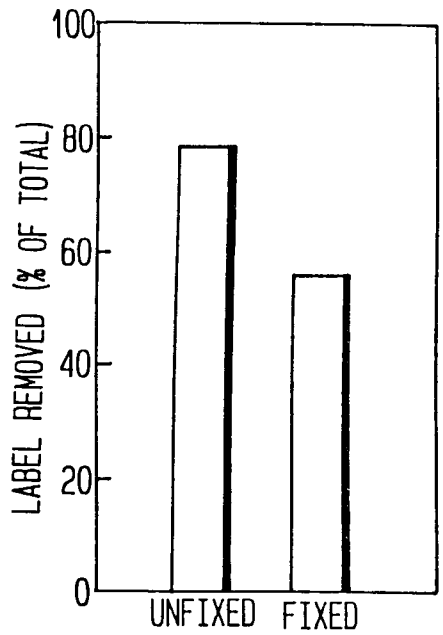


Fig. 15. Label removal by ß-galactosidase with fixed and unfixed platelets.

Platelets were labelled as described in Figure 1. Platelets were then incubated for 45 minutes at 37°C, in the presence of 3B3, after which they were cooled to 4°C. An aliquot was then fixed with 3% paraformaldehyde for 45 minutes. Platelets were then treated with ß-galactosidase in the cold, to selectively remove label bound on the surface of the cell, and not label in the canalicular system. Label removed was calculated as described in Figure 4.

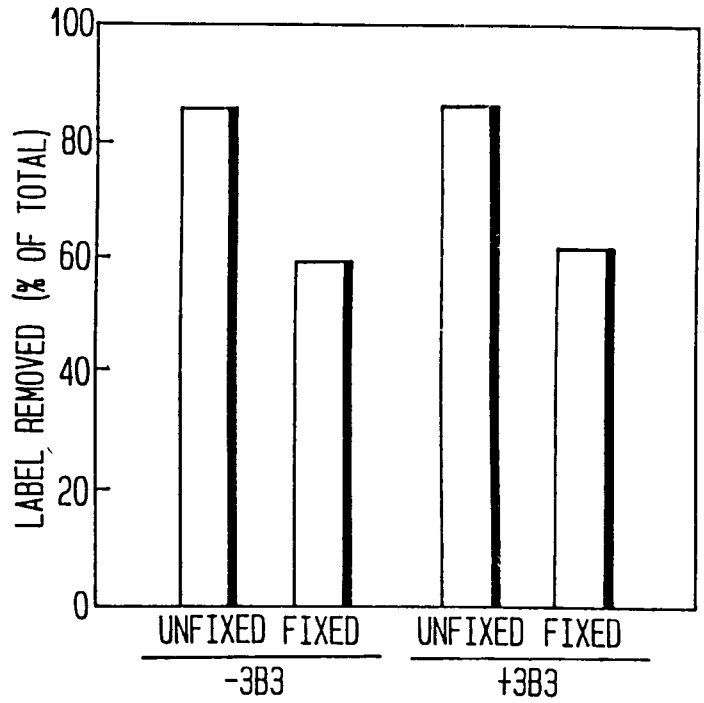


Fig. 16. Degree of label protected from ß-galactosidase removal when platelets are fixed: after incubation with and without 3B3.

Platelets were labelled and incubated as described in Figure 12, in the presence and absence of 3B3. The amount of label associated with the membranes of the canalicular system (measured as amount of label fixation-protected from ß-gal) can then be compared.

membranes of the canalicular system protected from enzymatic removal by glycosidases. With platelets cooled to 4°C, glycosidases are most likely able to diffuse into the channels and remove the label. This is shown by the findings in Figure 13. The fixed platelets show interior staining only after permeabilization. This is not because the 3B3 has been internalized (or endocytosed) but more likely because the staining antibody does not have access. Thus, the terms surface bound, and accessible do not necessarily refer to the same label. Surface-bound label refers to label on the plasma membrane of the platelet, while accessible label refers to label that is capable of removal with glycosidases.

3.4 ELECTRON MICROSCOPY AND IMMUNOCYTOCHEMISTRY

In order to visualise the intracellular compartment into which the 3B3 was transferred following its binding to platelet membrane GPIIbIIIa, it became necessary to observe the platelet ultrastructure at the electron microscope level. At the magnification possible it was hoped to provide further evidence that the interiorized 3B3 was in fact being sequestered in the canalicular system. If this could be demonstrated, it would strengthen the Model B hypothesis of Figure 14. Several workers have demonstrated immunocytochemical staining of antibodies and receptors, both internalized and on the platelet surface with cells embedded in conventional hydrophobic and hydrophilic resins as well as cryosections (10,17,40,42,46). To achieve this with the system and antibodies used in the present study it was necessary to:

(i) prepare human platelets for electron microscopy with clear ultrastructural detail especially with respect to cellular membranes

(ii) develop a system whereby an electron-dense immunogold probe could be used to target intracellular 3B3, either by labelling of the 3B3 itself, or application of a secondary or tertiary antibody system. Procedures using pre-embedding labelling as well as post-embedding labelling were established.

3.4.1 Preparation of platelets for electron microscopy and morphological assessment

Platelets were prepared as described in Materials and Methods. Results are shown in Figure 17. From the figure, the platelets exhibit many of their ultrastructural features. The membranes are clearly marked with the staining methods used. The buffers used in the preparation of the platelets were chosen for their suitability for the preservation of ultrastructural detail (5). Platelets were also fixed in suspension rather than as a pellet, after centrifugation. It has been demonstrated that the close cell contact in the pellet partially activated platelets and caused shape changes during fixation (5). Sodium cacodylate was used to buffer the osmium tetroxide during postfixation as it was found to have a beneficial effect of preserving internal membrane structures. Staining with tannic acid helped to render membranes of the canalicular system more conspicuous (52). Tannic acid staining did not mark the membranes as uniformly as expected, possibly due to the variation in the amount of membrane in the thin sections.

Depending on the orientation and aspect of the canalicular system membranes, some show more membrane area in the section and thus appear darker. Post-fixation with osmium tetroxide generally led to clearer membrane staining and higher contrast (Figure 17C,E).

Discussion

The protocol for electron microscopy of platelets was able to demonstrate ultrastructural morphology of the cells especially with respect to cell membranes and membranes of the canalicular system. The micrographs indicate the ramifying and extensive nature of the platelet canalicular system as well as its degree of morphological variation both in longitudinal and cross-section. Postfixation with osmium tetroxide as well as staining with tannic acid enhanced the clarity of the internal membrane structures, even the thin, finer membranes of the canalicular system. These membranes generally stained with a lower intensity than the plasma membrane and also appeared slightly thinner.

3.4.2 Radioactivity analysis of binding of gold-labelled 3B3

Colloidal gold with an average particle diameter of 10 nm was prepared as described in the Methods. At 100 000 x magnification under the transmission electron microscope, the gold was observed to be in the form of monodisperse, electron-dense particles of uniform diameter (results not shown).

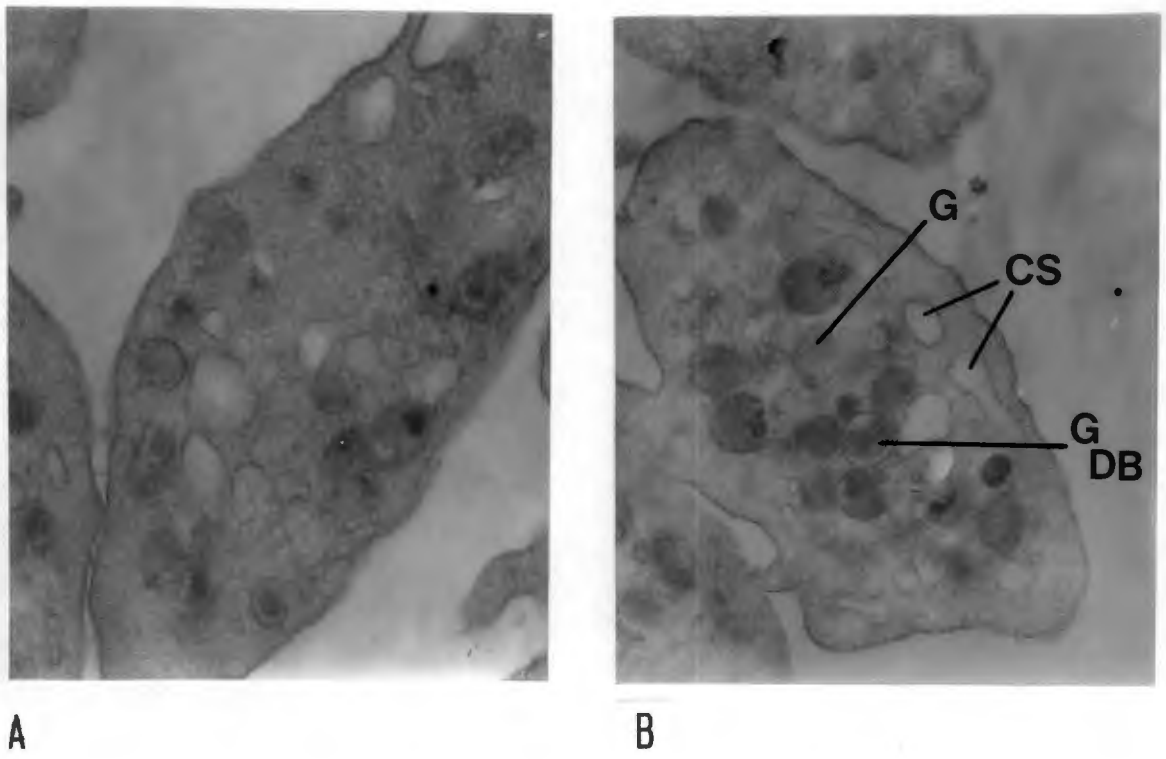


Fig. 17. Electron microscopy (TEM) of platelets for morphological assessment.

Platelets were incubated in HBSS P/B in the presence of 3B3 and prepared for electron microscopy as described in the Methods section. Most of the components of the conventional morphology of platelets can be seen: plasma membrane, membranes of the canalicular system, granules and dense bodies.

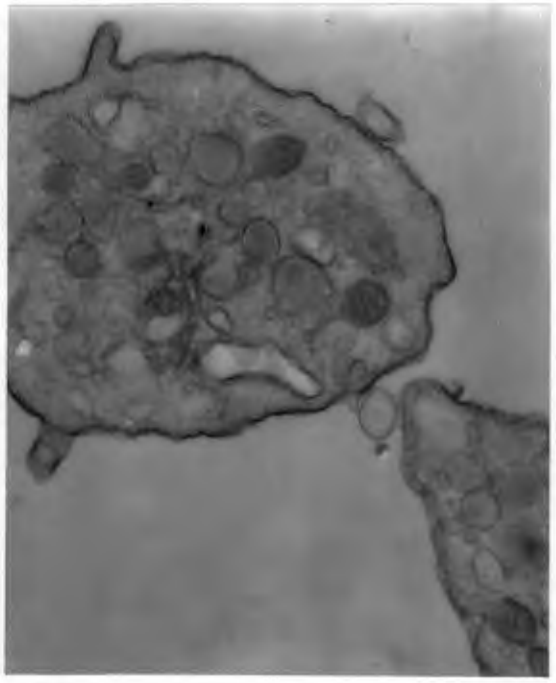
A,B Longitudinal section of resting platelets (G) α-granules, (CS) canalicular system, (G+DB) granule containing dense body. Mag. = 30 000 x. Postfixation with osmium tetroxide.

C Cross or transverse section of a platelet prepared with postfixation in osmium tetroxide to enhance membrane staining. Mag = 25 000 x.

D Cross or transverse section without osmium tetroxide postfixation. Mag = 20 000 x.

E Longitudinal section at high magnification with osmium postfixation showing marked staining of canalicular system membranes. Mag = 50 000 x.

F Longitudinal section at high magnification without osmium postfixation. Mag = 40 000 x.



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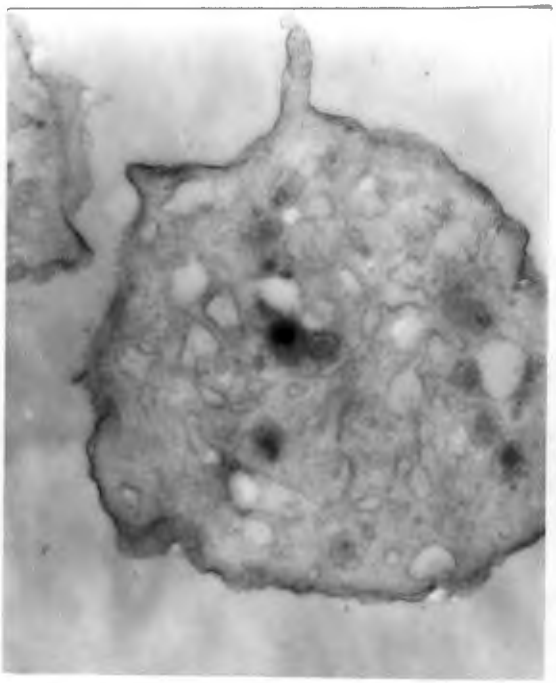

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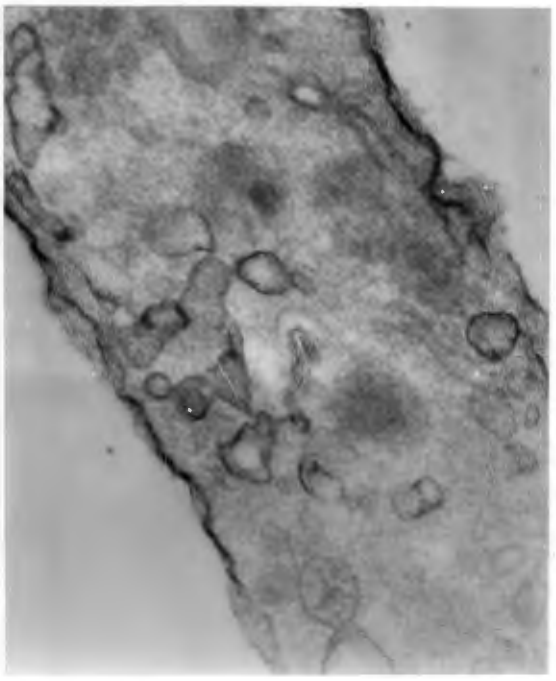

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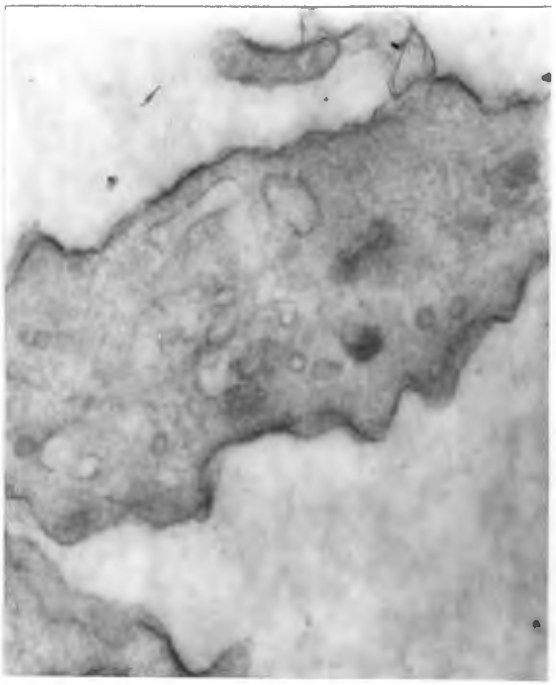


F

Iodination of 3B3 yielded a protein with a specific activity of 34 x 10^3 cpm/µg. After conjugation of 3B3 with colloidal gold, a theoretical specific activity of 4,5 x 10^3 cpm/µg protein was estimated based on the starting concentration of protein (protein determination after conjugation of gold was not performed; actual specific activity was probably somewhat higher).

Figure 18 shows the binding of the 3B3-gold conjugate in relation to the unconjugated 3B3. The conjugate bound to the platelets only slightly less efficiently than the unconjugated antibody.

3.4.3 Localization of the interiorized receptor-antibody complex using a pre-embedding technique

An attempt was made to visualise the gold-conjugated 3B3 bound to the platelets. The cells were labelled as in section 3.4.2 and the binding analysed as in Figure 19. Platelets were then processed for TEM as described in the Methods. The amount of radioactivity in the cell pellet contained in the resin blocks just prior to sectioning was analysed to ascertain if bound antibody was still present after the processing and embedding. This was found to be the case (Figure 19). The block was then sectioned for TEM.

Figure 20 shows micrographs of sectioned platelets stained with 3B3-gold. No gold particles could be seen in any of the micrographs. The pellet was sectioned several times and serial sections were observed to a total thickness of about 1,5 µm,

without visualisation of gold staining. Morphological analysis showed resting platelets with distended canalicular system structures (Figure 20). An opening to part of the canalicular system of one of the platelets can be seen as indicated by the large arrow in Figure 20.

Discussion

The micrographs in Figure 20 demonstrate that no gold was seen. Although this would indicate that binding of 3B3-gold did not occur, the radioactivity present, reflected in Figure 19, showed that the probe was present in the tissue. There are two possible explanations for this discrepancy. Firstly, it may be that the sections observed were from a part of the pellet that contained cells with few bound probes due to the binding being less than was previously thought. This is unlikely because a depth of 1,5 µm was scanned in the serial sections without noting any probes. Also, from the amount of radioactivity present in the tissue, the amount of probe was high. Secondly, it may be that the radioactive 3B3 is binding, but the gold either failed to conjugate properly with the antibody, or simply detached from it during manipulations. This is feasible as conjugation of gold with another monoclonal antibody was proved to be unsuccessful due to gold failing to bind.

3.4.4 Localization of the internalized receptor-antibody complex using a post-embedding technique

The pre-embedding technique employed in section 3.4.3 was unsuccessful in localizing the site of interiorization of the

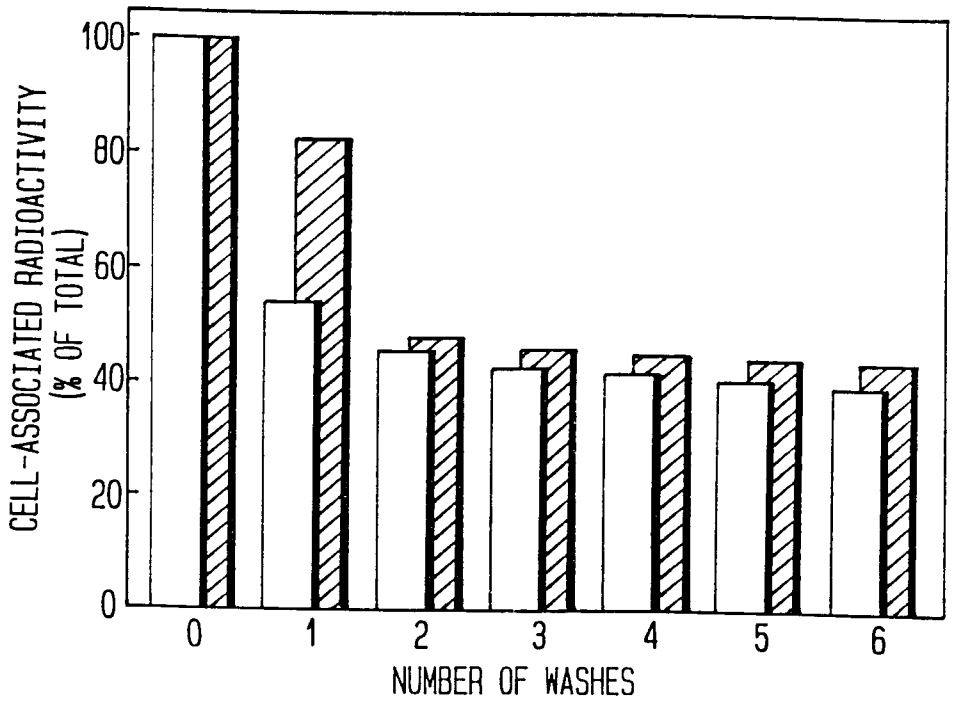


Fig. 18. Binding of 3B3 versus binding of gold-labelled 3B3.

Platelets were incubated at room temperature for 90 minutes with either iodinated 3B3 (hatched bars), or iodinated 3B3 conjugated with 10 nm gold (open bars). Efficiency of washing as well as the amount of cell-associated radioactivity was measured on a gamma counter.

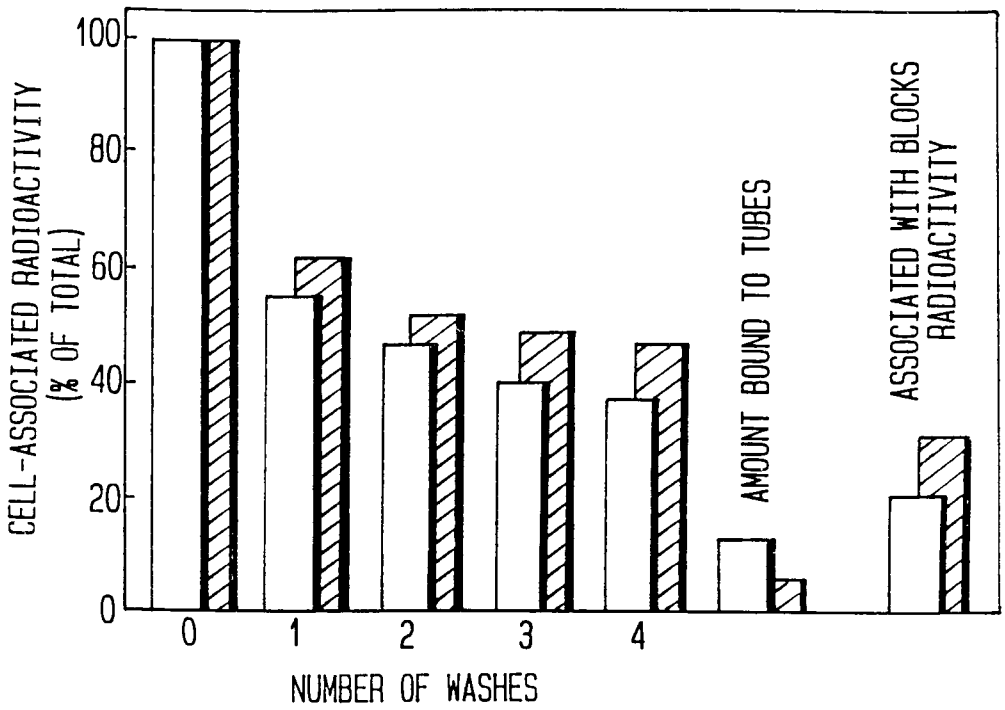


Fig. 19. Binding of 3B3 versus binding of gold-labelled 3B3: processing for electron microscopy.

Platelets were treated as described in Figure 22, those incubated with gold-labelled radioactive 3B3 being processed for TEM as described in Figure 15. Radioactivity associated with the cell pellets of two resin blocks was counted. The block with the largest cell pellet was sectioned for TEM. Hatched bars and open bars refer to iodinated 3B3 and iodinated 3B3-gold, respectively.

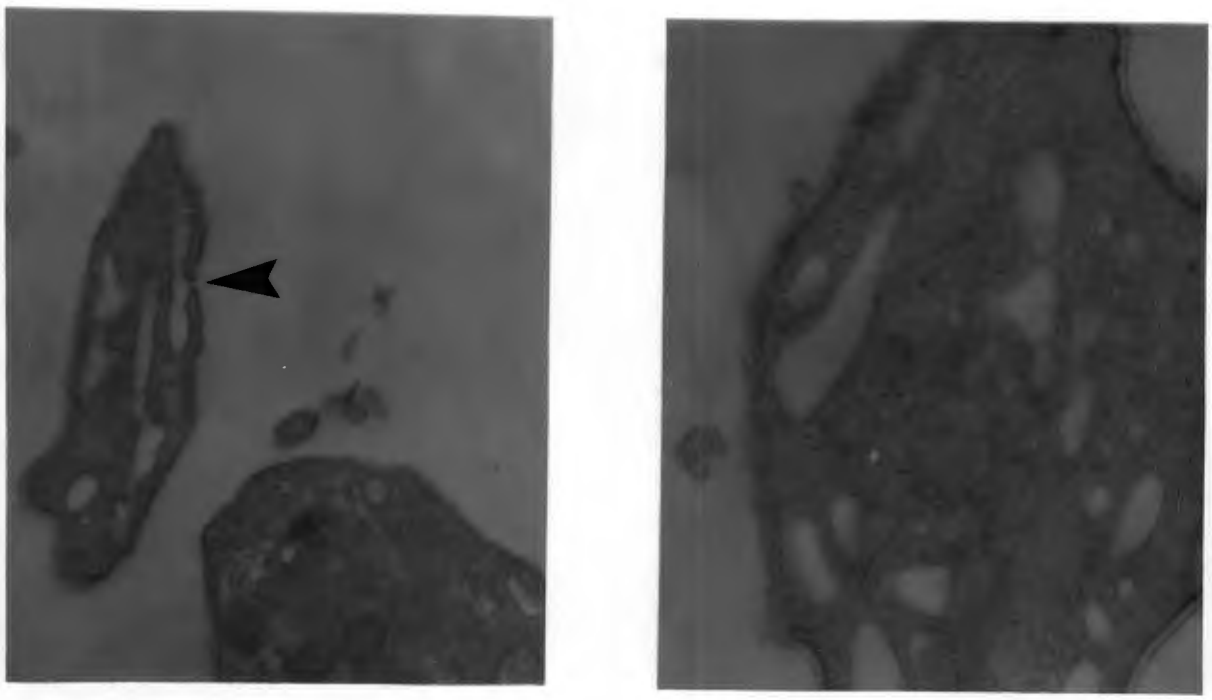


Fig. 20. Pre-embedding of platelets with 3B3-gold.

Platelets were incubated with iodinated 3B3 conjugated with 10 nm gold and processed for TEM as described in the Methods. Cells were then sectioned and viewed in an attempt to detect the gold probe. No probe can be seen in any of the sections viewed despite the radioactivity associated with the tissue, indicating the presence of 3B3. The preparation yielded good ultrastructural detail especially with respect to membranes. Magnification: micrograph on left (30 000x), micrograph on right (50 000x).

receptor-antibody complex. An alternative approach was to use a post-embedding method. The technique used is described in the Methods section. Usually, embedding the tissue in hydrophilic resins (such as LOWICRYL or LR WHITE) or using frozen, ultrathin sections processed on an ultracryomicrotome give the best staining of antigens when using an immunoprobe. These approaches were not feasible to the present study as facilities for cryosectioning were not available, and in any event, often provide poor membrane detail (42,46). Hydrophilic resins were not available for this study.

The post-embedding approach is in many ways preferable to a pre-embedding technique. For instance, for the localisation of intracellular (i.e. not on the cell surface) antigens accessibility of the probe is often a problem, especially when the gold is large (5 nm diameter gold or smaller particles give adequate penetration). Often, the tissue has to be permeabilized with detergent, destroying some membrane structures (60).

3.4.4 (i) Immunoreactivity of the antibodies: dot blot testing: Dot blots were performed as reported in Materials and Methods. Figure 21 shows results after testing the binding of a 5 nm gold-labelled goat anti-rabbit IgG (GAR-5) to a rabbit anti-mouse IgG (RAM). The RAM was used to target the 3B3 on the nitrocellulose. The blot indicated that the antibodies reacted specifically (control showed non-specific binding to be low) in binding 3B3.

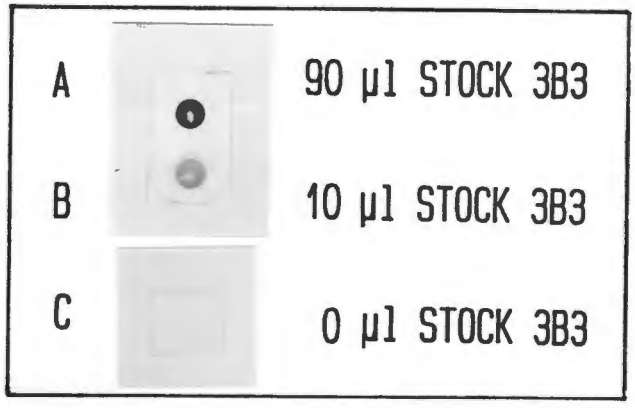


Fig. 21. Dot blot to test the immunoreactivity of a rabbit anti-mouse IgG against 3B3, visualised with 5 nm gold labelled Goat anti-rabbit IgG.

3B3 was applied to a nitrocellulose membrane on a dot-blot apparatus and allowed to dry (A,B). The nitrocellulose was blocked with BSA for 30 minutes at room temperature and stained with rabbit anti-mouse IgG in the presence of 1% normal goat serum. The nitrocellulose was then incubated overnight with a goat anti-rabbit IgG conjugated with 5 nm gold. The gold was enhanced as described in the Methods. A control of platelets without 3B3 was used to show non-specific binding of gold-labelled antibody (C).

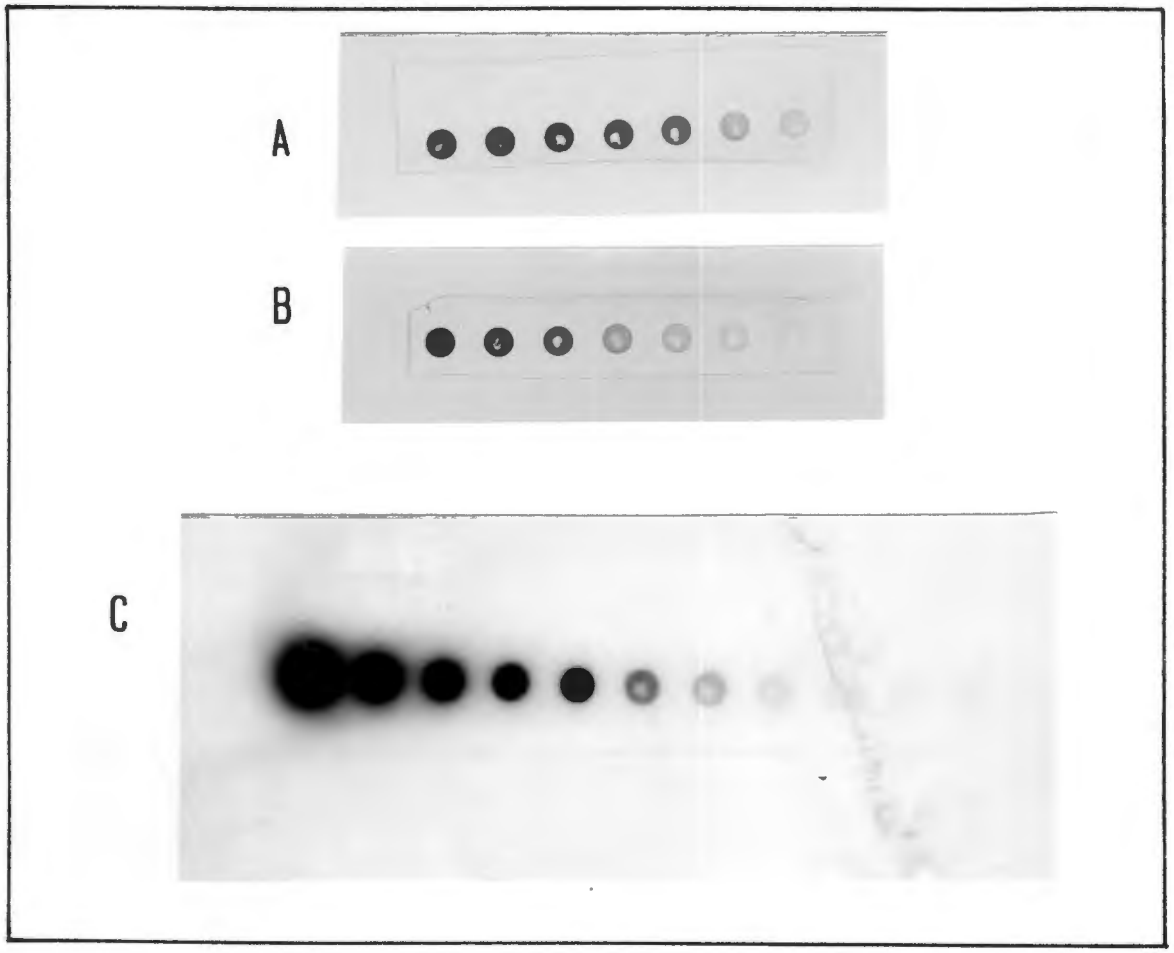


Fig. 22. Dot blot to test immunoreactivity of GAM-10 nm gold to 3B3.

Serial, double dilutions of affinity purified 3B3 (A), or affinity purified 3B3 labelled with radioactive iodine (B), were applied to a nitrocellulose dot blot apparatus and allowed to dry. The nitrocellulose was then blocked with BSA for 30 minutes at room temperature and stained with goat anti-mouse IgG conjugated to 10 nm gold (GAM-10) for 3 hours. The nitrocellulose was washed and developed with a silver enhancement stain in order to visualise the gold. Autoradiography was performed on the blot for 3 hours in order to visualise the radioactivity associated with the antibody (C).

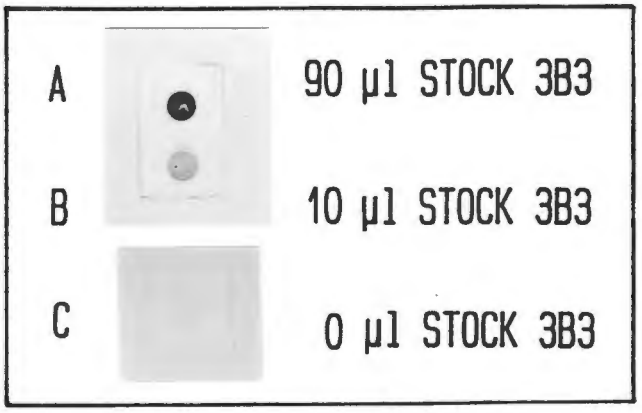


Fig. 23. Dot blot to test immunoreactivity of GAM-10 nm gold to 3B3 bound to whole platelets.

Platelets were labelled with two concentrations of 3B3 at 37°C for 45 minutes and washed. Platelets were then fixed with glutaraldehyde and incubated overnight with a lysine-containing solution to quench unlinked glutaraldehyde. Whole cells were then applied to a dot-blot apparatus and allowed to dry. The nitrocellulose was treated as described in Figure 19. Control platelets had no 3B3 in their incubation mix.

Figure 22 shows the reactivity of the 10 nm gold-labelled goat anti-mouse IgG (GAM-10) to 3B3 bound on the nitrocellulose. Both the 3B3, as well as the iodinated 3B3 were successfully targeted by the probe (A, 3B3; B, iodinated 3B3). (C) shows an autoradiograph of (B) which correlates the amount of gold with the amount of antibody present.

Figure 23 shows the reactivity of GAM-10 bound to whole cells. Binding was shown to be specific as demonstrated by the control.

Discussion

Two systems for post-embedding immunocytochemistry were tested. A system using GAR-5 with RAM to target 3B3 would be preferable as it would give improved accessibility of the RAM to the antigen embedded in the tissue, as compared with an antibody bound to a relatively large gold molecule which may cause steric hindrance effects (60). Also, non-specific binding of the gold-labelled antibody was reduced. The GAM-10 system also proved to be feasible on the blots. 10 nm gold would be more easily visible in the TEM but this may be offset due to binding problems caused by the size of the gold particle.

3.4.4 (ii) On-grid immunomarking of the receptor-antibody complex: Grids with ultrathin sections of platelets embedded in EPON-ARALDITE were treated as described. The sections stained with the RAM, GAR-5 system showed high levels of non-specific binding on the controls (sections of cells with no 3B3).

Lengthening the blocking time and altering the etching procedure could not reduce the non-specific binding (Results not shown).

The sections stained with GAM-10 are depicted in Figure 24. Binding of gold occurred in interior sites of the platelets in compartments morphologically similar to membranes of the canalicular system. The canalicular system appeared distended in many sections (similar to those in Figure 20). Some surface binding also occurred (arrows). The lumen of many of the canalicular system "vacuoles", especially the ones largely distended, contained a loosely packed, granular material that could either have been membrane of the canalicular system sectioned in the plane of its luminal surface, or interiorized material from the medium. The gold probe observed was in the form of single spheres (i.e. no clumping of gold had occurred) all on the luminal side of the membranes. The controls (platelets without 3B3) showed less than one probe bound per cell, with the gold often localized to the cytoplasm or a granule (Results not shown). Contrast of the cells in the sections was poor due to the bleaching effect of the etching agent, H_2O_2, on the osmium tetroxide and tannic acid stains. Apparent uniform staining of the extracellular environment was probably attributable to the lead citrate and uranyl acetate staining the etched resin.

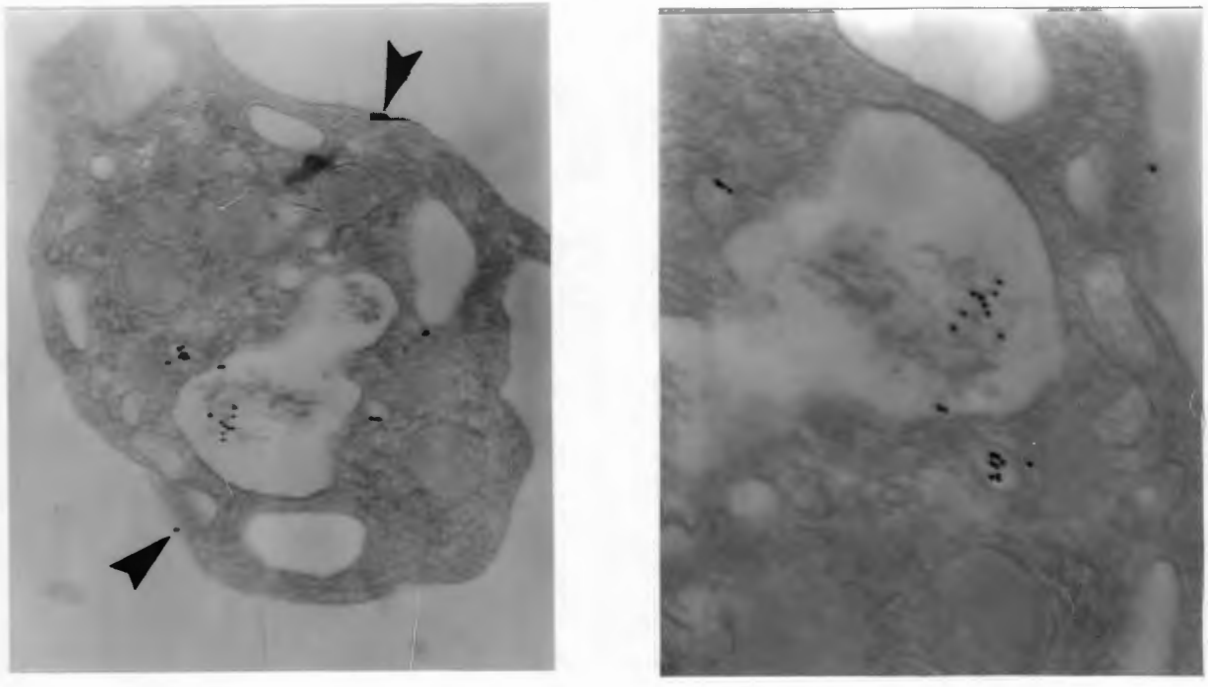
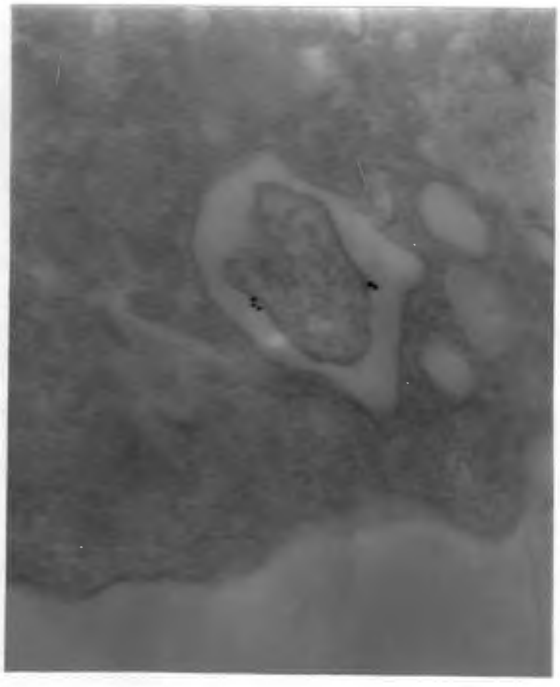


Fig. 24. Post-embedding immunocytochemistry.

Blocks containing platelets incubated in the presence and absence of 3B3, were sectioned onto nickel grids. Sections were etched with hydrogen peroxide and washed in Tris. Sections were then blocked with normal goat serum and stained with a polyclonal goat anti-mouse antibody conjugated with 10 nm gold. After washing with Tris, the sections were fixed with glutaraldehyde and washed with distilled water. Contrasting with uranyl acetate and lead citrate followed. Platelets processed for results shown in Figure 23 were used as 3B3-positive samples. B is an enlarged portion of A. Magnification: A (40 000x), B (80 000x), C (60 000x), D (40 000x), E (60 000x), F (100 000x). Arrows indicate surface binding (cf. Results).



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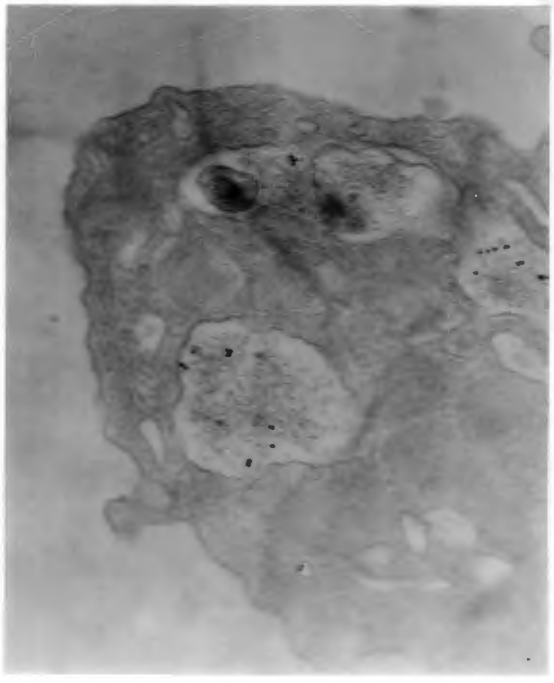

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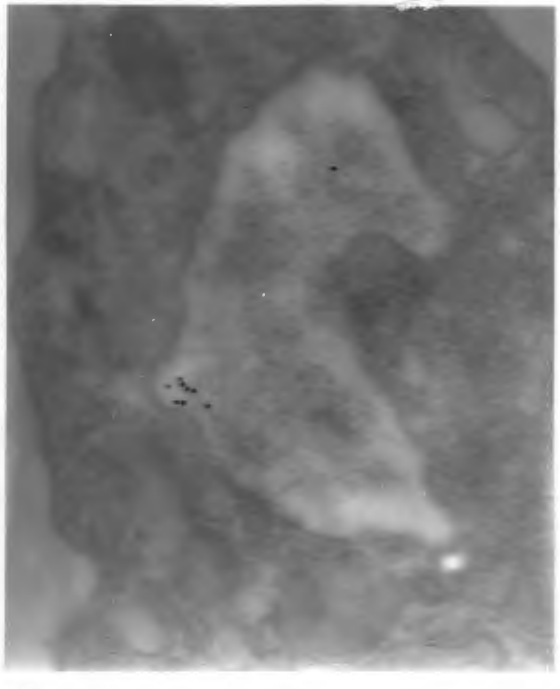
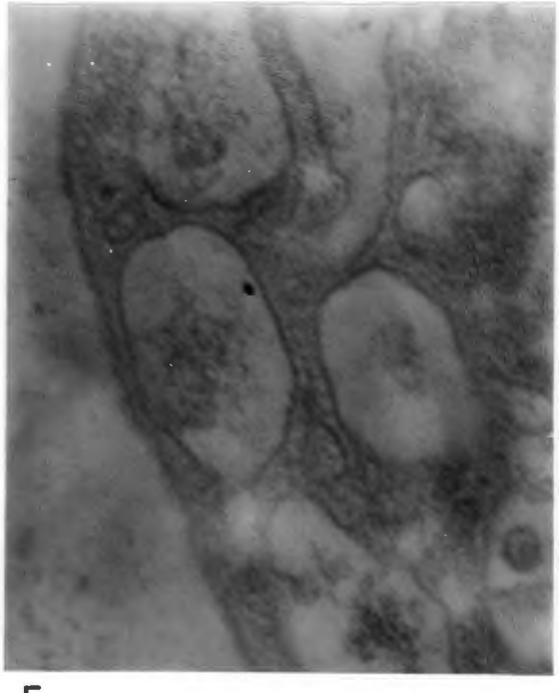


E



F

Discussion

Post-embedding immunocytochemistry of platelets incubated with 3B3, using GAM-10 as a probe, was able to detect interiorized antibody in structures that clearly resemble the canalicular system of platelets. These structures can easily be distinguished from granules that were stained in a darker, more uniform way. The binding of the probe was specific to cells incubated with 3B3. The probe was also exclusively found on the luminal aspect of the canalicular structures with no intracellular binding. The staining properties of all the structures in which probe was observed was consistent with that of the canalicular system. Very little probe was observed on the surface of the platelets.

3.5 FEASIBILITY OF SURFACE MEMBRANE BIOTINYLATION AS A MARKER TO STUDY MEMBRANE INTERNALIZATION

In order to reinforce the results obtained using the exogalactosylation technique, the feasibility of a biotinylation approach to membrane internalization was investigated. Biotinylation of membrane proteins with NHS-SS-Biotin would provide another covalent labelling technique.

The biotin derivative NHS-SS-Biotin is membrane impermeable and reacts with primary amines. Biotinylated protein could be detected by binding iodinated streptavidin. The avidin-biotin interaction is the strongest known noncovalent biological recognition ($Ka = 10^{15}\ M^{-1}$) between protein and ligand. Streptavidin was more suitable for iodination as it contains 6

tyrosines per subunit in contrast to avidin (1 tyrosine/subunit) (67). Streptavidin is a 60 kD protein consisting of four identical subunits of 15 kD. Each subunit contains a single biotin binding site. Iodinated streptavidin had a specific activity of $0,52 \times 10^5$ cpm/µg.

A reducing agent such as dithioerythritol (DTE) at a concentration above 0,37 µM (63) would then be used to remove accessible avidin-biotin by reduction of the disulphide bond in the spacer arm of the biotin analog. The amount of radioactivity remaining cell-associated would provide the basis for the internalization assay.

Figure 25 shows that label could not be removed by treatment with DTE as the amount of radioactivity released after DTE treatment was the same as after washing in the absence of DTE (approximately 0,9%). When mouse macrophages of the $P388D_1$ strain were used in an identical experiment, neither DTE nor 10 mM ß-mercaptoethanol were able to remove more label than was washed off under non-reducing conditions.

Discussion

The biotinylation technique cannot be employed to measure membrane internalization. Agents that should have reduced the disulphide linkage did not do so. Radioactivity from the streptavidin bound to biotin was clearly discernible from that bound non-specifically. It is unclear as to why the reducing agents failed to release the avidin-biotin.

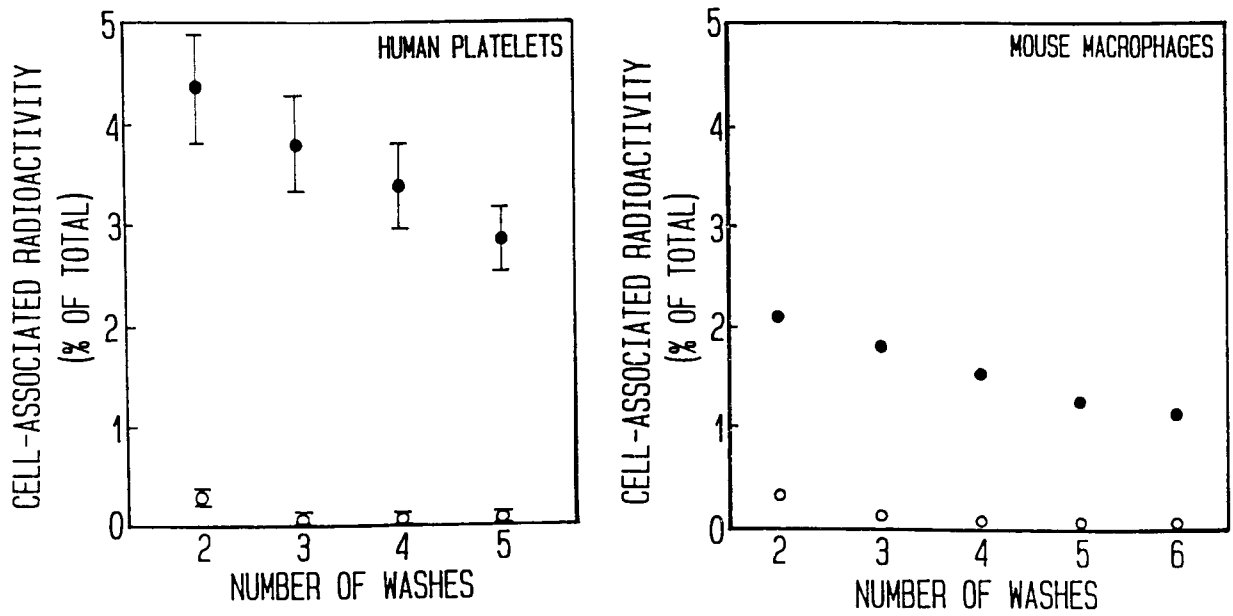


Fig. 25. Biotinylation of cell-surface membranes using NHS-SS-biotin.

Human platelets or mouse macrophages were incubated in the presence (●) and absence (o) of NHS-SS-Biotin, for 1 hour on ice. After washing, streptavidin labelled with radioactive iodine was added for 30 minutes on ice. Cells were then washed and the amount of radioactivity remaining was measured at each step. After 4 washes, dithioerythritol (DTE) was added for 15 minutes on ice, to remove accessible label. Wash number 5 indicates radioactivity remaining after treatment with DTE. With platelets, error bars indicate the variation between 2 samples. With macrophages, after the 5th wash, 10 mM ß-mercaptoethanol was added.

CHAPTER 4

CONCLUSION

There have been many studies involving platelets as endocytic and exocytic elements. It is apparent from morphometric studies that the platelet represents a vast area of membrane capable of interaction with elements in the surrounding plasma, especially if the membranes of the canalicular system are taken into account (4,32). A variety of ligands and particles have been used to study endocytic and pinocytic activity in platelets (9,11,12,19,36,38). Most of these workers indicate that platelets act in some way to take up particulate matter from surrounding fluid. More recent studies have involved uptake of specific plasma proteins by platelets and megakaryocytes (32,39,40,68) into intracellular sites. Coated pits and vesicles with an appearance different to clathrin-coated membrane have been demonstrated in mammalian platelets (41). These have been implicated in the traffic of endocytosed proteins to granules. The ability of cells to acquire such proteins into secretory granules rarely has been reported (32).

A recent review (32) has proposed a detailed and complex set of processes for the trafficking of endocytosed particulate and fluid-phase material within megakaryocytes and platelets. The role of the fibrinogen receptor, the GPIIbIIIa has long been understood to play a fundamental role with these processes (16,17,20,23,39,42).

Fibrinogen has been shown not to be synthesized by megakaryocytes. Its presence within secretory granules of platelets has led to the proposal that uptake occurred by endocytosis (39). The importance of the canalicular system has been discussed in this respect. It was demonstrated that fibrinogen-gold particles were cleared to the canalicular system, in a process independent of platelet activation (8).

Platelet endocytosis has been studied using monoclonal and polyclonal antibodies to some membrane receptors. Monoclonals against GPIIbIIIa are recognised by the receptor as a ligand, and the receptor reacts by undergoing redistribution to an intracellular compartment. The effect is specific to this receptor, as monoclonals to another glycoprotein, GPIb, do not cause any redistribution of the receptor with its "ligand". The mechanism proposed to account for this phenomenon is endocytosis (24,42).

The present study attempted to detect endocytic activity using covalent membrane markers and immunocytochemistry. A monoclonal antibody against the GPIIbIIIa, designated 3B3 (45), was used to detect the intracellular compartment to which the receptor was redistributed. Similar to a previous study (42), the 3B3 was shown to be internalized at 37°C to an intracellular compartment. Using a post-embedding immunocytochemical technique, the receptor-3B3 complex was demonstrated to be internalized to an intracellular, membrane-bound compartment. However, the study failed to demonstrate

any uptake of labelled membrane concomitant with uptake of receptor-3B3.

No membrane or fluid-phase internalization of a constitutive or basal nature could be observed in this study. The minute amount of fluid-phase uptake (Figure 10) was negligible when compared to pinocytic uptake measured in other cell types (cf. Table II).

A model, proposed to deal with this apparent contradiction, is similar to that proposed by Wencel-Drake (42). This model is summarized in Figure 14. The present findings were in support of model B: GPIIbIIIa-3B3 complexes are cleared from the platelet surface and sequestered in the canalicular system. That all the labelled membrane could be removed, supports sequestration into the canalicular system, rather than endocytosis, which would render the internalized label inaccessible to enzymatic removal. The principal finding in support of endocytosis as proposed by Wencel-Drake (42) is that immunofluorescent localization of the anti-GPIIbIIIa antibody was only possible when the membranes were permeabilised with the use of Triton. These findings can be accounted for by considering that fixation with paraformaldehyde probably resulted in closure of the channels at their point of access with the plasma membrane, by some kind of cross-linking effect. Thus, although the 3B3 was redistributing to the interior of the cell, the membranes that accompanied it were still accessible to the cell medium. The term "interiorization" is thus preferable to "internalization", the latter referring to

true endocytosis. Also, no difference in the labelling profile of membrane components could be demonstrated with cells labelled at 37°C (Figure 7), which is against a process of true endocytosis occurring. The observed redistribution of antibody-labelled GPIIbIIIa (cf. Figure 13) can also be explained in terms of uptake into the canalicular system.

The interior sites to which interiorized 3B3-receptor complexes were shown to redistribute are morphologically identical to structures of the canalicular system. It is thus proposed that the receptor-antibody complex undergoes a process of sequestration into the open canalicular system and not true endocytosis as was suggested by other authors. These findings are thus similar to studies on surface-activated platelets demonstrating sequestration of latex particles and fibrinogen-gold complexes in the canalicular system (9,23).

The present study did not attempt to measure interactions of platelets with fibrinogen. Other authors who propose that platelets acquire granule fibrinogen (and other plasma proteins) by endocytosis (32,39,41,68) have demonstrated this to occur at the stage of megakaryocytes only. It is very likely that the megakaryocyte possesses all the machinery for true endocytosis. It can also be accepted that these cells actively employ endocytic mechanisms to acquire plasma proteins they cannot synthesize. These proteins may subsequently be found in the secretory granules of platelets. It is more likely, therefore, that the granule proteins (as with other

platelet constituents) are inherited by the platelets when they are released into the circulation.

The results of the present study indicate strongly that in human platelets the fate of GPIIbIIIa, labelled with 3B3, appears to be sequestration into the canalicular system. It is possible that other mechanisms exist for dealing with different types of particulate matter. Platelets in the resting state may respond to and deal with different ligands and particles in a separate way to platelets activated or bound to surfaces. The evidence presented in this study should be considered when attempting to understand the role of the platelet as an endocytic cell.

ABBREVIATIONS

ß-Gal	ß-galactosidase
BSA	Bovine serum albumin
CCD	Citrate-citric acid-dextrose
cf	refer to
cpm	counts per minute
D	Daltons
DDSA	Dodecenylsuccinic anhydride
DPM	disintegrations per minute
DTE	Dithioerythritol
EDTA	Ethylenediaminetetraacetic acid
EM	electron microscope
$F(ab')^2$	Antibody binding fragments of IgG
MDCK	Madin-Darby canine kidney
M_r	molecular weight
mRNA	messenger ribonucleic acid
NHS	N-hydroxysuccinimide
ONPG	O-Nitrophenyl-ß-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
P/B	containing prostaglandin E_1 at 1 µg/ml and BSA at 0,1%
PBS	phosphate-buffered saline pH 7,4
PGE_1	Prostaglandin E_1
PRP	Platelet-rich plasma
RAM	Rabbit anti-mouse IgG
s	standard deviation (%)
SDS	Sodium dodecyl sulphate
TEM	Transmission electron microscope

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