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| <p>(21) International Application Number: PCT/US95/11986</p> <p>(22) International Filing Date: 20 September 1995 (20.09.95)</p> <p>(30) Priority Data:<br/>08/315,026 29 September 1994 (29.09.94) US</p> <p>(71) Applicant (for all designated States except US): MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave Levy Place, P.O. Box 1099, New York, NY 10029-6574 (US).</p> <p>(72) Inventor; and<br/>(75) Inventor/Applicant (for US only): COLLER, Barry, S. [US/US]; Apartment 6A, 1160 Park Avenue, New York, NY 10128 (US).</p> <p>(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith &amp; Reynolds, Two Militia Drive, Lexington, MA 02173 (US).</p>   |                  | <p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p><b>Published</b><br/><i>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> |
| <p>(54) Title: PLATELET BLOCKADE ASSAY</p>  |                  |   |
| <p>(57) Abstract</p> <p>The present invention is an assay for determining 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 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 period of time if the glycoprotein IIb/IIIa receptors are not blocked.</p> |                  |   |

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PLATELET BLOCKADE ASSAYBackground of the Invention

New agents that block the platelet glycoprotein IIB/IIIA (GPIIb/IIIA) receptor are being developed for use as antithrombotic agents in medical applications such as angioplasty and atherectomy. The doses of these agents required to achieve equivalent antiplatelet effects are likely to differ as a function of platelet count, glycoprotein IIB/IIIA ( GPIIb/IIIA surface density, and individual variations in drug pharmacokinetics. In fact, in one [(Simons et al. circulation, 89:596, (1994)) study, a patient with a platelet count greater than 900,000/ $\mu$ l, who received a standard dose of c7E3 Fab had virtually no antithrombotic effect. Since platelet counts within the normal range differ by more than 2-fold (150,000/ $\mu$ l - 350,000/ $\mu$ l), and platelet GPIIb/IIIA density per platelet also differs by about 2-fold, there may be as much as a four-fold difference in the total number of GPIIb/IIIA receptors requiring blockage even within the normal population. Little is known about individual differences in the pharmacokinetics of different GPIIb/IIIA blocking agents, but it is reasonable to expect variation based on renal function and perhaps other factors that act on low molecular weight compounds.

Several assays are currently available for evaluating GPIIb/IIIA receptor blockade, including platelet aggregation, and in the case of c7E3, either radiolabeled 7E3 binding or flow cytometry. These assays are time consuming, require special equipment and require extensive standardization and so are not suitable for bedside monitoring.

It would be desirable, therefore, to have a rapid and simple assay to assess the extent of receptor blockade in

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an individual. In the setting of angioplasty, it would be desirable to have a GPIIb/IIIa receptor assay conducted at the same time as determination of activated clotting time (ACT), which is an indicator of extent of

5 heparinization, that would indicate when the desired level of blockade had been achieved and that the patient was ready to undergo the angioplasty. During chronic infusions, periodic monitoring may also be desirable. Further, in certain circumstances (prior to surgery or an

10 invasive procedure) it may be desirable to rapidly determine whether the effect of the GPIIb/IIIa blocking drug has worn off.

#### Summary of the Invention

The present invention relates to a method of

15 determining the level of residual unblocked glycoprotein IIB/IIIa receptors in whole blood and, thus, the ability of the individual's platelets to undergo aggregation. Thus, the method refers to a method of determining whether an individual has reduced ability to form platelet thrombi

20 or has prolonged bleeding times. The present invention also relates to a kit comprising the reagents for carrying out the assay.

The method comprises a first step in which blood is withdrawn from an individual being assessed for GPIIb/IIIa

25 blockade and is mixed with an anticoagulant. The whole blood/anticoagulant mixture is combined with a buffer which maintains the pH and the salt concentration of the mixture at levels suitable for platelet aggregation, and with a glycoprotein IIB/IIIa ligand immobilized on a solid

30 surface. The ligand is immobilized on the solid surface such that platelet aggregation in the presence of platelets with unblocked GPIIb/IIIa is detectable. The resulting mixture is then agitated for a period of time that is sufficient to allow unblocked platelet

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glycoprotein IIb/IIIa receptors to bind with the glycoprotein IIb/IIIa receptor ligand. The absence of aggregation indicates that the GPIIb/IIIa receptors are blocked. In one aspect the solid surface is a small  
5 polymeric bead on which the ligand is immobilized at a density to allow the platelets to aggregate in the presence of unblocked GPIIb/IIIa receptor. Platelet aggregation is determined by bead agglutination. The absence of bead agglutination indicates that the  
10 GPIIb/IIIa receptors are blocked and the individual has reduced ability to form platelet thrombi. Optionally, a thrombin receptor activating peptide can be added, which causes the beads to agglutinate more quickly and causes agglutination to occur within a defined period of time.  
15 The amount of thrombin activating peptide can be adjusted so that agglutination occurs within a short time, such as two minutes to five minutes if blockade of GPIIb/IIIa receptors is less than 80%. The absence of agglutination indicates that the individual will have impaired platelet  
20 aggregation which generally correlates with prolonged bleeding times and reduced ability to form platelet thrombi. Alternatively, less thrombin receptor activity peptide can be used, such that agglutination will occur within a short period of time, such as two to five  
25 minutes, if blockade of GPIIb/IIIa receptors is less than about 50%. This indicates that the individual will have a normal or near-normal bleeding time.

The assay of the present invention has the advantage that it can be conveniently performed at the time of need,  
30 such as when a surgical procedure is being performed or about to be performed, in a matter of minutes without the need for expensive equipment or highly trained medical personnel. Such an assay has the further advantage that it is rapid enough to be used to indicate whether a  
35 patient undergoing antiplatelet therapy has achieved a

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sufficiently high level of glycoprotein IIb/IIIa blockade to proceed with a surgical procedure such as angioplasty, in which thrombosis must be avoided. In the event that a patient undergoing antiplatelet therapy requires invasive  
5 surgery, the assay is also useful to determine the extent to which the glycoprotein IIb/IIIa blocking drug is acting and, thus, whether a platelet transfusion is needed.

The present invention also relates to a kit that comprises the reagents for carrying out the assay of the  
10 present invention. The present invention further relates to a method of diagnosing an individual with Glanzmann thrombasthenia or with thrombocytopenia using the assay of the present invention.

#### Detailed Description of the Invention

15 Glycoprotein IIb/IIIa receptor proteins (GPIIb/IIIa), which are found on the cell surface of platelets, play a central role in thrombosis, i.e., coagulation (clotting) of blood. The binding of fibrinogen and perhaps other macromolecular liquids to GPIIb/IIIa leads to platelet  
20 aggregation and is consequently an essential step in the cascade that leads to platelet thrombosis formation and coagulation.

A large enough deficiency in the number of unblocked GPIIb/IIIa receptor proteins in an individual results in a  
25 decreased ability of platelets to aggregate, thus resulting in reduced ability to form platelet thrombi and abnormally long bleeding times. For example, blockade of about 80% or more of an individual's GPIIb/IIIa receptors, for example by the administration of a GPIIb/IIIa receptor  
30 blocking agent such as monoclonal antibody c7E3 Fab, abolishes platelet aggregation and prolonged bleeding times (period of time in which the individual will bleed from a wound before platelet thrombosis formation stops the bleeding), often to times greater than thirty minutes.

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Such individuals have an impaired ability to thrombose, i.e., to form blood clots. Certain diseases also result in decreased levels of unblocked GBII/IIIa receptors. For example, individuals who are homozygous for Glanzmann  
5 thrombasthenia have virtually no GPIIb/IIIa receptor proteins on their platelet cell surfaces and also have prolonged bleeding times (Coller et al. *Ann. N.Y. Acad. Sci* 614:193 (1993)). In addition, individuals who are severely thrombocytopenic, i.e., have platelet counts  
10 below 100,000 platelets/ $\mu$ l, have similar bleeding abnormalities. However, platelet aggregation is unaffected or minimally affected in individuals until about 40-50% of GPIIb/IIIa receptors are blocked and bleeding times are normal until as many as about 60% of  
15 GPIIb/IIIa receptors are blocked. Levels of GPIIb/IIIa blockade up to about 80% result in mild to moderate abnormalities in platelet aggregation and bleeding times (Coller et al. *Ann. N.Y. Acad. Sci* 614:193 (1993)). Consistent with these figures is the observation that  
20 individuals who are heterozygous for Glanzmann thrombasthenia and who have only 50-60% of the normal numbers of GPIIb/IIIa receptors show normal bleeding times. Consequently, the ability of the blood of an individual to undergo platelet aggregation and coagulate  
25 is dependent on the number of unblocked GPIIb/IIIa receptors.

One embodiment of the present invention is a method of assessing whether an individual will have severely impaired ability to form platelet thrombi or impaired  
30 platelet aggregation due to blockage of about 80% or more of the GPIIb/IIIa receptors or to a similar deficiency in the number of GPIIb/IIIa receptors. An individual with a blockage of 80% or more of the GPIIb/IIIa receptors will have prolonged bleeding times, i.e. bleeding times of  
35 greater than about thirty minutes, an impaired ability to

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form platelet thrombi and impaired platelet aggregation. The method comprises determining whether greater than 80% of the glycoprotein IIb/IIIa (GPIIb/IIIa) receptors are blocked in whole blood. Blockade of 80% or more of glycoprotein IIb/IIIa receptors in whole blood is  
5 determined by withdrawing blood from an individual being assessed for GPIIb/IIIa blockade and mixing the blood with an anticoagulant. The whole blood/anticoagulant mixture is then combined with 1) a buffer that is capable of  
10 maintaining the pH and salt concentrations of the blood at levels suitable for platelet aggregation and 2) a glycoprotein IIb/IIIa ligand immobilized on a solid surface such that platelet aggregation is detectable when the solid surface is combined with platelets having  
15 unblocked GPIIb/IIIa receptors. The resulting mixture is then agitated for a period of time that is sufficient to allow unblocked platelet glycoprotein IIb/IIIa receptors to bind with the glycoprotein IIb/IIIa receptor ligand. The absence of platelet aggregation indicates that more  
20 than about 80% of the GPIIb/IIIa receptors are blocked. The absence of platelet aggregation also indicates that the individual will have prolonged bleeding times and reduced ability to form thrombi. Platelet aggregation indicates that less than about 80% of the GPIIb/IIIa  
25 receptors are blocked. Preferably, the solid surface is a small polymeric bead. The GPIIb/IIIa ligand must be immobilized at a sufficient density on the surface of the beads so that the beads can agglutinate in the presence of platelets with GPIIb/IIIa receptors that are not blocked.  
30 The absence of agglutination indicates blockade of GPIIb/IIIa receptors. The absence of agglutination also indicates that the individual will have prolonged bleeding times due to GPIIb/IIIa blockade. Agglutination of the beads is indicative of less than 80% GPIIb/IIIa receptor  
35 blockade.

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As used herein, GPIIb/IIIa receptors are blocked when a drug, peptide, monoclonal antibody or small organic molecule binds to, complexes with or interacts with the GPIIb/IIIa receptor such that a natural ligand of the GPIIb/IIIa receptor, for example fibrinogen, cannot bind to the GPIIb/IIIa receptor.

Blood can be drawn from the individual being assessed for GPIIb/IIIa receptor blockade by any number of known techniques. Preferably, the blood is drawn into a Vacutainer or other closed tube to protect the individual who is drawing the blood from blood-borne infectious agents such as the hepatitis B virus or the HIV virus. The whole blood is then mixed with an anticoagulant. This can be carried out, for example, by drawing the blood into a tube containing anticoagulant or by adding anticoagulant to drawn blood. A sufficient amount of anticoagulant is used to prevent coagulation of the whole blood. Suitable anticoagulants include citrate, oxalate, ACD-A, heparin, hirudin or other antithrombin agent. A preferred anticoagulant is citrate. The final citrate concentration in the whole blood ranges from 0.3% to about 0.5% of the blood, preferably about 0.4%. Whole blood to which a sufficient amount of anticoagulant has been added to prevent coagulation is referred to as "anticoagulated blood".

The anticoagulated blood is mixed in an assay tube with a buffer solution and a suitable amount of small polymeric beads on whose surface a ligand that binds to the platelet GPIIb/IIIa receptor has been immobilized to produce an anticoagulated blood-bead mixture. The ligand is immobilized at a density sufficient to allow the beads to agglutinate in the presence of platelets with GPIIb/IIIa receptors that are not blocked.

A suitable buffer solution is capable of maintaining the pH and salt concentration of the blood within a range

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suitable for platelet aggregation. Suitable pH levels are about 6.6-8.2 and is preferably about 7.4. A suitable buffer comprises a concentration of a buffering composition such as HEPES, Tris or others that maintain  
5 the pH of the blood within the range suitable for platelet aggregation, as defined above. HEPES is a preferred buffer and is present at a concentration of 0.05M-0.25M, but generally at a concentration of 0.01 M. A suitable buffer also maintains the salt concentration of the blood  
10 within a range suitable for platelet aggregation. Consequently, the buffer contains a concentration of one or more salts, such as NaCl, that maintain the electrolytic balance of the blood within a range suitable for platelet aggregation. Suitable concentrations of NaCl  
15 in the buffer are between 0.10 M and 0.20 M, but typically 0.15 M. In addition, salts such as CaCl<sub>2</sub> are required for binding between the GPIIb/IIIa receptor and the GPIIb/IIIa receptor ligand such as fibrinogen. Suitable buffer concentrations of CaCl<sub>2</sub> range between about 1.0 mM and  
20 about 10.0 mM, and are preferably about 5.0 mM.

A GPIIb/IIIa ligand is a small organic molecule, polypeptide, protein, monoclonal antibody or nucleic acid that binds, complexes or interacts with GPIIb/IIIa receptors on the platelet surface. The aggregation of  
25 platelets results when the GPIIb/IIIa receptors on the surface of platelets bind, complex or interact with GPIIb/IIIa ligands bound to solid surfaces. Suitable GPIIb/IIIa ligands include fibrinogen, monoclonal antibody 10E5 (Coller et al. *J. Clin. Invest.* 72:325 (1983),  
30 monoclonal antibody c7E3 (The EPIC Investigators, *N.E. Journal of Med.*, 330:956 (1994), other monoclonal antibodies, Von Willebrand factor, fibronectin, vitronectin and ligands that comprise an arginine-glycine-aspartic acid sequence or other peptides

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or peptidomimetics that mimic this sequence ( Cook et al. *Drugs of the Future* 19:135 (1994)).

A solid surface is any surface onto which a GPIIb/IIIa ligand can be immobilized such that platelet aggregation is detectable when the solid surface is combined with platelets having unblocked GPIIb/IIIa receptors. Typically, the ligand is immobilized on the surface at a density such that platelet aggregation can occur by binding of the platelet GPIIb/IIIa receptor with the immobilized ligand. The ligand is immobilized on the solid surface by covalent binding, complexing or adsorption such that the ligand is capable of binding to GPIIb/IIIa receptor. Suitable solid surfaces include glass (for example, glass particles or test tubes), plastic or small polymeric beads. Small polymeric beads are preferred. The detection of platelet aggregation can be accomplished visually or by means of a magnification aid. Preferably, platelet aggregation is detected by observing some change in the solid surface which occurs as a consequence of platelet aggregation, for example, agglutination of small polymeric beads or plastic or glass microparticles.

A small polymeric bead, as used herein is any polymeric microparticle a GPIIb/IIIa receptor ligand can be covalently bound to or adsorbed on. The polymeric microparticles can be virtually any shape, but are generally spherical with uniform diameters ranging from about 0.1  $\mu\text{m}$  to about 10  $\mu\text{m}$  in diameter. Preferred diameters are from about 1.0  $\mu\text{m}$  to about 6.0  $\mu\text{m}$  in diameter. For example, the polymeric microparticle may be polymerized acrylonitrile 1-3  $\mu\text{m}$  beads with *N*-hydroxysuccinimide ester groups on their surface (e.g., Matrex 102 beads from Amicon Corporation) (Coller, *Blood*, 55:169 (1980)). The *N*-hydroxysuccinimide ester groups allow coupling of the *N*-terminus of the peptide, protein

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or monoclonal antibody to the surface of the bead (see example 2). Alternatively, the microparticle can be carboxylated polystyrene 3-6  $\mu\text{m}$  beads (Polysciences Inc.). The surface carboxyl groups of this bead can be coupled to the *N*-terminus of the protein, peptide or monoclonal antibody by means of a carbodiimide coupling (see Example 1). The beads may be colored to render the results of the agglutination reaction easier to interpret.

In carrying out the method of the present invention, 10  $\mu\text{l}$ -200  $\mu\text{l}$  of anticoagulated blood, 10  $\mu\text{l}$ -200  $\mu\text{l}$  of buffer solution and 1  $\mu\text{l}$ -50  $\mu\text{l}$  of small beads are mixed in the second step of the present invention. In one embodiment, about 50  $\mu\text{l}$  of anticoagulated blood, about 50  $\mu\text{l}$  of buffer solution, and about 5  $\mu\text{l}$  of small beads are used. Alternatively, the mixture can be scaled up or down, as long as the ratio of the components used remain within the limits described above.

As discussed previously, the GPIIb/IIIa receptor ligand can also be a ligand comprising an arginine-glycine-aspartic acid sequence, or mimic thereof, which is covalently bound to the solid surface through a suitable spacer. Examples of suitable ligands are disclosed in Beer et al., *Blood* 79:117 (1992), the contents of which are incorporated in their entirety herein by reference. Suitable GPIIb/IIIa receptor ligands include the peptide (glycine)<sub>n</sub>-arginine-glycine-aspartic wherein n is an integer from 2-20. The polyglycine portion of the ligand is the spacer and is covalently bound to the surface of the polymeric bead at the *N*-terminal amino group. The arginine-glycine-aspartic acid portion of the ligand bind to the GPIIb/IIIa receptor. Optionally, an additional amino acid or oligopeptide which does not significantly interfere with the binding of arginine-glycine-aspartic acid to the GPIIb/IIIa receptor may be bound to the C-terminus of

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aspartic acid by means of an amide bond. In one embodiment, the GPIIb/IIIa receptor ligand comprises (Glycine)<sub>9-11</sub>-arginine-glycine-aspartic acid-phenylalanine. Alternatively, the spacer portion of the ligand can  
5 comprise any moiety which causes the arginine-glycine-aspartic acid sequence to extend out from the surface of the microparticle sufficiently to allow binding between the ligand and GPIIb/IIIa receptors on the surface of platelets and does not significantly interfere with the  
10 ability of arginine-glycine-aspartic acid to bind with GPIIb/IIIa. Examples of suitable moieties include alkyl groups and polyglycol groups.

The mixture produced is agitated for a period of time sufficient to allow the platelet GPIIb/IIIa receptors to  
15 bind with the GPIIb/IIIa receptor ligand on the small polymeric beads and agglutination of the beads to occur. Agitation is preferably supplied mechanically, for example by placing the assay tube on a rocker. The assay tube can be rocked for up to twenty minutes at 5-40 cpm, but  
20 preferably about two minutes at about 20 cpm. Alternatively, the mixture produced can be agitated manually, for example by placing the mixture on a glass plate and rotating or by mixing with stick. Manual agitation can be continued for as long as twenty minutes,  
25 but preferably for about 2-3 minutes.

Subsequently, the mixture is assessed to determine whether agglutination of the beads has occurred and, optionally, the extent of the agglutination. Lack of agglutination of the beads indicates that the GPIIb/IIIa  
30 receptors on the platelets in the sample are blocked, i.e., greater than about 80% of the GPIIb/IIIa receptors are blocked. Lack of agglutination also indicates that the individual being assessed will have prolonged bleeding times due to about 80% or greater GPIIb/IIIa blockade.

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Agglutination of the beads indicates that less than about 80% of GPIIb/IIIa receptors on the platelets in the sample are blocked. The extent of agglutination is indicative of the level of blocked GPIIb/IIIa receptors.

5 A normal level of agglutination indicates that less than about 40-50% of the individual's GPIIb/IIIa receptors are blocked and that the individual will have a normal or near-normal bleeding times. A level of agglutination that is less than normal indicates that between about 50% and

10 about 80% of the individual's GPIIb/IIIa receptors are blocked, and that the individual will have a mild to moderately prolonged bleeding times, i.e., bleeding time that is longer than normal but less than about thirty minutes. Whether agglutination is less than normal is

15 determined by comparing the amount of agglutination from the blood of an individual being assessed with the amount of agglutination from a standard or control. The standard or control is the amount of agglutination that results from the blood of an individual with a normal platelet

20 count (150,000-350,000 platelets/ $\mu$ l) whose GPIIb/IIIa receptors are more than about 60% unblocked. The standard or control can be predetermined or run simultaneously.

Agglutination is determined visually by whether the beads clump, which is indicative of agglutination, or

25 whether they remain suspended in the solution, which is indicative of the absence of agglutination. Optionally, the beads can be colored to aid in visualizing agglutination, or clumping, of the beads. The level of agglutination is also determined visually by comparing

30 whether there is less clumping in the solution compared with the control or standard.

In the method of the present invention, a thrombin receptor activating peptide is optionally combined with the mixture of anticoagulated blood, buffer and small

35 polymeric beads to which a GPIIb/IIIa receptor ligand is

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bound or adsorbed. The thrombin receptor is a transmembrane protein that is present in platelets (Vu et al. *Cell* 64:1057 (1992)). A thrombin receptor activator, as defined herein, is a peptide, protein, antibody or  
5 small organic molecule that induces platelet activation, via the thrombin receptor i.e., which increases the rate of aggregation of platelets whose GPIIb/IIIa receptors are not blocked when the platelets are combined with a GPIIb/IIIa receptor ligand bound to solid surfaces. A  
10 suitable peptide is any peptide of appropriate sequence and size to activate platelets, as described above. The peptide can comprise thrombin, or a portion thereof, such that the amino acid sequence of the peptide or peptide mimic result in activation of the platelets. Vu et al.  
15 propose that the new amino terminus of the cleaved receptor protein functions as a tethered ligand which interacts with another region of the receptor to induce the activation signal. A fourteen amino acid peptide (T-14) (SEQ ID NO: 1) corresponding to the new N-terminus of  
20 the cleaved receptor protein is capable of aggregating platelets directly (Vu et al.). However, the entire peptide is not required for activity because an eleven amino acid peptide (T-11) (SEQ I.D. NO: 2) lacking the three C-terminal amino acids of T-14 is twice as potent as  
25 T-14 (see Coller et al. *Biochemistry* 31:11713 (1992), the contents of which are hereby incorporated by reference into this application in their entirety). In fact, a peptide comprising the first five or six amino acids has also been shown to be active. (Vassallo et al., *J. Biol. Chem.* 267: 6081 (1992), Hui et al., *Biochem. Biophys. Res. Commun.* 184:790 (1992), Sabo et al., *Biochem. Biophys. Res. Commun.* 188:604 (1992) and Scarborough et al., *J. Biol. Chem.* 267:13146 (1992)).

An important application of the assay of the present  
35 invention is for rapid monitoring of an individual during

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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." For example, monoclonal antibody c7E3 is given prior to  
5 angioplasty or atherectomy in high-risk clinical situations to reduce the risk of ischemic complications (The EPIC Investigators, *N.E. J. of Medicine* 330:956 (1994)). Monoclonal antibody c7E3 acts by blockading the GPIIb/IIIa receptor. Because GPIIb/IIIa blockade inhibits  
10 platelet thrombus formation, it is important that blockade of this receptor be reversed prior to invasive surgery in the event that invasive surgery is needed following c7E3 Fab injection. It is advantageous to have an assay that accurately and rapidly determines the extent to which  
15 GPIIb/IIIa platelet receptors in the patient's blood are blocked and, thus, the likelihood excessive bleeding will occur. When GPIIb/IIIa blockage is achieved, the patient is ready to undergo the procedure. The addition of a thrombin-activating peptide or other thrombin-activating  
20 agent has the advantage of inducing platelet aggregation, thereby increasing the rate at which the beads of the assay of the present invention agglutinate and, thus, decreasing the time required for the assay to be carried out.

25 The N-terminal serine group of the thrombin receptor activating peptides, e.g., T-14, T-11 and the 5-mer of T-14, is essential to these peptides' ability to induce platelet aggregation. This is based on the observation that acetylation of the N-terminal serine of T-11 results  
30 in loss of aggregating ability. In addition, T-11 and T-14 lose their ability to induce aggregation, when incubated in plasma, because aminopeptidase M, present in plasma, cleaves the N-terminal. Significant losses of platelet aggregating ability can occur in as little as ten  
35 minutes (Coller et al., *Biochemistry* 31:11713 (1992)).

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The presence of aminopeptidase M in whole blood can result in variability in the amount of time required for agglutination of the beads in the assay of the present invention.

5           The variability in the time required for agglutination can be avoided by carrying out the present method under conditions wherein the cleavage of the N-terminal serine of the thrombin receptor activating peptide is suppressed. This can be accomplished by  
10 employing a thrombin receptor activating peptide that is resistant to degradation by aminopeptidase M. A resistant thrombin receptor activating peptide should have the additional property of retaining sufficient activity such that when the resistant thrombin receptor activating  
15 peptide is added to the assay of the claimed invention, as described above, agglutination is rapid enough, when the platelet GPIIb/IIIa receptors are not blocked, that the assay can be used for bedside monitoring. An assay suitable for bedside monitoring results in agglutination  
20 in less than ten minutes, but preferably in less than five minutes. A preferred thrombin receptor activating peptide that is resistant to degradation by aminopeptidase M is racemic isoSer-Phe-Leu-Leu-Arg-Asp (SEQ ID NO: 3). This peptide is hereinafter referred to as T-6'. T-6' retains  
25 15-20% of the activity of the six amino acid thrombin receptor activating peptide Ser-Phe-Leu-Leu-Arg-Asp (SEQ ID NO: 4) while showing no significant loss of activity after two hours of incubation with platelet poor plasma (see Collier et al., *J. Biol. Chem.* 268:20741 (1993)), the  
30 contents of which are hereby expressly incorporated in their entirety into this application by reference). Other suitable thrombin receptor activating peptides that are resistant to aminopeptidase M include peptides comprise an N-terminus having the amino acid sequence of T-6', such  
35 that the peptide is resistant to aminopeptidase M

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degradation and retains sufficient platelet activating activity, as described above. Examples include analogues of T-11 (SEQ ID NO: 5) and T-14 (SEQ ID NO: 6), wherein the N-terminal serine of T-11 or T-14 is substituted with  
5 *iso*-serine. Although Applicant does not wish to be bound by any particular mechanism, it is believed that these peptides are resistant to deactivation by aminopeptidase M because isoserine is not cleaved from the N-terminus. The present invention also encompasses other thrombin receptor  
10 activating peptides such as analogues of T-14, T-11, the 6-mer of T-14 and the 5-mer of T-5 in which the N-terminal serine is substituted with a moiety, preferably an amino acid, that results in resistance of the peptide to cleavage by aminopeptidase M. Suitable thrombin receptor  
15 activating peptides can be prepared by peptide synthesis, according to methods known to those skilled in the art (See Example 3).

Alternatively, the variability can be avoided by including an inhibitor of aminopeptidase M in the assay.  
20 A suitable inhibitor of aminopeptidase M includes amastatin, which has been shown to enhance aggregation platelet aggregation in the presence of aminopeptidase M (Coller et al., *Biochemistry* 31:11713 (1992)).

Other platelet-activators can be used in place of the  
25 thrombin receptor activating peptides described above. For example, epinephrine, arachidonic acid or its metabolites, a thromboxane A<sub>2</sub>, platelet activating factor, plasmin, serotonin, vasopressin, tissue plasminogen activator, streptokinase and immune complexes can be  
30 added, alone or in combination with other platelet activators, to increase the rate of agglutination of the beads in the assay of the present invention.

A preferred embodiment of the present invention is based on the discovery that as a consequence of platelet  
35 activation by a thrombin receptor activating peptide, such

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as T-6', the time period required for agglutination of the small beads is rapid enough for bedside monitoring and is reproducible when the amounts of reagents and the percentage of blocked GPIIb/IIIa receptors in the blood being tested remain constant. For example, 70  $\mu$ l of small beads bound with fibrinogen in the presence of 100  $\mu$ l buffer (0.01 M HEPES, 0.15 M, NaCl, 5 mM CaCl<sub>2</sub>), 70  $\mu$ l anticoagulated whole blood and 5-10  $\mu$ l T-6' (see Example 5) will agglutinate in two minutes if high grade GPIIb/IIIa blockade has not been achieved, i.e. when less than 80% of the GPIIb/IIIa receptors are blocked. The concentration of T-6' in this assay can generally range from between about 2-5  $\mu$ M, which allows variations in the amounts of the other reagents. The skilled artisan can readily determine such variations. This provides a quick and simple assay that can be performed at the bedside to determine when a patient undergoing antiplatelet therapy has achieved GPIIb/IIIa blockade.

A bedside assay for GPIIb/IIIa can be used in conjunction with a variety of other medical procedures, for which levels of GPIIb/IIIa blockage may differ. For example, platelet transfusions may be needed prior to invasive surgery in order to prevent excessive bleeding if GPIIb/IIIa blockage is greater than 50% (Coller et al. *N.Y. Acad. Sci.*, 614:193 (1993)). Determining whether GPIIb/IIIa receptor blockage has returned to normal levels is particularly important if invasive surgery is needed immediately after a procedure such as angioplasty or atherectomy, in which the GPIIb/IIIa blockage has been elevated by antiplatelet therapy. Another embodiment refers to an assay to determine when 50% or less of the GPII/IIIa receptors of an individual are blocked. The same quantities of reagents are used as with the assay for determining 80% blockage, as discussed above, except that the concentration of isoSer-Phe-Leu-Leu-Glu-Arg is reduced

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to about 0.2-1  $\mu$ M. Agglutination within two minutes indicates that the individual will have a normal or near-normal bleeding time. Less than about 50% of the GPIIb/IIIa receptors are blocked in this individual.

5           Assays that are indicative of different levels of GPIIb/IIIa blockade can be readily determined by one of ordinary skill in the art. Whole blood is drawn from an individual with a normal platelet count, (150,000-300,000 platelets/ $\mu$ l in whom a known percentage of GPIIb/IIIa  
10 receptors are blocked. The amounts of reagents used in the assay, i.e. anticoagulated blood with the known percentage of GPIIb/IIIa blockage, buffer, small beads and T-6' or other platelet activator can be varied until a  
15 ratio of reagents is found that results in agglutination within a convenient period of time. Generally, it is most convenient to vary the amount of thrombin receptor activator in order to obtain an assay in which  
agglutination occurs within a desired period of time for a certain level of GPIIb/IIIa blockade. The percentage of  
20 GPIIb/IIIa receptors in the blood that are blocked can be determined by methods known to those skilled in the art, e.g. radiolabeled 7E3 binding (Coller et al., *N.Y. Acad. Sci.*, 614:193 (1993)) or flow cytometry (Coller et al. *Blood* 74:182, (1989)). This ratio of reagents can be used  
25 in the assay of the present invention to test the extent of GPIIb/IIIa blockade in a patient in whom the extent of GPIIb/IIIa blockade is unknown. Agglutination of the beads within the desired period of time indicates that extent of GPIIb/IIIa blockade is less than or equal to the  
30 indicated percentage. Failure of the beads to agglutinate indicates that GPIIb/IIIa blockade equals or exceeds the indicated percentage. If the platelet count is abnormally low, there may be no agglutination, even with there being relatively little receptor blockade. If is necessary to

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perform a platelet count to know that it is normal (150,000-350,000/ul).

A kit is provided herein, in which the reagents necessary for carrying out the assay of the present invention are provided. The kit comprises a blood, a  
5 buffer that maintains the pH and salt concentration of the blood sample assessed within ranges suitable for platelet aggregation and small polymeric beads coated with platelet glycoprotein IIb/IIIa receptor ligand. The buffer can be  
10 in solution, or can consist solely of the buffering composition and salts to which a known amount of water is added to give the desired buffer solution. Optionally, the kit can also comprise an anticoagulant. In one embodiment, the preferred buffer is HEPES; the  
15 anticoagulant is citrate; a GPIIb/IIIa receptor ligand is fibrinogen; small polymeric beads are polyacrylonitrile or carboxylated polystyrene in which a peptide GPIIb/IIIa receptor ligand, such as fibrinogen, is covalently bonded to the bead surface by means of a covalent bond between  
20 the N-terminus of the peptide and a carboxylate group on the bead surface.

In a further embodiment the kit additionally comprises a platelet activator, such as a thrombin receptor activating peptide. For example, the thrombin  
25 reactor activating peptide is isoSer-Phe-Leu-Leu-Arg-Asp. If the thrombin receptor activating peptide is not resistant to degradation by aminopeptidase M, the kit can additionally comprise an inhibitor of aminopeptidase M, such as amastatin.

30 As discussed above, it is advantageous for certain applications of the present invention that the assay of the present invention be used for bedside monitoring and that the assay indicate whether a certain level of GPIIb/IIIa receptor blockade has been achieved. As  
35 discussed earlier, the relative amounts of reagents can be

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adjusted so that agglutination of the beads results within a desired period of time if the desired level of GPIIb/IIIa receptor blockade is not achieved. A kit to be used in this context generally contains the quantity of  
5 buffer, thrombin receptor activating peptide, such as isoSer-Phe-Leu-Leu-Arg-Asp, and fibrinogen-coated polymeric beads such that agglutination of the beads will result within a known period of time suitable for bedside monitoring when mixed with a pre-determined amount of  
10 anticoagulated whole blood in which the desired level of GPIIb/IIIa receptor blockade has not been achieved. For example, if the method is to be carried out in approximately two minutes and it is to be determined whether the level of GPIIb/IIIa blockage is 80% or less,  
15 the kit comprises 100 parts by volume buffer to 10 parts by volume beads to 5-10 parts by volume isoSer-Phe-Leu-Leu-Arg-Asp. In another aspect, the desired period of time is two minutes and the level of GPIIb/IIIa blockage is 50% or less.

20 Another embodiment of the present invention refers to a method of diagnosing individuals with bleeding abnormalities due to reduced levels of unblocked GPIIb/IIIa receptors. For example, individuals who are homozygous for Glanzmann thrombasthenia have virtually no  
25 functional GPIIb/IIIa receptors and consequently have prolonged bleeding times. Individuals who are heterozygous for Glanzmann thrombasthenia have 50-60% of the normal number of GPIIb/IIIa receptors, but show normal bleeding times (Coller et al. *Ann. N.Y. Acad. Sci.* 614:193  
30 (1993)). Individuals with platelet levels below about 100,000 platelets/ $\mu$ l are severely thrombocytopenic and also have prolonged bleeding times. Consequently, when the blood of an individual who has not been administered an agent that blocks GPIIb/IIIa receptor and who is  
35 homozygous for Glanzmann thrombasthenia or is severely

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thrombocytopenic is tested for "blockade" of GPIIb/IIIa receptors by the assay of the present invention, no agglutination of the beads will be observed. The method of detecting an individual who is homozygous for Glanzmann  
5 thrombasthenia or thrombocytopenic comprises testing the blood of the individual for GPIIb/IIIa blockage by the assay described herein. The blood of the individual should be tested when the individual has not been administered an agent, e.g., an agent used for  
10 antiplatelet therapy, that blocks GPIIb/IIIa receptors. The absence of agglutination of the beads indicates that the individual should be tested further for Glanzmann thrombasthenia or thrombocytopenia.

The invention is further illustrated by the following  
15 examples, which are not intended to be limiting in any way.

#### EXEMPLIFICATION

##### Example 1 - Preparation of Fibrinogen Coated Carboxylated 20 Polystyrene Beads

0.5 ml of 2.5% carboxylated microparticles obtained from Polysciences Incorporated were placed into an Eppendorf centrifuge tube having a capacity of 1.5-1.9 ml. A sufficient amount of 0.1 M carbonate buffer, pH 9.6, was  
25 added to fill the tubes, which was then centrifuged for 5-6 minutes in a micro-centrifuge. The supernatant was carefully removed using a pasteur pipette and discarded. The pellet was then resuspended in carbonate buffer. To resuspend the pellet, the tube was filled halfway, capped,  
30 vortexed and then filled to capacity. The tube was then recentrifuged for 5-6 minutes and the supernatant removed and discarded. The pellet was then resuspended in 0.02 M sodium phosphate buffer, pH 4.5, and centrifuged for 5-6

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minutes. The supernatant was then removed and discarded. The resuspension in phosphate buffer and centrifugation was repeated two more times, after which the supernatant was removed and discarded.

5           A 2% solution of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride dissolved in 0.02 M sodium phosphate (2% carbodiimide) buffer was freshly prepared within 15 minutes of using. 0.625 ml of this 2% carbodiimide solution was added dropwise to the pellet.

10 The resulting suspension was mixed for 3-4 hours at room temperature using an end-to-end mixture. The suspension was then centrifuged for 5-6 minutes and the supernatant was removed and discarded. The resulting pellet was resuspended in 0.02 M phosphate buffer and centrifuged for

15 5-6 minutes. The supernatant was then removed and discarded. This process of resuspending in phosphate buffer and centrifuging was repeated two more times.

The resulting pellet was suspended in 0.2 M borate buffer, pH 8.5. 300  $\mu$ g of fibrinogen was added to the

20 suspension, which was then gently mixed overnight at room temperature on an end-to-end mixer. 50  $\mu$ l of 0.5 M ethanolamine was then added and the suspension mixed for 30 minutes.

The suspension was then centrifuged for 10 minutes,

25 and the supernatant was removed and discarded. The pellet was then resuspended in 1 ml of 10 mg per milliliter BSA solution in borate buffer. The mixture was capped, vortexed and then gently mixed for 30 minutes at room temperature. After centrifuging for 5-6 minutes the

30 supernatant is removed and discarded. This process of resuspending the pellet in BSA solution and centrifuging was repeated one time. The supernatant was then removed and discarded, and the pellet resuspended in 0.5 ml of

35 PBS, pH 7.4, containing 10 mg per milliliter BSA, 5% glycerol, and 0.1%  $\text{NaN}_3$ , and stored at 4°C.

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The extent of coupling was monitored by immunologic fibrinogen determination, optical density at 280nm and or protein assays (Lowry or Bio-Rad Protein Assay).

Example 2 - Covalent Coupling of Fibrinogen to Polymerized  
5 Acrylonitrile

Solid beads of polymerized acrylonitrile, primarily 1-3  $\mu\text{m}$  in diameter, with free carboxyl groups and *N*-hydroxysuccinimide carboxylate ester groups on their surface (Matrex 102, Amicon Corporation) come packed in  
10 dioxane to prevent hydrolysis of the esters. One milliliter of a well mixed suspension (containing 67 mg of beads) was centrifuged at 10,000 g for 1 minute, the supernatant dioxane removed, and the beads rapidly washed  
15 twice with 1.2 ml of 0.01 M Na acetate, pH 6. The dried, washed beads were then resuspended in 1.5 ml of either fibrinogen or albumin (2 mg/ml) solution in 0.03 M sodium citrate, pH 7.5, and allowed to rock overnight at 4°C. In  
20 some experiments, a smaller volume of a more dilute protein solution (0.7 mg/ml) was employed, but the same ratio of total protein to beads (0.045) was always maintained. The extent of coupling was monitored by  
immunologic fibrinogen determination, optical density at 280 nm and/or protein assays (Lowry or Bio-Rad Protein Assay) on the postcoupling supernatant after dialysis (to  
25 remove the interfering chromophore released by the reaction). In 8 experiments, the fibrinogen coupled with 88%-100% efficiency, whereas in 4 experiments, the albumin coupled at 17%-27% efficiency. Since complete coupling of  
30 fibrinogen required only ~0.1% of the total reactive sites, the reaction proceeded very rapidly and was complete in less than 7 minutes. Calculations based on the manufacturer's estimate of the surface area of the beads (6 sq. M/g) indicate that there is approximately 1 molecule of fibrinogen every 8000 square Å of bead

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surface, which translates into approximately 1 molecule of fibrinogen every 90 Å. On average, there were ~500,000 fibrinogen molecules coupled to each bead. When adjusted for the difference in molecular weight, virtually the same number of albumin molecules were bound to the beads as were fibrinogen molecules. After the coupling procedure was completed, the beads were washed extensively with 0.15 M NaCl, 0.01 M Tris-HCL, 0.05% azide, pH 7.4 until the supernatant buffer's absorbance at 280 nm reached baseline so as to remove the products of the reaction and any nonspecifically adsorbed fibrinogen. Both the fibrinogen and albumin beads were then resuspended in equal volumes of buffer (2-4 ml), giving a particle count of ~250,000/ $\mu$ l. To assess the amount of fibrinogen that adsorbed to the beads instead of being covalently coupled, beads were suspended in a solution of 3% SDS 8 M urea, 0.02 M NaPO<sub>4</sub> for 30 minutes at 37°C, and the protein content in the solution determined. Less than 2% of the bound protein could be eluted in this way.

### 20 Example 3 - Synthesis of Thrombin Receptor Activating Peptides

This example describes synthesis of thrombin receptor activity peptides, T-11, T-14 and T-6'. The methods described can be used to produce other thrombin receptor activity peptides. T-14 and T-11 were prepared on an automated peptide synthesizer (Applied Biosystems 430A; Foster City, CA) using t-Boc chemistry, 4-methylbenzhydrylamine resins and N-methylpyrrolidone as the coupling solvent as previously described (Beer et al., *Blood*, 79:117 (1992)). The protecting groups were  $\beta$ -benzyl ester for aspartic acid, benzyl for serine, 2-bromobenzyloxycarbonyl for tyrosine, 2-chlorobenzyloxycarbonyl for lysine, and tosyl for arginine. Arginine, asparagine, tyrosine, and selected

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proline and phenylalanine residues were double-coupled. Dimethylsulfide and anisole were included in all of the hydrogen fluoride cleavage solutions. Peptides were assessed by reverse-phase HPLC and selected peptides were purified by this technique. Acetylation of the N-terminus of T-11 to form Ac-T-11 was accomplished with acetic anhydride prior to cleavage from the resin. The mass of selected peptides was determined by fast atom bombardment mass spectrometry as previously described (Beer et al. 1992). T-6' was prepared by first synthesizing the peptide Phe-Leu-Leu-Arg-Asp by the automated method as described in the previous paragraph and then manually adding the t-butoxycarbonyl racemic isoserine in dichloromethane and dimethylformamide (2:1) to the peptide on the resin using 1-hydroxybenzotriazole to activate the isoserine and N,N'-diisopropylcarbodiimide to couple it to the peptide. The peptide was then cleaved from the resin as described in the previous paragraph.

Example 4 - Rapid Whole Blood Assay for Determining Blockage of Platelet Glycoprotein IIb/IIIa Receptors

Blood (9 volumes) is drawn from a patient by syringe and placed in a standard blue-top tube containing sodium citrate (1 volume) of 3.8% sodium citrate). Alternatively, blood may be drawn by vacut directly into a blue-top tube. The tube is then inverted to mix the anticoagulant with the whole blood. Then 50  $\mu$ l of this mixture is added to 50  $\mu$ l of buffer (0.15 M NaCl, 5 mM CaCl<sub>2</sub>, 0.05 M HEPES, pH 7.4) and to 5  $\mu$ l of fibrinogen coated beads prepared by the method of Example 2. The mixture is rotated on a glass plate for about two minutes. If GPIIb/IIIa receptors are blocked, the beads remain in suspension. If the GPIIb/IIIa receptors are not blocked, the platelets interact with the fibrinogen bound to the surface of the beads, resulting in clumping of the beads.

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Example 5 - Rapid Whole Blood Assay for Determining  
Whether 80% of Glycoprotein IIb/IIIa is Blocked

Blood (9 volumes) is drawn from a patient by syringe and placed into standard blue-top tube containing sodium  
5 citrate (1 volume of 3.8% sodium citrate). Alternatively, blood may be drawn by Vacutainer directly into a blue-top tube. The tube is then inverted to mix the anticoagulant with the whole blood. Then 70  $\mu$ l of this mixture is added to an assay tube containing to 100  $\mu$ l of buffer (0.15 M  
10 NaCl, 5 mM CaCl<sub>2</sub>, 0.01 M HEPES, pH 7.4), to 10  $\mu$ l of well-mixed fibrinogen coated beads prepared by the method of Example 1 and 5-10  $\mu$ l of isoSer-Phe-Leu-Leu-Arg-Asp.

The assay tube is then placed on a rocker and rocked at 20 cpm at room temperature for two minutes. The tube  
15 is removed from the rocker, held vertically to allow the blood to drain and viewed. If less than 80% of GPIIb/IIIa receptor blockage has been achieved in the patient's blood, agglutinated beads are readily seen in the streak marking where the blood tilted back and forth. If greater  
20 than 80% of GPIIb/IIIa receptor blockage has been achieved in the patient's blood, the streak does not contain bead agglutinates.

Example 6 - Correlation of Agglutination in the  
GPIIb/IIIa Blockade

25 Purified peak I-2 fibrinogen is coupled to 3  $\mu$ m, blue carboxylated beads (obtained from Polysciences, Inc.) using a water soluble carbodiimide (EDC) by the method of Example 1. The assay is conducted by adding to a 12 x 75 mm glass test tube: 100  $\mu$ l of buffer, 20  $\mu$ l of beads (2.5%  
30 slurry), and 5  $\mu$ l of isoSer-Phe-Leu-Leu-Arg-Gln. Blood is collected into citrate anticoagulant and 70  $\mu$ l of the whole blood is added to the test tube, which is then capped, placed on a rocking platform, and rocked at 16 cycles/min. When normal blood was tested using 1 or 2  $\mu$ M

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activating peptide, a 4+ agglutination endpoint was easily observed in all donors (n=10) by 2 min. Pretreating the blood with c7E3 Fab at 5 doses between 0.8 - 3.0 ug/ml for about 10 minutes at 22°C produced increasing GPIIb/IIIa receptor blockade and abolition of agglutination. Using 2  $\mu$ M activating peptide, the assay remained positive (i.e., 1-4+) at c7E3 Fab concentrations of 0.8 (n=1), 1.2 (n=1), 1.5 (n=3), and 1.8 (n=2)  $\mu$ g/ml, corresponding to GPIIb/IIIa receptor blockade of 56%; 77%; 68, 68, and 82%; and 66 and 73%. The assay became negative (i.e., 0+) at c7E3 Fab concentrations of 1.2 (n=1), 1.5 (n=1), 1.8 (n=3) and 2.4 (n=2)  $\mu$ g/ml, corresponding to GPIIb/IIIa receptor blockade of 77%; 92%, 83, 88 and 94%; and 88 and 89%. Aspirin (1 mM) and/or heparin (3.5 U/ml) did not appreciably affect the results. The percentage of receptor blockade was assayed by radiolabeled FE3 binding as reported in (Coller et al., N.Y. Acad. Sci., 614:193 (1993)).

#### Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

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CLAIMS

What is claimed is:

1. A method of determining whether an individual has reduced ability to form platelet thrombi due to GPIIb/IIIa blockade, comprising the steps of:
  - a) obtaining anticoagulated blood from the individual being assessed;
  - b) combining anticoagulated blood with 1) a sufficient volume of buffer that maintains the pH and salt concentration of the anticoagulated blood within a range suitable for platelet aggregation and 2) a ligand that binds to the platelet glycoprotein IIb/IIIa receptor, wherein the ligand is immobilized on a solid surface such that platelet aggregation can be detected when the solid surface is combined with platelets having unblocked glycoprotein IIb/IIIa receptors;
  - c) agitating the mixture formed in b) for a period of time sufficient for platelet glycoprotein IIb/IIIa receptors to bind with the platelet glycoprotein IIb/IIIa ligand attached to the small polymeric beads, thereby producing an agitated mixture;
  - d) assessing platelet aggregation in the agitated mixture, wherein the absence of aggregation indicates that the individual has reduced ability to form platelet thrombi.
2. The method of Claim 1, wherein the solid surface is a small polymeric bead, and wherein the ability to form platelet thrombi is determined by assessing agglutination of the beads, wherein the absence of agglutination of the beads indicates that the

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individual has reduced ability to form platelet thrombi.

3. The method of Claim 2 wherein the mixture of whole blood and anticoagulant is additionally combined in step b) with a thrombin receptor activating peptide.
4. The method of Claim 2 wherein the mixture of whole blood and anticoagulant is additionally combined in step b) with a thrombin receptor activating peptide and a plasma aminopeptidase M inhibitor.
5. The method of Claim 3 wherein the thrombin receptor activating peptide is resistant to deactivation by plasma aminopeptidase M.
6. The method of Claim 5 wherein the thrombin receptor activating peptide comprises isoSer-Phe-Leu-Leu-Arg-Gln.
7. The method of Claim 5 wherein the thrombin receptor activating peptide is isoSer-Phe-Leu-Leu-Arg-Gln.
8. The method of Claim 7 wherein the platelet glycoprotein IIb/IIIa receptor ligand is a synthetic peptide comprising the arginine-glycine-aspartic acid cell recognition sequence.
9. The method of Claim 8 wherein the synthetic peptide comprises (glycine)<sub>n</sub>-arginine-glycine-aspartic acid and wherein n is an integer from 2 to 20.
10. The method of Claim 7 wherein the small polymeric beads comprise a polymeric material selected from the group consisting of: polyacrylonitrile and

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carboxylated polystyrene, and wherein the peptide is covalently bonded to bead surfaces by means of an amide bond between the N-terminus amino acid of the peptide and a carboxylate group on the bead surface.

- 5 11. The method of Claim 10 wherein the small polymeric beads have a diameter from 3-6  $\mu\text{M}$ .
12. The method of Claim 7 wherein the platelet glycoprotein IIb/IIIa receptor ligand is fibrinogen.
- 10 13. The method of Claim 12 wherein the small polymeric beads comprise a polymeric material selected from the group consisting of: polyacrylonitrile and carboxylated polystyrene, and wherein fibrinogen is covalently bonded to the bead surface by means of an amide bond between the N-terminus of fibrinogen and a carboxylate group on the bead surface.
- 15 14. The method of Claim 13 wherein the small polymeric beads have a diameter from 3-6  $\mu$ .
- 20 15. The method of Claim 14 wherein the amounts of whole blood with anticoagulant, buffer, isoSer-Phe-Leu-Leu-Arg-Gln and fibrinogen-coated polymerized acrylonitrile beads are adjusted in such a manner that the absence of agglutination two minutes after mixing the anticoagulated blood, buffer, isoSer-Phe-Leu-Leu-Arg-Gln and beads indicates that the individual has prolonged bleeding times due to GPIIb/IIa blockade.
- 25 16. A method of determining whether about 80% or more of blockade of platelet glycoprotein IIb/IIIa receptors in whole blood are blocked, comprising the steps of:

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- a) obtaining anticoagulated whole blood from an individual being assessed for blockade of platelet glycoprotein IIb/IIIa receptor;
- b) combining anticoagulated blood with 1) a sufficient volume of a suitable buffer which maintains the pH and salt concentration of the anticoagulated blood within a range suitable for platelet aggregation and 2) a ligand which binds to the platelet glycoprotein IIb/IIIa receptor, wherein the ligand is immobilized on a solid surface such that platelet aggregation can be detected when the solid surface is combined with platelets having unblocked glycoprotein IIb/IIIa receptors;
- c) agitating the mixture formed in b) for a period of time sufficient for platelet glycoprotein IIb/IIIa receptors to bind with the platelet glycoprotein IIb/IIIa ligand attached to the small polymeric beads, thereby producing an agitated mixture;
- d) assessing platelet aggregation in the agitated mixture, wherein the absence of aggregation indicates that about 80% or more of the glycoprotein IIb/IIIa receptors on the platelets are blocked and the absence of aggregation indicates that less than about 80% of the glycoprotein IIb/IIIa receptors on the platelets are blocked.
17. The method of Claim 1, wherein the solid surface is a small polymeric bead, and wherein blockade of platelet glycoprotein IIb/IIIa is determined by assessing agglutination of the beads, wherein the absence of agglutination of the beads indicates that about 80% or more of the glycoprotein IIb/IIIa

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receptors on the platelets are blocked and wherein the presence of agglutination of the beads indicates that less than about 80% of the glycoprotein IIb/IIIa receptors on the platelets are blocked.

- 5 18. The method of Claim 17 wherein the mixture of whole blood and anticoagulant is additionally combined in step b) with a thrombin receptor activating peptide.
19. The method of Claim 17 wherein the mixture of whole blood and anticoagulant is additionally combined in  
10 step b) with a thrombin receptor activating peptide and a plasma aminopeptidase M inhibitor.
20. The method of Claim 17 wherein the thrombin receptor activating peptide is resistant to deactivation by plasma aminopeptidase M.
- 15 21. The method of Claim 20 wherein the thrombin receptor activating peptide comprises isoSer-Phe-Leu-Leu-Arg-Gln.
22. The method of Claim 20 wherein the thrombin receptor activating peptide is isoSer-Phe-Leu-Leu-Arg-Gln.
- 20 23. The method of Claim 22 wherein the platelet glycoprotein IIb/IIIa receptor ligand is a synthetic peptide comprising the arginine-glycine-aspartic acid cell recognition sequence.
24. The method of Claim 23 wherein the synthetic peptide  
25 comprises (glycine)<sub>n</sub>-arginine-glycine-aspartic acid and wherein n is an integer from 2 to 20.

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25. The method of Claim 22 wherein the small polymeric beads comprise a polymeric material selected from the group consisting of: polyacrylonitrile and carboxylated polystyrene, and wherein the peptide is covalently bonded to bead surfaces by means of an amide bond between the N-terminus amino acid of the peptide and a carboxylate group on the bead surface.
26. The method of Claim 25 wherein the small polymeric beads have a diameter of from about 3  $\mu$  to about 6  $\mu$ .
27. The method of Claim 22 wherein the platelet glycoprotein IIb/IIIa receptor ligand is fibrinogen.
28. The method of Claim 27 wherein the small polymeric beads comprise a polymeric material selected from the group consisting of: polyacrylonitrile and carboxylated polystyrene, and wherein fibrinogen is covalently bonded to the bead surface by means of an amide bond between the N-terminus of fibrinogen and a carboxylate group on the bead surface.
29. The method of Claim 28 wherein the small polymeric beads have a diameter of from about 3  $\mu$  to about 6  $\mu$ .
30. The method of Claim 29 wherein the amounts of whole blood with anticoagulant, buffer, isoSer-Phe-Leu-Leu-Arg-Gln and fibrinogen-coated polymerized acrylonitrile beads are adjusted so that the absence of agglutination two minutes after mixing the anticoagulated blood, buffer, isoSer-Phe-Leu-Leu-Arg-Gln and beads indicates that the GPIIb/IIIa receptors in the whole blood of the individual are blockaded.

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31. A method of determining the degree of GPIIb/IIIa receptor blockade in the blood of an individual, comprising the steps of:
- 5 a) obtaining anticoagulated blood from an individual being assessed for blockade of platelet glycoprotein IIb/IIIa receptor;
  - 10 b) combining anticoagulated blood with a sufficient volume of suitable buffer that maintains the pH and salt concentration of the anticoagulated blood within a range suitable for platelet aggregation, 2) a ligand that binds to the platelet glycoprotein IIb/IIIa receptor, wherein the ligand is immobilized on a solid surface such that platelet aggregation can be detected when the solid surface is combined with platelets having unblocked glycoprotein IIb/IIIa receptors;
  - 15 c) agitating the mixture formed in b) for a period of time sufficient for platelet glycoprotein IIb/IIIa receptors to bind with the platelet glycoprotein IIb/IIIa ligand attached to the small polymeric beads, thereby producing an agitated mixture;
  - 20 d) assessing platelet aggregation in the agitated mixture, wherein the absence of aggregation indicates that greater than about 80% of glycoprotein IIb/IIIa receptors are blocked and wherein normal aggregation indicates that less than about 50% of the glycoprotein IIb/IIIa receptors are blocked.
  - 25
  - 30
32. The method of Claim 31, wherein the solid surface is a small polymeric bead, and wherein the degree of glycoprotein IIb/IIIa receptor blockage is determined by assessing agglutination of the beads, wherein the

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- absence of agglutination of the beads indicates greater than about 80% of the glycoprotein IIb/IIIa receptors are blocked, and wherein normal agglutination of the beads indicates that less than about 50% of the glycoprotein IIb/IIIa receptors are blocked.
- 5
33. The method of Claim 32, where the mixture of whole blood and anticoagulant is additionally combined in step b) with a thrombin receptor activating peptide resistant to cleavage by aminopeptidase.
- 10
34. The method of Claim 33 wherein the thrombin activating receptor peptide is isoSer-Phe-Leu-Leu-Arg-Gln.
35. The method of Claim 34 wherein the amounts of anticoagulated whole blood, buffer, small polymeric beads, and isoSer-Phe-Leu-Leu-Arg-Gln are adjusted so that the absence of agglutination 2 minutes after mixing the anticoagulated blood, buffer, isoSer-Phe-Leu-Leu-Gln-Arg and beads indicates that about 80% or more of the GPIIb/IIIa receptors in the blood of the individual are blocked.
- 15
- 20
36. The method of Claim 34 wherein the amounts of anticoagulated whole blood, buffer, small polymeric beads, and isoSer-Phe-Leu-Leu-Arg-Gln are adjusted so that agglutination within 2 minutes of combining the anticoagulated whole blood, buffer, small polymeric beads, and isoSer-Phe-Leu-Leu-Arg-Gln indicates that less than about 50% of the GPIIb/IIIa receptors in the blood of the individual are blocked.
- 25

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37. A kit for determining the ability of an individual to form platelet thrombi, wherein the kit comprises:  
a) a buffer suitable for maintaining the pH and salt concentration of whole blood within a range suitable for platelet aggregation; and  
5 b) a ligand that binds to the platelet GPIIb/IIIa receptor, wherein the ligand is immobilized on the surface of small polymeric beads.
38. The kit of Claim 37 further comprising a thrombin  
10 receptor activating peptide.
39. The kit of Claim 38 further comprising an inhibitor of plasma aminopeptidase M.
40. The kit of Claim 38, wherein the thrombin receptor activating peptide comprise isoSer-Phe-Leu-Leu-Arg-Gln.  
15
41. The kit of Claim 38 wherein the thrombin receptor activating peptide is isoSer-Phe-Leu-Leu-Arg-Gln.
42. The kit of Claim 38 wherein the platelet glycoprotein IIb/IIIa receptor ligand is a synthetic peptide  
20 comprising the arginine-glycine-aspartic acid cell recognition sequence.
43. The kit of Claim 42 wherein the synthetic peptide comprises (glycine)<sub>n</sub>-arginine-glycine-aspartic acid and wherein n is an integer from 2 to 20.
- 25 44. The kit of Claim 43 wherein the small polymeric beads comprise a polymeric material selected from the group consisting of: polyacrylonitrile and carboxylated polystyrene, and wherein the peptide is covalently

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bonded to the bead surface by means of an amide bond between the N-terminus of the peptide and a carboxylate group on the bead surface.

45. The kit of Claim 44 wherein the small polymeric beads  
5 have a diameter of from about 3  $\mu\text{M}$  to about 6  $\mu\text{M}$ .
46. The kit of Claim 40 wherein the platelet glycoprotein IIb/IIIa receptor ligand is fibrinogen.
47. A method of diagnosing Glanzmann thrombasthenia or  
10 thrombocytopenia in an individual comprising the steps of:
- a) obtaining anticoagulated blood from an individual being assessed for Glanzmann thrombasthenia at a time when the individual has not been administered an agent that blocks  
15 platelet GPIIb/IIIa receptors;
- b) combining the blood obtained in step a) with a buffer which maintains the pH and electrolyte balance of the blood within a range suitable for platelet aggregation and a ligand that binds to  
20 the platelet glycoprotein IIb/IIIa receptor, wherein the ligand is immobilized on the surface of small polymeric beads;
- c) agitating the mixture formed in step b) for a period of time sufficient to allow the  
25 glycoprotein IIb/IIIa receptors on the platelets to bind with the platelet glycoprotein IIb/IIIa ligand attached to the small polymeric beads,
- d) assessing agglutination of the beads in the agitated mixture, wherein the absence of  
30 agglutination of the beads is indicative of Glanzmann thrombasthenia or thrombocytopenia.

## INTERNATIONAL SEARCH REPORT

International Application No

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/86

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 6 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)

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