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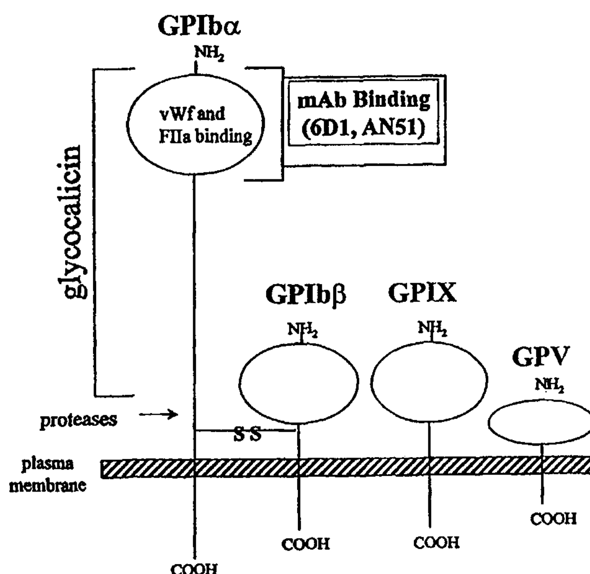

(57) Abstract

The present invention concerns methods for determining the number of platelets in a sample. A liquid medium comprising the sample such as a whole blood sample and a matrix such as particles with which is associated a binding molecule such as an antibody for a platelet cell surface glycoprotein receptor is subjected to agglutination conditions. The agglutination of the matrix is measured as an indication of the number of receptors present in the medium. The measurement above is then related to the number of platelets in the sample. Also disclosed are kits for carrying out an enumeration of platelets in a sample.

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METHOD FOR DETERMINING PLATELET COUNT

BACKGROUND OF THE INVENTION

1. Field of the Invention

5 This invention relates to the field of diagnostic assays and in particular to the determination of the number of platelets in a sample.

The role of platelets in mammalian physiology is extraordinarily diverse, but their primary role is in promoting hemostasis. In many situations, an evaluation of the ability of blood to clot is desired, a parameter that is frequently controlled by the ability of 10 platelets to adhere and/or aggregate. Thus, one may wish to assess the adhesive functions of platelets. For example, one may wish to know whether to administer drugs that will block, or promote, clot formation, or one may need to detect deficiencies in platelet function prior to surgical procedures. In other instances one may be interested in evaluating the effectiveness of a platelet inhibitor that is being tested as a new drug or is 15 being used as approved clinical treatment in a patient.

Platelets are known to aggregate under a variety of conditions and in the presence of a number of different reagents. Platelet aggregation is a term used to describe the binding of platelets to one another. The phenomenon can be induced by adding aggregation-inducing agents to platelet-rich plasma (PRP) or to whole blood. Platelet 20 aggregation *in vitro* depends upon the ability of platelets to bind fibrinogen to their surfaces after activation by an aggregation-inducing agent such as ADP or collagen.

Platelets play a critical role in the maintenance of normal hemostasis. When exposed to a damaged blood vessel, platelets will adhere to exposed sub-endothelial matrix. Following the initial adhesion, various factors released at the site of injury such 25 as thrombin, ADP and collagen activate the platelets. Once platelets are activated, a conformational change occurs in the platelet glycoprotein GPIIb/IIIa receptor allowing it to bind fibrinogen and/or von Willebrand factor.

It is this binding of the multivalent fibrinogen and/or von Willebrand factor molecules by GPIIb/IIIa receptors on adjacent platelets that results in the recruitment of 30 additional platelets to the site of injury and their aggregation to form a hemostatic plug or thrombus.

In vitro platelet aggregation is the laboratory method used to assess the *in vivo* ability of platelets to form the aggregates leading to a primary hemostatic plug. In this technique an aggregating agent such as ADP or collagen is added to whole blood or PRP and aggregation of platelets monitored. Platelet aggregometry is a diagnostic tool that
5 can provide insights difficult to obtain by other techniques, thus aiding in patient diagnosis and selection of therapy. These methods of monitoring platelet aggregation require expensive, laboratory dedicated instruments that are not easily portable and require standardization to ensure accurate quantitative results. In addition, unless performed using whole blood, results are unlikely to be available for several hours.
10 Currently there are two detection methods used in instruments with FDA clearance for performing platelet aggregometry: optical and impedance measurements.

The CHRONO-LOG Model 530 and Model 540 use the optical method for PRP and the impedance method for whole blood aggregometry. The impedance method has been shown to be substantially equivalent to the optical method for measuring platelet
15 aggregation in PRP.

A rapid platelet function assay has recently been developed and is described in U.S. Patent No. 5,763,199 (Coller). The assay determines glycoprotein IIb/IIIa receptor blockade in whole blood. Agglutination of small polymeric beads coated with a glycoprotein IIb/IIIa ligand such as fibrinogen results when the beads are contacted with
20 whole blood containing platelets with glycoprotein IIb/IIIa receptors that are not blocked. Failure to agglutinate indicates that blockade of the GPIIb/IIIa receptors has been achieved. In a preferred embodiment, the addition of a thrombin receptor activator results in an assay that is rapid and convenient enough to be performed at bedside and that results in agglutination of the small polymeric beads within a convenient, known
25 period of time if the glycoprotein IIb/IIIa receptors are not blocked. The assay includes the ability to transfer blood to be tested from a collection container to an assay device without opening the collection container. This platelet aggregation assay can be conducted at the same time as the activated clotting time (ACT), which is performed to assess the adequacy of heparinization. During chronic infusions of GPIIb/IIIa
30 antagonists, or with chronic oral therapy, periodic monitoring may also be desirable. In certain circumstances, as for example, prior to surgery or an invasive procedure, it may

be desirable to rapidly determine whether the effect of the GPIIb/IIIa antagonist has worn off sufficiently to allow the surgery or procedure to be performed without further interventions to reverse the effect of the GPIIb/IIIa inhibitor. Finally, in the event of bleeding complications, a rapid measure of platelet function may be helpful in determining whether the bleeding is due to a high or toxic level of platelet inhibition. The level of platelet inhibition may also be helpful in guiding whether to reverse the drug effect with platelet transfusions or look for other causes of bleeding.

In the performance of the above assay it is desirable to know whether a low result in the platelet function assay is due to low platelet function activity or simply to a low number of platelets. There are a number of approaches currently used to assess platelet number. For example, the number of platelets in a plasma sample may be determined by electornic particle enumeration using, for example, an instrument manufactured by Coulter Electronics (Hialeah, Florida). In another approach a whole blood analyzer such as, for example, an instrument manufactured by Sysmex Corporation of America (Long Grove, IL), is used. This instrument measures all cells in the whole blood.

In using the platelet function assay mentioned above, it is desirable to carry out both the platelet function assay and a platelet count measurement in the same instrument. This would avoid time delays in using the above instruments for platelet count measurement, which typically may not be present at the site of the platelet function assay, for example, a patient's bedside.

GPIbα is a component of the GPIb/IX-V complex that is involved in platelet adhesion to exposed subendothelium via interaction with von Willebrand factor (vWf). The GPIb/IX-V complex also serves as a high affinity thrombin (FIIa) receptor and is usually absent on platelets from patients having Bernard-Soulier syndrome. GPIb/IX is a member of the leucine-rich family of glycoprotein receptors and is highly negatively charged. GPIb is composed of GPIbα bonded by a disulfide linkage to GPIbβ. GPIb exists in a non-covalent 1:1 complex with GPIX. In addition, GPIb/IX forms a 2:1stoichiometric non-covalent complex with GPV. Evidence also suggests that GPIb/IX-V is in close proximity to and may associate with the platelet IgG receptor FcγRII.

GPIbα is a rod-like structure extending about 60 nm from the platelet surface and it is this long extension that likely facilitates interaction with the subendothelium. By comparison, the GPIIb/IIIa receptor extends only about 10 to 20 nm from the platelet surface. The extracellular portion of GPIbα is cleaved from the platelet surface by a
5 variety of proteases releasing a soluble fragment, glycocalicin, which retains the vWf and FIIa binding sites. A schematic of the GPIb-IX-V complex is depicted in Fig. 1.

2. Previous Disclosures

10 Tanaka, *et al.*, discusses a flow cytometric platelet enumeration utilizing monoclonal antibody CD42a in Clin. Lab. Haematol. (1996) 18:265-269.

Coller, *et al.*, discuss studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor in Blood (1983) 61:99-
15 110.

Ruan, *et al.*, disclose a monoclonal antibody to human platelet glycoprotein I in Br. J. Haematol. (1981) 49:501-509 and 511-519.

Michelson discusses flow cytometry: a clinical test of platelet function in Blood (1996) 87:4925-4936.

20 Fox, *et al.*, disclose a structure for the glycoprotein Ib-IX complex from platelet membranes in J. Biol. Chem. (1988) 263:4882-4890.

U.S. Patent No. 4,948,961 discloses hybridomas and monoclonal antibodies that specifically bind to GPIb on platelets and inhibit the binding of thrombin to platelets.

Ward, *et al.*, discuss mocarhagin, a novel cobra venom metalloproteinase that
25 cleaves the platelet von Willebrand factor receptor glycoprotein Ibα and the identification of the sulfated tyrosine/anionic sequence Tyr-276-Glu-282 of glycoprotein Ibα as a binding site for von Willebrand factor, α-thrombin, and several anti GPIbα monoclonal antibodies in Biochemistry (1996) 35:4929-4938.

Alboaggregins A and B, structure and interaction with human platelets is
30 discussed by Kowalski, *et al.*, in Thromb Haemost (1998) 79:609-612.

SUMMARY OF THE INVENTION

One aspect of the invention concerns a method for determining the number of platelets in a sample. A liquid medium comprising the sample and a matrix with which is associated a molecule that binds to a platelet cell surface glycoprotein receptor is subjected to agglutination conditions. The agglutination of the matrix is measured as an indication of the number of platelet-associated receptors present in the medium. The measurement above is then related to the number of platelets in the sample.

Another aspect of the present invention is a method for determining the number of platelets in a whole blood sample. A liquid medium is prepared comprising the sample and particles to which is bound an antibody to a GPIbα subunit of the GPIb/lx-v receptor. The liquid medium is subjected to agglutination conditions. The agglutination of the particles is measured as an indication of the number of platelet-associated receptors present in the medium and the measurement is related to the number of platelets in the sample.

Another aspect of the present invention is a kit for determining the number of platelets in a sample. The kit comprises in packaged combination (a) particles to which is bound an antibody to a GPIbα subunit of the GPIb/lx-v receptor and (b) a buffered medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic of the GPIb/IX-V complex.

Fig. 2A and 2B show the rate of agglutination of platelet rich plasma with covalent 6D1-microparticles.

Fig. 3A and 3B show the rate of agglutination of platelet rich plasma with anti-GPIbα mAbs coated indirectly on anti-mouse IgG microparticles.

Fig. 4 shows the kinetics of agglutination of platelets in whole blood with 6D1 microparticles.

Fig. 5 illustrates that immobilized 6D1-particles enumerate the platelets count in whole blood

Fig. 6 shows the agglutination of PRP by immobilized non-GPIb antibodies.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for enumeration of platelets in a whole blood sample. The method may be employed in conjunction with an assay for platelet function such as the rapid platelet function assay of U.S. Patent No. 5,763,199.

5 Before proceeding further with a detailed description of the present invention, a number of terms as used herein are defined.

Sample -- any solution, synthetic or natural, containing platelets, including body fluids such as, for example, whole blood, platelet-containing blood fractions such as platelet-rich plasma, and the like. The amount of the sample depends on the nature of the 10 sample. For fluid samples such as whole blood, the amount of the sample is usually about 30 μl to 300 μl, more usually, about 160μl. The term "sample" includes unprocessed samples directly from a patient or samples that have been pretreated and prepared in any convenient liquid medium although an aqueous medium is preferred. Preprocessing may include, for example, treatment with an anticoagulant. Preferably, the 15 sample is untreated whole blood or whole blood treated with an anticoagulant.

Blood can be drawn from an individual being assessed for enumeration of platelets by any number of known techniques. Preferably, the blood is drawn into a closed vessel such as a Vacutainer® tube to protect the individual who is drawing the blood from blood-borne infectious agents such as hepatitis B virus or the HIV virus. If 20 the blood is to be mixed with an anticoagulant, the blood may be drawn into a vessel containing anticoagulant or by adding anticoagulant to drawn blood. A sufficient amount of the anticoagulant is used to prevent coagulation of the whole blood as is well-known in the art. Suitable anticoagulants include EDTA, citrate, oxalate, heparin, hirudin or other antithrombin agents.

25 Platelet cell surface glycoprotein receptor – a carbohydrate-rich protein receptor on the surface of a platelet cell. The receptor usually contains at least about 5 to about 10 % by weight of carbohydrate, and may contain up to about 70 % or more of carbohydrate. Such receptors may be, for example, GPIbα, (about 60 % carbohydrate), GPIIb/IIIa (about 15% carbohydrate) and the like.

Binding molecule for a platelet cell surface glycoprotein receptor – a molecule that causes the aggregation of the receptor. The binding molecule may be a specific binding molecule or a non-specific binding molecule.

Specific binding -- the specific recognition of one of two different molecules for the other compared to substantially less recognition of other molecules.

Specific binding molecule -- one of two different molecules having an area on the surface or in a cavity that specifically binds to, and is thereby defined as, complementary with a particular spatial and polar organization of the other molecule. The specific binding molecule may be a ligand for the platelet cell surface glycoprotein receptor such as von Willebrand factor, thrombin, alboaggregins (reviewed in Kowalski, *et al.*, *supra*, and so forth. The specific binding molecule may be an antibody for the platelet cell surface glycoprotein receptor. In the case of the GPIbα receptor, such antibodies include but are not limited to AN51, sz2, AK2, VMI6d, 6D1, HPL11, 4H12, TM-60, LJ1610, ES85, C34, AP1, MB45 and the like.

Non-specific binding molecule – a molecule that is not a specific binding molecule but nonetheless binds to, or causes the aggregation of, the platelet cell surface glycoprotein receptor. Examples of such non-specific binding molecules include, by way of illustration and not limitation, wheat germ agglutinin, concanavalin A, poly-L-lysine, and the like. A particular requirement for the binding molecule is that it be substantially free from binding to molecules, which might be present in a sample, other than the platelet cell surface glycoprotein receptor.

Ligand – a molecule that is specifically recognized by and binds to another molecule.

Associated with – a molecule may be associated with a matrix such as a particle by being non-specifically bound or specifically bound to the matrix, by being physically adsorbed on the surface of the matrix or by being dissolved in the matrix. Non-specific binding of a molecule to a matrix may be achieved by covalently bonding or attaching the molecule to the matrix.

Matrix -- a support comprised of an organic or inorganic, solid or fluid, water insoluble material, which may be transparent or partially transparent. The matrix can have any of a number of shapes, such as particle, including bead, film, membrane, tube,

well, strip, rod, and the like. The surface of the matrix is, preferably, hydrophilic or capable of being rendered hydrophilic. The body of the matrix is, preferably, hydrophobic. The matrix may be suspendable in the medium in which it is employed. Examples of suspendable matrices in accordance with the present invention, by way of illustration and not limitation, are polymeric materials such as latex, lipid bilayers, oil droplets, cells and hydrogels. Other matrix compositions include polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), polysaccharides such as dextrans and modified dextrans, etc.; either used by themselves or in conjunction with other materials.

Binding of molecules to the matrix may be direct or indirect, covalent or non-covalent and can be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970).

The surface of the matrix may be polyfunctional or be capable of being polyfunctionalized or be capable of binding to a molecule, or the like, through covalent or specific or non-specific non-covalent interactions. Such binding is indirect where non-covalent interactions are used and is direct where covalent interactions are employed. A wide variety of functional groups are available or can be incorporated. Functional groups include carboxylic acids, aldehydes, amino groups, cyano groups, ethylene groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to surfaces is well known and is amply illustrated in the literature (see above). The length of a linking group to the molecule may vary widely, depending upon the nature of the molecule, the effect of the distance between the molecule and the surface on the specific binding properties and the like.

In general, the amount of the binding molecule employed is dependent on the size, affinity and structural characteristics of the molecule, and so forth and is usually best determined empirically. Usually, the molecule is present in from about 1 to about 100, more usually about 20 to about 100, frequently from about 30 to about 100, and in

some cases preferably close to about 90 to about 100 mole percent of the molecules present on the surface of the matrix.

Particles -- particles of at least about 0.1 microns and not more than about 10 microns, usually at least about 1 micron and less than about 6 microns. The particles can be virtually any shape, but are generally spherical with uniform diameters. The particle may have any density, but preferably of a density approximating water, generally from about 0.7 to about 1.5g/ml. The particles may or may not have a charge on the surface, either positive or negative, preferably negative. The particles may be solid (e.g., comprised of organic and inorganic polymers or latex), oil droplets (e.g., hydrocarbon, fluorocarbon, silicon fluid), or vesicles (e.g., synthetic such as phospholipid or natural such as cells and organelles).

The solid particles are normally polymers, either addition or condensation polymers, which are readily dispersible in the liquid medium. The solid particles will also be adsorptive or functionalizable so as to bind or attach at their surface, either directly or indirectly, a molecule for binding to the platelet cell surface glycoprotein receptor.

The solid particles can be comprised of polystyrene, polyacrylamide, homopolymers and copolymers of derivatives of acrylate and methacrylate, particularly esters and amides, silicones and the like.

Oil droplets -- are water-immiscible fluid particles comprised of a lipophilic compound coated and stabilized with an emulsifier that is an amphiphilic molecule such as, for example, phospholipids, sphingomyelin, albumin and the like that exist as a suspension in an aqueous solution, i.e. an emulsion.

The phospholipids are based upon aliphatic carboxylic acid esters of aliphatic polyols, where at least one hydroxylic group is substituted with a carboxylic acid ester of from about 8 to 36, more usually of from about 10 to 20 carbon atoms, which may have from 0 to 3, more usually from 0 to 1 site of ethylenic unsaturation and at least 1, normally only 1, hydroxyl group substituted with phosphate to form a phosphate ester. The phosphate group may be further substituted with small aliphatic compounds that are difunctional or of higher functionality, generally having hydroxyl or amino groups.

Emulsions comprising oil droplets can be made in accordance with conventional procedures by combining the appropriate lipophilic compounds with a surfactant, anionic, cationic, zwiterionic or nonionic, where the surfactant is present in from about 0.1 to 5, more usually from about 0.1 to 2 weight percent of the mixture and subjecting the mixture in an aqueous medium to agitation, such as sonication or vortexing. Illustrative lipophilic compounds include hydrocarbon oils, halocarbons including fluorocarbons, alkyl phthalates, trialkyl phosphates, triglycerides, etc.

The oil droplets can comprise a fluorocarbon oil or a silicone oil (silicon particle). Such droplets are described by Giaever in U.S. Patents Nos. 4,634,681 and 4,619,904 (the disclosures of which are incorporated herein in their entirety).

Molecules can be bound to the droplets in a number of ways such as, for example, as described by Giaever, *supra*.

Liposomes -- microvesicles comprised of one or more lipid bilayers having approximately spherical shape and one of the preferred materials for use in the present invention. The outer shell of a liposome consists of an amphiphilic bilayer that encloses a volume of water or an aqueous solution. Liposomes with more than one bilayer are referred to as multilamellar vesicles. Liposomes with only one bilayer are called unilamellar vesicles. The amphiphilic bilayer is frequently comprised of phospholipids. Phospholipids employed in preparing particles utilizable in the present invention can be any phospholipid or phospholipid mixture found in natural membranes including lecithin, or synthetic glyceryl phosphate diesters of saturated or unsaturated 12-carbon or 24-carbon linear fatty acids wherein the phosphate can be present as a monoester, or as an ester of a polar alcohol such as ethanolamine, choline, inositol, serine, glycerol and the like. Particularly preferred phospholipids include L-a-palmitoyl oleoyl-phosphatidylcholine (POPC), palmitoyl oleoylphosphatidyl-glycerol (POPG), L-α-dioleoylphosphatidylglycerol, L-α (dioleoyl)-phosphatidyl ethanolamine (DOPE) and L-α-(dioleoyl)-phosphatidyl-(4-(N-maleimidomethyl)-cyclohexane-1-carboxyamido) ethanol (DOPE-MCC).

Liposomes may be produced by a variety of methods including hydration and mechanical dispersion of dried phospholipid or phospholipid substitute in an aqueous solution. Liposomes prepared in this manner have a variety of dimensions, compositions

and behaviors. One method of reducing the heterogeneity and inconsistency of behavior of mechanically dispersed liposomes is by sonication. Such a method decreases the average liposome size. Alternatively, extrusion is usable as a final step during the production of the liposomes. U.S. Patent 4,529,561 discloses a method of extruding liposomes under pressure through a uniform pore-size membrane to improve size uniformity.

Liposomes and oil droplets will often have, for example, thiol or maleimide or biotin groups on the molecules comprising the lipid bilayer. Molecules may then be bound to the surface by reaction of the particles with one of these materials that is bound to a sulfhydryl reactive reagent, a sulfhydryl group, or avidin, respectively. Sulfhydryl reactive groups include, among others, activated disulfides such as 2-pyridyl disulfides and alkylating reagents such as bromoacetamide and maleimide.

Latex particles -- "Latex" signifies a particulate water suspendable water insoluble polymeric material usually having particle dimensions of about 0.1μ to about 30μ, more usually about 1μ to about 10μ, preferably, about 1 to about 6μ, in diameter. The latex is frequently a substituted polyethylene such as polystyrene-butadiene, polyacrylamide, polystyrene, polystyrene with carboxylic groups, polystyrene with amino groups, poly-acrylic acid, polymethacrylic acid, acrylonitrile-butadiene, styrene copolymers, polyvinyl acetate-acrylate, polyvinyl pyridine, vinyl-chloride acrylate copolymers, and the like. Non-crosslinked polymers of styrene and carboxylated styrene or styrene functionalized with other active groups such as amino, hydroxyl, halo and the like are preferred. Frequently, copolymers of substituted styrenes with dienes such as butadiene will be used.

Antibody -- an immunoglobulin that specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule such as a platelet cell surface glycoprotein. The antibody can be monoclonal or polyclonal and can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies

may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and $F(ab')_2$, Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

Antiserum containing antibodies (polyclonal) is obtained by well-established techniques involving immunization of an animal, such as a rabbit, guinea pig, or goat, with an appropriate immunogen and obtaining antisera from the blood of the immunized animal after an appropriate waiting period. State-of-the-art reviews are provided by Parker, "Radioimmunoassay of Biologically Active Compounds," Prentice-Hall (Englewood Cliffs, N.J., U.S., 1976); Butler, J. Immunol. Meth. 7: 1-24 (1975); Broughton and Strong, Clin. Chem. 22: 726-732 (1976); and Playfair, et al., Br. Med. Bull. 30: 24-31 (1974).

Antibodies can also be obtained by somatic cell hybridization techniques, such antibodies being commonly referred to as monoclonal antibodies. Monoclonal antibodies may be produced according to the standard techniques of Köhler and Milstein, Nature 265:495-497, 1975. Reviews of monoclonal antibody techniques are found in Lymphocyte Hybridomas, ed. Melchers, et al. Springer-Verlag (New York 1978), Nature 266: 495 (1977), Science 208: 692 (1980), and Methods of Enzymology 73 (Part B): 3-46 (1981). Samples of an appropriate immunogen preparation are injected into an animal such as a mouse and, after a sufficient time, the animal is sacrificed and spleen cells obtained. Alternatively, the spleen cells of a non-immunized animal can be sensitized to the immunogen in vitro. The spleen cell chromosomes encoding the base sequences for the desired immunoglobins can be compressed by fusing the spleen cells, generally in the presence of a non-ionic detergent, for example, polyethylene glycol, with a myeloma cell line. The resulting cells, which include fused hybridomas, are allowed to grow in a selective medium, such as HAT-medium, and the surviving immortalized cells are grown in such medium using limiting dilution conditions. The cells are grown in a suitable container, e.g., microtiter wells, and the supernatant is screened for monoclonal antibodies having the desired specificity.

Various techniques exist for enhancing yields of monoclonal antibodies, such as injection of the hybridoma cells into the peritoneal cavity of a mammalian host, which accepts the cells, and harvesting the ascites fluid. Where an insufficient amount of the monoclonal antibody collects in the ascites fluid, the antibody is harvested from the blood of the host. Alternatively, the cell producing the desired antibody can be grown in a hollow fiber cell culture device or a spinner flask device, both of which are well known in the art. Various conventional ways exist for isolation and purification of the monoclonal antibodies from other proteins and other contaminants (see Köhler and Milstein, supra).

In another approach for the preparation of antibodies the sequence coding for antibody binding sites can be excised from the chromosome DNA and inserted into a cloning vector which can be expressed in bacteria to produce recombinant proteins having the corresponding antibody binding sites.

In general, antibodies can be purified by known techniques such as chromatography, e.g., Protein A chromatography, Protein G chromatography, DEAE chromatography, ABx chromatography, and the like, filtration, and so forth.

Antibody to a platelet cell surface glycoprotein receptor – an antibody that specifically recognizes an epitope on a platelet cell surface glycoprotein receptor. Preferably, the receptor is GPIbα and, preferably, the N-terminal portion of GPIbα. Desirably, the portion of the receptor recognized should be at least about 5 nm to about 10 nm from the surface of the cell, preferably, at least about 20nm to about 30 nm from the cell surface. However, in some circumstances the portion of the receptor recognized by the ligand may be less than about 5 nm from the cell surface. It is usually preferred that the receptor be present at a density of at least about 5000 per cell, usually about 10,000 per cell, more usually, at least about 15,000 per cell, preferably, at least about 20,000 and more preferably, about 25,000 per cell. However, in some circumstances the density of the receptor may be less than about 10,000 per cell. Usually, the antibody when directed against GPIbα, substantially inhibits von Willebrand factor binding to the receptor, i.e., the antibody inhibits the binding of vWf to the receptor at least about 50 %, and preferably about 90 to about 100 %. The antibody preferably is substantially inhibitory of platelet aggregation induced by ristocetin or botrocetin, which means that

the antibody preferably inhibits platelet aggregation by at least about 50%, and usually and preferably by about 100 % at saturation. Still more preferably, the antibodies bind glycocalicin. More preferably, the antibody does not substantially interfere with functional aspects of the fibrinogen receptor. In other words the antibody does not inhibit platelet agglutination induced by ADP or be inhibited by IIb/IIIa antagonists, that is reagents that inhibit fibrinogen binding to the IIb/IIIa receptor. The antibody may also inhibit the binding of thrombin to GPIbα.

It is interesting to note that antibody from clone 6D1 has been used to assay for glycocalicin as discussed by Coller *et al.*, in J. Clin. Invest. (1984) 73:794-799. Furthermore, an antibody to GPIbα has been used in FACS analysis to determine the presence of and to sometimes enumerate platelets. However, it has been reported *in vitro* that the GPIbα receptor may be degraded by thrombolytic proteases (plasmin) or proteases released from activated platelets (calpains) and neutrophils (cathepsin G, elastase) and that thrombin-activated platelets show diminished surface GPI receptors which are internalized, but later reappear on the surface over time. We, however, have discovered that, although the above observations have been made, the present invention may be employed to accurately determine platelet count in a sample. Moreover, there is disagreement as to whether a significant portion of GPIbα receptors become degraded or internalized *in vivo*. Advantageously, antibodies directed against the GPIbα receptor permit significant improvement in system controls. In other words, they work well in agglutination reactions that reflect the platelet count. Without meaning to be held to any theory, the effectiveness may be due to the fact that GPIbα is a high-density receptor and that the immunogenicity of the receptor is preserved on fixed, lyophilized platelets.

Although antibodies against receptor GPIbα are preferred in the present invention, antibodies against other receptors may be useful. These receptors include GPIX, GPV, GPIbβ, GPIβ/IX, GPIIb/IIIa, $\alpha_2\beta_1$, $\alpha_5\beta_1$,$\alpha_6\beta_1$, PAR-1, and polyclonal anti-human platelet antibodies. Examples of antibodies that may be used in the present invention are the following by way of illustration and not limitation: monoclonal antibody from clone 6D1 as disclosed and described by Coller, 1983, *supra*; monoclonal antibody from clone AN51 as disclosed and described by Ruan, 1981, *supra*, and available from Dako, Carpenteria CA; monoclonal antibody from clone 4H12 as

disclosed in U.S. Patent No. 5,486,361; monoclonal antibody from clone TM-60 as discussed in U.S. Patent No. 5,486,361 referring to several publications therein; monoclonal antibody from clone SZ-2 as discussed in U.S. Patent No. 5,486,361 referring to C. Ruan, *et al.*, INSERM Symposium No. 27, pp. 59-68, ed. J.L. McGregor, c. 1986 by Elusive Science Publishers B.V.; monoclonal antibody from clone VM 16d as discussed in U.S. Patent No. 5,486,361 referring to C. Ruan, *et al.*, Thrombosis research (1991) 63:673-684; monoclonal antibody from clone LJ-Ib10 as discussed in U.S. Patent No. 5,486,361 referring to DeMarco, *et al.*, J. Biol. Chem. (1991) 266:23776-23783; ; monoclonal antibody from the clone HPL11 as discussed in Aihara et al., Thromb. Haemost. (1988) 60:182-187; monoclonal antibodies from the clones ES85, C34, and AK2, respectively, as discussed in Ward, *et al.*, in Biochemistry (1996) 35:4929-4938; monoclonal antibody from clone AP1 as discussed in Okita, *et al.*, J Cell Biol (1985) 100:317-321, and references therein; monoclonal antibody from clone MB45, which is commercially available from Caltag, Burlingame CA; monoclonal antibody from clone 5B12, which is commercially available from Dako; monoclonal antibody from clone 6F1 as described Coller, *et al.*, in Blood (1989) 74(1):182-192; and the like.

In the present method platelets are enumerated by combining in a liquid medium a sample suspected of containing platelets with a matrix with which the binding molecule for the platelet cell surface glycoprotein receptor is associated.

The matrix is preferably a particle. In preferred embodiments the composition of the particle may be any convenient composition, such as bioglas, organic polymers, e.g., polyacrylonitrile, polystyrene, polycarbonate, polymethacrylate, combinations thereof, or the like. Usually, at least about 50 weight %, preferably at least about 75 weight %, will be of a size or diameter within the range indicated. The particles may be modified in a variety of ways. The particles may be chemically activated by having functional groups present on the surface of the particles, or be coated with a compound, e.g. protein, which may serve to substantially irreversibly (under the conditions of the processing and assay) bind to the dye. The coating compound may be the binding component, which will be involved in the aggregation of the particles, or other compound, usually being a protein. Alternatively, depending on the nature of the particles, the particles may not have chemically active groups, but rather provide binding by adsorption.

In a particular embodiment of the present invention the matrix is a polymerized acrylonitrile bead that is about 1 to about 3 microns in diameter with N-hydroxysuccinimide ester groups on their surface (e.g., Matrex I02 beads from Amicon Corporation). The N-hydroxysuccinimide ester groups allow coupling of the N-terminus of an antibody to the surface of the bead. In another particular embodiment of the invention the matrix is a carboxylated polystyrene bead that is about 3 to about 6 microns in diameter (Polysciences Inc.). The surface carboxyl groups of the bead can be coupled to the N-terminus of the antibody by means of, for example, a carbodiimide coupling, N-hydroxysuccinimide coupling, and the like. The beads may be colored to render the results of the agglutination reaction easier to interpret. Coloring of the beads may be achieved by procedures well-known in the art.

In an alternative approach particles containing covalently attached anti-mouse (Fc)-specific antibody, which can serve as a capture antibody for a mouse monoclonal antibody used in the present invention. In this way potentially up to twice the amount of monoclonal antibody may be bound per particle and substantially all of the antigen binding sites of the antibody would be available for platelet binding. A further advantage of using a capture antibody is that non-specific agglutination between immobilized IgG and the platelet FcgRii receptor (about 1000/ cell) would be minimized. An additional advantage is that monoclonal antibody tissue culture supernatant can be utilized without further purification of the antibody.

The particles are preferably colored to render the results of the agglutination reaction easier to interpret.

Preferably, the medium for carrying out the methods in accordance with the present invention is an aqueous medium. Other polar cosolvents may also be employed in the medium, usually oxygenated organic solvents of from 1-6, more usually from 1-4 carbon atoms, including alcohols, ethers and the like. Usually, such cosolvents are present in less than about 70 weight percent, more usually, in less than about 30 weight percent. Additionally, various ancillary materials are frequently employed in the method in accordance with the present invention. For example, buffers are normally present in the assay medium, as well as stabilizers for the assay medium and the assay components;

surfactants, particularly non-ionic surfactants; binding enhancers, e.g., polyalkylene glycols; or the like.

The pH for the medium is usually in the range of about 2 to about 11, preferably, about 4 to about 9. The pH is chosen so as to maximize the extent of binding between the receptor and the binding molecule employed in the method and, thereby, maximize the extent of agglutination achieved. Various buffers may be used to achieve the desired pH and maintain the pH during the method. Illustrative buffers include HEPES, borate, phosphate, carbonate, Tris, barbital, and the like. The particular buffer employed is not critical to the method but one buffer may be preferred over others in certain circumstances. In some circumstances HEPES is preferred and is present at a concentration of about 0.05M to about 0.001M but generally at a concentration of about 0.01M. A suitable buffer also maintains the salt concentration of the liquid within a range suitable for agglutination. Thus, the buffer may contain a concentration of one or more salts such as sodium chloride that maintain the electrolytic balance of the blood within a range suitable for agglutination. Suitable concentrations of sodium chloride in the buffer are between about 0.10M and about 0.20M, typically about 0.15M. Other salts that may be present include, but are not limited to, calcium chloride and magnesium chloride.

The medium is subjected to agglutination conditions. Moderate temperatures are normally employed for carrying out the method. The temperature may be constant or may vary. Usually, a constant temperature is employed during the reaction step. The temperature employed is usually about 10 to about 80 °C, more usually, about 15 to about 45 °C, preferably, the temperature should be at least 25°C, more preferably in the range of about 30 to about 40°C, usually about 37°C.

The number of platelets to be determined usually varies between about 100,000/µl to about 400,000/µl. The amount to the sample employed varies from about 0.05 to about 0.5 ml. The concentration of the matrix with the binding molecule is usually about 0.01 to about1.0 % solids, more usually, from about 0.2 to about 0.6 % solids. The binding molecule is employed in a concentration of about 10^{-7} to about 10^{-9} M, more usually, from about 10^{-8} M. In many instances the final concentration of each of

the reagents is determined empirically to optimize the sensitivity of the method over the range of interest for the suspected platelet concentration.

The order of addition of the various reagents may be varied. Generally, the sample is combined with the liquid medium and the matrix is then added. However, the sample and the matrix may be combined with the liquid medium substantially simultaneously.

The time period for carrying out the present method is generally from about 30 seconds to about 1 hour, usually from about 10 seconds to about 2 minutes, more usually, about 20 seconds to about 1 minute. Basically, the time period for the reaction is sufficient to permit substantial agglutination of the matrix. During the period of the reaction, the medium may be agitated to facilitate binding of the binding molecule to the receptor. Agitation is preferably supplied mechanically, for example, by placing the reaction vessel on a rocker. The vessel may be rocked for the entire reaction period or for a shorter period as long as the basic objective of agglutination is achieved. The reaction vessel may be rocked at about 6 cpm to about 900 cpm, preferably, about 300 cpm and be controlled to ~ 0.1% of the preferable cpm. Alternatively, the reaction medium may be agitated manually, for example, by mixing with a mixing rod or by rotating on a glass slide. The time period for manual agitation is substantially the same as that for mechanical agitation.

Next, the agglutination of the matrix is measured as an indication of the number of receptors present in the medium. The presence of agglutination may be determined visually by observing clumping of the matrix, which would indicate agglutination. Optionally, as mentioned above, the matrix may be colored to aid in visualizing agglutination or clumping of the matrix. Useful dyes are those that absorb in the infrared. The extent of agglutination may be measured spectrophotometrically, turbidimetrically, nephelometrically, and so forth.

The level of agglutination is an indication of the number of receptors present, which is directly related to the number of platelets in the sample. The level of agglutination may be compared against a control or a standard of known platelet number to determine the number of platelets in the sample. Usually, the result will be compared

to a calibrator, which may be performed concomitantly or have been performed previously or may be provided as a standard curve.

An important application of the present invention is for rapid monitoring of an individual during a medical procedure or to determine whether an individual is ready to undergo a medical procedure. This type of monitoring is referred to as "bedside monitoring." As mentioned above, the present invention offers advantages over known platelet enumeration methods, which require sophisticated instrumentation. The present invention can be carried out without the need for such instrumentation. Thus, the determination of platelet count can be conducted at the bedside. If the determination indicates a low platelet count, the medical procedure may be postponed only until the situation has been corrected. For example, platelet transfusions may be administered immediately so that the medical procedure, e.g., invasive surgery, may be carried out as soon as possible and excessive bleeding may be avoided. The platelet count may be monitored during the platelet transfusions until a desirable level is achieved.

Another aspect of the present invention is a kit for determining the number of platelets in a sample. The kit comprises in packaged combination particles to which is bound an antibody to a GPIbα subunit of the GPIb/1x-v receptor and a buffered medium. The kit may also include, in predetermined amounts, additional reagents for carrying out the present method and/or for carrying out an assay for platelet function as mentioned above. The kit may also include a sample collection container and/or a device for carrying out the present method or a platelet function assay. The relative amounts of reagents may be varied widely to provide for concentrations in solution of the reagents that substantially optimize the sensitivity of a determination. Where appropriate, the reagents can be placed in an airtight package in order to maintain the activity of any reagents. The package may be, for example, a bag, pouch, or the like fabricated from a material that is substantially non-permeable to moisture. Such materials include, by way of example and not limitation, plastic, aluminum foil, and the like. For blood samples the kit may also include an article for piercing a person's skin, disinfectant or sterilizing pads and so forth. It will be evident that additional articles to be included in the kit of the present invention are dependent, among others, on the type of sample being collected.

These articles will be known to the skilled person. The kit may also include calibrators and standards.

EXAMPLES

The following examples are offered by way of illustration and not limitation.
5 Parts and percentages are by weight unless otherwise indicated. Temperatures are in degrees Centigrade (°C) unless indicated otherwise. The following preparations and examples illustrate the invention but are not intended to limit its scope.

EXAMPLE 1

10 Single Step Covalent Coupling of 6D1-Antibody to Carboxylated Infrared Polystyrene Beads.

1 ml of 2% Vanadyl-dyed 6μ Carboxyl Polystyrene microparticles, obtained from Interfacial Dynamics Corporation, Portland, Oregon, were centrifuged at 1500 x g for 5 minutes and resuspended in 0.1 ml (1/10 volume) of deionized and ultra-filtered (DIUF)
15 water. 6D1 monoclonal antibody (from Dr. Barry Coller, Mt. Sanai School of Medicine, New York, N.Y., purified by affinity chromatography on immobilized protein A as described by Scudder, *et al.*, Methods in Enzymology (1992) 215:295-311) was diluted to 0.8 to 0.025 mg/ml in 50 mM MES, pH 5.5, representing a 1:25 to 1:800 weight to weight (w/w) ratio of antibody to polystyrene, respectively. 1 ml of antibody was added
20 to 0.1 ml of the concentrated particles, followed immediately by the addition of 0.1 ml (1/10 volume) of freshly prepared Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride (EDC), obtained from either Pierce Chemical Co., Rockford IL, or Aldrich Chemical Co., Milwaukee WI, in DIUF water. The reaction was allowed to proceed for 2 hours at ambient temperature on a platform rocking apparatus (Vari-Mix,
25 Thermolyne, Dubuque IA) and then centrifuged as above. The supernatants were saved and the absorbance at 280 nm of the starting antibody solutions and supernatants recorded on a spectrophotometer in order to estimate the amount of protein coupled to the particles. The particle pellets were resuspended in 1 to 3 ml of 10 mM (HEPES)/NaOH, 150 mM NaCl, 0.02% NaN_3, pH 7.4 containing 2 mg/ml bovine serum
30 albumin (BSA) (Sigma Chemical Co., St. Louis MO) (HBS)/BSA) and washed three

times by centrifugation. The particles were suspended to 2% solids in the same buffer and stored at 2-8°C.

EXAMPLE 2

5 Two-Step Covalent Coupling of Anti-mouse (Fc) specific Antibody to Carboxylated Polystyrene Microparticles [(GAM)Fc-particles].

6 ml of 2% 6 μ Vanadyl-Carboxyl Polystyrene Particles were washed once by centrifugation at 1500 x g for 5 minutes in 0.01M sodium acetate buffer, pH 5.0 and then resuspended in about 1ml of the same buffer. Solid EDC was added (about 20 mg). The 10 sample was vortexed and then allowed to stand for about 1 hour at room temperature. Acetate buffer was added to the initial volume (6 ml) and the sample centrifuged as above to remove residual EDC. Goat antibody specific for the Fc portion of mouse IgG (Fortron Bio Science, Morisville, NC) was prepared at 0.25 mg/ml in the acetate buffer (4.8 ml) and used to resuspend the centrifuged particles. Under these conditions, the 15 particles were present at 2.5% solids (25 mg/ml) and the antibody at 0.25 mg/ml, representing a protein to polystyrene ration of 1:100 (w/w). The sample was allowed to incubate for 2 hours at room temperature on a Vari-Mix Rocker. The supernatants were saved for protein determination using a spectrophotometer at 280 nm and the pellets washed 3 times in and stored at 2% solids in HBSA buffer (10 mM HEPES/NaOH, 150 20 mM NaCl, 0.02% NaN_3, pH 7.4). The protein concentration of the antibody-coated particles was determined using Bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co.) as described by Seradyn Inc. (Indianapolis IN). A similar procedure was used for the direct two-step coupling of 6D1 and other antibodies to 3-6μ particles. In some cases, the beads were activated with both EDC and N-Hydroxy succinimide (NHS) 25 from Pierce Chemical Co. and then washed prior to the addition of antibody.

EXAMPLE 3

Affinity Adsorption of Anti-platelet Monoclonal Antibodies to Anti-mouse (Fc) Specific Carboxylated Polystyrene Microparticles.

30 Anti-platelet monoclonal antibodies were used as identified in Table 1 below, either purified by protein A chromatography or used in crude form from cell culture

supernatants. 6D1 monoclonal antibody was from Dr. Barry Coller as mentioned in Example 1; 5B12 monoclonal antibody was from Dako; AN51 monoclonal antibody was from Dako; and 6F1 monoclonal antibody (Coller, *et al.*, in Blood (1989) 74(1):182-192). Each antibody was added at 1.25 molar excess to GAM particles described in
5 Example 2 above and allowed to incubate for overnight with gentle rocking. Unbound antibody was removed by washing three times in HBSA and the final particles stored at 2% solids in HBS/BSA at 4°C. A table outlining some anti-platelet monoclonal antibodies, the receptors they interact with, and the approximate receptor density/platelet is depicted below in Table 1.

10 Table 1

Antibody	Receptor	Receptors/Cell
6D1, AN51	GPIbα	~ 25,000
6F1	GPIa/IIa	~ 1,000
5B12	GPIIb/IIIa	~ 50,000

15 EXAMPLE 4

Use of Immobilized-6D1 Microparticles to Estimate Platelet Count in PRP.

Monoclonal antibody 6D1 was coated by a covalent two-step EDC procedure, as described in Example 2 above, to 3 μ blue carboxylated polystyrene microparticles (Polysciences, Warrington, PA) and stored at 2.5% solids. Platelet rich plasma (PRP)
20 was prepared from whole anticoagulated blood by centrifugation at 750 x g for 4 minutes and enumerated using an electronic particle counter (Coulter Corporation, Hialeah, FL). Platelet poor plasma (PPP) was obtained by centrifugation of whole blood at about 2,000 x g for 10 minutes. PRP was diluted into autologous PPP to obtain different platelet counts. 50 μl of platelets were placed in wells of a microtiter plate. 5-10 μl of about 2.5%
25 6D1-particles were added and the reaction monitored on a plate reader (Molecular Devices) at 650 nm. The results are displayed in Fig. 2 and provide proof-of concept that

the concentration of platelets in a sample can be enumerated based upon the rate of agglutination of the platelet-containing sample with immobilized 6D1-coated particles.

EXAMPLE 5

5 Use of Monoclonal anti-GPIb Antibodies Indirectly Immobilized on GAM-particles to Enumerate Platelets in PRP.

PRP and PPP were prepared as described above in Example 4. 50 μl of PRP was placed in triplicate wells of a Costar # 3369 microplate. 10 μl of 2 % 6μ dyed GAM(Fc) particles or such particles containing adsorbed monoclonal antibodies (mAbs) 6D1 or 10 AN51 were added and the reaction allowed to proceed for a period of 10 minutes at a temperature of 25°C. The reaction was monitored at 750 nm. The results are illustrated in Fig. 3 and demonstrate that anti-GPIb antibodies other than 6D1 can be used to enumerate platelets. Top panel - mAb 6D1. Bottom panel - mAb AN51. In addition, other anti-platelet mAbs 6F1 and 5B12 were immobilized analogously and tested as 15 described and the results are shown in Fig. 6. The results shown in Fig. 6 illustrate several points. One point is that antibodies against receptors other than GPIb (e.g., mAb 5B12, GPIIb/IIIa receptor) can be utilized in the assay to enumerate platelets. Another point is that antibodies against receptors of low density (e.g., mAb 6F1,GPIaIIa receptor, density of about 1,000/cell) may not yield adequate rates of agglutination to detect the 20 normal physiologic platelet count range (about 100,000 to 400,000/μl). Thus, a minimum receptor density of about 5,000/cell, preferably, about 10,000/cell is desirable and, as mentioned above, one such receptor is represented by GPIb (25,000/cell).

EXAMPLE 6

25 Kinetics of the Agglutination of GAM(Fc):6D1-particles with Platelets in Whole Blood.

Citrate-anticoagulated whole blood (50μl) was placed into individual microtiter plate wells. 10 μl of 6 μ dyed GAM(Fc)-particles (negative control) or GAM(Fc):6D1-coated particles were added and the reaction monitored at 750 nm at a temperature of 25°C for 10 minutes with continuous shaking, taking data points every 10 seconds. The 30 OD at 750 nm was plotted at each time point. The results are shown in Fig. 4 and demonstrate that immobilized anti-platelet GPIb antibodies can agglutinate platelets and

that the agglutinated particles are detected in undiluted whole blood. In contrast, the non-specific GAM(Fc)-coated particles themselves did not cause significant agglutination of blood platelets.

5 EXAMPLE 7

Correlation between the Rate of Agglutination of 6D1-particles in Whole Blood with the Blood Platelet Count

Whole citrated blood was separated into packed red blood cells, PRP and PPP, by standard centrifugation techniques. Samples were prepared at constant packed red cell 10 volume (hematocrit, 40%) while varying the PRP/PPP ratio (platelet count). The approximate hematocrit was verified using a micro-hematocrit apparatus (CLAY ADAMS®, Becton Dickinson Co., Sparks, MD) according to the manufacturer's instructions. 50 μl of the mixture was placed in triplicate wells of the microtiter plate and the reaction initiated with 10 μl of covalent 2% 6D1 Vanadyl 6μ particles, conducted at a 15 temperature of 25°C for a period of 10 min. and recorded at 750 nm. The results conclusively demonstrated that the immobilized anti-GPIbα mAb 6D1 resulted in the enumeration of platelets in whole blood based upon the rate of agglutination of such particles with blood platelets. The results are summarized in Fig. 5.

It is evident from the above results that a simple method is provided for 20 enumeration of platelets. A whole blood sample may be used and the method can be carried out in conjunction with a rapid platelet function assay such as that described above in U.S. Patent No. 5,763,199.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were 25 specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit 30 or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining the number of platelets in a sample, said method comprising:

(a) subjecting to agglutination conditions a liquid medium comprising said sample and a matrix with which is associated a binding molecule for a platelet cell surface glycoprotein receptor,

(b) measuring the agglutination of said matrix and

(c) relating the measurement of step (b) to the number of platelets in said sample.

2. A method according to claim 1 wherein said receptor is present in at least about 5,000 per cell.

3. A method according to claim 1 wherein said receptor is not subject to substantial interference from ligand binding to said receptor

4. A method according to claim 1 wherein said binding molecule is an antibody.

5. A method according to claim 1 wherein said binding molecule is a snake venom protein which interacts with GP1b.

6. A method according to claim 1 wherein said binding molecule is an alboaggregin.

7. A method according to claim 4 wherein said antibody is an antibody to CD42.

8. A method according to claim 4 wherein said antibody is an antibody to GPIb-IX.

9. A method according to claim 4 wherein said antibody is an antibody to GPIbα.

10. A method according to claim 9 wherein said antibody is a monoclonal antibody designated 6D1.

11. A method according to claim 9 wherein said antibody is a monoclonal antibody designated AN51, AP1, 4H12, TM-60, SZ2, HPL11, ES85, C34, AK2.

12. A method for determining the number of platelets in a sample, said method comprising:

(a) subjecting to agglutination conditions a liquid medium comprising said sample and particles to which is bound an antibody to a GPIbα receptor,

(b) measuring the agglutination of said particles and

(c) relating the measurement of step (b) to the number of platelets in said sample.

13. A method according to claim 12 wherein said antibody is a monoclonal antibody designated 6D1.

14. A method according to claim 12 wherein said antibody is a monoclonal antibody designated AN51, AP1, 4H12, TM-60, SZ2, HPL11, ES85, C34, AK2.

15. A kit for determining the number of platelets in a sample, said kit comprising in packaged combination:

(a) particles to which is bound an antibody to a GPIbα receptor,

(b) a buffered medium.

16. A kit according to claim 15 wherein said antibody is a monoclonal antibody designated 6D1.

17. A kit according to claim 11 wherein said antibody is a monoclonal antibody designated AN51, AP1, 4H12, TM-60, SZ2, HPL11, ES85, C34, AK2.

Schematic of the GPIb/IX-VComplex (CD42)

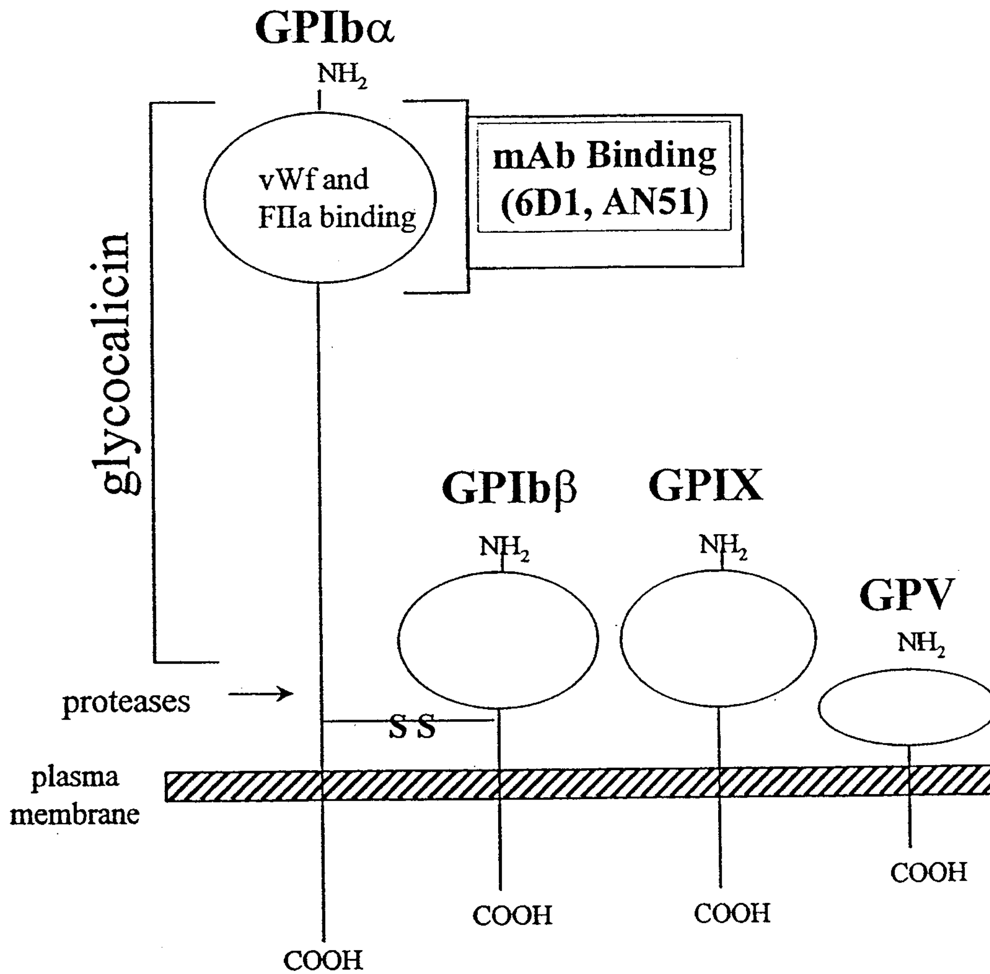

Fig. 1

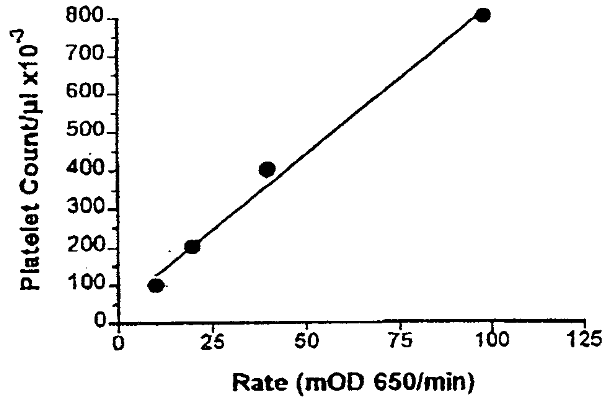

Fig. 2A

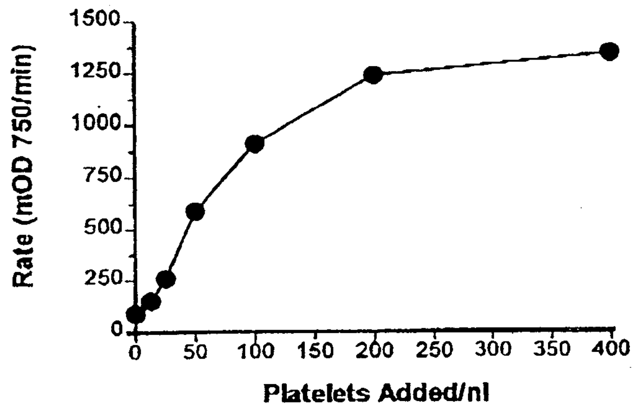

Fig. 2B

Platelet Count Assay in PRP using anti-GPIbα mAb 6D1 immobilized on IR-dyed 6µ GAM(Fc)-particles (0.3%)

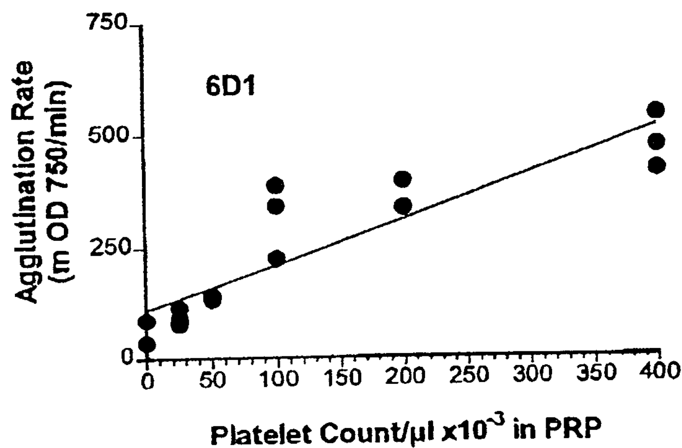

Fig. 3A

Platelet Count Assay in PRP using anti- GPIbα mAb AN51 immobilized on IR -dyed 6µ GAM(Fc)-particles (0.3%)

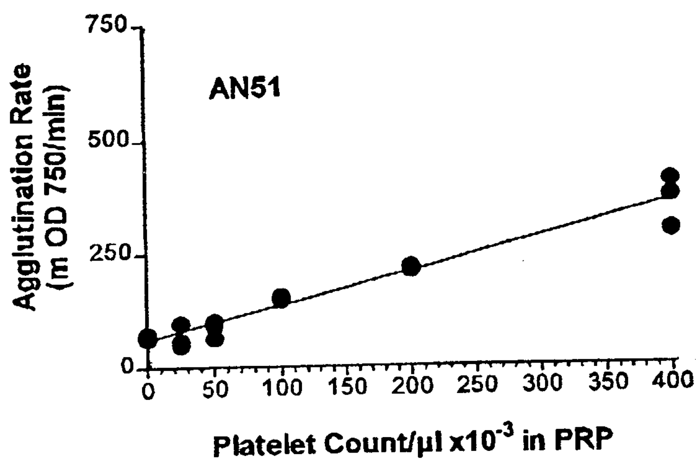

Fig. 3B

Agglutination of Platelets in Citrated Blood by 6D1 Immobilized on 6μIR-dyed GAM(Fc) Particles

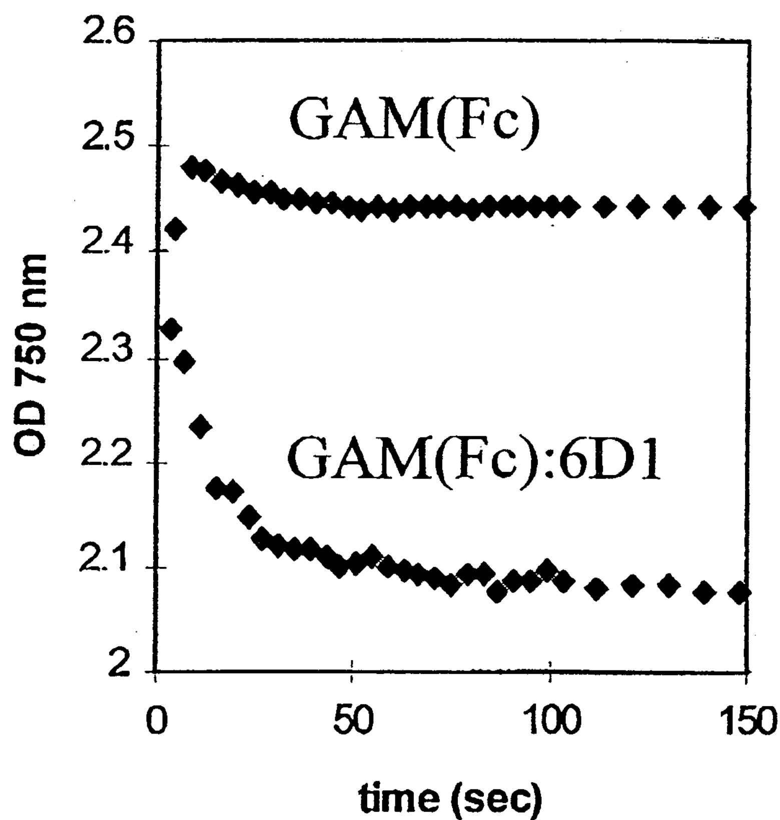

Fig. 4

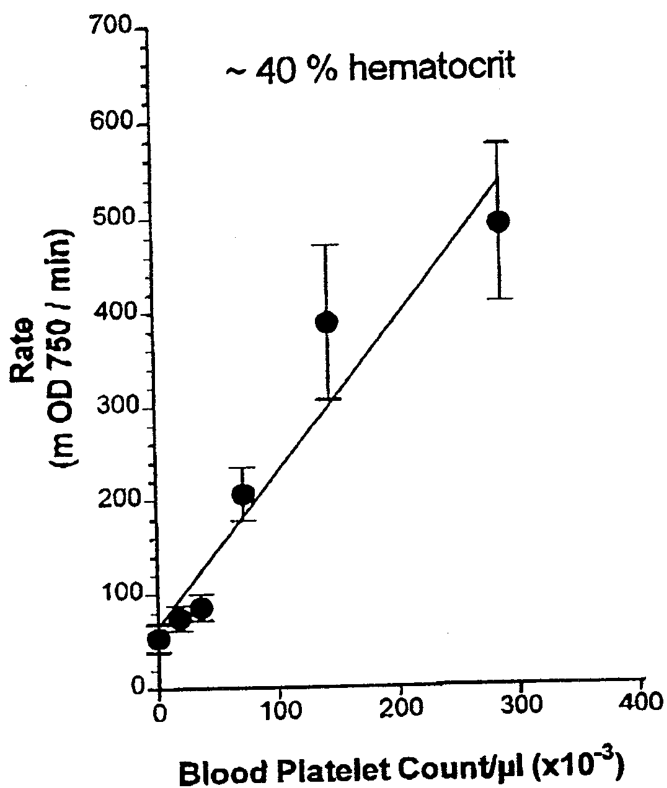

Fig. 5

Agglutination of Citrated PRP by Anti-Platelet mAbs Bound to IR- dyed GAM(Fc) Microparticles

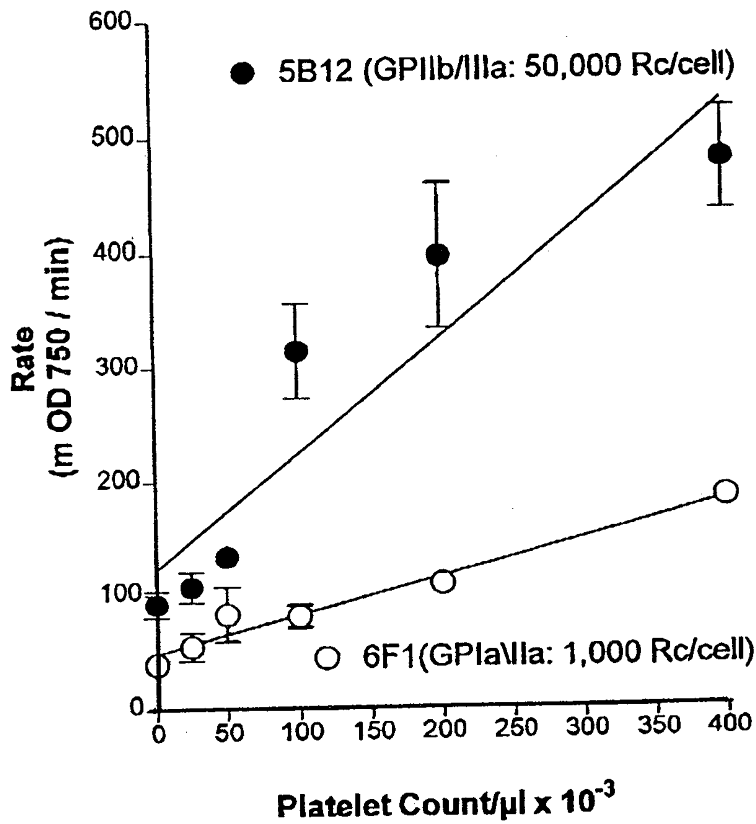

Fig. 6

INTERNATIONAL SEARCH REPORT

Intern. ...al Application No
PCT/US 99/24670

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/86 G01N33/80 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 03655 A (LILL HELMUT ;DONIE FREDERIC (DE); BOEHRINGER MANNHEIM GMBH (DE); F) 8 February 1996 (1996-02-08) page 16, line 7-32 page 17, line 1-6 ---	1-17
X	WO 98 41868 A (ACCUMETRICS INC) 24 September 1998 (1998-09-24) page 23, line 20-28 claim 11 ---	1-17
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[X] Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

Inten   nal Application No
PCT/US 99/24670

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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